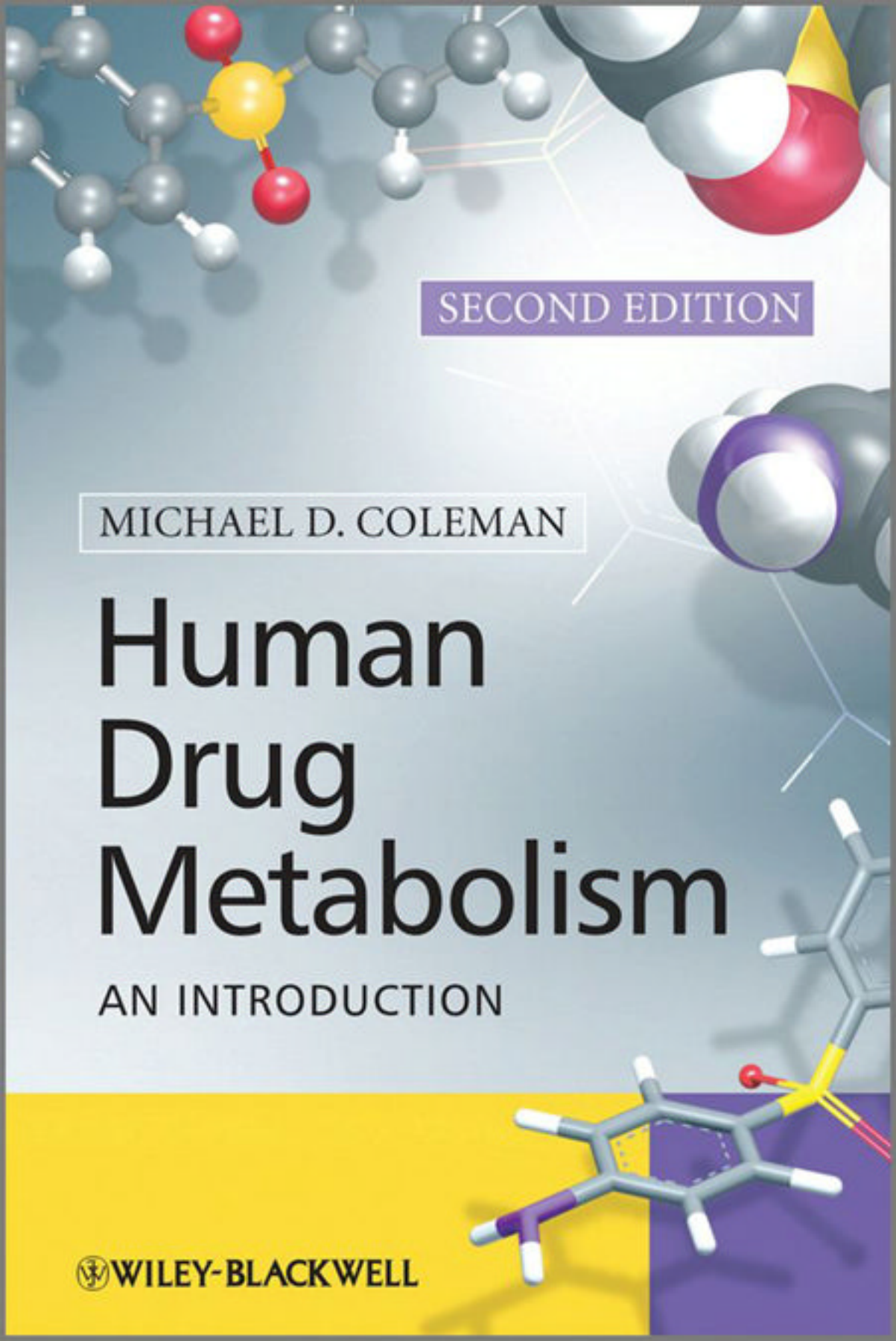
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MICHAEL D. COLEMAN
Human Drug Metabolism
AN INTRODUCTION
WILEY-BLACKWELL

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An Introduction

Second Edition

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Michael D. Coleman
Aston University, Birmingham, UK

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First impression – 2010

For Mark, Carol and Devon

Contents

Preface

In the five years since I wrote the first edition, it is not surprising that advances in the understanding of drug metabolism and toxicity have been rapid and wide-ranging, both experimentally and in patients. *In vitro*, the refinement of many analytical techniques has illuminated how the major drug metabolising enzymes, the cytochrome P450s, accomplish their catalytic activities on a molecular level. This is reflected in the considerable expansion of detail available on how these isoforms manage to combine the apparently contradictory features of selectivity and flexibility. Aside from the role of the CYPs, Chapter 3 now includes more focus on other enzyme systems involved in oxidative drug metabolism, such as the flavin monooxygenases. Since the first edition was written, the interdependence and communication of the various nuclear and cytoplasmic receptor systems that control CYP expression is now better understood and this area has been broadened in Chapter 4. The marked expansion of clinical knowledge of the impact made on co-administered drugs by the selective serotonin reuptake inhibitors has been addressed more fully in Chapter 5. More is also understood about conjugative systems, not only with regard for their ability to accelerate the clearance of drugs, but also their important role in detoxification, which is underlined in the updated Chapter 6.

With respect to the situation *in vivo*, one of the most important issues clinically is the relevance of human polymorphisms in drug metabolism to the 'real world'. Again, the scientific and medical literature has grown considerably in recent years and surprisingly, polymorphisms can be beneficial in several therapeutic areas and in others, the anticipated impacts on drug efficacy and toxicity have not been as severe as expected. Hence, Chapter 7 is now more than twice the length of its predecessor. Regarding Chapter 8, the areas concerning the competing theories on drug hypersensitivity have been expanded and the powerful impact of microarrays in the possible prediction of future hepatotoxins is underlined. Appendix A provides an updated summary of some major methods in drug metabolism pertaining to drug discovery and Appendix B describes advances in our understanding of the metabolism of drugs of abuse, although some are clinically essential. Appendix C remains as an uncompromising guide to those who wish to excel academically and Appendix D is retained and slightly expanded. The further reading section has also been updated and widened in scope with the intention of providing improved insights into the detail of the different areas.

Overall, I have tried to expand the clinical aspects of drug metabolism, whilst retaining some of the scientific detail that informs the clinical drug disposition process. An understanding and appreciation of biotransformation remains crucial in the perpetual struggle

to harness safely the sheer power of modern drug efficacy. Again, I must thank my wife Clare for her tolerance and my mother Jean for her continued encouragement while I have been updating this book, which I hope will be of help to your studies.

M.D. Coleman, DSc.
September 2009

Preface to First Edition

'Throw physic to the dogs; I'll none of it' exclaims the eponymous Macbeth in Act 5, Scene 3, in one of Shakespeare's shortest and most violent plays. This response to the lack of efficacy and severe toxicity of early seventeenth-century therapeutics unfortunately has some resonance today. Despite the spectacular advances made in the last 50 years, many medicines in practice are neither beneficial nor safe. Indeed, increasing numbers of patients are dying as a result of their treatment, rather than their condition. There are many reasons for our inability to eradicate 'iatrogenic' (literally, physician induced) disease; these might include pharmacological interactions or factors relating to the patient's condition. However, the metabolism of drugs by the patients' own systems can have a powerful influence on the success of treatment.

This book is intended to provide a basic grounding in human drug metabolism, although it is useful if the reader has some knowledge of biochemistry, physiology and pharmacology from other sources. In addition, a qualitative understanding of chemistry can illuminate many facets of drug metabolism and toxicity. Although chemistry can be intimidating, I have tried to make the chemical aspects of drug metabolism as user-friendly as possible.

Regarding the layout of the book, Chapter 1 uses the idea of the therapeutic window to outline how both efficacy and toxicity are dependent on drug concentration, which is in turn linked to the rate of drug removal from the system. Biological systems actively eliminate small xenobiotic (foreign) molecules and how quickly this happens is a strong determinate of treatment outcome. Chapter 2 tries to put the metabolism of drugs in the context of other biological processes. Human metabolizing systems must synthesize endogenous molecules, inactivate them when their purpose is served and defend the body from foreign molecules. Drugs fit into the latter category and are treated by biological systems as foreign and unwelcome. Chapter 3 outlines how human metabolizing systems have availed themselves of highly specialized metabolizing enzymes of bacterial and eukaryotic origin, particularly cytochrome P450s. Phase I, the initial, mainly oxidative, phase of metabolism, begins the process of the conversion of lipophilic drugs to easily excreted water-soluble metabolites. The chapter considers the remarkable flexibility and capability of these oxidative enzymes. Chapter 4 reveals the mechanisms whereby the presence of some drugs can induce a massive adaptive increase in the metabolizing capability of cytochrome P450s. The threat to clinical drug efficacy posed by the resulting acceleration of drug removal from the body is outlined in a number of drug classes. By contrast, the inhibition of drug-metabolizing systems described in Chapter 5 is shown to cause life-threatening drug accumulation in a very short space of time. The mechanisms of cytochrome P450 inhibition are explained in the context of the main pharmacological features of enzyme inhibition. Chapter 6 illustrates the processes of conjugation, which can either act as companion

processes for oxidative metabolism, or eliminate drugs in their own right. In conjugative metabolism, large hydrophilic molecules are either attached directly to drugs or oxidized metabolites with the object of increasing their water solubility and molecule weight. This process, in concert with Phase III efflux pump systems, facilitates the removal of the metabolites from cells to the urine and the bile. Chapter 7 discusses other factors that influence drug-metabolizing processes, such as genetic polymorphisms, age, gender, diet, alcohol intake and disease. Chapter 8 explains some of the toxicological consequences of xenobiotic metabolism. The roles of cytochrome P450s in the origins of reversible and irreversible effects on the body are discussed. Irreversible events associated with reactive species formation due to cytochrome P450 metabolism include necrosis, immune-related toxicity and cancer.

At the end of the book, in Appendix A, there is a brief discussion of the role of drug metabolism in the commercial development of new therapeutic agents. The increasing popularity of illicit drugs makes it interesting to include some background on the metabolism of some major drugs of abuse in Appendix B, although it does include clinically useful agents such as opiates. Many readers of this book will be studying for formal examinations of some type, so some accumulated general advice on the preparation for examinations is supplied in Appendix C. Appendix D contains a brief list of cytochrome P450 substrates, inhibitors and inducers, and finally there is a list of suggested reading for those interested in a deeper, more detailed knowledge of the subject.

Whilst no human effort is without error and this book is no exception, it is hoped that it will facilitate understanding of the impact of metabolizing systems on drug therapeutic outcomes. All of us eventually participate in healthcare in some capacity, if not professionally, then as patients. Therefore, it is our duty to constantly update our therapeutic knowledge to liberate the full potential of the many remarkably effective drugs currently available.

I am very grateful to Mr Graham Smith for drawing the detailed figures. I would like to acknowledge the support and encouragement of my wife Clare, as well as my mother Jean, during the writing process and I very much hope you, the reader, find this book useful.

M.D. Coleman, DSc.

1 Introduction

1.1 Therapeutic window

1.1.1 Introduction

It has been said that if a drug has no side effects, then it is unlikely to work. Drug therapy labours under the fundamental problem that usually every single cell in the body has to be treated just to exert a beneficial effect on a small group of cells, perhaps in one tissue. Although drug-targeting technology is improving rapidly, most of us who take an oral dose are still faced with the problem that the vast majority of our cells are being unnecessarily exposed to an agent that at best will have no effect, but at worst will exert many unwanted effects. Essentially, all drug treatment is really a compromise between positive and negative effects in the patient. The process of drug development weeds out agents that have seriously negative actions and usually releases onto the market drugs that may have a profile of side effects, but these are relatively minor within a set concentration range where the drug's pharmacological action is most effective. This range, or 'therapeutic window' is rather variable, but it will give some indication of the most 'efficient' drug concentration. This effectively means the most beneficial pharmacodynamic effects for the minimum side effects.

The therapeutic window (Figure 1.1) may or may not correspond exactly to active tissue concentrations, but it is a useful guideline as to whether drug levels are within the appropriate range. Sometimes, a drug is given once only and it is necessary for drug levels to be within the therapeutic window for a relatively brief period, perhaps when paracetamol (acetaminophen) is taken as a mild analgesic. However, the majority of drugs require repeated dosing in time periods which range from a few days for a course of antibiotics, to many years for anti-hypertensives and antithyroid drugs. During repeated intermediate and long-term dosing, drug levels may move below or above the therapeutic window due to events such as patient illness, changes in diet or co-administration of other drugs. Below the lowest concentration of the window, it is likely that the drug will fail to work, as the pharmacodynamic effect will be too slight to be beneficial. If the drug concentration climbs above the therapeutic window, an intensification of the drug's intended and unintended (off-target) pharmacodynamic actions will occur. If drug levels continue to rise, irreversible damage may occur which is usually described by the word 'toxicity'. To some extent, every patient has a unique therapeutic window for each drug they take, as there is such huge variation in our pharmacodynamic drug sensitivities. This book is concerned with what systems influence how long a drug stays in our bodies.

Human Drug Metabolism 2E, Michael D. Coleman

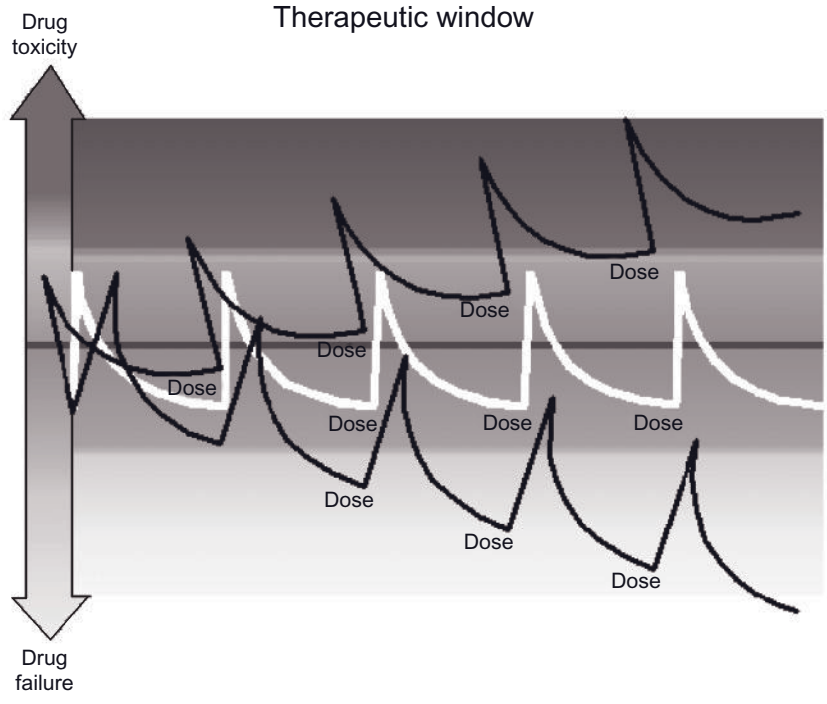

Figure 1.1 The 'therapeutic window', where drug concentrations should be maintained for adequate therapeutic effect, without either accumulation (drug toxicity) or disappearance (drug failure). Such is human variation that our personal therapeutic windows are effectively unique for every drug we take.

Whether drug concentrations stay in the therapeutic window is obviously related to how quickly the agent enters the blood and tissues prior to its removal. When a drug is given intravenously, there is no barrier to entry, so drug input may be easily and quickly adjusted to correspond with the rate of removal within the therapeutic window. This is known as 'steady state', which is the main objective of therapeutics. The majority of drug use is by other routes such as oral or intramuscular rather than intravenous, so there will be a considerable time lag as the drug is absorbed from either the gastro-intestinal tract (GIT) or the muscle, so achieving drug levels within the therapeutic window is a slower, more 'hit and miss' process. The result from repeated oral dosing is a rather crude peak/trough pulsing, or 'sawtooth' effect which you can see in the diagram (Figure 1.1). This should be adequate, provided that the peaks and troughs remain within the confines of the 'therapeutic window'.

1.1.2 Therapeutic index

Drugs vary enormously in their toxicity and the concentrations at which one drug might cause potentially lethal effects might be 10 or 100 times lower than a much less toxic drug. A convenient measure for this is the 'therapeutic index'. This has been defined as

the ratio between the lethal or toxic dose and the effective dose that shows the normal range of pharmacological effect.

In practice, a drug (such as lithium) is listed as having a narrow TI if there is less than a twofold difference between the lethal and effective doses, or a twofold difference in the minimum toxic and minimum effective concentrations. Back in the 1960s, many drugs in common use had narrow TIs, such as barbiturates, that could be toxic at relatively low levels. Over the last 30 years, the drug industry has aimed to replace this type of drug with agents with much higher TIs. This is particularly noticeable in drugs used for depression. The risk of suicide is likely to be high in a condition that takes some time (often weeks) to respond to therapy. Indeed, when tricyclic antidepressants (TCAs) were the main treatment option, these relatively narrow TI drugs could be used by the patient to end their lives. Fortunately, more modern drugs such as the SSRIs (selective serotonin reuptake inhibitors) have much higher TIs, so the risk of the patient using the drugs for a suicide attempt is greatly diminished. However, there are many drugs (including the TCAs to a limited extent), which remain in use that have narrow or relatively narrow TIs (e.g. phenytoin, carbamazepine, valproate, warfarin). Therefore the consequences of accumulation of these drugs are much worse and happen more quickly than drugs with wide TIs.

1.1.3 Changes in dosage

If the dosage exceeds the rate of the drug's removal, then clearly drug levels will accumulate and depart from the therapeutic window towards toxicity. If the drug dosage is too low, levels will fall below the lowest threshold of the window and the drug will fail to work. If a patient is established at the correct dose that does not change, then this is the oral version of 'steady state'. So, theoretically, the drug should remain in its therapeutic window for as long as therapy is necessary unless other factors change this situation.

1.1.4 Changes in rate of removal

The patient may continue to take the drug at the correct dosage, but drug levels may drop out of, or exceed, the therapeutic window. This could be linked with redistribution of the drug between bodily areas such as plasma and a particular organ, or protein binding might fluctuate; however, the major factor in the maintenance of drug levels within the therapeutic window is the rate of removal and/or inactivation of the drug by bodily processes.

1.2 Consequences of drug concentration changes

If there are large changes in the rate of removal of a drug, then this can lead *in extremis* to severe problems in the outcome of the patient's treatment: the first is drug failure, whilst the second is drug toxicity (Figure 1.2). These extremes and indeed all drug effects are directly related to the blood concentrations of the agent in question.

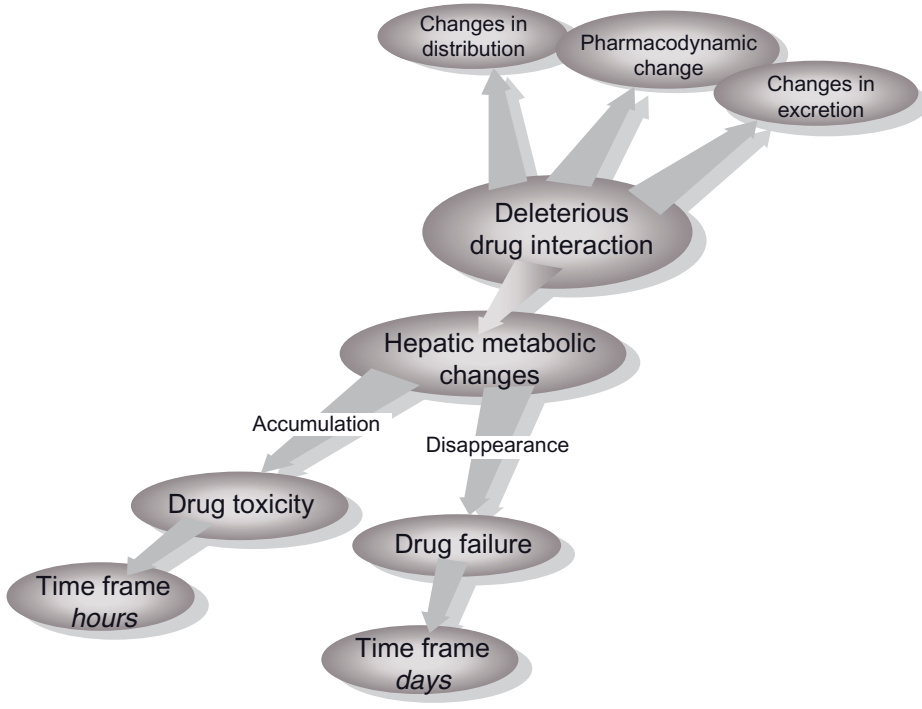

Figure 1.2 Consequences of drug interactions in terms of metabolic changes and their effects on drug failure and toxicity

1.2.1 Drug failure

Although it might take nearly a decade and huge sums of money to develop a drug that is highly effective in the vast majority of patients, the drug can only exert an effect if it reaches its intended target in sufficient concentration. There may be many reasons why sufficient concentrations cannot be reached. Drug absorption may have been poor, or it may have been bound to proteins or removed from the target cells so quickly it cannot work. This situation of drug 'failure' might occur after treatment has first appeared to be successful, where a patient becomes stabilized on a particular drug regimen, which then fails due to the addition of another drug or chemical to the regimen. The second drug or chemical causes the failure by accelerating the removal of the first from the patient's system, so drug levels are then too low to be effective. The clinical consequences of drug failure can be serious for both for the patient and the community. In the treatment of epilepsy, the loss of effective control of the patient's fits could lead to injury to themselves or others. The failure of a contraceptive drug would lead to an unwanted pregnancy and the failure of an antipsychotic drug would mean hospitalization for a patient at the very least. For the community, when the clearance of an antibiotic or antiparasitic drug is accelerated, this causes drug levels to fall below the minimum inhibitory concentration, thus selecting drug-resistant mutants of the infection. Therapeutic drug failure is usually a gradual process, where the time frame may be days before the problem is detected (Figure 1.2).

1.2.2 Drug toxicity

If a drug accumulates for any reason, either by overdose or by a failure of drug removal, then serious adverse reactions will result. A reduction in the rate of removal of the drug from a system (often due to administration of another drug), will lead to drug accumulation. Toxicity can be an intensification of a drug's therapeutic action, or an unrelated damaging effect on a tissue or organ system. If the immunosuppressive cyclosporine is allowed to accumulate, severe renal toxicity can lead to organ failure. Excessive levels of anticonvulsant and antipsychotic drugs cause confusion and drowsiness, whilst the accumulation of the antihistamine terfenadine, can lead to lethal cardiac arrhythmias. In contrast to drug failure, drug toxicity may occur much more rapidly, often within hours rather than days.

1.3 Clearance

1.3.1 Definitions

The consequences for the patient when drug concentrations either fall below the therapeutic window or exceed it can be life threatening. The rate of removal of the drug from the body determines whether it will disappear from, or accumulate in the patient's blood. A concept has been devised to understand and measure rate of removal; this is known as 'Clearance'. This term does not mean that the drug disappears or is 'cleared' instantly. The definition of clearance is an important one that should be retained:

Clearance is the removal of drug by all processes from the biological system.

A more advanced definition could be taken as:

A volume of fluid (plasma, blood or total body fluid) from which a drug is irreversibly removed in unit time.

Clearance is measured in millilitres of blood or plasma per min (or litres per hour) and is often taken to mean the 'clearance' of the drug's pharmacological effectiveness, which resides in its chemical structure. Once the drug has been metabolized, or 'biotransformed', even though only a relatively trivial change may have been effected in the structure, it is no longer as it was and products of metabolism, or metabolites as they are known, often exert less or even no therapeutic effect. Whether or not they retain some therapeutic effect, metabolites are usually removed from the cell faster than the parent drug and they will eventually be excreted in urine and faeces. There are exceptions where metabolites are as effective as the parent drug (some tricyclic antidepressants, such as desipramine and morphine glucuronides), and there are metabolites that are strangely even less soluble in water and harder to excrete than the parent compound (acetylated sulphonamides), but in general, the main measure of clearance is known as total body clearance, or sometimes, systemic clearance:

$$Cl_{\text{total}}$$

This can be regarded as the sum of all the processes that can clear the drug. Effectively, this means the sum of the liver and kidney contributions to drug clearance, although the lung and other organs can make some contribution.

For drugs like atenolol or gabapentin, which unusually do not undergo any hepatic metabolism, or indeed metabolism by any other organ, it is possible to say that:

$$Cl_{\text{total}} = Cl_{\text{renal}}$$

So renal clearance is the only route of clearance for these drugs, in fact it is 100 per cent of clearance.

For paracetamol and for most other drugs, total body clearance is a combination of hepatic and renal clearances:

$$Cl_{\text{total}} = Cl_{\text{hepatic}} + Cl_{\text{renal}}$$

For ethanol, you will probably already be aware that there are several routes of clearance, including hepatic, renal and the lung, as breath tests are a well-established indicator of blood concentrations.

$$Cl_{\text{total}} = Cl_{\text{hepatic}} + Cl_{\text{renal}} + Cl_{\text{lung}}$$

Once it is clear what clearance means, then the next step is to consider how clearance occurs.

1.3.2 Means of clearance

In absolute terms, to clear something away is to get rid of it, to remove it physically from the system. The kidneys are mostly responsible for this removal, known as elimination. The kidneys cannot filter large chemical entities like proteins, but they can remove the majority of smaller chemicals, depending on size, charge and water solubility. The filtrate eventually reaches the collecting tubules that lead to the ureter and the bladder. As the kidney is a lipophilic (oil-loving) organ, even if it filters lipophilic drugs or toxins, these can easily leave the urine in the collecting tubules and return to the surrounding lipophilic tissues and thence back to the blood. So the kidney is not efficient at eliminating lipophilic chemicals.

One of the major roles of the liver is to use biotransforming enzymes to ensure that lipophilic agents are made water soluble enough to be cleared by the kidney. So the liver has an essential but indirect role in clearance, in that it must extract the drug from the circulation, biotransform (metabolize) it, then return the water-soluble product to the blood for the kidney to remove. The liver can also actively clear or physically remove its metabolic products from the circulation by excreting them in bile, where they travel through the gut to be eliminated in faeces. Bacterial effects on this process can lead to the reabsorption of the metabolite or parent drug into the gut, a process known as enterohepatic recirculation (Chapter 6, section 6.2.9).

The liver has an impressive array of enzymatic systems to biotransform drugs, toxins and other chemical entities to more water-soluble products. However, the ability of the

liver to metabolize a drug can depend on the structure and physicochemical characteristics of the agent, so some drugs are easy for it to clear and some are difficult.

1.4 Hepatic extraction and intrinsic clearance

1.4.1 High extraction drugs

Hepatic extraction is a useful term to measure how easily the liver can process, or metabolize, a given drug or toxin. The term 'hepatic extraction' effectively means the difference between the drug level in blood that enters the liver (100 per cent) and the amount that escapes intact and unmetabolized (that is, 100 per cent minus the metabolized fraction).

Extraction is usually termed E and is defined as the extraction ratio, or

$$\text{Extraction Ratio } (E) = \frac{\begin{array}{l}\text{Concentration entering the liver} \\ -\text{ concentration leaving the liver}\end{array}}{\text{Concentration entering the liver}}$$

Clinically, most drugs' hepatic extraction ratios will either be high ($E > 0.7$), or low ($E < 0.3$), with a few agents falling into the intermediate category (E is >0.3, but <0.7). For high extraction drugs, the particular enzyme system that metabolizes this drug may be present in large amounts and drug processing is very rapid. This often happens if the drug is very similar in structure to an endogenous agent, which is normally processed in great quantity on a daily basis. Hence, the early anti-HIV drug AZT (zidovudine), is a close structural analogue of the DNA constituent thymidine and so possesses a half-life of an hour or less in man. In the case of a high extraction drug, the inbuilt or 'intrinsic' ability of the liver to metabolize the drug means that the only limitation in the liver's ability to metabolize this type of drug is its rate of arrival, which is governed by blood flow.

So, in the case of a high clearance drug, where the liver's intrinsic ability to clear it is very high:

$$Cl_{\text{hepatic}} = Q\,(\text{liver blood flow}) \times \text{Extraction ratio } E$$

i.e.

$$Cl_{\text{hepatic}} = QE$$

So, basically, hepatic clearance is directly proportional to blood flow:

$$Cl_{\text{hepatic}}\ \alpha\ Q$$

During intensive exercise, human liver blood flow can fall temporarily by more than 70%, but during normal day-to-day living, blood flow through the liver does not normally vary that much. This means that a high extraction drug will be cleared at a fairly predictable rate. However, hepatic blood flow can be significantly reduced in old age (Chapter 7, section 7.3.1) and end-stage cirrhotic alcoholism (Chapter 7, section 7.7.7). Patients with impaired cardiac output, either as a result of congestive heart failure or myocardial

infarction, also experience marked reductions in liver blood flow. All these circumstances have been shown to reduce the clearance of high extraction drugs clinically and should be borne in mind during drug dosage determination in these patients.

Many drugs are bound in plasma to proteins such as human serum albumin (HSA) or alpha-1 acid glycoprotein (AAG). HSA usually transports endogenous acidic agents, such as fatty acids, bilirubin and bile acids, although it also binds drugs such as warfarin, ibuprofen and diazepam. The endogenous function of AAG is not fully understood, but may involve modulation of the immune system. AAG will bind basic drugs such as erythromycin and protease inhibitors.

Usually, for any given drug, there is equilibrium between protein-bound and free drug. In effect, high extraction drugs are cleared so avidly, that the free drug disappears into the metabolizing system and the bound pool of drug eventually becomes exhausted. As the protein binding of a high extraction drug is no barrier to its removal by the liver these drugs are sometimes described as undergoing 'unrestricted' clearance. Drugs in this category include pethidine (known as meperidine or Demerol in the US), metoprolol, propranolol, lignocaine, nifedipine, fentanyl and verapamil.

You also might see the term 'intrinsic clearance' which reflects the inbuilt ability of the liver (independent from other variables like blood flow) to remove a drug; high extraction drugs have a high intrinsic clearance. As mentioned above, the only limitation in clearance for these drugs is how much drug the blood can deliver. If blood flow was to be infinite, then hepatic clearance would be the same as intrinsic clearance.

1.4.2 Low extraction drugs

On the opposite end of the scale ($E < 0.3$), low extraction drugs are cleared slowly, as the metabolizing enzymes have some difficulty in oxidizing them, perhaps due to stability in the structure, or the low capacity and activity of the metabolizing enzymes. The metabolizing enzymes may also be present only in very low levels. These drugs are considered to be low intrinsic clearance drugs, as the inbuilt ability of the liver to remove them is relatively poor.

If a low extraction drug is not extensively bound to protein (less than 50 per cent bound) then how much drug is cleared is related directly to the intrinsic clearance of that drug. In the case of a low extraction, strongly protein bound drug, then the liver finds clearance even more difficult, as the affinity of drug for the protein is much greater than the liver's affinity for the drug. The anticonvulsants phenytoin and valproate are both highly protein bound (~90 per cent) and low extraction drugs and so the amount of these drugs actually cleared by the liver really depends on how much unbound or free drug there is in the blood. This means that:

$$Cl_{\text{hepatic}} \propto Cl_{\text{intrinsic}} \times \text{fraction unbound}$$

Therefore, clearance is proportional to the ability of the liver to metabolize the drug ($Cl_{\text{intrinsic}}$) as well as the amount of unbound or free drug in the plasma that is actually available for metabolism. Hepatic blood flow changes have little or no effect on low extraction drug plasma levels, but if the intrinsic ability of the liver to clear a low extraction drug falls even further (due to enzyme inhibition or gradual organ failure), there will

be a significant increase in plasma and tissue free drug levels and dosage adjustment will be necessary. Conversely, if the intrinsic clearance increases (enzyme induction, Chapter 4) then free drug levels may fall and the therapeutic effects of the agent will be diminished.

It is worth noting, that with drugs of low extraction and high protein binding such as phenytoin and valproate, a reduction in total drug levels due to a fall in protein binding (perhaps due to renal problems or displacement by another, more tightly bound drug) will actually have no sustained effect on free drug plasma and tissue levels, as the 'extra' free drug will just be cleared or enter the tissues and the bound/unbound drug ratio will quickly re-assert itself. Since the free drug is pharmacologically active and potentially toxic whilst the bound drug is not, it is not usually necessary to increase the dose in these circumstances. The concentration of the free drug has the greatest bearing on dosage adjustment considerations and laboratory assay systems are now routinely used to determine free drug levels with highly bound, low extraction drugs which are therapeutically monitored, such as with phenytoin and valproate. Other examples of low extraction drugs include paracetamol, mexiletine, diazepam, naproxen and metronidazole. The term 'restrictive' clearance is also used to describe these drugs, as their clearance is effectively restricted by their protein binding.

1.5 First pass and plasma drug levels

Clearance is the removal of drug from all tissues and usually the liver is seen as the major force in the clearance of drugs. However, this is an oversimplification, as other tissues can clear drugs and in the real world of a drug entering the body, the gut makes a significant contribution to clearance (Figure 1.3). To be absorbed from the gut, the drug must pass through the gut mucosal epithelial cells and enter the hepatic portal circulation, which leads directly to the liver. A drug may diffuse past the membranes of the gut epithelial cells passively, due to its relative lipophilicity or if it is more water soluble, it may require 'help' from transporter systems called solute carriers (Chapter 2, section 2.6.2). These transporters normally convey vital nutrients such as amino acids as well as drugs with similar physicochemical characteristics (like some statins). However, once in the gut epithelial cells, a drug can be pumped back out into the lumen by efflux proteins (Chapter 4, section 4.4.7) and/or metabolized by various enzymes in the gut wall cells. In the case of some drugs, this can account for a high proportion of the dose before it reaches the liver. The fraction of the original dose left then enters the liver and following hepatic extraction, most of the dose will have been inactivated. This is particularly apparent with high extraction drugs. This process, where an oral dose is metabolized by various systems, is termed 'first pass'.

In some drugs, the vast majority of the dose is lost before it reaches the systemic circulation. The amount that actually reaches the plasma can be measured and the amount that was dosed is also known, so an equation can be produced which gives us how much enters the system. This is known as the 'absolute bioavailability' of the drug and is termed *F*. It can be defined as

$$F = \frac{\text{Total amount of drug in the systemic circulation after oral dosage}}{\text{Total amount of drug in the systemic circulation after intravenous dose}}$$

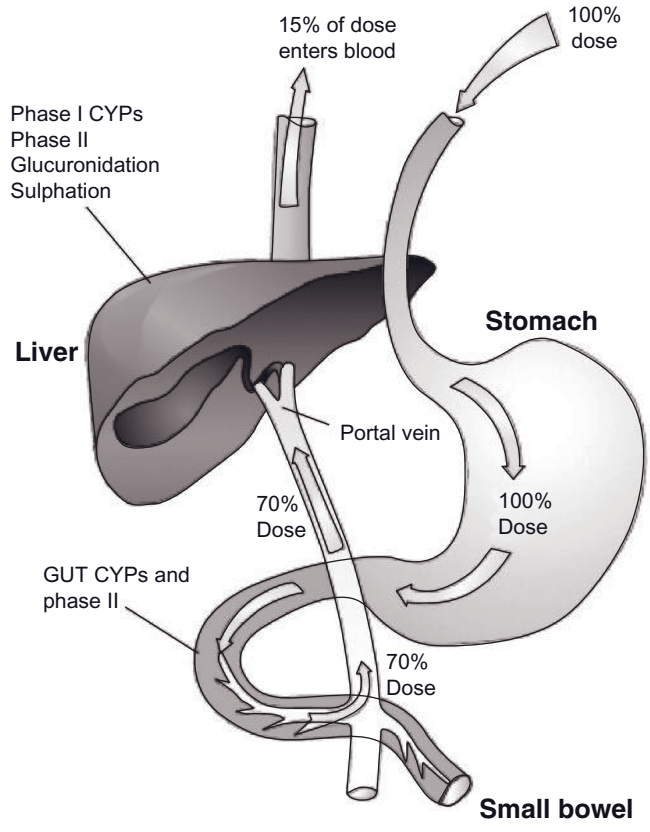

Figure 1.3 The 'first pass' of an orally dosed highly cleared drug showing the removal of drug by the gut and liver, leading to relatively low levels of drug actually reaching the circulation

Highly extracted drugs are often stated to have a 'poor bioavailability'. This means that the oral dose required to exert a given response is much larger than the intravenous dose. If the bioavailability is 0.2 or 20 per cent, then you might need to administer about five times the intravenous dose to see an effect orally.

1.5.1 Changes in clearance and plasma levels

Consider an extreme example; if the intravenous dose of a poorly bioavailable ($F = 0.2$), narrow TI drug X was 20 mg and the usual oral dose was 100 mg, it is clear that if the whole oral 100 mg were to reach the plasma, the patient would then have plasma levels far in excess of the normal intravenous dose, which could lead to toxicity or death. This could happen if the first pass effect was reduced or even completely prevented by factors that changed the drug's clearance.

Similarly, if the clearance of the drug was to be accelerated, then potentially none of the 100 mg would reach the plasma at all, so causing lack of efficacy and drug failure.

1.6 Drug and xenobiotic metabolism

From the therapeutic point of view, it is essential to ensure that drug concentrations remain within the therapeutic window and neither drug failure, nor drug toxicity, occur in the patient. To understand some of the factors related to drug metabolism that can influence the achievement of these aims, there are several important points to consider over the next few chapters of this book.

- What are the metabolic or biotransformational processes that can so dramatically influence drug concentrations and therefore drug action?
- How do these processes sense the presence of the drugs and then remove these apparently chemically stable entities from the body so effectively?
- What happens when these processes are inhibited by other drugs, dietary agents and toxins?
- What is the effect of illness, genetic profile and other patient circumstances on the operation of these processes?
- How can these processes of removal of a drug lead to toxicity?
- What were these processes originally designed to achieve and what is their endogenous function?

The next chapter considers the last point and illustrates that in a subject usually termed ‘drug metabolism’, modern drugs are newcomers to an ancient, complex and highly adaptable system that has evolved to protect living organisms, to control instruction molecules and carry out many physiological tasks.

2 Drug Biotransformational Systems – Origins and Aims

2.1 Biotransforming enzymes

John Lennon once said 'Before Elvis, there was nothing'. Biologically, this could be paraphrased along the lines of 'Before bacteria, there was nothing'. Bacterial life has existed on this planet for more than 3.5 billion years and it first emerged in a far more hostile environment than that of today. Bacteria would have had to survive above and below the earth whilst exposed to corrosive/reactive chemicals, heat and lack of oxygen. The phenomenal growth and generation rates of bacteria enabled them to evolve their enzyme systems quickly enough to not only survive but prosper in all environmental niches. Cell structures eventually settled around the format we see now, a largely aqueous cytoplasm bounded by a predominantly lipophilic protective membrane. Although the membrane does prevent entry and exit of many potential toxins, it is no barrier to other lipophilic molecules. If these molecules are highly lipophilic, they will passively diffuse into and become trapped in the membrane. If they are slightly less lipophilic, they will pass through it into the organism. So aside from 'housekeeping' enzyme systems, some enzymatic protection would have been needed against invading molecules from the immediate environment. Among the various molecular threats to the organism would have been the waste products of other bacteria in decaying biomass, as well as various chemicals formed from incomplete combustion. These would have included aromatic hydrocarbons (multiples of the simplest aromatic, benzene) that can enter living systems and accumulate, thus deranging useful enzymatic systems and cellular structures. Enzymes that can detoxify these pollutants such as aromatics are usually termed 'biotransforming enzymes'.

2.2 Threat of lipophilic hydrocarbons

Organisms such as oysters that cannot rid themselves of lipophilic aromatic and non-aromatic hydrocarbons tend to accumulate these chemicals to toxic levels. Mudskippers, however, are less vulnerable to such toxicity as they use their biotransforming enzymes to remove these chemicals from their systems. With the advent of human dependence on petrochemical technology, vast amounts of lipophilic hydrocarbons are now a fixture of the air we breathe, as well as our food and drink. Dioxin (2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin; TCDD) is part of a series of polychlorinated dibenzo derivatives and it is one the best studied toxic lipophilic hydrocarbons. This herbicide contaminant demonstrates

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perhaps an extreme form of the threat of these molecules to all life on earth. Dioxins are not only carcinogenic and teratogenic endocrine disruptors, but also their half-lives in man can exceed 10 *years*. What is particularly worrying is that we have created molecules like dioxins of such stability and toxicity that despite the fact they actually trigger a potent cellular response intended to metabolize and clear them (Chapter 4), we still cannot get rid of them quickly enough to protect ourselves.

Although dioxins are for humans a 'worst case scenario', the majority of living organisms including ourselves now possess some form of effective biotransformational enzyme capability which can detoxify and eliminate most hydrocarbons and related molecules. This capability has been effectively 'stolen' from bacteria over millions of years. The main biotransformational protection against aromatic hydrocarbons is a series of enzymes so named as they absorb UV light at 450 nm when reduced and bound to carbon monoxide. These specialized enzymes were termed cytochrome P450 monooxygenases or sometimes oxido-reductases. They are often referred to as 'CYPs' or 'P450s'. CYPs may have evolved at first to accomplish reductive reactions in the absence of oxygen and they retain this ability, although their main function now is to carry out oxidations. These enzymes are part of a family whose functional characteristics are reminiscent of a set of adjustable spanners in a tool kit. All the CYPs accomplish their functions using the same basic mechanism, but each enzyme is adapted to dismantle particular groups of chemical structures. It is a testament to millions of years of 'research and development' in the evolution of CYPs, that perhaps 50,000 or more man-made chemical entities enter the environment for the first time every year and the vast majority can be oxidized by at least one form of CYP.

2.3 Cell communication

2.3.1 Signal molecule design

At some point in evolution, single-cell life forms began to coalesce into multi-cell organizations, allowing advantages in influencing and controlling the cells' immediate environment. Further down this line of development, groups of cells differentiated to perform specialized functions, which other cells would not then need to carry out. At some point in evolution, a dominant cellular group will have developed methods of communicating with other cell groups to coordinate the organisms' functions. Once cellular communication was established, other cell groups could be instructed to carry out yet more specialized development. In more advanced organisms, this chain of command and control has two main options for communication: either by direct electrical nervous impulse or instruction through a chemical. Neural impulse control is seen where the sympathetic nervous system influences the adrenal gland by direct enervation. For an instructional chemical such as a hormone (from the Greek meaning to urge on) to operate, its unique shape must convey information to a receptor, where the receptor/molecule complex is capable of activating the receptor to engage its function. An instructional molecule must possess certain features to make it a viable and reliable means of communication. Firstly, it must be stable and not spontaneously change its shape and so lose the ability to dock accurately with its receptor. Secondly, it must be relatively resistant to reacting with various other cell enzymes or chemicals it might contact, such as proteolytic enzymes on the cell surface or

in the cytoplasm. Finally, it must be easily manufactured in large amounts with the components of the molecule being readily available. It is immediately obvious that the pharmaceutical industry uses the same criteria in designing its products that often mimic that of an endogenous molecule. The final feature of an instruction molecule is that it must also be *controllable*. It is no use to an organism to issue a 'command' that continues to be slavishly obeyed long after the necessity to obey is over. This is wasteful at best, and at worst seriously damaging to the organism which will then carry out unnecessary functions which cost it energy and raw materials which should have been used to address a current, more pressing problem. The chemical instruction must be controlled in a period that is appropriate for its function. This might range from seconds to many years.

There are contradictions in this approach; the formation of a *stable* molecule which will be easily and quickly disposable. To make a stable compound will cost energy and raw materials, although to dismantle it will also cost the organism. It all hinges on for what purpose the instruction molecule was designed. For changes that are minute by minute, second by second, then perhaps a protein or peptide would be useful. These molecules can retain information by their shape and are often chemically stable, although the large numbers of various protease and other enzymes present at or around cell membranes mean that their half-lives can be exceedingly short. This allows fine control of a function by chemical means, as rate of manufacture can be adjusted to necessity given that the molecule is rendered non-functional in seconds.

2.3.2 Lipophilic hydrocarbons as signal molecules

Unlike short-term modulations of tissue function, processes like the development of sexual maturity require long-term changes in tissue structure as well as function and these cannot be achieved through direct neural instruction. Chemical instruction is necessary to control particular genes in millions of cells over many years. To induce these changes, hormone molecules need to be designed and assembled to be stable enough to carry an instruction (the shape of the molecule) and have the appropriate physicochemical features to reach nuclear receptors inside a cell to activate specific genes.

Lipophilic hydrocarbon chemicals have a number of advantages when acting as signalling molecules. Firstly, they are usually stable, plentiful and their solubility in oils and aqueous media can be chemically manipulated. This sounds surprising given that they are generally known to be very oil soluble and completely insoluble in water. However, those enzymes we inherited from bacteria such as CYPs have evolved to radically alter the shape, solubility and stability of aromatic molecules. This is in effect a system for 'custom building' stable instructional small molecules, which are easiest to make if a modular common platform is employed, which is usually the molecule cholesterol. From Figure 2.1 you can see the position of cholesterol and other hormones in relation to oil and water solubility, relative to a detergent, which is amphipathic, i.e. soluble in oil and water. The nearest agents with a detergent-like quality in biological systems are bile salts, which use this ability to break large fat droplets into smaller ones to aid absorption.

Cholesterol itself is very soluble in lipids and has almost zero water solubility so it requires a sophisticated transport system to move it around the body. Although a controversial molecule for its role in cardiovascular disease, it has many vital functions such as

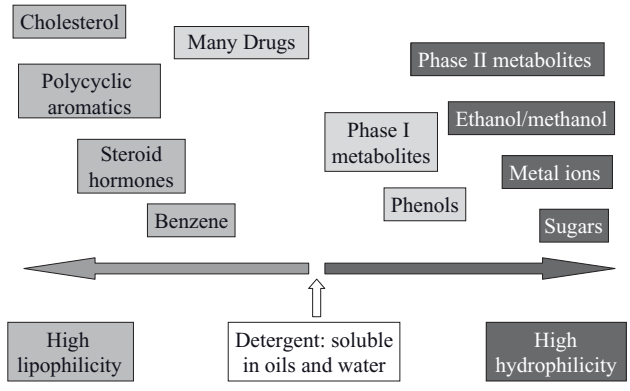

Figure 2.1 The lipophilicity (oil loving) and hydrophilicity (water loving) of various chemical entities that can be found in living organisms

the formation of bile acids as well as maintenance of cell membrane fluidity. This latter function shows that cholesterol itself is so lipophilic that it is trapped in membranes. However, steroid hormones built on the cholesterol 'platform' are much less lipophilic than their parent molecule so they do not get trapped in lipid-rich areas, although from Figure 2.1 it is clear they are still not water-soluble. Highly lipophilic pollutant molecules like large polycyclic hydrocarbons are trapped within membranes and fatty tissue. Steroid hormones are synthesized so that they move through the circulation bound to the appropriate carrier molecule and then they can leave the blood to enter cells without being trapped within membranes.

They can then progress through the cytoplasm, binding various sensor molecules associated with the nucleus. Thus, their information is conveyed intact to instruct the cell. Once the stable steroid platform has been built by CYPs and served its purpose, the final link in the process is the use of various other CYPs to ensure the elimination of these molecules. The complete synthesis and degradation system is fully adjustable according to changing circumstances and can exert a remarkably fine control over steroid molecules. Such is the efficiency of this system that early human contraception studies showed that after an oral dose of oestradiol-17β, systemic bioavailability was virtually zero.

2.4 Potential food toxins

As living organisms developed in complexity and their diets expanded to include many types of plants and animals, it was clearly necessary to evolve a system that would protect an organism from food toxins. This process was probably greatly accelerated by the evolution of land animals from their sea-going ancestors. Diets rich in plant material led to the consumption of high numbers of lipophilic agents with long half-lives, including many aromatic-based compounds. To counter this, it has been suggested that plants evolved chemical defence agents like toxic alkaloids to avoid being eaten. In response, animals probably developed enzymes such as CYP2D6 to metabolize the alkaloids (Chapter 3, section 3.6.2, and Chapter 7, section 7.2.3). Aside from direct lipophilic toxins, more

long-term threats to animals in this regard would be the large number of plant hormone-like chemicals, such as phytoestrogens. Animals and humans can have enough problems regulating their own hormone levels to ensure timely and appropriate reproduction and maintenance of reproductive tracts, without exogenous hormones deranging function, just because the organism prefers a diet rich in hormone-laden plants. It is now clear that oestrogen receptors will bind and function in response to a wide variety of chemicals. This is mainly because large numbers of molecules have an aromatized ring in a similar orientation to a steroid. Regarding diet, there are so many oestrogens and other female hormones in some foods that diet alone has been successfully used to control the menopause in women as an alternative to drug therapy. There are commercial sources of plant oestrogens that are sufficiently potent to be marketed as human breast size enhancers that actually work. It is also clear that long-term exposure to inappropriate hormone levels can lead to cancer in vulnerable tissues such as the endometrium, breasts and ovaries. CYPs are a major defence against such unwanted molecules and they actively protect us from exogenous hormone-like molecules. Interestingly, the fact that plant phytoestrogen breast enhancers and other hormone 'mimics' do exert effects in humans indicates that these agents partially thwart CYP systems, as they are not easy to metabolize and inactivate rapidly enough to prevent interference in human hormone balances.

As has been mentioned, plants synthesize many protective toxin-like agents, but in a harsh environment, to be able to consume such plants without toxicity provides an animal with a significant advantage in its survival prospects. CYPs have also evolved to protect us from such molecules, such as coumarin anticoagulants in some plants. If these cannot be quickly rendered safe and eliminated, severe haemorrhaging can result. The evolutionary 'arms race' between animals, plants and fungi is fiendishly inventive, as agents such as mycotoxins can use our own CYPs to cause lethal toxicity and carcinogenicity (Chapter 8, section 8.6.5). Figure 2.2 illustrates the varying roles of CYPs in living systems.

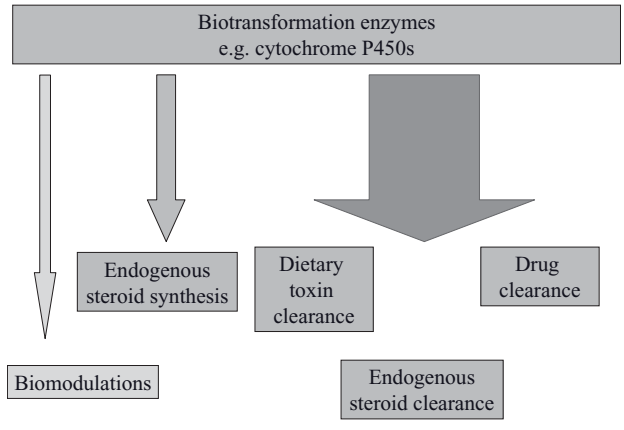

Figure 2.2 Various functions of biotransformational enzymes, from assembly of endogenous steroids, modulation of various biological processes as well as the clearance of drugs, toxins and endogenous steroids

2.5 Sites of biotransforming enzymes

Aside from their biotransformational roles in steroid biosynthesis and drug/toxin clearance, CYPs carry out a wide array of metabolic activities that are essential to homeostasis. This is not surprising, as they are found in virtually every tissue. The main areas are the liver and gut that have the highest concentrations of biotransformational capability. These CYPs are mainly concerned with the processing and clearance of large amounts of various endogenous and exogenous, or 'xenobiotic', chemicals. The CYPs and other metabolizing systems in organs such as the lung and kidney make relatively little contribution to the overall clearance of a drug, but are relevant in the formation of toxic species from drugs and xenobiotics. The brain is a good example of this; CYPs are often found in particular areas, rather than universally distributed. They are found at very low levels, often less than 2 per cent of hepatic P450 levels. Their central nervous system (CNS) role involves catalyzing specific neural functions by regulating endogenous entities such as neurosteroids, rather than larger-scale chemical processing. A number of CYPs are also engaged in the regulation of vascular tone through arachidonic acid metabolism in the periphery as well as the brain. To date, nearly 60 human CYPs have been identified and perhaps surprisingly, about half of them have highly specific biomodulatory roles that are distinct from high volume chemical oxidation. It is likely that hundreds more CYP-mediated endogenous functions remain to be discovered.

2.6 Biotransformation and xenobiotic cell entry

2.6.1 Role of the liver

Drugs, toxins and all other chemicals can enter the body through a variety of routes. The major route is through the digestive system, but chemicals can by-pass the gut via the lungs and skin. Although the gut metabolizes many drugs, the liver is the main biotransforming organ and the CYPs and other metabolizing enzymes reside in the hepatocytes. These cells must perform two essential tasks at the same time. They must metabolize all substances absorbed by the gut whilst also processing all agents already present (from whatever source) in the peripheral circulation. This would not be possible through the conventional way that organs are usually supplied with blood from a single arterial route carrying oxygen and nutrients, leading to a capillary bed that becomes a venous outflow back to the heart and lungs. The circulation of the liver and the gut have evolved anatomically to solve this problem by receiving a conventional arterial supply and a venous supply from the gut *simultaneously* (Figure 2.3); all the blood eventually leaves the organ through the hepatic vein towards the inferior vena cava.

The hepatic arterial blood originates from the aorta and the venous arrangement is known as the hepatic portal system, which subsequently miniaturizes inside the liver into *sinusoids,* which are tiny capillary blood-filled spaces. This capillary network effectively routes everything absorbed from the gut direct to the hepatocytes, which are bathed at the same time in oxygenated arterial blood. Metabolic products can leave the hepatocytes through the hepatic vein or by a separate system of *canalicali*, which ultimately form the bile duct, which leads to the gut. So, essentially, there are two blood routes into the hepatocytes and one out, which ensures that no matter how a xenobiotic enters the body, it will be presented to the hepatocytes for biotransformation.

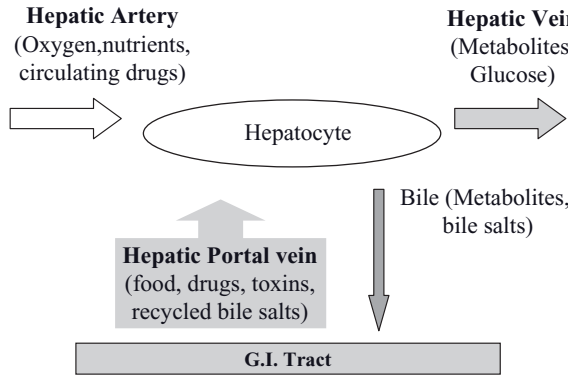

Figure 2.3 The hepatocytes can simultaneously metabolize xenobiotics in the circulation and those absorbed from the gut through their dual circulation of venous and arterial blood. Metabolites escape in the hepatic vein for eventual renal excretion, whilst biliary metabolites reach the gut

2.6.2 Drug and xenobiotic uptake: transporter systems

Although an agent might be presented to the vicinity of a hepatocyte, there is no guarantee it will enter the cell. This depends on the lipophilicity, size, charge and other physiochemical properties of the agent. If an agent is too lipophilic, as described in section 2.3, it may enter a cell and become trapped in the membrane. Alternatively, if a drug is very water soluble, it would not be capable of crossing the lipid membrane bilayer of the cell. Until the last decade or so, it was often assumed that drug absorption would usually be simply through passive diffusion from high to low concentration. It is now apparent that many drugs and toxins which are charged or amphipathic diffuse rather poorly across lipid membranes and their successful cellular and systemic absorption is in a large part due to their exploitation of the complex membrane transport systems which are found not only in the gut, but also on the sinusoidal (sometimes called the basolateral) membranes of hepatocytes, which are bathed in blood from the portal circulation direct from the gut, as well as arterial blood. These membrane transporters regulate cellular entry of amino acids, sugars, steroids, lipids and hormones which are vital for homeostasis. We know this because if the hepatocyte transporters are inhibited, the bioavailability of several drugs increases because they escape hepatic clearance by the CYPs and other systems. Transporter proteins are found in all tissues and can be broadly categorized into two ‘superfamilies’; those that assist the entry of drugs, toxins and nutrients into cells (uptake, or influx transporters) and those that actively pump them out using ATP in the process, usually against concentration gradients (efflux transporters: Chapters 4 and 5).

Hepatic and gut uptake (influx) transporter systems

These transporters, usually known as the solute carriers (SLCs), are found in the liver, gut, brain, kidney and the placenta. These systems operate without using ATP and transport everything from small peptides to anions like bilirubin-related metabolites. The main

hepatic uptake transporters are known as organic anion transporting peptides, or OATPs. These transporters originate from a gene known as SLCO1B1 which is found on chromosome 12. OATPs are sodium independent and they effectively operate a process of facilitated diffusion, known as electroneutral exchange. For every amphipathic molecule they pump in, they expel a neutralizing anion, like glutathione (GSH), bicarbonate or even a drug metabolite. The system is rather like a revolving door and many drugs enter gut epithelial cells and hepatocytes this way, particularly the more hydrophilic statins. The best documented OATPs are OATP1A2, OATP1B1 and OATP1B3. These transporters are vital to the uptake of several classes of drugs and OATP1B1 can be inhibited by gemfibrozil, rifampicin, cyclosporine and by the anti-HIV protease inhibitors such as ritonavir (Chapter 5, section 5.4.1). In Chapter 4, it will be described how metabolizing systems respond in concert to changes in concentrations of substrates and the degree of OATP expression is modulated by the nuclear PXR receptor system, which controls the expression of many CYPs and detoxifying enzymes.

Regarding other hepatic transporters, NTCP (sodium taurocholate cotransporting polypeptide) transports bile salts, but also can handle rosuvastatin and NTCP has also been used to selectively target liver tumours by linking cytotoxic agents to bile salts. There are several other uptake transporters which are of most relevance in tissues other than the liver, such as the kidneys and the gut. The OATs pump small anions mainly in the kidney, but OAT2 and OAT5 are hepatic. OATs can be inhibited by the cephalosporin antibiotics, which may be linked with their renal toxicity.

2.6.3 Aims of biotransformation

Once drugs or toxins enter the hepatocytes, they are usually vulnerable to some form of biotransformation. Although you can see some of the many functions of CYPs and other biotransformational enzymes (Figure 2.2), it is essential to be clear on what they have to achieve with a given molecule. Looking at many endogenous substances like steroids or xenobiotic agents, such as drugs, all these compounds are mainly lipophilic. Drugs often parallel endogenous molecules in their oil solubility, although many are considerably more lipophilic than these molecules. Generally, drugs, and xenobiotic compounds, have to be fairly oil soluble or they would not be absorbed from the GI tract. Once absorbed these molecules could change both the structure and function of living systems and their oil solubility makes these molecules rather ‘elusive’, in the sense that they can enter and leave cells according to their concentration and are temporarily beyond the control of the living system. This problem is compounded by the difficulty encountered by living systems in the removal of lipophilic molecules. As previously mentioned in Chapter 1, section 1.3.2, even after the kidney removes them from blood by filtering them, the lipophilicity of drugs, toxins and endogenous steroids means that as soon as they enter the collecting tubules, they can immediately return to the tissue of the tubules, as this is more oil-rich than the aqueous urine. So the majority of lipophilic molecules can be filtered dozens of times and only low levels are actually excreted. In addition, very high lipophilicity molecules like some insecticides and fire retardants might never leave adipose tissue at all (unless moved by dieting or breast feeding, which mobilizes fats). Potentially these molecules could stay in our bodies for years. This means that for lipophilic agents:

- the more lipophilic they are, the more these agents are trapped in membranes, affecting fluidity and causing disruption at high levels;
- if they are hormones, they can exert an irreversible effect on tissues that is outside normal physiological control;
- if they are toxic, they can potentially damage endogenous structures;
- if they are drugs, they are also free to cause any pharmacological effect for a considerable period of time.

The aims of a biotransformational system include assembly of endogenous molecules, as well as clearance of these and related chemicals from the organism. These aims relate to *control* for endogenous steroid hormones (assembly and elimination), as well as *protection*, in the case of highly lipophilic threats, like drugs, toxins and hormone 'mimics' (endocrine disruptors). Metabolizing systems have developed mechanisms to control balances between hormone synthesis and clearance so the organism can finely tune the effects of potent hormones such as sex-steroids. These systems also actually detect the presence of drugs and act to eliminate them.

2.6.4 Task of biotransformation

Essentially, the primary function of biotransforming enzymes such as CYPs is to 'move' a drug, toxin or hormone from the left-hand side of Figure 2.1 to the right-hand side. This means making very oil-soluble molecules highly water-soluble. This sounds impossible at first and anyone who has tried to wash their dishes without using washing up liquid will testify to this problem. However, if the lipophilic agents can be structurally altered, so changing their physicochemical properties, they can be made to dissolve in water. Once they are water-soluble, they can easily be cleared by the kidneys into urine and they will finally be eliminated.

2.6.5 Phase's I–III of biotransformation

Most lipophilic agents that invade living systems, such as aromatic hydrocarbons, hormones, drugs and various toxins, vary in their chemical stability, but many are relatively stable in physiological environments for quite long periods of time. This is particularly true of polycyclic aromatics. This means that a considerable amount of energy must be put into any process that alters their structures. This energy expenditure will be carried out pragmatically. This means that some molecules may require several changes to attain water solubility, such as polycyclics, whilst others such as lorazepam and AZT, only one. The stages of biotransformation are often described as 'Phases' I, II and III. Phase I metabolism mainly describes oxidative CYP reactions, but non-CYP oxidations such as reductions and hydrolyses are also sometimes included in the broad term 'Phase I'. This has been highlighted as rather arbitrary and inconsistent and it is recommended that it is more accurate to refer to a particular process specifically, rather than using the loose term 'Phase I'.

The term ‘Phase II’ describes generally conjugative processes, where water-soluble endogenous sugars, salts or amino acids are attached to xenobiotics or endogenous chemicals. The very term ‘Phase II’ suggests that ‘Phase I’ processes must necessarily occur prior to conjugative reactions with a molecule. Although this does often happen, conjugation also occurs directly without prior ‘preparation’ by oxidative processes. The products of ‘Phase II’ tend also to be strongly associated with detoxification and high water solubility. This is not always the case either and it is important to realize that some conjugative ‘Phase II’ processes can form either toxic species, or metabolites even less water-soluble than the parent drug. The more recent term ‘Phase III’ describes the system of efflux pumps that excludes water-soluble products of metabolism from the cell to the interstitial fluid, blood and finally the kidneys. The efflux pumps can also exclude drugs as soon as they are absorbed from the gut, as well as metabolites. Although the Phase I–III terminology remains popular and thus is sometimes used in this book, it is important to recognize the limitations of these terms in the description of many processes of biotransformation.

Biotransformation has a secondary effect, in that there is so much structural change in these molecules that pharmacological action is often removed or greatly diminished. Even if the metabolite retained some potential pharmacodynamic effects, its increased polarity compared with the parent drug means that the Phase III systems are likely to remove it relatively quickly, so diminishing any effects it might have exerted on the target tissue.

The use of therapeutic drugs is a constant battle to pharmacologically influence a system that is actively undermining the drugs’ effects by removing them as fast as possible. The processes of oxidative and conjugative metabolism, in concert with efflux pump systems, act to clear a variety of chemicals from the body into the urine or faeces, in the most rapid and efficient manner. The systems that manage these processes also sense and detect increases in certain lipophilic substances and this boosts the metabolic capability to respond to the increased load. The next chapter will outline how mainly CYP-mediated oxidative systems achieve their aim of converting stable lipophilic agents to water-soluble products.

3 How Oxidative Systems Metabolize Substrates

3.1 Introduction

It is essential for living systems to control lipophilic molecules, but as mentioned earlier, these molecules can be rather 'elusive' to a biological system. Their lipophilicity means that they may be poorly water-soluble and may even become trapped in the first living membrane they encounter. To change the physicochemical structure and properties of these molecules they must be conveyed somehow through a medium that is utterly hostile to them, i.e. a water-based bloodstream, to a place where the biochemical systems of metabolism can physically attack these molecules.

3.2 Capture of lipophilic molecules

Virtually everything we consume, such as food, drink and drugs that are absorbed by the gut will proceed to the hepatic portal circulation. This will include a wide physicochemical spectrum of drugs, from water-soluble to highly lipophilic agents. Charged or water-soluble agents (if they are absorbed) may pass through the liver into the circulation, followed by filtration by the kidneys and elimination. The most extreme compounds at the end of the lipophilic spectrum will be absorbed with fats in the diet via the lymphatic system and some will be trapped in membranes of the gut. The majority of predominantly lipophilic compounds will eventually enter the liver. As mentioned in the previous chapter, the main functional cell concerned with drug metabolism in the liver is the hepatocyte. In the same way that most of us can successfully cook foodstuffs in our kitchens at high temperatures without injury, hepatocytes are physiologically adapted to carry out millions of high-energy, potentially destructive and reactive biochemical processes every second of the day without cell damage occurring. Indeed, it could be argued that hepatocytes have adapted to this function to the point that they are biochemically the most resistant cells to toxicity in the whole body – more of those adaptations later.

In the previous chapter it was outlined how the circulation of the liver and gut had evolved to deliver xenobiotics to the hepatocytes. The next task is 'subcellular', that is, to route these compounds to the CYPs themselves inside the hepatocytes. To attract and secure highly physicochemically 'slippery' and elusive molecules such as lipophilic drugs requires a particular subcellular adaptation in hepatocytes, known as the smooth endplasmic reticulum (SER; Figure 3.1). You will be aware of the rough endoplasmic reticulum (RER) from biochemistry courses, which resembles an assembly line where ribosomes

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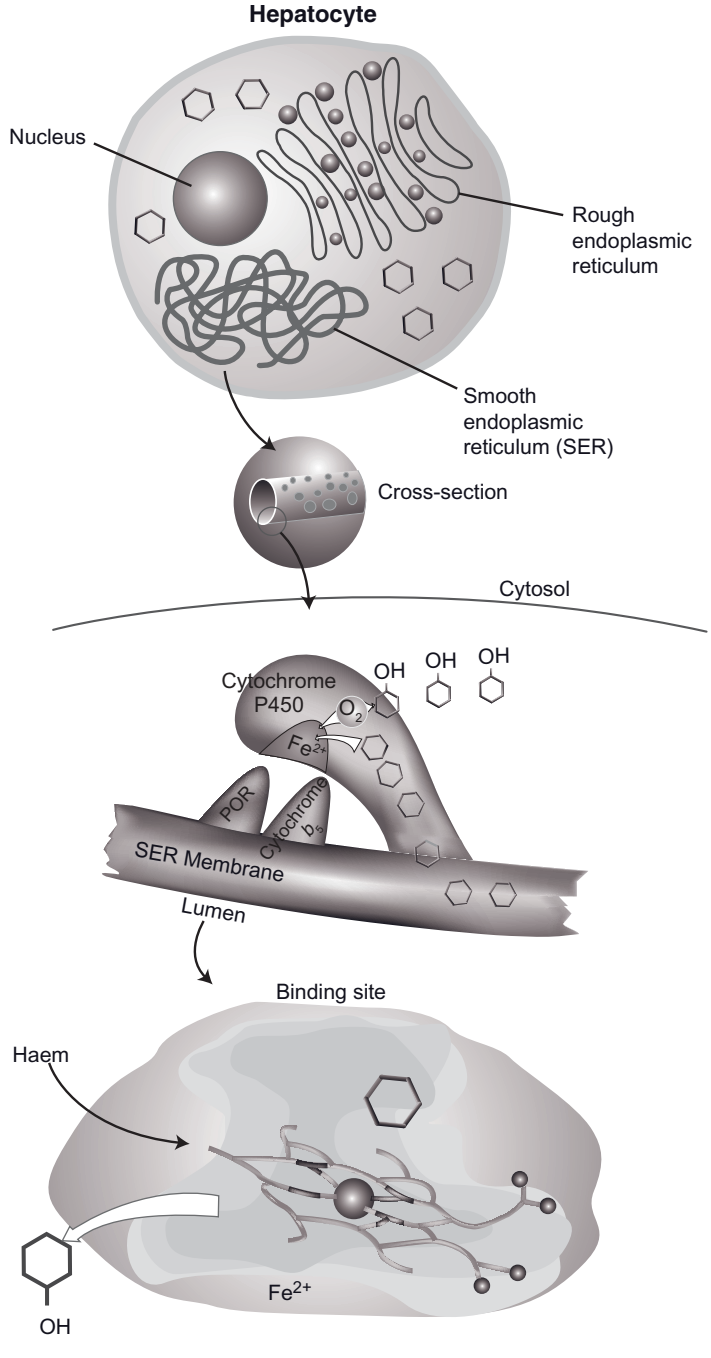


Figure 3.1 Location of CYP enzymes and their REDOX partners, cytochrome b_5 and POR (P450 oxidoreductase), in the hepatocyte and how lipophilic species are believed to approach the enzymes' active site

'manufacture' proteins. Regarding the SER, pictures of this organelle's structure resemble a spaghetti-like mass of tubes. The most lipophilic areas of the SER are the walls, that is, the membranes of these interconnected tubes, rather than the inside (lumen). The drugs/toxins essentially flow along inside the thickness of the walls of the SER's tubular structure (Figure 3.1) straight into the path of the CYP monooxygenase system. This is a highly lipophilic environment in a lipid-rich cell within a lipid-rich organ, so in a way, it is a 'conveyor belt' along which lipophilic molecules are drawn along once they enter the liver for two reasons. Firstly, their lipophilicity excludes them from the aqueous areas of the cell and secondly, the CYPs metabolize them into more water-soluble agents. This 'repels' the metabolites from the SER walls, so they enter the lumen or the cytoplasm, so creating and maintaining a concentration gradient, which causes the lipophilic agents to flow towards the P450s in the first place.

3.3 Cytochrome P450s: classification and basic structure

CYPs belong to a group of enzymes which all have similar core structures and modes of operation. Although these enzymes were discovered in 1958, vast amounts of research have not yet revealed all there is to know of their structure and function. Their importance to us is underlined by their key role in more than 75 per cent of all drug biotransformations. In all living things, over 7,700 individual CYPs have been described so far, although humans make do with just 57; of these, only 15 metabolize drugs and other xenobiotics. Many of the other CYPs are poorly understood in terms of their physiological function and regulation and have been termed 'orphan' CYPs.

In principle, to understand how CYPs operate, it is first necessary to discover their detailed structure. Currently, every CYP from any source is classified according to its amino acid sequence homology, that is, if two CYPs have 40 per cent of the full length of their amino acid structure in common they are assumed to belong to the same 'family'. To date, more than 780 CYP families have been found in nature in total, but only 18 have been identified in humans. The families are numbered, such as CYP1, CYP2, CYP3, etc. Subfamilies are identified as having 55 per cent sequence homology; these are identified by using a letter and there are often several subfamilies in each family. So you might see CYP1A, CYP2A, CYP2B, CYP2C, etc. Regarding the individual CYP enzymes themselves, these 'isoforms' originate from alleles, or slightly different versions of the same gene. They are given numbers within the subfamily, such as CYP1A1 or CYP1A2 and these isoforms have 97 per cent of their general sequences in common. From a practical point of view, differences in the binding site amino acid sequences of the isoforms rather than the full length structure are likely to be more relevant in terms of which specific molecules these enzymes can actually metabolize. With CYP2D6, it is known that change in just one amino acid residue in the binding site is crucial in substrate binding. The amino acid sequences of many bacterial, yeast and mammalian enzymes are now well known and this has underlined some large differences, as well as surprising similarities between the structures of our own CYPs and those of animals, eukaryotes and bacteria. Interestingly, the same metabolite of a given substance will be made by different CYPs across species. Ideally, it's often easiest to understand how machines work by watching cutaway models, like the ones seen of engines at motor shows. With enzymes in living systems, options are much more limited and the most practical method is to 'catch it in the act', that is, to

crystallize it when it is bound to a substrate. Then, the technique of X-ray crystallography can be used to explore and map the contours and features of the enzyme. Some CYPs are water-soluble, such as P450cam, which was crystallized relatively early on, affording the opportunity to study it in detail. As mammalian CYPs function in a lipid environment, this renders crystallization exceedingly difficult. However, by making certain minor external structural modifications to improve water solubility, in 2000 a rabbit CYP (2C5) was crystallized, followed in 2003 by the crystallization of some of the most important human CYPs, CYP2C9, and CYP3A4. Since then, other human drug metabolizing CYPs have also been crystallized, such as CYP2D6, CYP2C8 and CYP2A6.

However, in some ways, using crystallography in this context is like studying how an animal runs or how a machine operates through a series of 'freeze frames', rather than being able to watch the process operating in real time in a 'natural' environment. Indeed, with any research technique, findings are influenced by the limitations of that technique and X-ray crystallography requires the subjection of the CYP isoforms to extremely unphysiological conditions. In some cases, such as CYP2C9, several crystal structures exist, each capturing the isoform binding a different substrate. Nonetheless, great progress has been made in our understanding of external CYP structure, including details of the access and egress pathways for substrates and products, as well as the dimensions of the inner structure such as the active site. The many basic structural similarities between the CYPs have also been revealed using crystallography. As mentioned above, because crystallography is a 'freeze frame' technique, it has been much harder to determine how CYPs actually operate catalytically and it has also been particularly difficult to determine the key to their flexibility; that is, how they can apparently recognize and bind so many disparate groups of substrates. These range from large molecules such as the immunosuppressant cyclosporine, down to relatively small entities such as ethanol and acetone. The advent of many *in silico*, or computer-based techniques such as molecular dynamics, have allowed researchers to understand in much more detail how the protein structures of CYPs unwind and unfold to provide that remarkable degree of flexibility during substrate binding.

Although CYPs in general are capable of metabolizing almost any chemical structure, they do have a number of features in common:

- Most mammalian CYPs exist in a so-called 'lipid microenvironment'. This means that the CYP is partially embedded in the lipophilic membrane of the SER and their access channels are actually positioned inside the membrane ready to receive lipophilic substrates, rather like having an underwater entrance to a tropical island cave.

- CYPs feature a haem group in their active site which contains iron, which is a crucial and highly conserved part of their structures. This area is quite rigid, but it is surrounded by much more flexible complex binding areas.

- All CYPs contain at least one binding area in their active site, which is the main source of their variation and their ability to metabolize a particular group of chemicals.

- To catalyze substrate oxidations and reductions, CYPs exploit the ability of a metal, iron, to gain or lose electrons, rather like a rechargeable battery in a cordless drill. (Figure 3.1).

- They all have closely associated 'REDOX partners', which are P450 oxidoreductase (POR) and cytochrome b_5, that supply them with electrons to 'fuel' their catalytic activities (Figure 3.1).

- They all bind and activate oxygen as part of the process of metabolism.

- They are all capable of reduction reactions that do not require oxygen.

3.4 CYPs – main and associated structures

3.4.1 General structure

There are many complex and detailed three-dimensional structures of the CYPs available online which are worth viewing. However, there is perhaps a simpler way to help you visualize some idea of CYP flexibility and structure at the same time. If you place a small coin a little off centre of the palm of your hand towards your first finger, you could imagine that this is the haem iron catalytic centre, the active site of the CYP. If your hand is partially clenched, but not forming a tight fist around the coin, you might see how there are various flexible 'access channels' or entrances where a 'substrate' can enter and a 'product' leave. You can also see how flexible your fingers are in assisting the 'binding' of various substrates of different shapes to the less mobile 'catalytic centre' of the palm of your hand. In fact, to some extent, we can superimpose the actual generic structural features of real CYP isoforms onto this basic 'CYP hand' analogy. The haem – iron active site 'palm and coin' is set inside what is sometimes termed the CYP protein 'fold' (your hand). This consists of many coils of protein, known as α helices. The whole enzyme structure is usually anchored (the wrist) in the membrane of the smooth endoplasmic reticulum (SER) by an N-terminal α helix, rather like the legs of an oil rig reaching down to the seabed. The structural features of a CYP are often referred to as distal (far from) or proximal (close to) the haem-iron. Hence, the substrate enters the distal area of the isoform (wrist/palm edge/fingers), whilst the REDOX partners which provide the electrons to operate the enzyme, are proximal to the haem iron (near the thumb and first finger).

3.4.2 Haem moiety

Among the major core α helix sub-structures of CYPs, the backbone of these enzymes is known as the 'I' helix, which has a kink in it which locates an area called the 'cys pocket', which in turn holds the haem-iron active site in place. This could be the 'palm and coin' of the CYP hand analogy. CYPs such as 3A4 and 2C9 have some flexibility in the movement of the haem, but in most CYPs this is a relatively rigid part of the protein's structure. The haem structure is also known as ferriprotoporphyrin-9 (F-9; Figure 3.2). The F-9 is the highly specialized lattice structure that supports a CYP iron molecule, which is the core of the enzyme, which catalyzes the oxidation of the substrate. This feature is basically the same for all CYP enzymes; indeed, F-9 is a convenient way of positioning and maintaining iron in several other enzymes, such as haemoglobin, myoglobin and catalase. The iron is normally secured by attachment to five other molecules; in the horizontal plane,

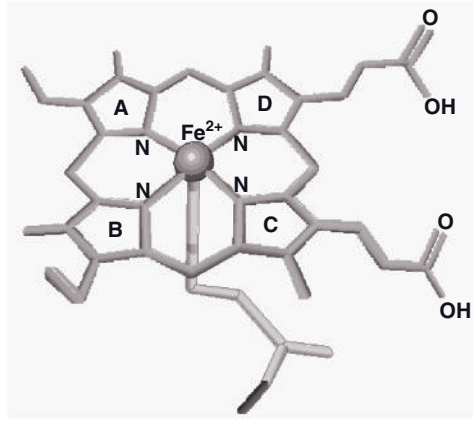


Figure 3.2 Main structural features of ferriprotoporphyrin-9, showing the iron anchored in five positions (pentacoordinate form). The cysteinyl sulphur holds the iron from below

four of them are pyrrole nitrogens, whilst the fifth group, a sulphur atom from a cysteine amino acid residue holds the iron in a vertical plane. This is known as the 'pentacoordinate' (five-position) state and could be described as the 'resting' position, prior to interaction with any other ligand (Figure 3.2).

The pentacoordinate state appears to show iron bound tightly to the sulphur and below the level of the nitrogens. When the iron binds another ligand, it is termed 'hexacoordinate' and iron appears to move 'upwards' and draws level with the nitrogens to bind a water molecule which is hydrogen bonded to a threonine amino acid residue that is located just above the iron, which is linked with proton movements during operation of the enzyme. The F-9 is held in place by hydrogen bonding and a number of amino acid residues, particularly an argenine residue, which may also stabilize the F-9 molecule. As mentioned previously, the iron is crucial to the catalytic function of CYP enzymes, and the process whereby they oxidize their substrates requires a supply of electrons, which is sourced by the dual 'fuel pumps' of the system, POR and cytochrome b_5. These REDOX partners are sited extremely close to a relatively flat area of the proximal side of the CYP and we will look at them in more detail later on, in section 3.4.6.

3.4.3 CYP flexible regions

Running across and over the 'I' helix/haem active site, is a series of helices which form a cover or lid on the active site. These helices are usually described as the F and G 'domain'. This domain consists of an F helix, and F/G loop structure and a G helix. A B/C helix loop is also part of the cover of the active site. Human CYPs contain extra F and G helices (usually termed F′ and G′) which give them added flexibility in uncovering the active site enough to accommodate large molecules. To follow the CYP hand analogy, these flexible regions to the structures, the various F/G helices, their loops and the B/C helix loop, could be regarded as the 'fingers' which are normally partially clenched, but can open out to form an access pathway to accommodate large substrates. As the human

hand can grasp a pin or a beach ball, the small substrate metyrapone binds to CYP3A4 without any visible movement in the 'fingers', whilst erythromycin requires them to stretch out widely to allow binding to the active site. Indeed, CYP3A4 increases its active site area by 80 per cent to accommodate erythromycin. To try to see how the whole CYP isoform is oriented in the SER membrane, then you could imagine the lipophilic substrates diffusing through the membrane and entering the isoform through the access path, which includes the highly lipophilic opening 'fingers'. In any CYP, the access path is generally defined as the widest, shortest and usually the most lipophilic route to the haem iron active site. The active site is supplied with electrons through another access channel from the other side of the CYP by POR and cytochrome b_5. As mentioned above, in the CYP hand analogy, this area could be visualized as located between the thumb and first finger. These REDOX partners are also embedded in the SER membrane right next to the CYP. Finally, there is also what is often termed an egress channel (between the 'fingers'), which is routed away from the SER membrane into either the lumen of the SER or the cytosol, where the more hydrophilic product will naturally exit the isoform, as the other paths are so lipophilic they effectively repel the product.

3.4.4 Substrate binding in CYPs

The term 'active site' of an enzyme usually means the area where structural changes in the substrate are catalyzed with the help of various co-factors. This term can encompass a binding area which locates and holds the substrate in such an orientation that the appropriate moiety of the molecule is presented to the structures on the enzyme that catalyze the reactions the enzyme is intended to accelerate. In many enzymes, the dimensions and properties of the active and binding sites are quite well defined and mapped in detail. With acetylcholinesterase for example, the anionic site is mainly responsible for attracting and locating the substrate acetylcholine and the esteratic site is intended to catalyze the hydrolysis of the substrate. This is not the case in CYPs, as crystallographic studies have shown that what constitutes the 'active and binding sites' of a CYP can be a very broad area indeed. To date, it has been generalized that CYP3A4, CYP2C8 and CYP2C9 have very large active sites, whilst that of CYP2D6 is intermediate and CYP2A6's site is quite small. However, with the larger sited CYPs like CYP3A4 and CYP2C9, they can still bind very small substrates alongside the giant ones. If we return to the 'CYP hand' analogy, if you grasp an object like a door key, which is similar in size to the coin at the 'catalytic centre', the 'binding site' is a relatively small area on the palm and little hand/finger movement is required to grasp it. If you grasp a dinner plate, you can easily hold it in such a way that the edge contacts the coin, although all your fingers and thumb are now required to articulate to grasp the plate and the CYP 'binding site' is pretty much your whole hand. With real CYPs, they undergo similar huge changes in movement and binding area to accommodate substrates of differing sizes like the contrasting agents metyrapone and erythromycin mentioned earlier. Essentially, what constitutes the 'binding site' of any given CYP is very difficult to define. Examination of crystallized CYPs bound to different substrates have shown that CYPs do contain small-intermediate hydrophobic pockets, as well as a capability of the rest of the F and G helices to act as 'extending and enclosing fingers' to bind larger substrates. What is usually described as the hydrophobic pocket in a CYP comprises many amino acid residues that can bind a molecule by a number

of means, including weak van der Waals' forces, hydrogen bonding, as well as other interactions between electron orbitals of phenyl groups, such as 'pi-pi bond stacking'. This provides a grip on the substrate in a number of places in the molecule, preventing excessive movement. Interestingly, when not binding substrates, CYP active site areas are full of water molecules, which are displaced upon substrate binding.

In effect, crystallographic studies have shown that the type of hydrophobic amino acid residues seen in the smaller, 'internal' CYP hydrophobic pockets are also found on the 'fingers' of CYPs, such as CYP3A4. These are the F and F′, G and G′ helices (among others) and they are capable of binding a hydrophobic molecule by using the same pi-pi bond stacking and van der Waals forces. This effect is borne out by observations of the binding of progesterone, which appears to be held between the 'fingers' of CYP3A4, rather than in the 'palm'. Technically, progesterone actually appears to be stuck to the outside of the enzyme. So it is not unusual for many CYPs, that large swathes of the interior and exterior of the isoforms are available for substrate binding.

It is also apparent from crystallographic studies, that there is much more about CYP binding to discover. Studies have shown that substrate binding is not always 'productive', in that the substrate may not be bound near enough to the active site to actually be oxidized (such as a published crystallographic models of CYP2C9 and CYP3A4 with S-warfarin and testosterone binding respectively). Hence, binding may occur in non-productive and productive stages, involving internal rearrangement of the isoform, or may involve simultaneous binding of other substrates. There is also evidence that other conformational changes in the F and G helices occur in contact with an oxidized product molecule, which effectively facilitates its passage out of the CYP.

3.4.5 CYPs: summary of structure and function

CYPs such as CYP3A4 and CYP2C9 managed to combine binding features which at first appear to be contradictory. These and other CYPs can not only bind a range of sizes of substrates, but their sophisticated binding control processes also enable them to even distinguish between left and right-handed versions of the same molecule. This is achieved because we have seen that their structures maintain some areas of relative rigidity and others of constant fluidity, like the relatively rigid palm and flexible fingers of the human hand. Rigidity in the haem promotes oxidation of the substrates, whilst the proximal side of the CYP is sufficiently flexible to link with the REDOX partners, but rigid enough to conduct electrons from the partners to the haem iron. The highly flexible and lipophilic distal side buried in the membrane allows the entrance of virtually any size and shape of molecule and can even facilitate simultaneously the temporary binding of the main body of the molecule during orientation of the target moiety to the haem. In essence, CYPs manage paradoxically to be both broad and highly specific in terms of substrates by ensuring that flexibility in one area does not impair rigidity in the others and vice-versa.

In modern cars, when you turn the key in the ignition, fractionally before the starter motor turns the engine, the electronic management system engages the fuel pump to raise the fuel system to the correct pressure for start up. Similarly, when a substrate binds a CYP, it causes the CYP to become 'coupled' with one or other of its REDOX partners which then supply it with sufficient reducing power to oxidize the substrate. The next two sections describe these vital components of CYP function, the REDOX partners.

3.4.6 CYP REDOX partners (i) P450 oxidoreductase (POR)

P450 oxidoreductase (POR) is an NADPH reductase that is a separate entity from mammalian CYPs but it is indispensible to them (Figure 3.3). NADPH reductases in general are found in most tissues, but they are particularly common in the liver. POR is essential to life, as removal of the gene from animal embryos is lethal. There is a rare human condition known as Antley-Bixler Syndrome (ABS) which is linked with POR mutations and in severe form results in major structural malformations which are associated with disordered steroid metabolism. Perhaps logically, the expression of POR is mainly under the control of the same nuclear receptors that control CYP expression, such as HNF4α and CAR (see Chapter 4). The reductase is a flavoprotein complex, which consists of a large vaguely butterfly-shaped protein 'framework', which locates and binds two equal components, FAD (flavin adenine dinucleotide, an electron carrier) and FMN (flavin mononucleotide). The structure is unusual in enzymology, as FAD and FMN do not usually function together in the same enzyme, except in the various nitric oxide synthetases. Although in tissues, NADH (used in oxidative metabolic reactions) can be plentiful, FAD has evolved to discriminate strongly in favour of NADPH, which fuels reductive reactions. NADPH is formed by the consumption of glucose by the pentose phosphate pathway in the cytoplasm. This oxidative system, which can consume up to 30 per cent of the glucose in the liver, produces NADPH to power all reductive reactions related to CYPs, fatty acid and steroid synthesis, as well as the maintenance of the major cellular protectant thiol, glutathione (Chapter 6, section 6.4.2).

POR will not pass electrons to a CYP unless it is embedded in the SER membrane very close to the CYP. The enzyme complex operates as follows: FAD is reduced by NADPH, which is then released as NADP+ (Figure 3.3). FAD then carries two electrons as $FADH_2$ that it passes on to FMN, forming $FMNH_2$, which in turn passes its two electrons to the CYP. In the presence of high substrate concentrations, POR is required to provide a 'current' of electrons to sustain continuous CYP catalytic activity, rather like a machine tool would need electricity in a factory (Figure 3.4). Interestingly, POR is probably not the only source of electrons for the CYP catalytic cycle, which is discussed in the next section.

In the lung, POR is toxicologically relevant, as it mediates the metabolism of the herbicide paraquat, which occasionally features in accidental and suicidal poisonings. The

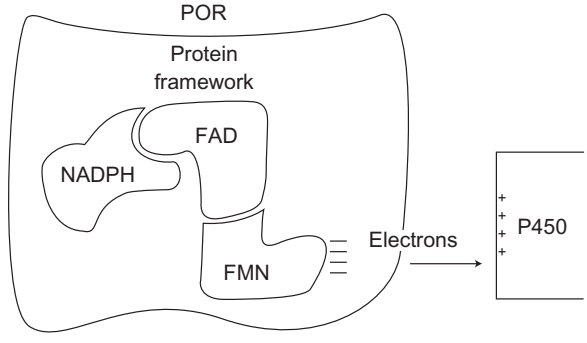


Figure 3.3 Position of CYP reductase in relation to CYP enzyme and the direction of flow of electrons necessary for CYP catalysis

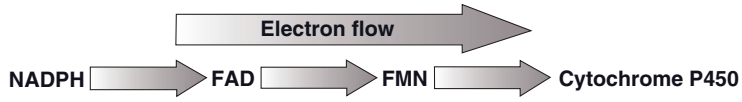


Figure 3.4 Direction of electron flow in P450 oxidoreductase (POR) supply of reducing power to CYP-mediated metabolic processes

reduction of paraquat by POR leads to a futile cycle that generates vast amounts of oxidant species, which destroy the non-ciliated 'Clara' cells of the lung leading to subsequent death several days later. To date there is still no known antidote to paraquat poisoning and it is an agonizing and drawn-out method of suicide. Oxidoreductases are also implicated in the reduction of nitroaromatic amines to carcinogens (Chapter 8).

3.4.7 CYP REDOX partners (ii) Cytochrome b_5

Cytochromes b_5 are electron transport haemoproteins which strongly resemble the active sites of CYP P450's, in that they also are built around a central F-9 haem group. These proteins are ubiquitous in nature, where they convert plentiful cellular supplies of NADH to NAD+, so building up proton gradients which in turn stimulate the flow of electrons. There are numerous forms and structures of cytochrome b_5; a soluble form is found in erythrocytes, where it is known as NADH-dependent methaemoglobin reductase, or sometimes NADH diaphorase. This version of cytochrome b_5 converts methaemoglobin (Chapter 8, section 8.2.2), which is formed normally in small amounts and cannot carry oxygen, back into haemoglobin. Interestingly, those with a rare genetic absence of NADH diaphorase spend their whole lives with blue cyanotic skin. Other forms of the enzyme are lipophilic and membrane bound, such as a variant which is found in the outer membrane of hepatic mitochondria (known as OM cytochrome b_5) which is functionally very different to microsomal cytochrome b_5 , which is of greatest interest in drug metabolism.

Our understanding of the role of microsomal cytochrome b_5 in the operation of CYPs is far from complete but has advanced considerably over the last few years. Microsomal cytochrome b_5 is anchored by a helix which penetrates deep into the SER membrane, but its haem structure is cytosolic (it stands clear of the SER membrane) and it is physically linked with its CYP isoform and it is also closely associated with POR. Until relatively recently, it was widely assumed that the flow of electrons needed to 'power' CYPs was entirely dependent on POR, which required an adequate supply of NADPH to operate. However, it appears that cytochrome b_5 also has a complex but essential role in CYP function. The standard CYP catalytic cycle (see section 3.7 and Figure 3.5) requires two electrons to undergo a complete 'turn'. The second electron is the 'rate limiting step', in that the speed of the CYP operation depends on how rapidly this electron can be supplied. It is now clear that cytochrome b_5 can supply this electron just as quickly and possibly even quicker than POR/NADPH. This suggests that CYPs can source their electron flow indirectly from NADH as well as NADPH.

Although work has been carried out using antibodies to inactivate cytochrome b_5 which has shown it is likely to be essential for CYP activity, a key study in 2008 actually deleted the expression of hepatic microsomal cytochrome b_5 expression in mice. This work showed firstly that the mice suffered no ill-effects and developed normally, which suggests

that other enzyme systems can carry out the main functions of cytochrome b_5 in cellular housekeeping. What was particularly interesting about this study was that both *in vitro* and *in vivo*, drug clearance by CYPs was greatly reduced by up to 90 per cent of normal with some drugs. This means that in the mouse, not only do most CYPs rely on cytochrome b_5 to be part of the process of electron supply, but also the utilization of NADPH and the efficient function of POR is also conditional on the presence of functional cytochrome b_5. If these processes occur in man, then it is likely that the two REDOX partners are not only interdependent, but their individual contributions to the supply of electrons may be exquisitely modulated. Indeed, there is some evidence in bacterial CYPs that REDOX activity is coupled to substrate binding and cytochrome b_5 binding can even change the structure of the main CYP, changing dimensions of access channels and even binding characteristics. It is likely that this process operates both ways as CYPs and REDOX partners couple and uncouple according to substrate binding and processing.

The full complexity and flexibility of the modulation of CYP substrate binding and the resultant internal enzymatic conformational changes remain to be uncovered. It is likely that the CYP's response to a particular substrate is effectively 'customized' in terms of binding, electron supply and catalytic activity. In some ways CYPs can be regarded as highly sophisticated pumps, which draw up lipophilic agents from the SER membrane 'conveyor belt' and expel them as processed and more hydrophilic products into the lumen, thousands of times per second.

3.5 Human CYP families and their regulation

As we have seen, despite their common structure and function, CYP substrate specificity varies enormously within the main families of these enzymes. Aside from CYPs that are involved in steroid synthesis and arachidonic acid metabolism, there are only three CYP families which are relevant to humans in terms of drug and toxin biotransformations. These include:

CYP1 family (CYP1A1, CYP1A2, and CYP1B1);

CYP2 family (CYP2A6, CYP2A13, CYP2B6 CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1);

CYP3 family (CYP3A4, CYP3A5, CYP3A7).

It is believed that 9 out of 10 drugs in use today are metabolized by only five of these isoforms; CYPs 1A2, 2C9, 2C19, 2D6 and 3A4/5. CYP2E1 is interesting mostly from a toxicological perspective and the internal regulation of small hydrophilic molecules. Each CYP has its own broad substrate 'preferences', and in some cases they may not be expressed in some individuals at all, or in very low levels (CYP2D6 polymorphisms; Chapter 7, section 7.2.3). Using immunologically based methods which employ specific antibodies raised to bind to the various CYPs in ELISA and Western Blotting systems, it has been a remarkable achievement that these often extremely similar isoforms have been distinguished, structurally as well as functionally using other assay systems. However, their respective levels of expression in man are often difficult to determine, particularly concerning members of the same family and subfamily.

3.5.1 CYP regulation-transcriptional

As befits such important and complex enzyme systems, there are multiple layers of CYP modulation, which are far from fully understood. Timeframes of control of CYP expression and functionality range from weeks to fractions of a second. An organism may need to build and/or destroy new hormones, such as during the onset of puberty and the regulation of sexual cycles. In addition, xenobiotic 'threat' molecules such as toxins or drugs must also be biotransformed. All these instances require a sustained increase in the expression of a CYP or CYPs which processes these particular molecules in response to their changing concentrations. This process of specific isoform enzyme induction is discussed in detail in Chapter 4 and is governed through sensor molecules in the cytoplasm or more usually the nucleus, which then bind to response elements on the genes which code for the particular CYPs. This transcriptional control thus involves the massive upregulation of the CYP expression system, which includes changes in DNA and various types of RNA activity, which results in increased ribosomal protein manufacture. This process is reversible but rather slow, taking place in humans over 1–3 weeks.

It is now established that there are intermediate steps controlling the CYP transcription process. CYP mRNAs possess sites which can bind a number of regulator molecules which can prevent or reduce translation of the protein (CYP2B1, CYP2E1: see below). Indeed, with the CYP1A family, the ratio between CYP mRNA activity and functional protein produced can be up to 100 : 1, whilst with CYP2C9 it is closer to 1 : 1. In addition, a series of quality control 'chaperone' molecules exist (such as BAP31) which assist in the normal assembly, folding and delivery of CYPs to the ER. Misfolded or faulty CYPs are 'rejected' and degraded. Indeed, molecules like BAP31 also play a role in how CYPs are finally located in the ER, as well as how long they remain anchored and functional. These steps may provide another opportunity for modulation over days rather than weeks.

3.5.2 CYP Regulation-post-translational

Once assembled and operational, it is logical that there should be further apparatus governing CYP which can respond to 'fine tuning' necessary in processes which are controlled over hours and minutes rather than days and weeks. In terms of post-translational modification of a finished protein, one of the most important means is phosphorylation of key amino acids and/or peptides which confer selectivity and specificity on an enzyme. Phosphorylation may control enzymatic activity as well as stability and consequent functional capability, rather like changing the 'sell by date' on a foodstuff. Several CYPs may be influenced to some extent post-translationally through phosphorylation, such as CYP3A4, CYP2E1 and CYP1A2 and it is likely that most human CYPs are modulated in this way to some extent and this may provide a control mechanism over timeframes of hours and minutes.

As already described, there is also evidence that CYPs may 'internally' automatically regulate their own ability to process substrates, by altering the conformations of their access and egress channels, in response to either the number of substrates binding (see testosterone and CYP3A4 in the next section) and/or the changing physicochemical properties of the substrate as it becomes a product during biotransformation. Binding in the general area of the hydrophobic pockets and access channels can accelerate processing of

similar substrates actually at the haem active site and some substrates may impede or accelerate the processing of others, which may be linked with endogenous processes. It is also apparent that the degree of coupling between CYPs and their REDOX partners is also highly variable; indeed, CYP3A4 is usually uncoupled until more than one of several similar substrate molecules is bound. This makes sense, as it is wasteful in traffic to keep the engine 'in gear' unless you intend to move off reasonably quickly. Overall, these control mechanisms at the enzymatic level may modulate CYP processing over infinitesimal fractions of a second, as required for endogenous processes such as steroid metabolism.

The main human families of CYPs have been extensively studied over the last 15 years and a summary of what is known of these enzymes is given below. In Appendix D, a more extensive list of substrates, inhibitors and inducers of the main clinically relevant human CYPs can be found.

3.6 Main human CYP families

3.6.1 CYP 1A series

CYP 1A1

The gene that codes for CYP1A1 is on chromosome 15. This isoform binds and oxidizes planar aromatic, essentially flat, lipophilic molecules. The most common representatives of these compounds are multiples of benzene, such as naphthalene (two benzenes), and what are usually termed polycyclic aromatic hydrocarbons (PAHs) that are many benzene molecules in chains. There is evidence that CYP1A1 also processes many other variants of planar aromatics, such as the polybrominated diphenylethers (PBDEs), known for their carcinogenicity and endocrine disruptor effects. Interestingly, this isoform is hepatically 'non-constitutive', i.e. it is not normally expressed or found in the liver. This is probably because the accumulation of large amounts of planar aromatics in the liver should not normally occur. CYP1A1 is inducible (Chapter 4) in all tissues, and this occurs in the lung in response to aromatics encountered in industrial and traffic pollution. Tobacco smokers exhibit high lung levels of this CYP due to the PAHs and other aromatics present in the smoke. Interestingly, non-smokers exposed to environmental tobacco smoke also show increased levels of CYP1A1. Metabolic products of CYP1A1, which are often epoxides, vary in their stability and the most reactive, such as those from benzpyrene derivatives, are carcinogenic. There is also evidence of higher levels of CYP1A1 in breast cancer sufferers. Experimentally, this CYP is often studied through its ability to O-deethylate the test substrate 7-ethoxyresorufin. In general, CYPs have partly evolved to clear potential threats to the organism, so the metabolism and clearance of toxic agents such as the PDBEs by CYP1A1 would be necessary and beneficial. However, the isoform is polymorphic (Chapter 7, section 7.2.3) and its absence may predispose to toxicity with such agents. In addition, CYP1A1-mediated production of reactive metabolites is likely to be more of a threat than a protection, as it is often overexpressed in the vicinity of carcinogenesis. Hence, whether CYP1A1-mediated clearance of xenobiotics is beneficial or deleterious may change from day to day according to the individual. The proton pump inhibitor omeprazole induces this CYP in man (Chapter 4) and *in vitro* studies suggest it also inhibits it, thus possibly protecting cells from CYP1A1-formed reactive species.

CYP1A2

This CYP is also found on chromosome 15, only 25 kilobases away from CYP1A1 in man and it is linked with oestrogen metabolism. Increased levels of this enzyme are also associated with colon cancer. CYTP1A2 oxidizes planar aromatic molecules that contain aromatic amines, which its relative CYP1A1 does not. CYP1A2 orientates aromatic amines, some of which are quite large, in such a way as to promote the oxidation of the amine group. Consequently, this enzyme is able to metabolize a variety of drugs that resemble aromatic amines: these include caffeine, β-naphthylamine (a known human bladder carcinogen; Chapter 8, section 8.6.4) and theophylline. The enzyme is also capable of oxidizing several tricyclic antidepressants (TCAs). It tends to be inhibited by molecules that are planar and possess a small volume to surface area ratio. It can be inhibited by the methylxanthine derivative furafylline, as well as ciprofloxacin, enoxacine, cimetidine, mexiletine and fluvoxamine. The CYP1A1 inducer omeprazole promotes a similar response in CYP1A2. Other inducers of this CYP include TCDD and (probably fairly heavy) consumption of broccoli (Chapter 7, section 7.4.3).

CYP1B1

To provide a perspective on the vast stretches of time over which CYPs have evolved, it is thought that CYP1B1 became distinct from the CYP1A sub-family over 300 million years ago. The isoform CYP1B1 is found in most tissues expressed at a modest or low level and it does not appear to make much contribution to drug clearance. CYP1B1 is the only member of its sub-family discovered so far and the gene that codes for it is found on chromosome 2. It is inducible through the same pathway as CYP1A1/2 (Chapter 4, section 4.4.2) and it can form reactive and carcinogenic species from endogenous oestrogens as well as from PAHs and it also metabolizes the anti-oestrogenic drug tamoxifen. Recent research has uncovered several endogenous roles for CYP1B1, which paradoxically may even include suppression of tumorigenesis, if work in mice is confirmed in man. It is now established that CYP1B1 is vital in eye development and it catalyzes the production of an arachidonic acid metabolite which maintains the transparency of the cornea, as well as the regulation of its aqueous humour. Indeed, mutations in this CYP are linked with congenital glaucoma, where intra-ocular pressure is excessive and can lead to early blindness. CYP1B1's role in oestrogen metabolism is linked strongly with its overexpression in malignant breast tissue. Indeed, CYP1B1 is overexpressed in tumours of so many differing tissues (particularly the lung and pancreas) that it is being considered as a therapeutic target, as the effects of anticancer prodrugs which are activated by this CYP would be confined to the tumours, as the CYP is poorly expressed in bystander tissues. Interestingly, the messenger RNA which codes for CYP1B1 has a site which binds a microRNA known as MIRN27B, which is thought to inhibit the amount of active CYP produced. In patients with breast cancer, a study has shown that these patients possessed low levels of MIRN27B, which suggests that defective post-transcriptional regulation of CYP1B1 by MIRN27B was a major factor in the development of their malignancy.

3.6.2 CYP2 series

Around 18–30 per cent of human CYPs are in this series, making it the largest single group of CYPs in man. They appear to have evolved to oxidize various sex hormones, so

their expression levels can differ between the sexes. As with many other CYPs, they are flexible enough to recognize many potential xenobiotics and they are thought to oxidize as much as half of all administered drugs.

CYP2A6

This CYP was originally of interest as it is partly responsible (with a cytosolic aldehyde oxidase) for the metabolism of nicotine to its much less pharmacologically active metabolite, cotinine. CYP2A6 is also known for its small binding site area in relation to other CYPs. More recently, studies of the polymorphisms (Chapter 7, section 7.2.3) associated with this CYP have indicated that low expression leads to reduced smoking behaviour and it is much easier for these individuals to stop the habit. CYP2A6 comprises up to 10 per cent of total liver CYP content and it uniquely clears coumarin to 7-hydroxycoumarin, which has been used as the major marker for this CYP for many years. Methoxsalen (an antipsoriatic agent) is a potent mechanism-based (Chapter 5, section 5.3.5.) inhibitor of 2A6, as is grapefruit juice, although it is also weakly inhibited by imidazoles (e.g. ketoconazole). Methoxsalen will inhibit CYP2A6 in man and it prolongs the plasma survival of nicotine, so reducing smoking. Interestingly, CYP2A6 also has toxicological significance, in that it oxidizes carcinogens and mutagens, such as aflatoxins, 1, 3 butadiene and nitrosamines, which are all discussed in Chapter 8. It tends to have a small role in the metabolism of a number of drugs (pilocarpine, letrozole and valproate), but it is often difficult to determine how large a contribution CYP2A6 is making in the metabolism of these substrates. This CYP is clinically inducible by anticonvulsants such as phenobarbitone and the antibacterial rifampicin, amongst others.

CYP2B6

This originates from a gene found on chromosome 19; the 2B series have been extensively investigated in animals, but CYP2B6 is the only 2B form found in man. This isoform is found in all human livers and comprises around 3-10 per cent of total hepatic CYPs. Its level of expression varies by more than 100-fold and it may be subject to sex differences, being more common in women than men. CYP2B6 may be the most polymorphic of all CYPs in man (see Chapter 7, section 7.2.3). It is thought to be implicated in the metabolism of more than 70 xenobiotics, including amfebutamone (bupropion), mephenytoin, some coumarins, cyclophosphamide and its relatives, the antimalarial artemisinine, selegiline (l-deprenyl), as well as methadone and ketamine. Three agents have been shown to inhibit this isoform, the antiplatelet drugs clopidogrel and ticlopidine and the antineoplastic drug thiotepa. It is inducible by rifampicin, phenobarbitone, the anti-HIV reverse-transcriptase inhibitor efavirenz and also the DDT substitute pesticide methoxychlor. This pesticide acts as an endocrine disruptor when it is oxidized to pro-oestrogenic metabolites by a number of human CYPs including CYP2B6 itself. CYP2B6 prefers non-planar neutral or weak bases which accept hydrogen bonding. It tends to hydroxylate at highly specific areas of molecules, particularly close to methoxy groups, which suggests that it may have a biosynthetic role in the assembly of specific endogenous molecules.

CYP2C8

This polymorphic CYP originates on chromosome 10 and is one of four CYP2C isoforms (the others are CYP2C9, 18 and 19) which comprise about 18 per cent of total CYP content which share 80 per cent amino acid sequence identity. CYP2C8 tends to bind and metabolize relatively large molecules which are also weak acids. It is known to clear the anticancer agent taxol as well as verapamil, cerivastatin (now withdrawn), amodiaquine and rosiglitazone. Although CYP2C8 is similar in structure to CYP2C9, it differs catalytically concerning several drugs, metabolizing tolbutamide more slowly than 2C9, but clearing trans-retinoic acid more efficiently. Warfarin is cleared by CYP2C9 to a 4-hydroxy metabolite, whilst 2C8 forms a 5-hydroxy derivative. Ibuprofen is mainly cleared by CYP2C9, whilst 2C8 plays a minor role. The different binding orientations of substrates such as diclofenac that both 2C9 and 2C8 metabolize are related to differences between the hydrophobicity and geometry of the respective binding sites. CYP2C8 can be inhibited by quercetin, the glitazone drugs, gemfibrozil and diazepam in high concentrations, as well as the leukotriene receptor antagonist montelukast and the antibacterial trimethoprim. The antiretroviral agents efavirenz and saquinavir are also inhibitors of this isoform. It is inducible by rifampicin and phenobarbitone.

CYP2C9

CYP2C9 has been particularly heavily studied in terms of its structure and function. It has evolved to process relatively small, acidic and lipophilic molecules that accept hydrogen bonds. It is thought that the structure of the access channel area may be responsible for this enzyme's preference for acidic molecules. There are a large number of substrates for this CYP, which include tolbutamide, dapsone, phenytoin, valproate, rosuvastatin and warfarin. Substrates of CYP2C9 often have relatively narrow TIs, so polymorphisms and inhibition of the CYP can have serious clinical consequences. As described above in section 3.4.4, the active site is flexible, extensive and capable of great binding 'plasticity'. Indeed, initial binding is not always productive, in the case of warfarin. This CYP is a particular focus for many analytical and theoretical studies to uncover CYP mechanisms of action, such as how substrate binding affects REDOX electron supply, subsequent substrate binding and promotion of product egress. Sulphafenazole is a potent inhibitor of this polymorphic enzyme and other inhibitors include amiodarone and fluconazole. It is inducible by the usual suspects (rifampicin and phenobarbitone) as well as the steroids prednisone and norethindrone.

CYP2C18

This CYP does not have any real role in either the metabolism of drugs or environmental toxins, as its mRNA is found in the liver in plentiful amounts, yet very little actual protein appears. Studies with the CYP when it is expressed have shown that it can clear some CYP2C substrates such as tolbutamide and ifosfamide, as well as some steroids. It is

polymorphic, but there is relatively little literature on it and it does not appear to be linked with any disease states or conditions.

CYP2C19

This is also inducible and differs by only around 10 per cent of its amino acids from CYP2C9, but it does not oxidize acidic molecules, indicating that the active sites and access channels are subtly different. CYP2C19 prefers weak bases such as amides which have at least two hydrogen bond acceptors. It metabolizes omeprazole and its related proton pump inhibiting anti-ulcer agents, as well as S-mephenytoin and diazepam. CYP2C19 is inducible (rifampicin and carbamazepine) and polymorphisms in this gene exist and there is a higher incidence of poor metabolizer phenotypes in Chinese/Japanese than Caucasians. This means that parent drug can accumulate in poor metabolizers, sometimes advantageously in the case of ulcer therapy (Chapter 7, section 7.2.3). Tranylcypromine acts as a potent inhibitor of 2C19, as well as the antiplatelet drugs (clopidogrel, ticlopidine) and fluvoxamine. Paradoxically, clopidogrel's clinical anti-platelet response is linked strongly with CYP2C19 status (Chapter 7, section 7.2.3).

CYP2D6

The gene which codes for this isoform is on chromosome 22; CYP2D6 processes basic drugs which feature a nitrogen atom which can be protonated, so it is responsible for myriad N-dealkylation reactions. Indeed, this CYP is responsible for more than 70 different drug oxidations and is particularly noteworthy as it is non-inducible, which is very unusual for human CYP isoforms. Famously, it was the first CYP to reveal the clinical effects of a genetic polymorphism (Chapter 7, section 7.2.3), with around 7–10 per cent of Caucasians expressing poorly or even non-functioning enzyme. As with CYP2C9 substrates, in cases of polymorphism or enzyme inhibition, serious clinical problems can arise if CYP2D6 is the main route of clearance of a low TI drug. As many as 15 per cent of prescribed drugs are cleared by this isoform, including:

- antiarrhythmics: (flecainide, mexiletine);
- TCAs, SSRI and related antidepressants: (amitriptyline, paroxetine, venlafaxine, fluoxetine);
- antipsychotics: (chlorpromazine, haloperidol);
- beta-blockers: (labetalol, timolol, propanolol, pindolol, metoprolol);
- analgesics: (codeine, tramadol, oxycodone, hydrocodone, meperidine [pethidine]).

It is important to realize that the clinical risks of polymorphisms are difficult to assess conclusively and to some extent remain controversial. They are discussed in more detail in Chapter 7, in sections 7.2.2 and 7.2.11. CYP2D6 also forms the metabolites necessary for the optimal anti-oestrogenic actions of tamoxifen. Quinidine, fluoxetine, propoxyphene, celecoxib and paroxetine inhibit this enzyme. Relatively little is found in the gut, and it comprises about 2–4 per cent of the CYPs in human liver.

CYP2E1

This comprises around 7 per cent of human liver P450 and is the only human member of the CYP2E subfamily. It is found in a number of extra-hepatic sites, such as the lung, kidney and lymphocytes. Curiously, CYP2E1 has been detected in cellular areas besides the endoplasmic reticulum, such as the Golgi apparatus and the plasma membrane. CYP2E is also found in mitochondria, although it is not clear why it is there but it is functional and uses adrenodoxin reductase to supply it with reducing power, as this variant does not use NADPH.

Generally, this CYP is also unusual in that it oxidizes small, often water soluble heterocyclic agents, ranging from pyridine through to ethanol, acetone and other small ketones such as methyl ethyl ketone and methyl isobutyl ketone (MEK and MIBK). Ethanol and acetone are strong inducers of this isoform and CYP2E1 is found in 5–10 fold greater quantities than normal in heavy drinkers, compared with those who imbibe moderately or lightly. Indeed, this ethanol-mediated induction leads to much increased vulnerability to CYP2E1-mediated drug and toxin damage, most notably paracetamol-induced liver failure in alcoholics (Chapter 7, section 7.7.5). The muscle relaxant drug chlorzoxazone is cleared by this CYP and is a marker for CYP2E1 capability in man.

Many of the substrates of CYP2E1 are implicated in toxicity and/or carcinogenicity, as the metabolites formed can be highly reactive. It also seems that CYP2E1 produces large amounts of reactive oxygen species and it has been suggested that oxygen is its main substrate. This process underlines the significant role CYP2E1 has in oxidative stress in alcoholics. CYP2E1 is also linked with hepatotoxicity due to trichloroethylene, which was used in manufacturing as a degreaser and also in dry cleaning. It is also partly responsible for the oxidation of paracetamol (Chapter 8) and it is thought to convert acrylamide into its carcinogenic epoxide glycidamide. Several dietary N-nitrosamines are activated by CYP2E1, as is the tobacco procarcinogen NNK (4-[methylnitrosamine]1-[3-pyridyl]-1-butanone). This is one of a number of CYP isoforms that may be related to smoking-induced cancers (see CYP1A1/2 above). Many sulphur-containing agents block this enzyme, such as carbon disulphide, diethyl dithiocarbamate and the ethanol abstinence-inducing drug antabuse (disulfiram; Chapter 5 section 5.6.4; Chapter 7, section 7.7.3). There is evidence in mice that CYP2E1 mRNA is tightly regulated around 24 circadian rhythms by hepatic nuclear factor-1α (HNF-1α) and molecular clock genes such as CRY-1. It is probable that a similar system may exist in man and regulates more than one CYP.

3.6.3 CYP3A series

CYP3A4, 3A5, 3A7 and 3A43

All the CYP3A protein gene loci are found on chromosome 7. CYP3A5 exhibits only about a 10 per cent difference in its sequence homology with CYP3A4 and the two isoforms are also difficult to distinguish catalytically, as they metabolize mostly the same substrates at similar rates. CYP3A5 appears to be in the minority, as only about a fifth of human livers express it, making CYP3A4 the major human biotransformational CYP by some margin. In contrast, CYP3A5 is found in greater quantity than CYP3A4 in human lung. Overall, the two main CYP3A isoforms are responsible for the metabolism of more than 120 drugs

and they comprise more than half of our hepatic CYP content. Indeed, the colour of a healthy liver with normal blood flow is actually partly due to these enzymes. CYP3A isoforms are also found in our intestinal walls in considerable quantity. The major endogenous function of the CYP3As is to metabolize steroids, but their active sites are so large and flexible that a vast array of different molecules can undergo at least some metabolism by these enzymes. Crystallographic studies have shown the three-dimensional structure of human CYP3A4 to resemble most closely bacterial CYPBM-3, but there are similarities with other bacterial CYPs that can metabolize erythromycin. It is clear that human CYP3A isoforms have much larger active sites compared with bacterial enzymes, which underlines their evolution to oxidize such a wide variety of exogenous substrates. As discussed above, like CYP2C9, CYP3A4 has a large access channel and hugely flexible active site with many points of potential hydrophobic binding. Binding studies with steroids and erythromycin again show the similarities with other CYP enzymes across various species.

Studying CYP3A4 can be difficult, as its expression is linked closely with the efflux pump p-glycoprotein (Chapter 4, section 4.4.7; Chapter 5, section 5.4.2) and many drugs are substrates for the CYP and p-glycoprotein, so it is difficult to determine their respective contributions to drug clearance. Drugs used as 'probes' for CYP3A include the short-acting benzodiazepine midazolam, as well as erythromycin and alfentanil. The 6β-hydroxylation of endogenous cortisol can also be used reliably to measure the effects of induction and inhibition of CYP3A activity. Total gut and hepatic CYP3A activity can be differentiated from just hepatic activity by using oral and intravenous midazolam administration respectively. Although some reactions are specific to CYP3A5, such as the demethylation of the anti-rejection drug tacrolimus *in vitro*, it is usually very difficult *in vivo* to determine the relative contributions of CYP3A4 and CYP3A5.

CYP3A4 and CYP3A5 are inducible through several agents, such as anticonvulsants, St John's Wort, phenytoin, dexamethasone, clotrimazole (also an inhibitor of CYP3A activity), sulfinpyrazone, and rifampicin. Interestingly, with some inducers, the tissue concerned, as well as the isoform involved may be crucial to the inductive effect. *In vitro* studies with human cell models suggest that rifampicin and phenobarbitone preferentially induce CYP3A4, with little or no effect on CYP3A5. Conversely, phenobarbitone is a potent inducer of lung CYP3A5, with a minor effect on CYP3A4.

Inhibitors of the CYP3A isoforms include verapamil, the azole antifungals (ketoconazole, itraconazole, fluconazole, voriconazole), as well as the macrolides (erythromycin, clarithomycin, troleandomycin) and various citrus juices, most notably grapefruit. Fascinatingly, verapamil will not inhibit CYP3A5 if cytochrome b_5 is absent, which underlines the complex relationship between CYP REDOX-partners and substrate/inhibitor CYP binding (see end of section 3.4.7). Ketoconazole is thought to inhibit CYP3A4 more strongly than CYP3A5, whilst troleandomycin inhibits both isoforms to a similar degree, but through different mechanisms. Hence, although there are clearly differences between CYP3A4 and 3A5 in terms of their catalytic behaviour as brought out by the effects of various inhibitors and inducers, the net clinical impact of these differences is probably marginal at best.

Of the other CYPs in the 3A sub-family, CYP3A7 is mainly foetal, where it hydroxylates retinoic acid and steroids. It has been found in adults, mainly extrahepatically. CYP3A43 is a relatively recent discovery and it is found in the testis and prostate, although its level of expression is linked with a risk of cancer in these organs. Neither CYPs 3A7 nor 3A43 are involved with biotransformations on the scale of CYP3A4 and CYP3A5.

CYP3A4 binding characteristics-cooperativity

As mentioned previously, CYP3A4 and 3A5 are the most flexible CYP isoforms in terms of their ability to bind a vast range of different sized substrates, although this has been bought at the price of reduced stability of the structure of these proteins compared with other CYPs. Before CYP crystal structures were available, the active sites were studied through a combination of mutagenesis and binding studies. Using bacterial or insect cell expression systems, human genes that expressed CYP3A4 were damaged by the use of mutagens in areas coding for specific amino acids that corresponded to the active site of the enzyme. This would, of course, change the binding characteristics of the damaged enzyme. The information gained from these and other studies show that the CYP3A isoforms can bind up to three substrate molecules at a time, in a similar manner to CYP2C9. Clearly, in this area, our basic perception of enzymes just binding one substrate at a time is far too simplistic. CYPs have evolved the capacity to bind multiple substrates probably for efficiency, as it utilizes resources (reducing power) in the most rapid and economical way, in the same way an assembly line is more efficient than hand building cars. An added bonus, as we have seen, is that the space and flexibility in CYP3A4 and CYP2C9 allow the option of processing of very large molecules as well as a number of smaller ones.

Regarding the processing of multiple small molecules, if the substrates are the same, the process of one molecule being metabolized appears to allosterically adjust the mobility of the molecule on its binding site, making it easier or sometimes more difficult, for it to be oriented for oxidation, thus modulating the process of CYP function. This is termed a 'homotropic' effect. With CYP3A isoforms, the active site is large as we have seen and testosterone, for example, is a small molecule. When a single testosterone molecule binds there is no actual catalytic activity in the isoform. Indeed, the CYP is not even coupled to its REDOX partner and in effect, nothing happens. Once a second testosterone molecule binds, the CYP is coupled with its REDOX partner and is now 'in gear' and operational and ready to process at maximum capacity as the testosterone molecules displace bound water molecules in the CYP and cause each other to be 'pushed' towards the haem iron to be oxidized, rather like bullets packed into a magazine as they feed into a machine gun.

These multi-site bindings can be yet more complex; studies with flavonoids have shown that they can bind at one place in the active site and at the same time stimulate the metabolism of a different type of molecule (PAHs) in another area of the active site. This simultaneous binding at two distinct but adjacent areas of the same broad site also is thought to be responsible for the inhibitory effects of one compound on the metabolism of another, which is termed a 'heterotropic' effect. Testosterone metabolism is partially competitively inhibited by erythromycin. Whilst in another chapter it will be explained how the amount of CYPs present in the liver is a response to substrate pressure (Chapter 4), it is now clear that there is a high degree of sophistication in control of function of all CYPs in all tissues. This is especially important in the liver, where fine-tuning of steroid levels is necessary in response to changes in menstrual cycles or pregnancy in women, as well as spermatogenesis formation in men, where various steroid molecules are required to maintain different levels relative to each other at specific times in the cycles. This allosterically based process of multi-site internal regulation of CYP function is just one of the mechanisms whereby hormone metabolism is controlled. As the presence and binding of one CYP substrate could markedly influence the clearance of another, it is clear

how xenobiotics can and do exert very complex disruptive effects on hormone regulatory processes. In a sense, this is a potential 'Achilles Heel' of CYP regulation and function.

As has been mentioned, the range of substrates that can be oxidized by CYP3A4 runs from bulky molecules such as cyclosporine A (molecular weight 1202), to small phenolics such as paracetamol. That a molecule of fungal origin such as cyclosporine can be easily and rapidly metabolized by CYP3A4, underlines the evolution of these enzymes to cope with the possibility of the ingestion of exogenous toxin molecules in the diet of humans. Other substrates include: codeine (narcotic), diazepam (tranquillizer), erythromycin (antibiotic), lidocaine (anaesthetic), lovastatin (HMGCoA reductase inhibitor, a cholesterol-lowering drug), taxol (cancer drug), warfarin (anticoagulant).

Azole antifungal agents, such as ketoconazole and fluconazole, as well as anti-HIV agents such as ritonavir, inhibit CYP3A4. Due to the importance of this enzyme in the endogenous regulation of steroid metabolism, any inhibition can have serious consequences in the form of disruption of hormone control and more immediately, marked changes to the clearance of drugs metabolized by this CYP.

3.7 Cytochrome P450 catalytic cycle

Having established the phenomenal multiplicity and flexibility of these enzymes, it should be a relief to learn that all these enzymes essentially function in the same way, although again we do not fully understand the process yet. CYPs can carry out reductions (see later on) and these occur after substrate binding and before oxygen binding. However, their main function is to insert an oxygen molecule into a usually stable and hydrophobic compound. Many textbooks present this cycle, but it appears intimidating due to the many details involved in this complex process. However, it is important to understand that there are only five main features of the process whereby the following equation is carried out:

$$\text{Hydrocarbon}\,(-\text{RH}) + \text{O}_2 + 2\ \text{electrons} + 2\ \text{H}^+\ \text{ions gives: alcohol}\,(-\text{ROH}) + \text{H}_2\text{O}$$

1. Substrate binding (reduction may happen after this stage).
2. Oxygen binding.
3. Oxygen scission (splitting).
4. Insertion of oxygen into substrate.
5. Release of product.

3.7.1 Substrate binding

The first step, as covered in the previous section, is the binding and orientation of the molecule. This must happen in such a way that the most vulnerable part of the agent must be presented to the active site of the enzyme, the iron, so the molecule can be processed with the minimum of energy expenditure and the maximum speed. The iron is usually (but not always) in the ferric form when the substrate is first bound:

$$\text{Fe}^{3+} — \text{RH}$$

Once the substrate has been bound, the next stage is to receive the first of two electrons from the REDOX partners, so reducing the iron:

$$Fe^{2+} — RH$$

3.7.2 Oxygen binding

The next stage involves the iron/substrate complex binding molecular oxygen sourced from the lungs. This process runs faster than the substrate binding to the iron, as there is much more oxygen present in the cell than substrate.

$$\begin{array}{l} Fe^{2+} — RH \\ \quad | \\ \quad O_2 \end{array}$$

You will note that oxygen does not just exist as one atom. It is much more stable when it is found in a molecule of two oxygen atoms, O_2. Indeed, oxygen is almost never found in nature as a single atom as its outer electron orbitals only have six instead of the much more stable eight electrons. To attain stability, two oxygen molecules will normally covalently bond so sharing four electrons, so this gives the same effect as having the stable eight electrons. So therefore, to split an oxygen molecule requires energy, but this is like trying to separate two powerful electromagnets – the oxygen will tend to 'snap back' immediately to reform O_2 as soon as it is separated. Two problems thus arise: first, how to apply reducing power to split the oxygen and second, how to prevent the oxygen reforming immediately and keeping the single oxygen atom separate long enough for it to react with the vulnerable hydrocarbon substrate.

3.7.3 Oxygen scission (splitting)

To split the oxygen molecule into two atoms firstly requires a slow rearrangement of the Fe^{2+} O_2 complex to form

$$\begin{array}{l} Fe^{3+} — RH \\ \quad | \\ \quad O_{2-} \end{array}$$

The next stage is the key to whether the substrate will be oxidized or not. This is the rate-limiting step of the cycle. A second electron from supplied by the REDOX partners feeds into the complex and forms

$$\begin{array}{l} Fe^{3+} — RH \\ \quad | \\ \quad O^2_2 \end{array}$$

Or

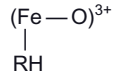
$$\begin{array}{l} Fe^{3+} — RH \\ \quad | \\ \quad O\text{-}O_{2-} \end{array}$$

As stated earlier, it has been suggested that of the two REDOX partners, cytochrome b_5 may supply this second electron more quickly than NADPH reductase. As this stage of

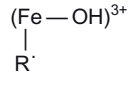
the process is so rapid it is not feasible to detect experimentally, so the most likely pathway has been worked out which corresponds to what is possible and what happens in terms of the products that we can actually measure. Certainly an oxygen atom with two spare electrons is a very attractive prospect to two hydrogen atoms and water is formed, leaving a single oxygen atom bound to the iron of the enzyme. This solves the two problems described above; the oxygen molecule has been split, but it cannot just 'snap back' to form an oxygen molecule again, as water is stable and takes an oxygen molecule away from the enzyme active site.

3.7.4 Insertion of oxygen into substrate

The remaining oxygen is temporarily bound to the iron in a complex, which is sometimes termed a 'perferryl' complex (below).



It has been suggested that the perferryl complex may operate in low or high spin states and also that oxygen is bound in different ways to the iron. FeO_2^+ (peroxo-iron) and even a peroxide ($FeOOH^{2+}$) have both been suggested to take part in some CYP reactions such as oestrogen aromatizations. The perferryl is thought to be the main method of oxygen binding to the CYP and it is exceedingly reactive, as it activates the substrate by either removing hydrogen (hydrogen abstraction) or an electron (e.g. from nitrogen atoms) from part of the substrate molecule. These steps are not necessarily in that order and multiple electron or abstractions can take place. It is apparent that the hydrogen abstraction part of the process takes longer than the subsequent processes and is thought to be the rate-limiting step in the oxidation process. The hydrogen to be removed will be closest to the carbon to be oxidized. The abstracted hydrogen is then bound to the perferryl complex. This leaves the carbon with a spare electron, which makes it a reactive radical, as seen below. The substrate, i.e. the carbon atom, has been activated which makes sense as it is now much more likely to react with the hydroxyl group.



The final stage is the reaction between the newly created hydroxyl group and the carbon radical, yielding the alcohol, as seen below. The entry of the oxygen atom into the substrate is sometimes called the 'oxygen rebound' reaction.

Fe^{2+} and ROH

3.7.5 Release of product

The whole CYP catalytic process could be described as complex yet dramatic. Although a 'pump' analogy has been used previously in this chapter, in some ways, CYPs could also be likened to the cycling of an automatic weapon, with the 'load, fire, extract, eject,

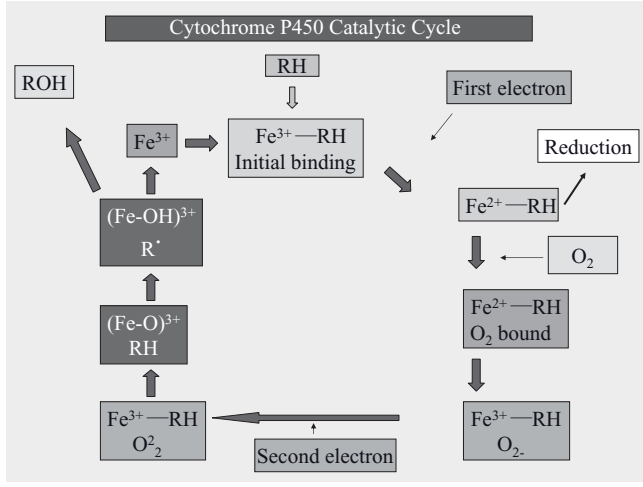


Figure 3.5 Simplified scheme of cytochrome P450 oxidation

reload' stages analogous to CYP substrate conversion to product. This analogy is not perfect, as the coupled CYP provides all the energy to sustain the process rather than the 'substrate' of a machine gun, but it conveys something of the rapidity and violence of the process. Once the substrate has been converted to a metabolite, it has changed both structurally and physicochemically to the point that it can no longer bind to the active site of the CYP. The metabolite is thus released and the CYP isoform is now ready for binding of another substrate molecule. It is important to break down the function of CYPs to separate stages, so it can be seen how they operate and overcome the inherent problems in their function. However, students often find the catalytic cycle rather daunting to learn and can be intimidated by it. It is much easier to learn if you try to understand the various stages, and use the logic of the enzyme's function to follow how it overcomes the stability of substrate and oxygen by using electrons it receives from the adjacent reductase systems to make the product. A simplified cycle is shown on Figure 3.5. As more research is carried out, fine details may change in the cycle, but the main features, the substrate binding, option for reduction, oxygen binding and activation, perferryl complex formation, abstractions of hydrogen or electrons and finally substrate release are well established.

3.7.6 Reductions

As mentioned earlier, CYPs probably evolved to reduce chemical agents before oxidation could occur and once an agent is bound to the haem iron, there is an opportunity for a reduction reaction to occur prior to the oxygen binding stage. This is because a REDOX partner, usually NADPH reductase, supplies an electron which can be used to reduce the substrate. There are several enzyme systems that can effect reductions, such as NADPH reductases themselves, which are found in virtually all tissues and are relevant in

aromatic amine-mediated carcinogenicity (Chapter 8, Figure 8.12). CYPs are just one of many systems which can reduce xenobiotics and interestingly, there are examples where drugs undergo reductions and oxidations sometimes by the same CYPs. This is thought to occur with the muscle relaxant eperisone and human CYPs are capable of reducing the antibiotic chloramphenicol also (see Chapter 8).

3.8 Flavin Monooxygenases (FMOs)

3.8.1 Introduction

Alongside the CYPs, other cellular systems can accomplish oxidations of endogenous molecules and these systems are usually widespread in many tissues. Of these systems, the flavin monooxygenases (FMOs) have not received anything like the attention directed at CYPs, although realization of their significance in drug clearance is growing rapidly. Originally known as 'Ziegler's enzyme', after its discoverer, the FMOs are actually the second most prolific oxidizers of drugs and xenobiotics after the CYPs. In humans, the five functional forms of FMO (designated 1-5) are coded for by separate genes found on chromosome 1. The nomenclature for FMOs is based around amino acid sequence commonality as with the CYPs. Individual isoforms are regarded as belonging to the same family if they possess greater than 82 per cent amino acid sequence homology. Of the five FMOs, FMO-3 is the most important in human liver and it is thought to be expressed at nearly 60 per cent of the level of CYP3A4, which gives some idea of its potential contribution to drug oxidation. FMO-5 is also found in the liver in similar proportions to FMO-3, but FMOs-1, 2 and 4 are not usually found in the adult liver. FMO-3 is not expressed in neonates at all (see Chapter 7, section 7.2.4) and it seems that FMO-1 takes its place. FMO-1 is found mainly in the adult kidney and intestine, whilst FMO-3 is virtually absent in the kidney. A lack of selective experimental substrates for FMO-4 and 5 have made it difficult to study them, although they are not believed to contribute to xenobiotic clearance in man. FMOs are subject to genetic polymorphisms, and they are linked with the condition trimethylaminuria (TMAU), otherwise known as fish-odour syndrome (Chapter 7, section 7.2.4).

3.8.2 Structure

The main features of these enzymes are pretty well conserved and are virtually 'standard issue' across the animal kingdom. FMOs have some structural resemblance to oxidoreductases (PORs) and a yeast FMO has been crystallized and through similar techniques used with CYPs, the sequence of its operations can be worked out through a series of 'freeze-frames' where FMO can be 'observed' binding its co-factor and substrate. The main structure of the enzyme could be described as clam-shaped, with a small 'top shell' and a larger bottom 'shell'. The access channel is obviously between the two 'shells' (Figure 3.6). On the floor of the bottom 'shell' there is a depression to which FAD is anchored firmly. On the top 'shell', there is a binding site which will fairly loosely hold NADPH which is the main co-factor. The NADPH is bound in the orientation where the adenine portion faces the upper shell, whilst the nicotinamide portion is aimed at the flavin of FAD.

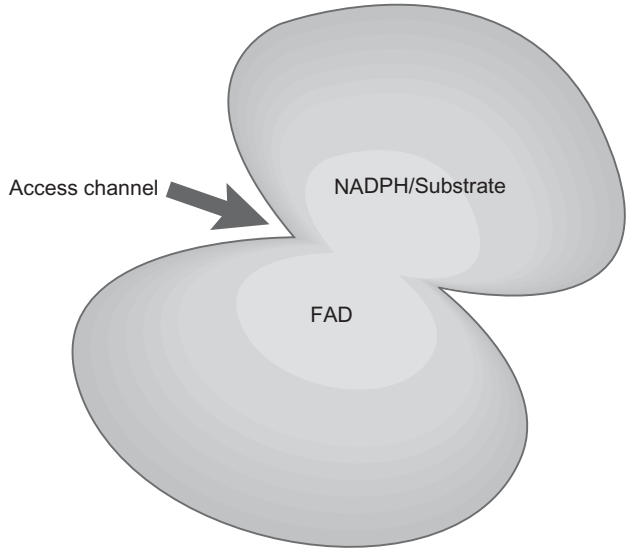


Figure 3.6 Generic structure of a Flavin monooxygenase (FMO)

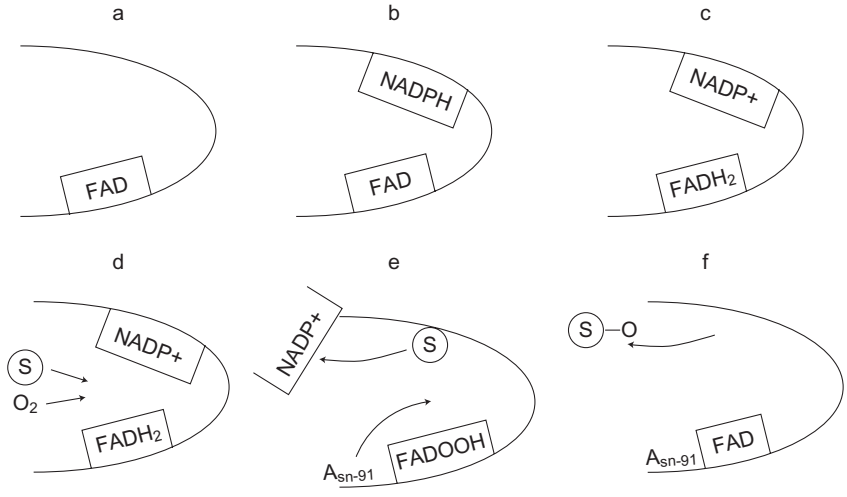


Figure 3.7 Process of Flavin monooxygenase catalysis: a-c outlines the first step or priming of the enzyme. The second step (d-f) involves oxygen binding and substrate (S) displacement of NADPH, followed by reaction of oxygen and substrate and release of the oxide

3.8.3 Mechanism of catalysis

FMOs basically use the co-factor NADPH as an electron donor and FAD as the built in electron carrier, which is similar to POR. The first step or 'priming' of FMO, involves NADPH transferring a hydride ion to the FAD, forming a $FADH_2$ and NADP+ complex (Figure 3.7). It is believed that this is probably the 'resting' state of the enzyme. The

presence of a nucleophilic (electron rich) substrate seems to initiate step two, which involves $FADH_2$ reacting with molecular oxygen (O_2) to form FADOOH, which is called a hydroperoxyflavin. The substrate then displaces the NADP+ and is held surrounded by a sort of 'jacket' of water molecules. This means that it does not actually make contact with the enzyme. A crucial amino acid on the lower 'shell' of the FMO, called asparagine-91 (Asn-91) is instrumental in catalyzing the reaction of the two oxygen molecules held as FADOOH with the nucleophilic portion of the substrate, a nitrogen lone pair of electrons. This leads to one oxygen molecule reacting with the substrate forming an oxide, whilst the other forms water. The enzyme is eventually regenerated to FAD and then it 'reloads' itself with more NADPH ready to oxidize another substrate molecule.

The oxidation process is a single step transfer of two electrons, rather than the CYP system of two successive single electron oxidations. Unlike many CYPs, which do not initiate their catalytic cycle unless more than one substrate molecule binds, FMOs are ready primed as the $FADH_2$ and NADP+ complex can oxidize immediately; in fact, aside from nitrogen, they specialize in the oxidation of other nucleophiles, like sulphur and phosphorus. These groups are found in various drugs and xenobiotics and the respective oxides formed by the FMOs are more water soluble than the parent drugs. FMOs can also metabolize tertiary amines to form N-oxides in drugs such as TCAs, as well as morphine, methadone and pethidine (meperidine). They also can form the 1 and 3-N-oxide metabolites of the 2, 4, diaminopyrimidine antiparasitics (trimethoprim and pyrimethamine), as well as many other sulphoxide metabolites such as that of cimetidine. There is relatively little known to date as to details of substrate preferences for the different FMOs, although FMO-3 oxidizes generally smaller nucleophilic heteroatoms than FMO-1 which prefers drugs with bulkier side-chains, forming N-oxides of chlorpromazine, imipramine and orphenadrine. FMO-2 is sporadically expressed man (Chapter 7, section 7.2.4) and when it is expressed, it oxidizes sulphur, rather than nitrogen containing agents. There is no evidence so far that FMOs can be inhibited as drastically as CYPs and any relatively mild inhibition that might occur is more likely to be competitive (nitrogen/sulphur containing nucleophiles) rather than mechanistic inhibition. If inhibition occurs *in vivo*, it is thought it may be related to the influence of the combination of dietary factors and particular polymorphic variants of FMOs 1, 2 and 3.

3.8.4 Variation and expression

FMOs display some interesting differences and similarities in their expression and operation in comparison with CYPs. Like CYPs, their metabolites are usually more hydrophilic than the parent, although unlike CYPs, they are less likely to produce reactive species and their metabolites tend to be low in toxicity, such as the various N-oxides. Our knowledge of the regulation of FMOs is much less detailed than with CYPs, but their expression can be regulated hormonally, which accounts for the marked switch from predominant expression of hepatic FMO-1 to 3, which appears to be accelerated by the birth process.

As FMOs are considered by many to have evolved as a detoxification system, it seems inexplicable that FMO expression does not appear to be capable of responding to xenobiotic challenge over, say days or weeks, which is the hallmark of the complex and highly efficient CYP enzyme induction system (Chapter 4). It is apparent that the

variation in FMO expression is mostly genetic and it has been suggested that separate human populations may have evolved to express particular variants of these isoforms which detoxify flora and fauna of specific geographical regions. Clearly this theory suggests that individuals would be at significant risk if they strayed to a different area where potential local toxins could not be cleared by their FMO expression profile.

3.8.5 FMOs in drug development

Clinically, FMOs do represent a potentially attractive prospect in drug development. As mentioned above, they are not easily inhibited and their general lack of induction response makes their expression stable and not subject to unexpected and potentially dangerous changes induced by diet, alcohol/drug consumption or other small-molecule induced stimuli. From a practical standpoint, it is also useful that FMO-3 is expressed in quantities capable of clearing drugs in its own right. Additionally, if a new drug was to be cleared by a combination of FMOs and CYPs, the introduction of a potent CYP inhibitor to a clinical regime would not completely shut down all drug clearance, so preventing accumulation-mediated toxicity, as the FMO would continue to eliminate the drug. This has been demonstrated already to some extent by the family of gastroprokinetic agents, cisapride, mosapride and itopride. Poor gastric motility leads to accumulation of stomach acid, which causes pain and inflammation of gastro-oesphageal tissues. These drugs relieve these symptoms by improving gastric motility, although not without side-effects. In the presence of a CYP3A4 inhibitor cisapride can accumulate and extend the cardiac QT interval (Chapter 5, section 5.5.2) potentially lethally. The drug has been withdrawn in most countries, although apparently it remains highly effective for constipation in cats. In contrast, itopride, is cleared by FMO-3, thus making the drug potentially ‘idiot proof’, that is, it would be significantly safer and easier to use in complex real-world clinical regimens.

However, in common with CYPs, it is apparent that there is a very wide variation in the expression of FMOs between racial groups and individuals. There are also a large number of genetic polymorphisms for these enzymes (Chapter 7, section 7.2.4) and the clearance of some drugs, such as ranitidine, is related to FMO-3 expression in particular ethnic groups such as Koreans. Hence, the variation in expression of FMOs may complicate or even ultimately frustrate the strategy of designing drugs which are cleared exclusively by FMOs.

3.9 How CYP isoforms operate *in vivo*

The detailed processes on how living systems operate are sometimes focused on at the expense of a global understanding of how these systems might operate in the tissue. It is useful to try to visualize how CYPs process massive numbers of molecules from hydrophobic to at least partially hydrophilic products every second. If you visualize just one hepatocyte, and imagine the smooth endoplasmic reticulum, with its massive surface area, with vast numbers of CYP and REDOX partners embedded in its tubing, then you can see how the liver can sometimes metabolize the majority of drugs and endogenous substrates in a given volume of blood in just one passage through the organ, rather like a automotive

catalytic converter forms water and CO_2 and nitrogen oxides from a hundreds of combustion products.

3.9.1 Illustrative use of structures

Most textbooks at this point show a large number of chemical reactions that highlight how CYP isoforms metabolize specific drugs/toxins/steroids, etc and this one is no exception. However, it is appreciated that many students might not have studied chemistry, or struggle with it as a subject and feel intimidated by chemical structures. This is worth overcoming, as some qualitative understanding of basic organic chemistry really pays off in illustrating, understanding and appreciating how CYP enzyme systems operate at the molecular level. If you study the diagrams it should not be too difficult to eventually see a molecule in a way that approaches how a CYP enzyme might 'see' it. After all, it is worth the effort, as we thrive as a species in part due to these remarkable enzymes.

3.9.2 Primary purposes of CYPs

As mentioned before, CYP isoforms have evolved to:

- make a molecule less lipophilic (and often less stable) as rapidly as possible;
- make some molecules more vulnerable to conjugation.

The first step is the binding of the substrate. As you will have seen, individual CYPs bind groups of very broadly similar chemical structures. This is partly achieved by the size and physicochemical characteristics of the molecule, as we have seen. For example, the entrance to CYP2C9 is not wide enough to bind part of a large molecule like cyclosporine, so this molecule is virtually excluded from all the CYPs, except the one with the largest and most flexible entrance and binding area, CYP3A4. The processes involved in the orientation of substrates to proximity with the haem iron are complex as mentioned previously, but once an agent is presented to the iron, oxidation and occasionally reduction, can then occur.

3.9.3 Role of oxidation

CYP metabolism is almost always some form of oxidation, which can achieve their main aims. Oxidizing a molecule can have three main effects on it, as follows.

Increase in hydrophilicity

Forming a simple alcohol or phenol is often carried out to make a molecule soluble in water so it can be eliminated without the need for any further metabolic input.

Reduction in stability leading to structural rearrangement

Obviously some chemical structures are inherently less stable than others and any prototype drugs that are unstable and have the potential to react with cellular structures are weeded out in the drug discovery process. However, the process of CYP-mediated metabolism, where a stable drug is structurally changed, can form a much more reactive and potentially toxic product (Chapter 8). A very young child hitting objects randomly with a piece of metal will not be able to discern the difference between an inert object and an extremely dangerous one (electrical equipment or an explosive device). In the same way, a molecule may be bound and metabolized by CYPs, irrespective of the impact these processes may have on the stability and potential toxicity of the product. There is a risk that the new molecule may be very reactive and dangerous indeed and may attack the CYP itself or the surrounding cellular structures. Although this does happen, evolution has retained the advantages of CYPs, such as their ability to process virtually any required molecule, through the appearance of conjugation and detoxification systems that contain and usually quench the reactivity of these agents (Chapter 6). This could be compared with the evolution of the Porsche 911. The weight of the engine over the driven rear-wheels offers tremendous traction and thus acceleration. However, intensive modification of the car over many years has counteracted the 911's inherent tendency to carry straight on through corners and vastly increased its safety whilst retaining its performance. With CYP oxidation processes, the evolution of attendant detoxification systems ensures that the risk to the cell of creating a reactive species usually pays off and a molecule can be quite radically changed in terms of its physicochemical properties without problems. For example, a lipophilic functional group might be oxidized to an alcohol, which may be so unstable that it breaks off. This has the dual advantage of removing a lipophilic structure that leaves the molecule more hydrophilic (see the oxidation of terfenadine). It can also pave the way for further metabolism, such as conjugation (Chapter 6).

Facilitation for conjugation

Many oxidative metabolites are much more vulnerable than their parent molecules to reaction with water-soluble groups such as glucuronic acid and sulphates. Once a conjugate is formed, this vastly improves water solubility and Phase III transport systems will generally remove it from the cell and into the blood.

3.9.4 Summary of CYP operations

A sculptor was once asked how he would go about sculpting an elephant from a block of stone. His response was 'knock off all the bits that did not look like an elephant'. Similarly, drug-metabolizing CYPs have one main imperative, to make molecules more water-soluble. Every aspect of their structure and function, their position in the liver, their initial selection of substrate, binding, substrate orientation and catalytic cycling, is intended to accomplish this deceptively simple aim.

With experience, you should be able to look at any drug or chemical and make a reasonable stab at suggesting how a CYP enzyme might metabolize it. It is important to see these

enzymes not as carrying out thousands of different reactions, but as basically carrying out only two or three basic operations on thousands of different molecules every second.

3.10 Aromatic ring hydroxylation

3.10.1 Nature of aromatics

Large, highly lipophilic, planar and stable molecules with few, if any, vulnerable functional groups look to be a difficult proposition to metabolize (Figure 3.8). Indeed, if there are any aliphatic groups, or non-aromatic rings associated with an aromatic molecule, these will often be attacked rather than the aromatic group. The ring hydroxylation of amphetamines is the exception to this. Polycyclic hydrocarbons are not easy to clear and they are perceived by living systems as a potent threat. This is reflected in the elaborate expression system (Ah/ARNT; Chapter 4, section 4.4.2) which modulates the non-constitutive isoform CYP1A1, which has evolved to deal with them, which is highly effective. These include molecules such as those shown in Figure 3.8. The simplest aromatic is benzene and this can be oxidized by CYP1A1 eventually to phenol, which is more reactive, but more water-soluble than benzene and vulnerable to sulphation and glucuronidation during conjugative metabolism.

3.10.2 The oxidation of benzene

There are several intermediates formed during the oxidation of benzene (Figure 3.9). The two main routes are the cyclohexadienone and an epoxide; in the presence of water both stages will rearrange to form the phenol. During this process, the hydrogen atom close to the oxygen will sometimes be moved around on the ring, or even lost. This is known as the NIH shift.

Epoxidation is defined chemically as a reaction where an oxygen atom is joined to an unsaturated carbon to form a cyclic, three-membered ether. Epoxides are also known as arene oxides and vary enormously in their stability, which depends on the electron density

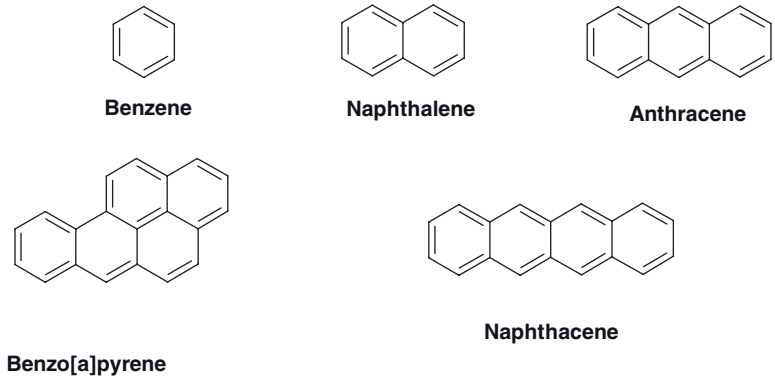


Figure 3.8 Some aromatic hydrocarbon molecules

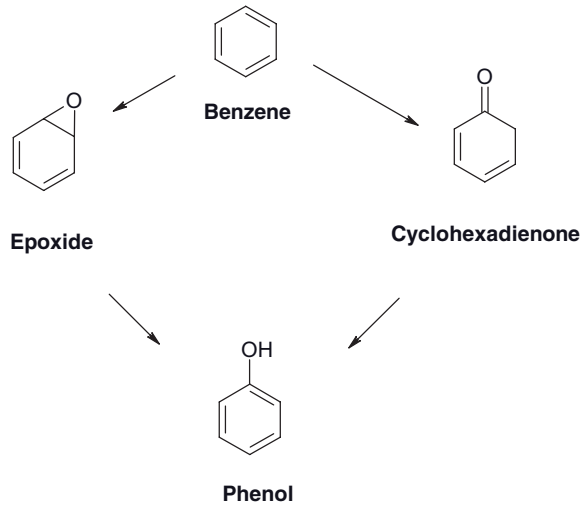


Figure 3.9 Main pathways of benzene hydroxylation

of the double bond being oxidized: the higher the density, the more stable the epoxide. This means that epoxides of varying stability can be formed on the same molecule, due to differences in electron densities and this is most apparent in benzpyrene. The anticonvulsant carbamazepine forms a number of epoxides and the 10, 11 derivative is stable enough to be pharmacologically active, whilst bromobenzene 3,4 epoxide's half-life in blood is less than 14 seconds. Generally, arene oxides form phenols or diols in the presence of water (as does carbamazepine 10, 11 epoxide), although the cytosolic enzyme epoxide hydrolase (Chapter 6, section 6.6, and Chapter 7, section 7.2.4) is present to accelerate this process.

The phenols and diols are usually substrates for sulphation or glucuronidation. Although the process of aromatic hydroxylation is difficult to achieve and the phenolic product is more hydrophilic, the structural features of the larger polycyclics mean that this process can lead to the formation of unstable carcinogenic reactive intermediates.

3.11 Alkyl oxidations

The saturated bonds of straight chain aliphatic molecules are very stable; indeed, they can be even harder to break into from the thermodynamic point of view than aromatic rings, whilst molecules with unsaturated bonds are the easiest to oxidize. Straight chain aliphatic molecules are easier to oxidize if they have an aromatic side chain. Alkyl derivatives are generally oxidized by the routes briefly described below.

3.11.1 Saturated alkyl groups

The oxidation of a saturated alkyl group can lead to the alcohol being inserted in more than one position (Figure 3.10). The 'end' carbon group of the molecule is sometimes

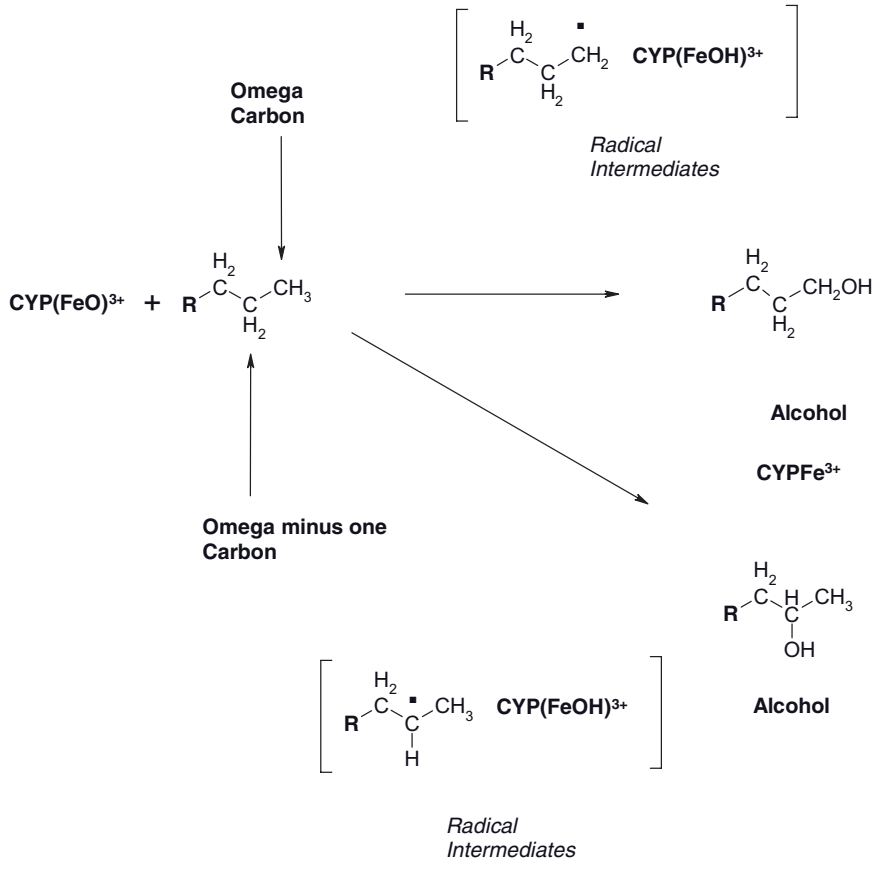


Figure 3.10 Omega and omega minus one carbon oxidation of aliphatic saturated (single bond) hydrocarbons by CYP isoforms

called the 'omega' group and the oxidation can result in this group being turned into an alcohol (omega oxidation) or alternatively, the penultimate group (omega minus one).

During the oxidation of saturated molecules (Figure 3.10) the CYP will operate as described in section 3.7, abstracting hydrogen and causing the carbon molecule to form a radical. The carbon radical and the hydroxyl group then react to form the alcohol. Even though alkanes like hexane are very simple structures, they can be metabolized to a large number of derivatives (see section 3.11.3, 'Pathways of alkyl metabolism').

As well as the formation of alcohols, CYP isoforms can desaturate carbon–carbon double bonds to single unsaturated bonds (Figure 3.11). This process can occur alongside alcohol formation and is a good example of a CYP-mediated process that leads to quite considerable rearrangement of the molecule's structure. The first hydrogen is abstracted by the CYP isoform and may leave the FeO^{3+} complex, allowing it to grab a second hydrogen atom. The highly unstable adjacent carbon radicals rearrange to form an unsaturated product. The two hydrogens and the single oxygen atom that the CYP enzyme used to accomplish this effect form water.

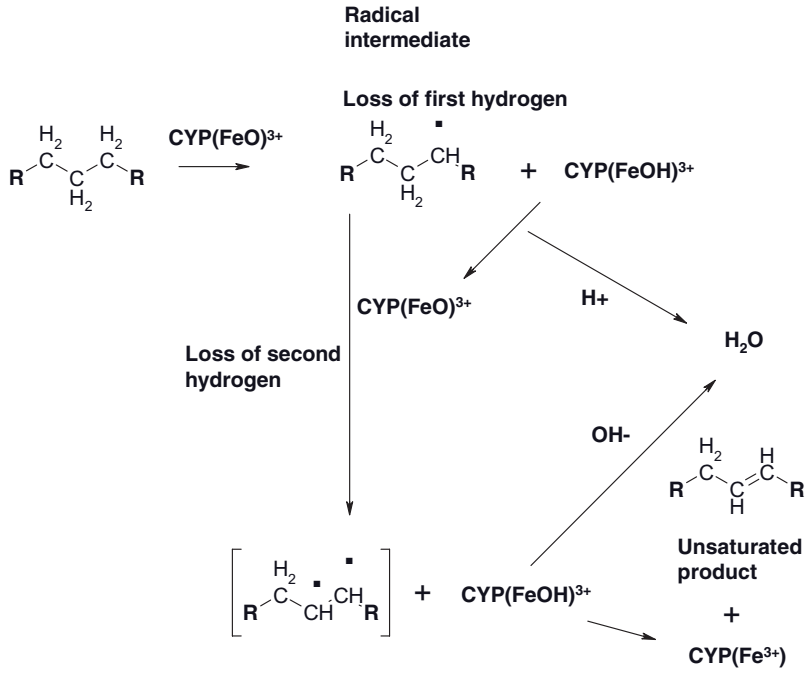


Figure 3.11 Formation of unsaturated bonds from a saturated starting point

3.11.2 Unsaturated alkyl groups

Unsaturated or double bonds are more electron-rich than saturated bonds, and as mentioned earlier, this makes them easier to oxidize and several possible products can be formed (Figure 3.12). These include an epoxide called an 'oxirane', as well as two carbonyl derivatives, or aldehydes, which can split the molecule.

3.11.3 Pathways of alkyl metabolism

A good example of where these pathways can lead is the complex metabolism of an otherwise apparently simple molecule, hexane (Figure 3.13). This hydrocarbon was once used as a volatile component of several adhesive mixtures, which were extensively applied in the leather and shoe industries. If you want an adhesive or paint to dry or cure quickly, the volatility of the carrier solvent is crucial. This makes the adhesive or paint easier to use in a mass production setting. However, it was gradually realized that many people who used hexane-based adhesives in the leather industry were suffering from damage to the peripheral nervous system, known as peripheral neuropathy. This was a progressive effect and was traced to the hexane itself. In humans, hexane is cleared at first to several hexanols, which is logical, as a volatile, water-insoluble and highly lipophilic agent

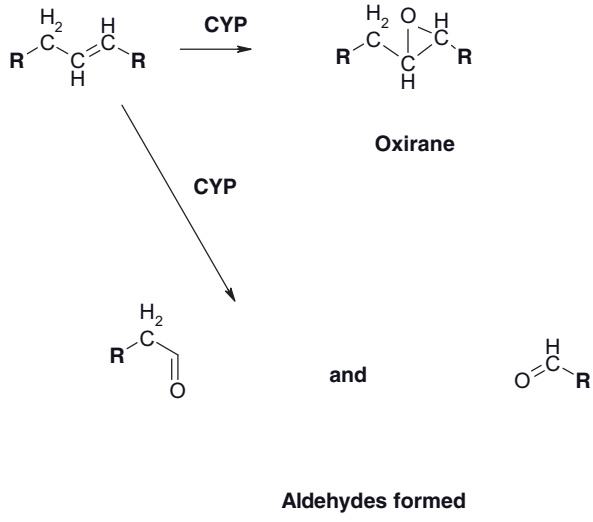


Figure 3.12 Metabolism of unsaturated alkyl groups

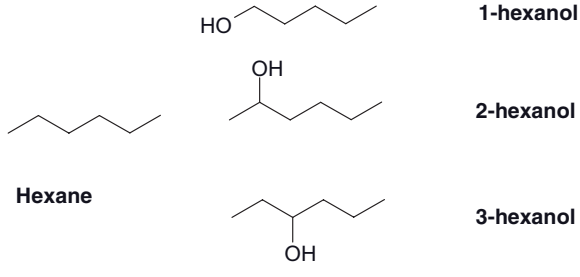


Figure 3.13 Oxidation of hexane to hexanols

capable of causing intoxication will be a strong candidate for rapid clearance to an albeit only slightly water-soluble alcohol.

The 2-hexanol derivative undergoes further oxidative metabolism, initially to a diol, the 2,5 derivative (Figure 3.14), which can undergo further CYP isoform-mediated (probably by 2E1) oxidation to a di-ketone which is the 2,5 hexanedione derivative. This compound is unusual in that it is a specific neurotoxin. It interferes with microtubule formation in neural fibres, causing gradual loss of neural function. It is also cytotoxic to neuronal cells and its relatives, the 2,3 and 3,4 diones, are cytotoxic to cell cultures. Consequently, *n*-hexane is banned from use in adhesives and should only be used where the fumes cannot be inhaled. The 2,3 and 3,4 hexanediones are used as food colourings and flavourings, although it is unlikely they can be formed in human liver. Several isomers of hexane have been used as substitutes for hexane, although the potential neurotoxicity of adhesives that use volatile alkanes should never be underestimated.

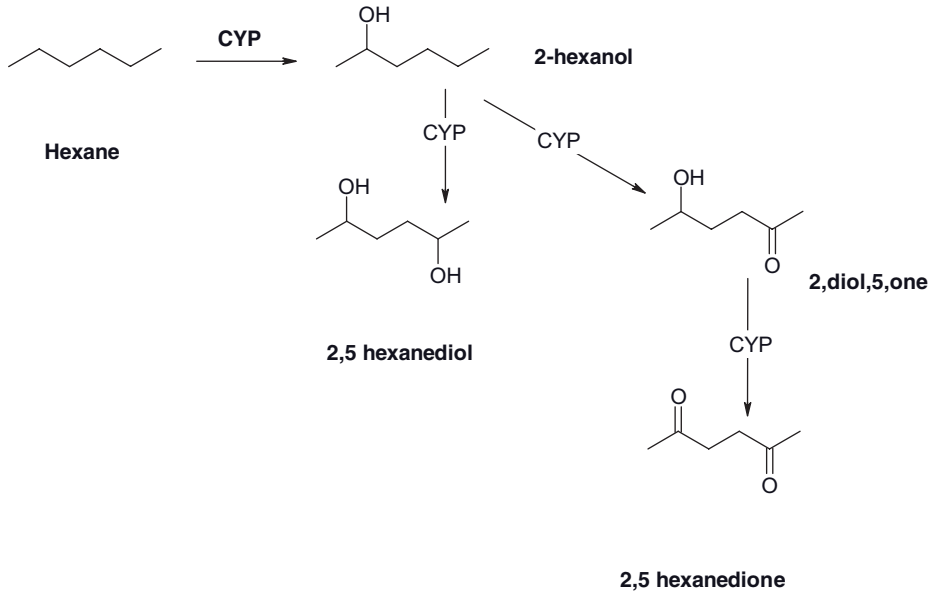


Figure 3.14 Formation of the neurotoxin 2,5 hexanedione by CYP oxidations

3.12 'Rearrangement' reactions

The use of oxidation as a tool to rearrange molecules to less lipophilic products has the added benefits of unmasking other vulnerable groups and making the products simpler to conjugate. There are several CYP-mediated oxidations that have this effect on molecules.

3.12.1 Dealkylations

Alkyl groups, especially bulky ones, are very lipophilic and often are attached to drugs through 'hetero' atoms, i.e. nitrogens, oxygens and sulphurs. From the perspective of biotransformation, it makes sense to remove the alkyl group, leaving the hetero group vulnerable for conjugation with glucuronides or sulphates (Figure 3.15). The quickest way to remove the alkyl group is to oxidize it to an alcohol. This should be a win–win situation, whether the product is stable or unstable, the alcohol (called a carbinolamine in the case of N-dealkylation) is usually unstable and splits off, forming an aldehyde. This reveals a less lipophilic heteroatom 'handle' for conjugation. If the alcohol is stable, then the drug is still more hydrophilic than it was and that might also be a target for subsequent conjugation. With substituted aromatic compounds it is easier for the CYP to oxidize an alkyl substituent group than the ring. Another result of dealkylation can be the splitting of a large lipophilic molecule into two smaller more hydrophilic ones (Figure 3.16).

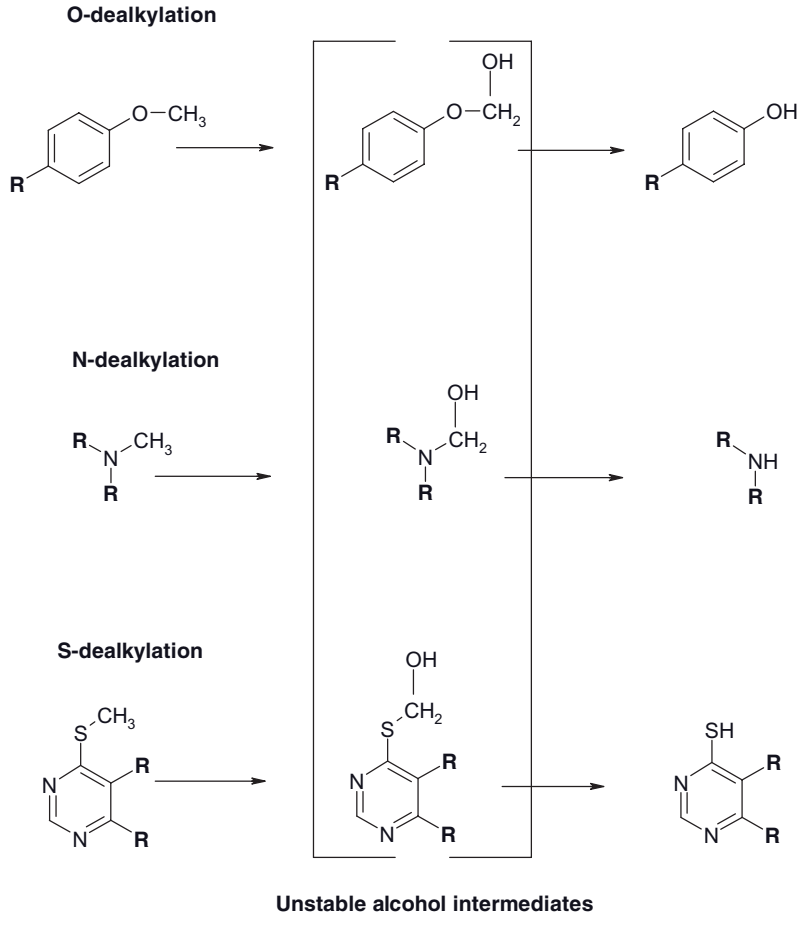


Figure 3.15 Rearrangement reactions caused by the CYP-mediated oxidation of an alkyl group leading to the formation of a more water-soluble product, which is also more vulnerable to Phase II. The 'waste products' of the reactions are usually small aldehydes or ketones

There are many examples of drugs that undergo this type of dealkylation. Imipramine, the TCA, is demethylated to form desmethyl imipramine, which also has pharmacological potency and is usually known as desipramine. The removal of one methyl group may not make much difference to the lipophilicity of a large molecule, although it may change its pharmacological effects. More than one alkyl group may have to be removed to make the compound appreciably less lipophilic. On the other hand, the N-dealkylation reaction of the antihistamine terfenadine has a much more dramatic effect (Figure 3.16). The oxidation of the alkyl group adjacent to the nitrogen causes an unstable alcohol to be formed, which splits away, taking half the molecule with it. Virtually the same reaction of alkyl oxidation at the other end of the parent molecule results in a stable alcohol that is then oxidized to a carboxy derivative (fexofenadine) which is not metabolized further and is less toxic than the parent drug (Chapter 5, section 5.1).

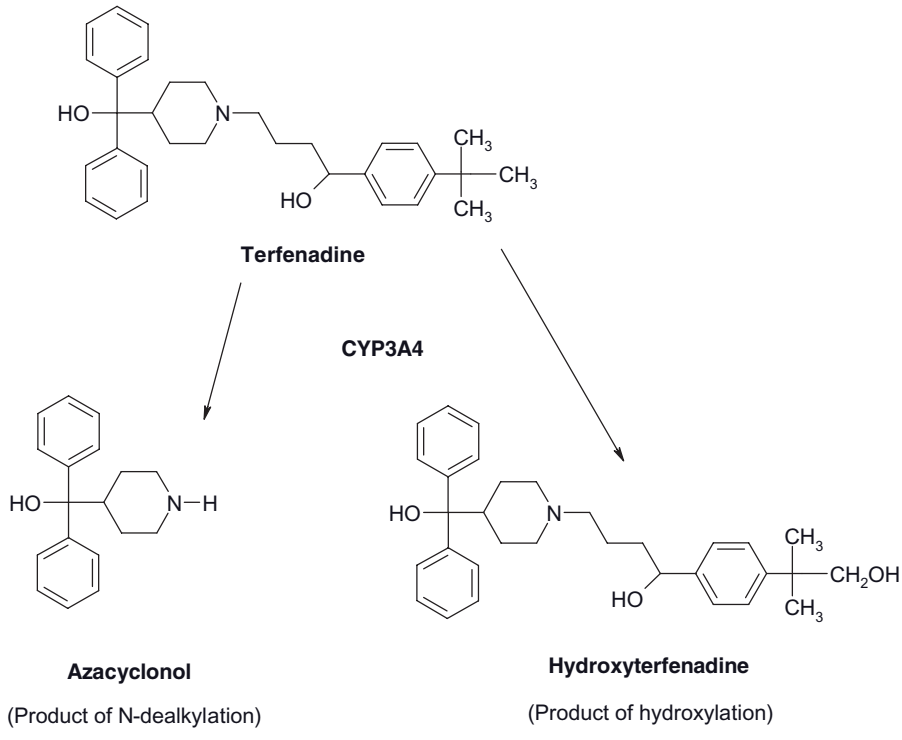


Figure 3.16 Metabolism of terfenadine: essentially the same oxidation reaction applied in two different areas of the molecule leads to vastly different effects on the structure

N-dealkylation mechanisms

N-dealkylation is only part of the picture of the metabolism of how CYPs can oxidize heteroatoms. Before N-dealkylation occurs, CYPs have the option of oxidizing the substituted nitrogen itself to form an N-oxide (Figure 3.17). If N-oxide formation does not occur, then N-dealkylation can proceed. Again, this is a 'win–win' process, as N-oxides are more water-soluble than the parent drug. Generally, FMOs are credited with the vast majority of N-oxidations, but it has become apparent that CYPs can also accomplish them. Whether N-oxide formation or dealkylation occurs is dependent on factors such as the surrounding groups on the molecule and the CYP itself. The mechanism of N-oxidation and N-dealkylation is now believed to differ slightly from the majority of CYP-mediated hydrogen abstractions/oxygen rebound reactions. It begins with the CYP perferryl complex abstracting one of the nitrogen's lone pair of electrons (Figure 3.17), forming an aminium ion (N^+). Once this has been created, either the oxygen reacts with the N^+ giving the N-oxide, or the perferryl complex can abstract a hydrogen atom from one of the adjacent carbons forming a carbon radical. The reaction then proceeds as with most CYP oxidations, where the hydroxyl group bounces off the haem iron to react with the carbon radical to make the (usually unstable) alcohol, or carbinolamine. Chlorpromazine and the TCAs can undergo N-oxidation or N-dealkylations, as well as sulphoxide formation (Figure 3.18)

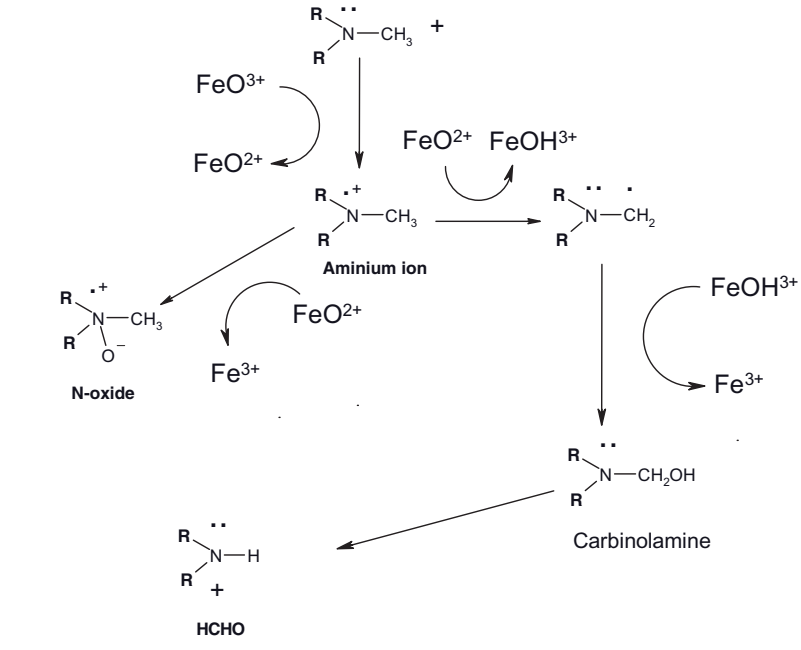


Figure 3.17 Pathways of CYP-mediated N-oxidation and N-dealkylation

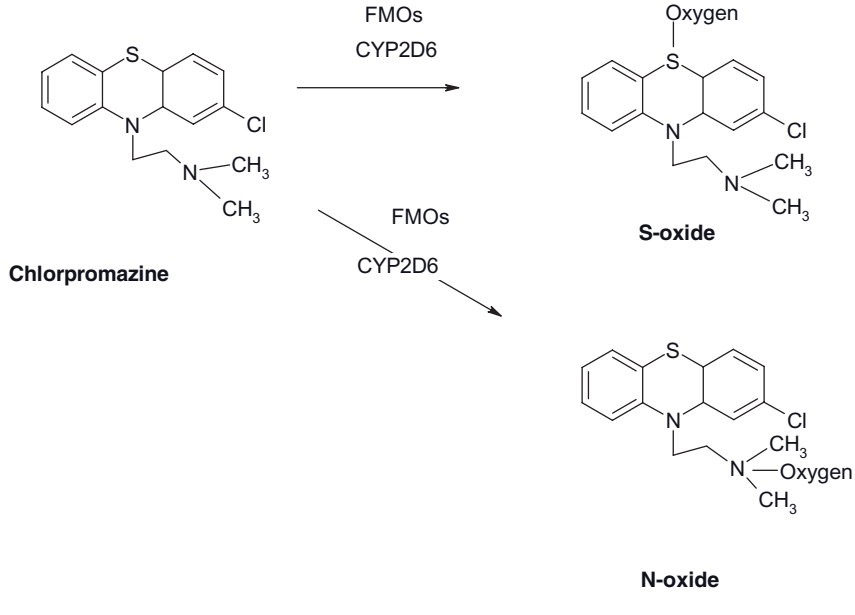


Figure 3.18 Sulphoxide and N-oxide formation with chlorpromazine, by either CYPs or FMOs

and as mentioned in section 3.8, flavin monooxygenases (FMOs) can accomplish these reactions also.

3.12.2 Deaminations

Amine groups in drugs can be primary, secondary or tertiary. Primary amines can be removed completely thorough conversion of the carbon—nitrogen single to a double bond, where the nitrogen loses an electron. Via a hydrogen atom from water, ammonia is formed with a ketone product. This is one of the fates of amphetamine (Figure 3.19). More amphetamine metabolism can be found in Appendix B.

3.12.3 Dehalogenations

Using the same basic tool, oxidation to an alcohol, it is possible for CYPs to remove halogens (chloride, bromide or fluoride) from molecules, forming a ketone and a halogen ion. A number of volatile general anaesthetics are subject to this route of metabolism. The adjacent carbon to the halide is oxidized to a short-lived alcohol, which causes the movement of electrons towards the halogen, which dissociates (Figure 3.20).

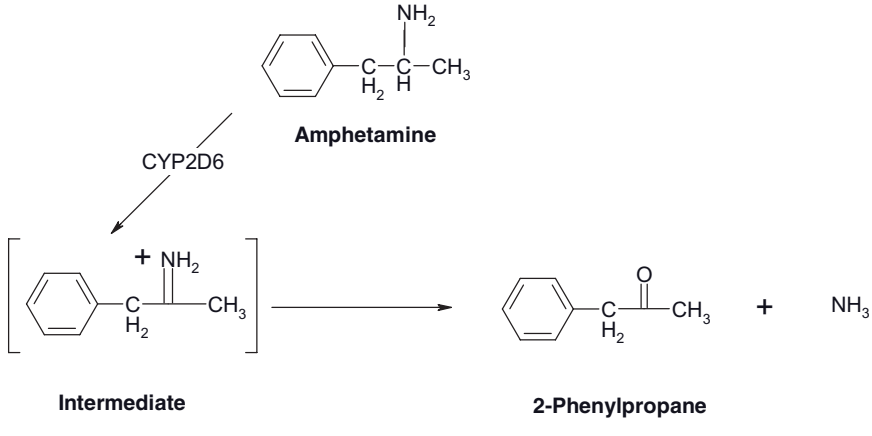


Figure 3.19 Oxidative deamination of amphetamine

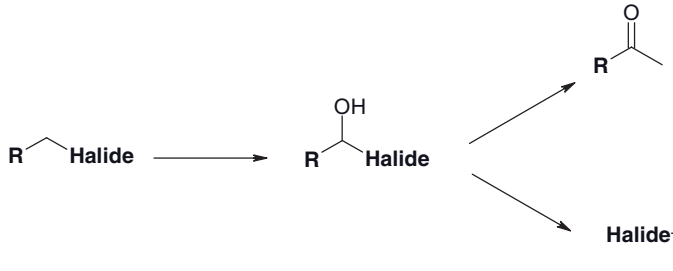


Figure 3.20 Removal of halides through an unstable alcohol intermediate

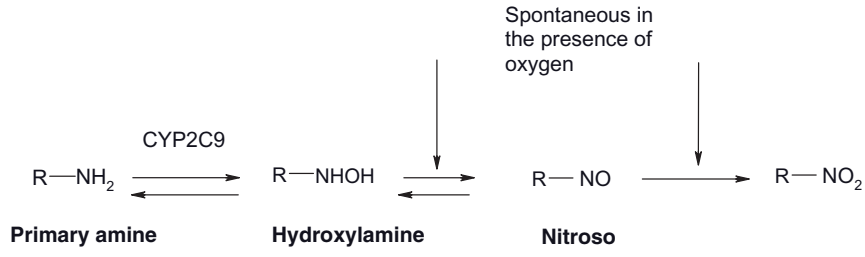


Figure 3.21 Primary amine oxidation

3.13 Other oxidation processes

3.13.1 Primary amine oxidations

Primary amines found in sulphonamides and sulphones can be metabolized to hydroxylamines and their toxicity hinges on these pathways (Figure 3.21 and see Chapter 8, section 8.6.4). The hydroxylamines formed are often reactive and although they can be stabilized by glutathione (GSH) and other cellular antioxidants, they can spontaneously oxidize in the presence of oxygen to nitroso and then nitro-derivatives. The nitro forms are usually stable, but are vulnerable to reductive metabolism that drives the process shown in Figure 3.21 in the opposite direction. Secondary amines can also be oxidized to hydroxylamines.

3.13.2 Oxidation of alcohol and aldehydes

Although CYP2E1 is induced by ethanol, the vast majority of ethanol clearance (90 per cent) is normally by oxidation to acetaldehyde by another group of enzymes, the alcohol dehydrogenases (ADHs). These enzymes are found in the cytoplasm and they are NAD^+ dependent zinc metalloenzymes. They form NADH from NAD+ in the process of alcohol oxidation. There are five classes of ADH isoforms. Class I (ADH1, ADH2, ADH3) isoforms have a high affinity for ethanol and can be blocked by pyrazoles. Classes II and III are more suited to the metabolism of longer chain alcohols and cannot be blocked by pyrazole.

Aldehydes are formed from many reactions in cells, but they are oxidized to their corresponding carboxylic acid by several enzyme systems, including aldehyde dehydrogenase ALDH's, xanthine oxidase and aldehyde oxidase. These enzymes are usually detoxifying, as many aldehydes, such as formaldehyde, are cytotoxic by-products of CYP and other oxidative reactions. Of the three aldehyde dehydrogenase classes, two are relevant to alcohol metabolism. Class I are found in the liver cytosol and specialize in acetaldehyde. Class II ALDHs are found in the liver and kidney mitochondria and metabolize acetaldehyde and several other substrates. More about ADH and ALDH in alcoholism is to be found in Chapter 7 (section 7.7.2).

3.13.3 Monoamine oxidase (MAO)

Yet another important oxidative enzyme system that processes endogenous and exogenous substrates is monoamine oxidase (MAO), which exists in two isoforms, MAO A and MAO B. Both are found in the outer membrane of mitochondria in virtually all tissues. They have evolved to become two separate enzymes with similar functions and they originate from different genes in man. They use FAD as a cofactor and are capable of oxidizing a very wide variety of endogenous biogenic amines as well as primary, secondary and tertiary xenobiotic amines. They accomplish their removal of amine groups through an initial reductive half-reaction, followed by an oxidation half-reaction. The reductive half oxidizes the amine and the FAD is reduced. The second half of the process involves the use of oxygen to reoxidize the FAD, leaving hydrogen peroxide and an aldehyde as products. Clorgyline blocks MAO A, whilst deprenyl is a potent inhibitor of MAO B. In the 1960s, irreversible MAO inhibitors were used as antidepressants, aimed at increasing biogenic amine levels. Unfortunately, they could cause hypertensive crises (sufficient to cause a stroke) through ingestion of other amines, such as tyramine from cheese and a long list of other foods. MAO inhibitors are still used, but only in a minority of patients.

3.14 Control of CYP metabolic function

Although CYPs appear to be part of an impressive and flexible system for the oxidation of drugs, it is not enough just to process endogenous and xenobiotic molecules at a set rate. Endogenous and exogenous CYP substrates can vary enormously in their concentrations within the body, even on a day-to-day basis. As we have seen, steroid hormone levels must be matched to accomplish specific tasks in narrow time frames, so production and destruction must be under exceedingly fine control. This is apparent during the menstrual cycle and pregnancy. Our exposure to various exogenous chemicals, including drugs, is also variable in terms of concentration and physicochemical properties. As an advertising campaign once said, ‘power is nothing without control’. It is essential for the CYP system to be finely controllable to respond to the often extreme changes in the small-molecular weight chemical presence in cells. This process of CYP induction mentioned briefly earlier will be discussed in detail in terms of mechanism and clinical consequences in the next chapter.

4 Induction of Cytochrome P450 Systems

4.1 Introduction

The aim of drug therapy is to provide a stable, predictable pharmacological effect that can be adjusted to the needs of the individual patient for as long is deemed clinically necessary. The physician may start drug therapy at a dosage that is decided on the basis of previous clinical experience and standard recommendations. At some point, the dosage might be increased if the desired effects were not forthcoming, or reduced if side effects are intolerable to the patient. This adjustment of dosage can be much easier in drugs that have a directly measurable response, such as a change in clotting time. However, in some drugs, this adjustment process can take longer to achieve than others, as the pharmacological effect, once attained, is gradually lost over a period of days. The dosage must be escalated to regain the original effect, sometimes several times, until the patient is stable on the dosage. In some cases, after some weeks of taking the drug, the initial pharmacological effect seen in the first few days now requires up to eight times the initial dosage to reproduce. It thus takes a significant period of time to create a stable pharmacological effect on a constant dose. In the same patients, if another drug is added to the regimen, it may not have any effect at all. In other patients, sudden withdrawal of perhaps only one drug in a regimen might lead to a gradual but serious intensification of the other drug's side effects. These effects are shown by some illustrative histories, as detailed below.

History 1

After suffering a head trauma in a motorcycle accident, a 22-year-old male was subject to recurrent grand-mal convulsions that were treated with carbamazepine. After starting on 200 mg daily, this dose had to be gradually increased stepwise over four weeks to maintain plasma levels within the therapeutic window to 1200 mg daily.

Analysis

Plasma levels were not maintained within the therapeutic window at each dose level for more than a week or so, as carbamazepine clearance appeared to gradually increase, until

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a dosage was reached where clearance stabilized so that drug levels remained within the therapeutic window.

History 2

A 23-year-old male epileptic was prescribed phenytoin (300 mg/day) and carbamazepine (800 mg/day). The laboratory assays showed that phenytoin was in the therapeutic range, while carbamazepine was undetectable in the plasma. A 50 per cent reduction of the phenytoin dosage allowed the carbamazapine plasma concentrations to rise to therapeutically effective levels.

Analysis

The lack of carbamazepine in the plasma at a dosage which is known to exert a reasonable therapeutic effect in other patients implied that the drug's clearance was much higher than normal, to the point where bioavailability was almost zero. Cutting the phenytoin dosage slowed the high rate of clearance of carbamazepine, allowing plasma levels to ascend to the therapeutic window.

History 3

A 49-year-old male epileptic was prescribed phenytoin at 600 mg/day and carbamazepine at 2000 mg/day. The patient's condition was controlled with minimal side effects for three months. The phenytoin was then abruptly discontinued; within four days, the patient became gradually more lethargic and confused, until one week later hospitalization was necessary. The carbamazepine dosage was reduced to 1200 mg/day and the confusion and sedation gradually disappeared.

Analysis

A stable co-administration of two drugs implies that despite the high dose of carbamazepine, blood levels for both drugs were initially in the therapeutic window. The removal of the phenytoin led to gradual increase in the symptoms of carbamazepine overdose, without any change in the dose. This indicates that carbamazepine blood levels climbed way above the therapeutic window into toxicity. This was caused by a marked, but gradual, fall in carbamazepine clearance when the phenytoin was withdrawn.

History 4

A 64-year-old obese male was prescribed simvastatin 10 mg daily. Over the next three months, lack of clinical response led to a fivefold increase in dosage. He was then admitted to hospital with rhabdomyolysis. On his own initiative he had self-administered St John's

Wort, which he discontinued when his mood was sufficiently elevated, around 10 days prior to the toxicity manifesting itself.

Analysis

The statin was not effective unless considerably higher doses than normal were used, indicating that the drug was being cleared at a higher rate than normal. The general practitioner was unaware that the patient was taking St John's Wort extract. The patient abruptly stopped taking the herbal extract and the clearance of the statin gradually fell while the dose did not, so the drug accumulated and exerted toxicity.

History 5

A 47-year-old female was stabilized on phenobarbitone and warfarin and her prothrombin time was optimized by substantial increase over the normal dosage of anticoagulant, although blood levels were within normal limits. Within 10 days of the abrupt withdrawal of phenobarbitone the patient suffered a mild haemorrhage.

Analysis

As a higher than normal dosage of warfarin was necessary to maintain its plasma levels in the therapeutic window in the presence of the phenobarbitone, it suggests that the latter drug was accelerating the clearance of warfarin. Once the phenobarbitone was stopped, this accelerating effect was lost too, leading to accumulation of the warfarin to the point that blood levels rose above the therapeutic window leading to toxicity, in this case an exaggerated therapeutic effect.

History 6

A 55-year-old male being treated for tuberculosis was taking rifampicin (600 mg daily), isoniazid (400 mg daily), ethambutol (200 mg daily) and pyrazinamide (400 mg daily), was also epileptic and was taking carbamazepine (2000 mg daily). The patient decided to stop all medication over the Christmas period to enter his annual seasonal alcohol binge, where he drank heavily for several days. After approximately 13 days, he resumed his drug regimen and before the end of the first day, he was drowsy, lethargic, confused and eventually difficult to wake and was hospitalized. Some of the symptoms of the tuberculosis resumed, such as fever, chills and cough.

Analysis

The patient was suffering from carbamazepine toxicity and very high plasma levels were found on blood analysis. This indicates that the cessation of all drug intakes over the

Christmas period of 13 days had led to a marked reduction in the clearance of carbamazepine, and the resumption of his previous high dosage caused drug accumulation and significant CNS toxicity. The absence of pressure of the anti-tuberculosis drugs had also allowed the disease to partially re-establish itself and may well have led to selection of partly drug-resistant forms of the bacteria.

History 7

A terminally ill cancer patient stabilized on methadone (250 mg/day) for pain relief contracted an infection soon after another patient arrived from a major hospital. The infection was determined to be MRSA (methicillin-resistant *Staphylococcus aureus*) and rifampicin (600 mg/daily) was started for a projected period of 14 days. At day two, the patient complained of severe breakthrough pain and the methadone dose was increased. By day 14 the infection had been cleared and the patient was taking methadone at 600 mg/day to control their pain. After rifampicin was stopped, the methadone dose was tapered to its original level over 21 days with minimal toxicity or pain breakthrough.

Analysis

The rifampicin accelerated the clearance of methadone to the point that analgesia was lost extremely rapidly and had to be restored with more than a doubling of the dose. The reversal of the induction effect appeared to take somewhat longer than the onset of the initial effect, which may have been related to the highly variable nature of methadone's half-life and the pathology of the terminal patient.

4.1.1 Summary

In all five cases, there are a number of common features:

- Some of the drugs' clearances were not stable until a relatively high dose was employed.
- One drug (or herbal preparation) was able to grossly accelerate the clearance of another agent (s).
- The changes in plasma levels were sufficiently great to either lead to toxicity or total loss of efficacy.
- The toxic effects occurred gradually over days, rather than hours.
- The increase in drug clearance caused by other drugs was fully reversible.

4.2 Causes of accelerated clearance

A number of explanations could be put forward for the effects seen above. There could be a reduction in drug absorption in the presence of another agent, possibly linked to

inhibition of intestinal drug uptake transporters (Chapter 2, section 2.6.2), although this is relatively rare and would happen within hours rather than days of the regimen change. Perhaps the renal clearance of the drug could be accelerated in some way, although this too is unlikely. To enter the circulation from an oral dose, the drugs must pass through the gut, the portal circulation and then into the liver itself. In Histories 1 and 2, the clearance of carbamazepine was initially unstable and in the presence of phenytoin, virtually 100 per cent cleared before it reached the circulation.

Since the liver's blood flow is not likely to undergo a sustained increase, then the only way such a large effect on drug levels can occur is that the liver is extracting much more of the drug than usual in the presence of the other drug. This acceleration of drug metabolism as a response to the presence of certain drugs is known as 'enzyme induction' and drugs which cause it are often referred to as 'inducers' of drug metabolism. The process can be defined as: '*An adaptive increase in the metabolizing capacity of a tissue*'; this means that a drug or chemical is capable of inducing an increase in the transcription and translation of specific CYP isoforms, which are often (although not always) the most efficient metabolizers of that chemical. Usually, hepatic induction has the most significant clinical impact, although other tissues are also involved, such as the lung, intestine and the kidneys.

4.3 Enzyme induction

4.3.1 Types of inducer

There are several broad groups of drugs and chemicals capable of inducing hepatic metabolism: these include:

- *Anticonvulsants*, mainly the older established drugs, such as phenytoin, carbamazepine and phenobarbitone. Topiramate and oxcarbazepine are weaker inducers. These drugs induce many CYP isoforms, including CYPA2, CYP2C8/9, CYP2C19 and CYP3A4/5.

- *Steroids*, such as dexamethasone, prednisolone and various glucocorticoids, induce CYP3A4/5.

- *Polycyclic aromatic hydrocarbons (PAHs)*; these are found in atmospheric pollution, cigarette smoke, industrial solvents and barbecued meat. They also contaminate foodstuffs and watercourses (particularly dioxins and polycyclic chlorinated biphenyls). These compounds induce the normally non-constitutive CYP1A1 in the liver, as well as CYP1A2, which specializes in polycyclic aromatic amines. Induction of CYP1A1 is also very strong in the lungs in smokers and is a standard marker for heavy tobacco use.

- *Antibiotics*, such as the rifamycins (rifampicin, rifabutin, rifapentin) and griseofulvin, induce most CYPs including CYP1A2, CYP2C9, CYP2C19 CYP2B6 and CYP3A4. Flucloxacillin is believed to induce CYP3A4/5. The azole CYP inhibitor clotrimazole, is actually an inducer of CYP3A4, although this is not clinically

relevant due to very low absorption from an oral dose and the usual application is topical, with virtually zero systemic absorption.

- *Recreational agents*; nicotine and PAHs in tobacco products are known inducers of CYP1A2, actually causing plasma levels of the SSRI fluvoxamine to be around half those of non-smokers, Clozapine and warfarin clearance may also be accelerated. Heavy alcohol consumption will induce CYP2E1, which is relevant to chlorzoxazone.

- *Herbal remedies*; although more research must be conducted into the various herbs on the market, St John's Wort is the most clinically relevant and investigated herbal inducer (CYP3A4/5).

- *Protease inhibitors:* agents such as ritonavir and nelfinavir are potent inducers of CYP3A4, but CYP3A5 is induced much less. Paradoxically, ritonavir in particular is a powerful inhibitor of these CYPs.

- *Miscellaneous inducers:* the atypical stimulant modafinil, used by narcoleptics and some helicopter pilots, is a mild CYP3A4 inducer (greater than 400 mg/day), but inhibits CYP2C19. The non-nucleotide reverse transcriptase inhibitors nevirapine and efavirenz induce CYP3A4 and CYP2B6 in a mild to moderate fashion.

4.3.2 Common features of inducers and clinical significance

A new drug is generally regarded as an inducer if it produces a change in drug clearance which is equal to or greater than 40 per cent of an established potent inducer, usually taken as rifampicin. Looking at the structures of the strongest hepatic enzyme inducers there are apparently few common features. These chemicals range in size from very small and water-soluble (ethanol) to very large and lipophilic (PAHs, rifampicin-related agents). However, inducers are usually (but not always) lipophilic, contain aromatic groups and consequently, if they were not oxidized, they would be very persistent in living systems. CYP enzymes have evolved to oxidize this very type of agent; indeed, an elaborate and very effective system has also evolved to modulate the degree of CYP oxidation of these agents, so it is clear that living systems regard inducers as a particular threat among lipophilic agents in general.

The process of induction is dynamic and closely controlled. The adaptive increase is constantly matched to the level of exposure to the drug, from very minor almost undetectable increases in CYP protein synthesis, all the way to a maximum enzyme synthesis that leads to the clearance of grammes of a chemical per day. Once exposure to the drug or toxin ceases, the adaptive increase in metabolizing capacity will subside gradually to the previous low level, usually within a time period of a few days. This varies according to the individual and the drug. Studies with smokers have shown than their enzyme induction subsides in two to four weeks, whilst rifampicin and anticonvulsant induction can subside at similar or slightly quicker timescales.

Estimating the comparative potencies of various commonly used inducing drugs is complicated by inter-individual and ethnic differences. However, predictions based on

many clinical studies show rifampicin as the most powerful inducer, followed by phenytoin at around 60-70 per cent of rifampicin's effect, whilst carbamazepine and phenobarbitone's potencies lie around the 40 per cent level. Many other drugs, toxins and over the counter herbal remedies also exert inductive effects within this range, although these have not been as well documented. It is important to note that clinically, there may be some reduction in plasma levels caused by one inducer on another drug, but it may or may not be significant enough to lead to the drug falling out of the therapeutic window. Rifampicin, phenytoin, carbamazepine or phenobarbitone are almost always capable of reducing a co-administered drug's plasma levels by more than half. Hence, when a patient has spent at least two or three weeks on a regimen containing one or other of these drugs, careful consideration should be given to the appropriate dosage of an additional drug to ensure adequate systemic exposure.

4.4 Mechanisms of enzyme induction

4.4.1 Introduction

The process by which enzyme induction occurs has three main requirements:

- The hepatocyte must detect the presence of particular potentially persistent lipophilic drugs and/or toxins and correctly sense their concentration.

- The process of detection is translated into an increase in the capability of the appropriate metabolic system or systems within the cell, which will clear the drug and/or toxin as efficiently as possible.

- The complete (detection and action) system should be dynamic and reversible, so it is sensitive to further changes in drug concentration.

It is apparent that the main inducible CYPs, CYP1A1/1A2, CYP2C8/9 and CYP3A4, employ broadly similar systems to regulate their ability to respond to increases in drug concentration. Indeed, this commonality is borne out by observations that agents that don't induce CYP3A4, don't induce CYPs 2C8/9 and 2C19 either. The exception to this rule seems to be CYP2E1, which appears to have a unique system of induction. On a cellular level, we know that the type of induction mechanism involved with a given CYP is closely related to a combination of endogenous as well as xenobiotic-responsive functions. Indeed, the networks of various nuclear and cytoplasmic receptor systems described below can 'cross-talk', in that different receptors may modulate each other and even activate the same gene. This multiple system approach is used in aircraft to maximize safety and in the cell, to reduce the possibility of potentially disruptive chemicals remaining in the body unchallenged. Indeed, the various receptor systems which govern CYP expression act in concert with other aspects of biotransformation relevant to the inducing chemical. These include stimulating the proliferation of the SER to provide space for the CYPs to be anchored, as well as the production of sufficient quantities of REDOX partners to fuel the CYPs. The expression of conjugation and Phase III transporters is also up-regulated (Chapter 6). This all translates into relatively rapid and profound effects on drug clearance. Clinically,

rifampicin or a potent St John's Wort extract can cause other drugs to fall out of their therapeutic windows in a couple of days. With anticonvulsants the process may take a few days longer and be complete in most patients in around three to five weeks. There is, however, a huge variation in particular CYPs and in individual clinical responses.

4.4.2 CYPs 1A1/1A2 and 1B1 induction

The AhR system

Although enzyme induction has been known clinically since the 1960s, it was not until the 1970s that the cellular basis of the process was unravelled by studying the effects of dioxin (TCDD; Chapter 2, section 2.2). In the cytoplasm of most cells a receptor complex can be found which consists of four components (Figure 4.1): the aryl hydrocarbon receptor, or 'AhR', heat-shock protein (Hsp90), co-chaperone p23 and an immunophilin called XAP2. Many receptors are complexed with several 'chaperone'-like molecules mainly to ensure they keep a certain shape so they can bind their substrate, rather like polystyrene packing is used to help fragile articles survive postal systems. TCDD binds to the AhR complex which then migrates towards the nucleus. The AhR/TCDD complex then breaks away and it alone enters the nucleus and heterodimerizes (two different proteins form a

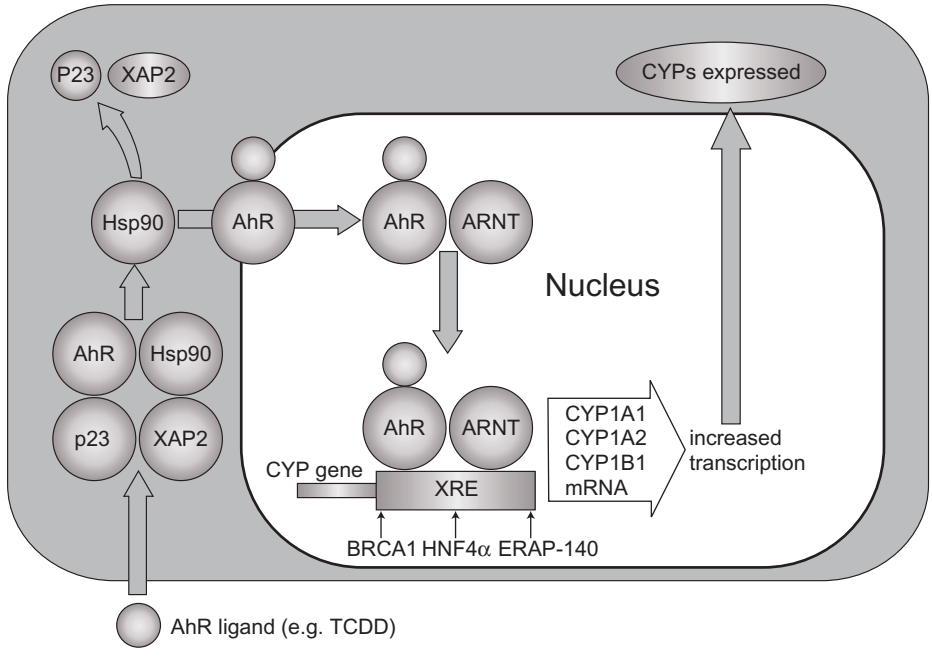

Figure 4.1 Basic mechanism of CYP1A1 and 1A2 induction: the AhR receptor binds the inducer alongside Hsp90, but only the AhR receptor and the inducer cross into the nucleus to meet ARNT and together they bind to DNA xenobiotic response elements (XRE). Co-activators which induce expression of the CYP isoforms include BRCA1, ERAP-140 and HNF4α

complex) with the nuclear protein ARNT (aryl hydrocarbon nuclear receptor translocator) and this new complex binds to specific DNA sequences upstream of the CYP1A1/1A2 and 1B1 genes, which are termed xenobiotic-responsive elements (XRE) or sometimes DREs (drug or dioxin-responsive elements). Several other nuclear proteins are also required before CYP transcription and translation are fully engaged, such as oestrogen receptor associated protein (ERAP-140) and it is apparent that BRCA1 may also be important at this stage.

BRCA1 (breast cancer 1 early onset) is a tumour suppressor gene which has myriad functions and is found on chromosome 17. Defects in this gene are associated with the early onset of breast cancer, mainly because the BRCA1 is vital in DNA repair. It is thought that healthy BRCA1 may be part of the process that ensures that CYP1A1/1A2/1B1 induction efficiently responds to challenge from xenobiotics like PAHs and TCDD. Defects in the gene do affect the response to TCDD in experimental breast cancer lines and it is possible that in addition to a lack of DNA repair in mutated BRCA1 individuals, there is also insufficient detoxification of potential carcinogens which would normally be oxidized and cleared. This is yet to be established clinically, although it is conceivable that with carcinogens that require CYP-mediated activation, defective BRCA1 could disable CYP induction and actually reduce toxic metabolite formation.

CYP1 family induction is an exceedingly sensitive system, as it responds powerfully to single figure nanomolar concentrations of TCDD. Interestingly, although CYPs 1A1/2 and 1B1 are in the same family, it appears that their expression can be controlled differentially through a complex interplay between the various co-activators such as ERAP, BRCA and the response elements on different areas of the respective isoform genes.

It is thought that a healthy AhR system obeys the law of mass action, so providing there are enough AhR receptors in the cell cytoplasm, then the more TCDD-like inducer molecules that appear in the cell, then more TCDD/AhR complexes will migrate to the nucleus and bind to the DREs. This will in turn increase CYP expression. At rest, this system operates in an analogous way to an idling engine, with a low level of 'revolutions' connected to a 'throttle', or the AhR receptor. A sudden influx of PAHs or related chemicals 'floors' the throttle, leading to increased CYP synthesis, which is capable of eventually clearing (by CYP-mediated oxidation) large amounts of the toxins from the cell. Release the 'throttle', the system subsides, saving energy and raw materials. It is rather ironic that in contrast to rats, humans barely metabolize TCDD itself and as mentioned in Chapter 2, section 2.2, this toxin is breathtakingly persistent in man. Its clearance can be accelerated in cases of human poisoning by the consumption of foodstuffs which include the fat substitute Olestra, which will speed up the TCDD elimination half-life from seven to ten to 'only' one to two years.

The AhR system-endogenous function

The AhR/ARNT system is found in all tissues and is involved with the development of organs such as the liver, as well as the immune system. Indeed, part of the toxicity of polycyclic aromatics is linked with their disruption of AhR/ARNT controlled cellular processes. The AhR and ARNT receptor system is multifunctional, as TCDD induces other enzyme systems as well as CYP1A1/1A2 and CYP1B1, such as glutathione-S-transferase, aldehyde dehydrogenase and NADPH reductase (known as NADPH oxidoreductase, or

NQO1). Therefore, it is easy to see that exposure to compounds which resemble TCDD will have a significant impact on the concentrations of many other endogenous molecules which are cleared by these enzyme systems. ARNT itself participates in many other cellular responses to hypoxia and hypoglycaemia through HIF proteins. There is some evidence that overactivity of the AhR system, unrelated to xenobiotics, is actually carcinogenic in itself, particularly in the lung and the pancreas. Indeed, the high level of CYP1B1 expressed in lung cancers is now thought to be a consequence of AhR overactivity, rather than an inductive effect of carcinogens, partly as considerable numbers of non-smokers develop lung cancer. This is probably not the case for CYPs 1A1 and CYP1A2 expression, which appears to be directly linked with exposure to cigarette-linked xenobiotics.

The AhR system-control and toxicological significance

As with all induction effects, CYP1 induction begins within hours of exposure to the toxin or drug, but it may take from several days to weeks before CYP expression is maximally induced. As well as TCDD, it is thought that PAHs and heterocyclic amines induce CYP1 isoforms in this way, as does the anti-ulcer agent omeprazole. Essentially, the 'default mode' for hepatic expression of CYPs 1A1/2 and CYP1B1 is in the 'off' or very low level position, as the liver does not normally encounter planar aromatics such as TCDD in serious quantities. Even after induction, the CYP1 family is subject to further post transcriptional regulation (MIRN27B and CYP1B1 in Chapter 3, section 3.6.1) and there is also evidence that the CYP1A1/2 system can be switched off by other agents, such as the 'orphan' (currently function unknown) nuclear receptor, the short heterodimer partner (SHP). This receptor can bind to a number of nuclear receptors such as RXR (retinoic acid X receptor) and several other receptors which control thyroid and oestrogen levels. SHP can also block the response of CYP1A1 to TCDD, by directly blocking ARNT. There are also likely to be a number of compounds that bind to SHP and regulate CYP1A1 activity. Interestingly, several flavonoids can also block the AhR system, preventing CYP1 induction and thus protecting cells from the consequences of reactive species formation. In the future, the AhR induction system could well be successfully therapeutically targeted to intercept the development of early stage lung tumours.

CYP1A1/2 and CYP1B1 induction is of high toxicological significance in the lung in non-ciliated 'Clara' cells, which are in the forefront of the detoxification of pollutants in inspired air. These cells have more than half their volume given over to SER and induction of CYP1A1/2 by PAHs in tobacco leads to the formation of reactive epoxides, which attack DNA, forming 'adducts' or small PAH-related structures which are covalently bound to DNA and are strongly linked with lung carcinogenicity. It has been suggested that the high state of lung induction of CYP1A1 leads to an increased Clara cell exposure to reactive species of oxygen generated by 1A1 even when it is not metabolizing substrates. This is because CYPs 1A1 and 1A2 are thought to 'leak' reactive oxygen species and this 'drip–drip' effect might make as great a contribution to DNA damage as the reactive aromatic metabolites. As mentioned in Chapter 3, CYP1A1 induction has also been implicated in the metabolism of smoking related nitrosamines like NNK which in animal studies, led to DNA damage from NNK metabolites resulting in an O^6-methylguanine adduct. Other lung-specific toxins include CYP2E1-activated vinylidene chloride. The likelihood of the development of lung tumours in response to metabolism depends on the

degree of CYP induction, the amount of carcinogenic species produced, their detoxification and the efficiency of DNA repair mechanisms.

4.4.3 CYP2B6 2C8/2C9/C19 and 3A4 Induction

The Nuclear Receptor System

Unlike the cytosolic AhR system described above, these CYPs are controlled by transcription factors which are usually (but not always) found in the nucleus, so they are termed nuclear receptors (NR). These receptors are the means by which a number of steroid hormones. vitamins, mineralocorticoids and glucocorticoids translate commands from other tissues to activate specific genes to express a variety of proteins as well as the CYPs.

The NRs are regulated by master controlling nuclear receptors, known as hepatocyte nuclear factors (HNFαs). The most important identified so far is HNF4α that is continuously active and regulates so many processes, (organ development as well as lipid and insulin metabolism) that it is probably the key to normal liver function, although it is found in other tissues. HNF4α is so important that gene knockout studies have shown that animal embryos do not survive without it. It is likely that HNF4α directly or indirectly regulates all endobiotic and xenobiotic biotransformational processes.

NRs are fairly similar in structure, containing an N-terminal DNA binding domain (DBD) and a C-terminal hormone/chemical-binding domain, rather like one of those international electric plug appliance adaptors. When the hormone or xenobiotic is absent, the C-terminal domain, sometimes called the ligand binding domain (LBD) is locked in the 'off' position by a co-repressor protein complex. The binding of the appropriate ligand to the LBD causes it to rearrange and release its co-repressor. The receptor then attracts and binds a co-activator complex. There are several co-activators, part of a series of proteins known as p160s, such as SRC-1 (steroid receptor co-activator 1) and the splendidly named GRIP1 (glucocortoid receptor interacting protein 1). Once the activated complex is formed, it seeks to bind a specific DNA hormone response element (HRE), also termed XRE, or xenobiotic response element.

Among all the various nuclear receptors, there is further subdivision, in that some NR receptors such as the thyroid and vitamin D receptors (TR & VDR), CAR (constitutive androstane receptor) and PXR (pregnane X-receptor) form complexes with RXR (the retinoic acid receptor) in order to bind HREs. The presence of HNF4α is then required to make all these binding processes productive and activate gene transcription. Of the NRs, CAR and PXR are the focus of particular attention, as they control the expression of the CYPs among other biotransformational systems. (Figures 4.2a and b and Figure 4.3).

CAR mediated control of CYP expression

CAR is also known under nuclear receptor classification as NR1I3 and it modulates basal biotransformational metabolism (among many other processes) and it is under the direct control of HNF4α as well as the glucocorticoid receptor. In common with the AhR receptor, it too is found in the cytoplasm, complexed with Hsp90, p23, an immunophilin

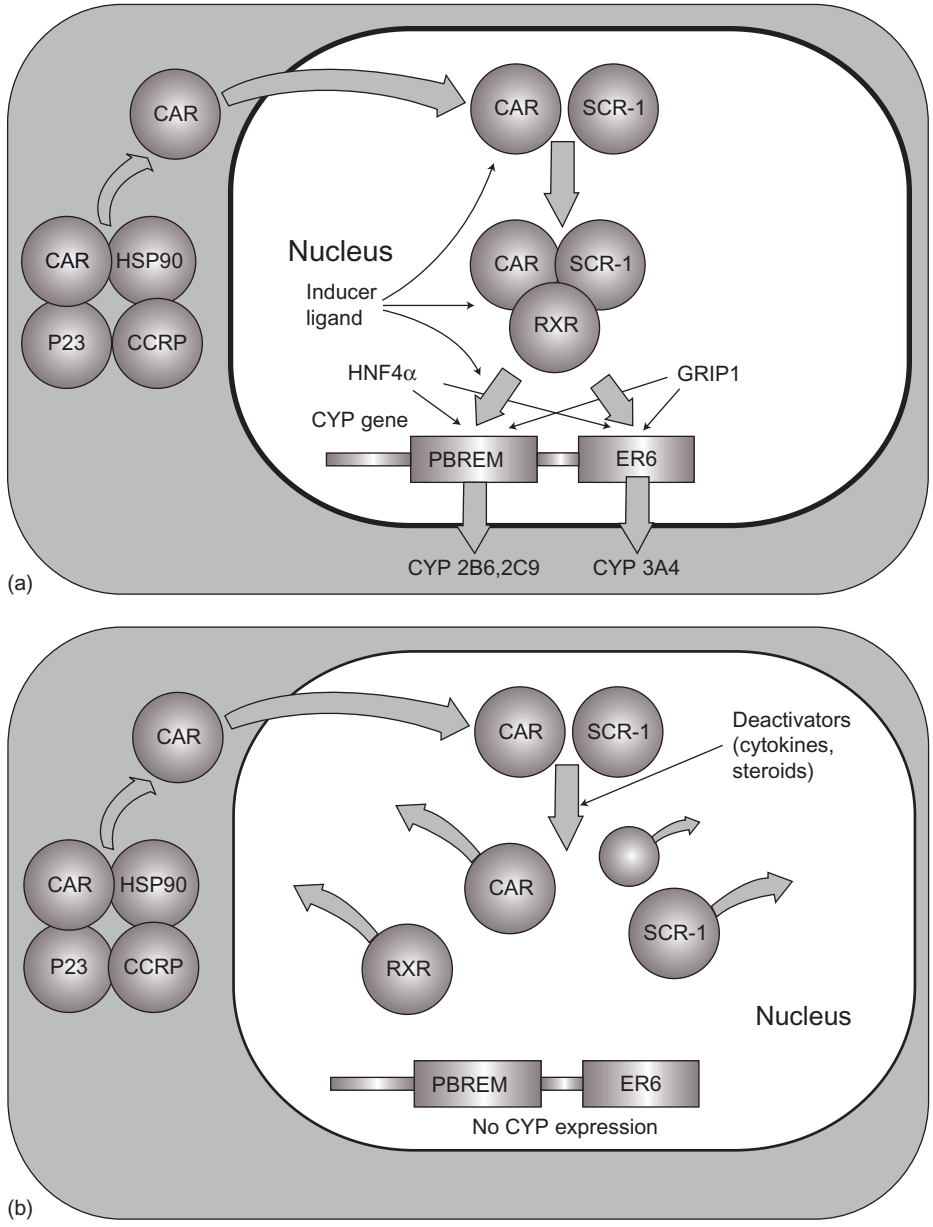

Figure 4.2 (a) Constitutive androstane receptor (CAR)-mediated control of CYP2 series and CYP3A4. CAR and SCR-1 bind the inducer ligand inside the nucleus, bind retinoic acid X receptor (RXR) and activate the CYP expression (b) Possible mechanism for the modulation of CAR-ligand activated CYP induction: a series of endogenous deactivators cause break up of the CAR/RXR/SCR-1/ligand complex and induction is switched off

and a protein called CCRP (CAR retention protein; Figure 4.2a). However there is a vital difference. To follow the idling engine analogy used above of AhR, CAR operates like the throttle is held half way down by itself, running the engine at half its capable number of revolutions. CAR does not need to be stimulated into action by agonists, it is already, as its name suggests, a 'constitutive' receptor, driving the expression and activity of CYPs and conjugation systems (Chapter 6) as well as transporter systems such as the OATPs and NTCP (Chapter 2, section 2.6.2). The presence of inducers, such as steroid hormones and drugs (phenobarbitone, primidone, phenytoin and rifampicin) merely speeds up the process, forcing the 'throttle' towards the floor and there are also several steroid inhibitory factors which cause the throttle to be released, sometimes as far back as 'idle speed'.

CAR operates the induction pathways through changes in its binding affinities for RXR, its co-activator (SRC-1, or steroid receptor co-activator-1) and DNA. The CAR/RXR complex is a heterodimer (like AhR and ARNT) and basic CAR function comprises the continuous recruitment of RXR by CAR followed by association with SRC-1 into a complex. The CAR/RXR/SRC-1 complex binds to DNA (Figure 4.2) at the PBREM (phenobarbitone-responsive enhancer module) in the CYP2B gene (particularly CYP2B6) and to the Everted Repeat 6 (ER6) element of the CYP3A4 and CYP2C9 genes. HNF4α binding to the CYP promoter sites then ensures protein synthesis will occur. Other co-activators like GRIP1 may also be involved. This is the 'half-way down throttle' stage. If inducers of CAR appear, they increase the stability of the rather 'wobbly' CAR/RXR complex making it more rigid and thus promoting a better fit with SRC-1 followed by more productive PBREM binding, pushing the throttle towards the floor-proportionally to the quantity and potency of the inducer. Why some chemicals are very potent inducers and others less so is not entirely clear, as exactly how inducers interact with CAR is not fully understood. It is likely to involve the agent dephosphorylating the receptor rather than full binding of the inducer to CAR. The more the chemical stabilizes CAR throughout its recruitment and binding processing of its co-activators, the more potent the inducer. It some ways, the system is rather like gradually engaging the clutch in a car with manual transmission. There are several mechanisms for restricting or even switching off CAR operation, and some steroids such as progesterone and various androgens inhibit CAR, so 'lifting the foot off the throttle (Figure 4.2b). Variation in CAR expression is one of the main reasons why there is so much interindividual expression in biotransformation. Overall, CAR also regulates CYP2C8, CYP2C9 and CYP2C19 as well as CYP2A6 and UGT1A1 (Chapter 6), as well as various transporters. As mentioned in Chapter 3, CAR and HNF4α control POR, one of the CYP 'fuel pumps'. In the light of the emerging role of cytochrome b_5 as an additional significant supplier of CYP reducing power, it would be surprising if CAR and HNF4α did not control the expression of this REDOX partner also.

PXR mediated control of CYP expression

The main NR usually associated with the control of the CYP3A series is PXR, although as mentioned above CAR is also heavily involved. PXR is also known as the SXR (steroid and xenobiotic receptor) and is classified in the nuclear receptor family as NR1I2. Although PXR is a nuclear receptor, some aspects of its behaviour resemble AhR rather than CAR.

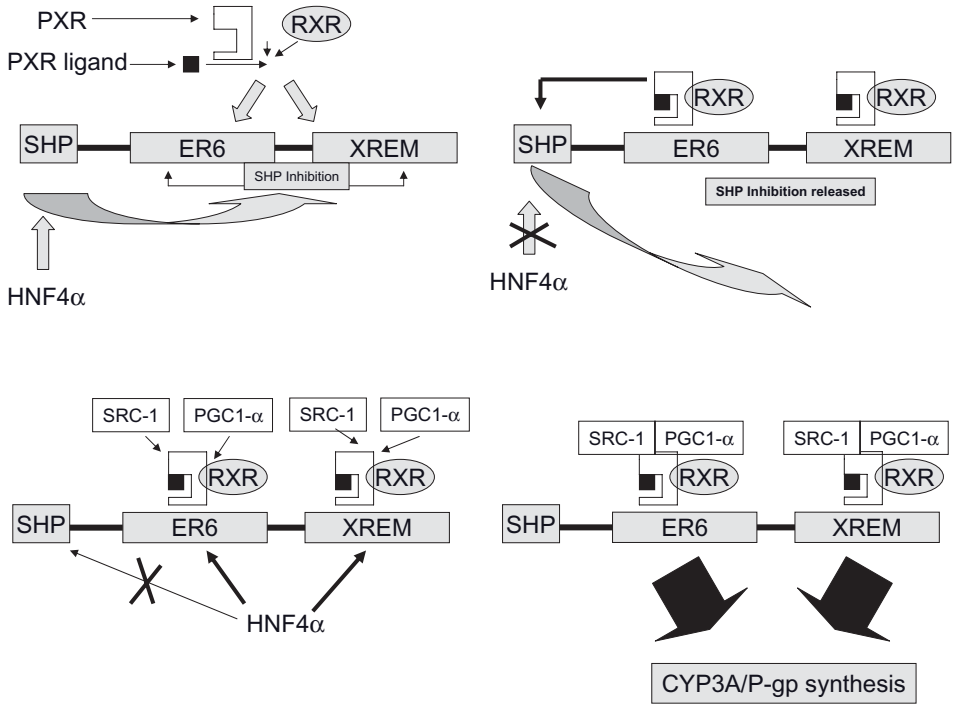

Figure 4.3 Mechanism of CYP3A induction through the pregnane X receptor (PXR) and retinoic acid receptors (RXR). HNF4α causes SHP to 'lock' the system. The PXR/inducer ligand binding shuts off SHP and allows HNF4α to promote the binding of the co-activators, such as SRC-1 and PGC-1, so triggering full induction.

As with AhR, PXR appears to lie dormant in the absence of any binding hormones, toxins or drugs. Unlike CAR, it has a large LBD, or binding site, which is rather quaintly termed 'highly promiscuous', in that it will bind a very wide range of chemical structures of all shapes and sizes. There is some degree of specificity, as rifampicin will only induce through PXR and not CAR, despite some agents which act through both NRs. This is reflected in the remarkable variation in the structures and sizes of the many clinical inducers of the CYP3A series. These include bulky antibiotics like rifampicin, several steroids as well as imidazole antifungals like clotrimazole, barbiturates and some organophosphate pesticides. It is important to note that the induction process is tailored to species, so animal studies have been less helpful in assessing the possible human enzyme-inducing properties of a novel chemical agent; rifampicin is a potent inducer of human and rabbit 3A enzymes, but it is without effect in the rat. PXR, alongside HNF4α., has an important role in controlling bile acid and cholesterol metabolism (Chapter 6) and it also part of the process where the liver displays one of its most remarkable abilities, which is to grow back after partial hepatectomy.

In terms of day-to-day operations, PXR function is a multi-stage almost simultaneous process, rather like moving off in a car (Figure 4.3). You start with the car parked with the brakes engaged, then you engage the transmission, release the brake and then press the throttle and go, more or less at the same time. Firstly, the PXR 'transmission' must be engaged: after the PXR receptors bind ligands, they recruit RXRs forming heterodimers. These then migrate to DNA and binding occurs at the CYP3A gene in two separate

areas. One binding site is an ER6 in the proximal promoter of the gene and at the same time, PXR/RXR complexes also bind to another ER6 and a DR3 (Direct Repeat 3) in a second area called the XREM, or xenobiotic responsive enhancer module. The system requires both the proximal promoter and the XREM to be bound by PXR/RXR heterodimers before induction can proceed. The 'transmission' is engaged, but the brake must be released before the 'throttle' (co-activator binding) can be pressed.

Normally, CYP3A transcription is kept locked up by the presence of SHP, which performs the same role in the AhR/CYP1A induction story. The expression of SHP is controlled by none other than HNF4α and SHP's 'brake' role is to prevent HNF4α from binding to the PXR/RXR bound promoter and XREM complexes, which in turn prevents the recruitment of the co-activators SRC-1 and PGC-1α. The presence of the inducer/PXR complex is thought to release the brake effectively.

The system will not fully launch transcription and translation unless HNF4α. binds the PXR/RXR complexes already bound to both the promoter and the XREM. As SHP is no longer present, HNF4α. binding then promotes recruitment of at least two co-activators, SRC-1 (also used by CAR) and PGC-1α (peroxisome proliferator-activated receptor co-activator 1). The throttle is being pressed, the brakes are off and we are in gear.

PXR system modulation

How rapidly the PXR system 'accelerates', that is, how potent an effect a particular inducer exerts on CYP expression seems to depend on several factors. As the process outlined above has so many steps, you would expect inducers to either compete for binding to PXR, or affect the co-repressor recruitment steps or all of the above. This is what seems to happen and given that the whole process is controlled by HNFα it is clear there is almost limitless capacity for variation in terms of the basic pre-set responsiveness of the system as well as its susceptibility to different inducers and groups of inducers. Indeed, induction in different patients has been observed to differ by more than 20-fold. Although azoles are inhibitors of CYPs, they can nevertheless modulate CYP expression through the NR system. Clotrimazole binds to PXR and promotes SRC-1 recruitment, whilst ketoconazole, known for its potent clinical CYP inhibition, can also partly block CYP3A induction as it disrupts SRC-1 binding to PXR. From a clinical point of view, the various induction processes look complex, but in some ways the process is rather like television. You can watch what is happening on the screen without entirely understanding how it works. In the same way, it is important to be aware of the drugs which cause induction and note what actually happens to drug clearance in the patient.

Interestingly, the PXR system is also implicated in drug resistance in anti-cancer therapy and is likely to be how tumour cells detect chemotherapeutic agents and respond by accelerated biotransformation and detoxification of the drugs. As PXR also controls MDR genes, which code for membrane transport systems like P-glycoprotein (section 4.4.7 and Chapter 5, section 5.4.2) which can eject a drug as soon as it enters a cell, this nuclear receptor is vital for cancer cells to protect them from the therapeutic agent. Owing to the broadness of PXR's binding abilities, it is thought to be a difficult receptor to block therapeutically, although it is believed sulforaphane, which is found in broccoli, is a PXR antagonist and is actually capable of down regulating CYP3A expression in human hepatocytes. Although the levels of sulforaphane required to cause this effect are in the range which is theoretically achievable through eating broccoli, this is untested in man at the time of writing. As PXR controls

various transporter systems as well as CYPs, in the future, it may be possible to design agents which will prevent drug resistance in cancer therapy through selective NR antagonism.

Receptor cross-talk and CYP capabilities

Regarding CYPs and general biotransformational capability, there is a great deal of overlap in the nuclear receptor-mediated control of CYP expression. HNF4α modulates at virtually all levels simultaneously, controlling the expression and specific activities of NRs like CAR, PXR and AhR as well as expression of a large number of genes ranging from CYPs and their REDOX partners, through to conjugation systems and transporters (Chapter 6). CAR appears to run basal 'housekeeping' metabolism, whilst PXR and AhR respond to both 'emergencies' such as the appearance of xenobiotics, as well as housekeeping in terms of bile salts and cholesterol. PXR-mediated induction, for example, is credited with the prevention of drug-induced cholestasis (cessation of bile flow) that can be caused by more than 20 drugs, including oral contraceptives, anabolic steroids and some antibiotics.

The different induction systems modulate each other also, as PXR can control AhR activity and CAR is in turn partly influenced by AhR. It is not yet established whether CAR actually modulates PXR activity. As mentioned above, CAR predominantly regulates CYP2C9 and CYP2B6, but PXR also has a role in controlling these CYPs and CAR also partly regulates CYP3A4/5, which is predominantly operated by PXR. In terms of clinical effects, many agents such as rifampicin and phenobarbitone promote the appearance of several CYPs through stimulation of both the CAR and PXR systems, whilst there are some experimental agents such as CITCO that are very specific and potent CAR stimulators, like rifampicin is to PXR. This overlap provides a 'safety net' to ensure that 'threat molecules' as well as endogenous agents that have outlived their usefulness will be controlled one way or another. It is also important to reiterate, as detailed in Chapter 6, the NR-mediated control of CYPs also extents to conjugation and transport process, providing a complete modulatory system for ensuring the detection, metabolism and excretion of hormones, drugs and toxins. There is also evidence that the drugs may influence the sensitivity of the immune system through their ability to stimulate NRs. Rifampicin's action on PXR causes down regulation of the important immune system transcription factor NFκB, which leads to the reduction in the expression of cytokines such as TNFα. Interestingly, activation of NFκB can down regulate PXR and lead to reduced expression of CYPs. This reciprocal relationship underlines the complexity of the cross-modulation between xenobiotics and the immune system's sensitivity and vice versa.

4.4.4 CYP2E1 induction

CYP2E1 is of relatively minor interest from the standpoint of drug metabolism (it oxidizes isoniazid, paracetamol and chlorzoxazone), but it is of major interest in hepatotoxin activation (paracetamol, carbon tetrachloride, thioacetamide) and carcinogen activation (*N*-nitrosodimethylamine, benzene, vinyl chloride and trichloroethylene). This isoform undergoes induction by apparently disparate factors like small hydrophilic molecules, such as ethanol, acetone and pyridine, as well as by systemic stresses, such as obesity, diabetes and starvation. In principle, the main points of CYP2E1 induction are now under-

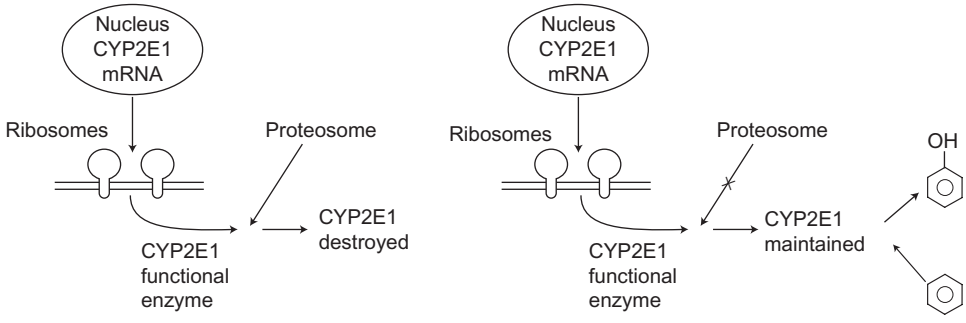

Figure 4.4 CYP2E1 induction: this CYP is not controlled by nuclear receptors and CYP enzyme is made in large quantities constantly, but in the absence of substrate, the proteosome system destroys the enzyme. The presence of the substrate effectively induces CYP2E1 by preserving it from the proteosome.

stood, although the details and its main physiological purpose remain to be fully explored. When animals are exposed to 2E1 inducers, functional CYP2E1 protein levels are increased up to eightfold, although the CYP2E1 mRNA levels remain the same, showing that 2E1 is not induced with a nuclear receptor regulated system like other CYPs. It is apparent that the main regulatory step of CYP2E1 is after transcription and translation are complete and the protein is actually fully assembled and shipped to the ER (or the mitochondria).

In human cellular systems, CYP2E1 remains functional for only a couple of hours before it is degraded. The presence of substrate 'induces' by increasing functional CYP2E1 survival time by twentyfold in comparison with substrate free cells. This suggests that CYP2E1 might be inherently structurally unstable and so is directly stabilized by its substrate, or perhaps that the substrate somehow prevents the usual rapid destruction of the CYP through another mechanism. It is thought currently that the latter explanation is more likely as CYP2E1, together with most other cellular proteins is regulated by the proteasome (Figure 4.4). This structure is often likened to the cellular equivalent of a paper shredder. The proteasome is vaguely tube-shaped and contains an internal protein 'slice 'n' dice' mechanism which reduces proteins to peptides and amino acids for recycling. The proteosome works in tandem with a series of ligases which attach the protein ubiquitin to any unwanted proteins or cellular structures. The proteasome recognizes the ubiquitin-label and destroys the protein. Inhibitors of this system are actually inducers of CYP2E1 by preventing its destruction and it is thought that Hsp90 is involved. It is likely that substrate-free CYP2E1 normally contains some sort of label which the proteasome reads and automatically destroys the protein. Binding of the substrate prevents the proteosome reading the label and the protein survives as long as it binds the substrate.

It is unusual amongst normally tightly conserved and complex processes such CYP regulation, that in the absence of substrate, considerable effort is being made to produce large amounts of CYP2E1 which are more or less immediately destroyed. Why 2E1 might function in such an apparently wasteful way could lie in the specific triggers of its induction and the nature of the chemicals 2E1 is designed to oxidize. CYP2E1 is very sensitive to diet, even becoming induced by high fat/low carbohydrate intakes. Surprisingly, starvation and diabetes also promote CYP2E1 functionality. Insulin levels fall during diet restriction, starvation and in diabetes and the formation of functional 2E1 is suppressed by

insulin, so these conditions promote the increase of 2E1 metabolic capability. One of the consequences of diabetes and starvation is the major shift from glucose to fatty acid/triglyceride oxidation, of which some of the by-products are small, hydrophilic and potentially toxic 'ketone bodies'. These agents can cause a CNS intoxicating effect which is seen in diabetics who are very hypoglycaemic, they may appear 'drunk' and their breath will smell as if they had been drinking. In the non-diabetic individual who is in a state of starvation, any ketone-mediated intoxication would obviously hamper the search for food, so these molecules must be cleared rapidly. The key factor here is the speed at which these compounds could accumulate – the nuclear-receptor mechanism of induction, with its time frame of days, might be too slow to cope with the accumulation of ketone bodies in starvation, so the much quicker 'substrate-mediated protein preservation' system perhaps might be rapid enough to ensure that adequate levels of CYP2E1 were present to prevent intoxication of the CNS. It is conceivable that the highly responsive control system of this CYP may also be linked with its propensity for the production of oxidative species which can promote cellular damage. This is less of a problem in the presence of high levels of a potent inducer such as ethanol, as alcoholics invariably ensure that sufficient substrate is imbibed to fully occupy the CYP and production of oxidative species might be limited. However, in the absence of such a substrate to occupy the active site CYP, a long-lived CYP2E1 might pose severe problems for a cell, producing intolerable levels of reactive oxidative species, so it is logical to degrade it quickly in the absence of an intended substrate. If this were to be the case, then the process of CYP2E1 binding and activation of oxygen should not cause enough change in the structural conformation of the CYP to stave off the remorseless proteasome shredding machine.

4.4.5 Non-inducible CYPs: CYP2D6

CYP2D6 is important as the main source of clearance for tricyclic antidepressants, some antipsychotics (haloperidol, risperidone), some beta-blockers and SSRIs. It is not thought to be inducible, and in cases where the clearance of a CYP2D6 substrate is accelerated in the presence of a known inducer, it is usually because CYP3A4 has been induced and this isoform is responsible for the increased clearance. CYP2D6 substrates dextromethorphan and mirtazapine clearances are markedly increased by rifampicin and carbamazepine respectively in this way. Interestingly, some studies such as those with the benzodiazepines and citalopram have shown that CYP2D6 activity increases in pregnancy. Whether this is a true induction process remains to be determined. The nearest to an induction effect seen in CYP2D6 is the expression of multiple copies of the gene which causes an ultra-fast clearance in some African and Middle Eastern ethnic groups (Chapter 7, section 7.2.3). Although technically this could be termed an adaptive increase in CYP expression, it is not responsive to a specific drug or toxin and neither is it reversible, so it is not a true induction effect. It probably linked with diet and environment.

4.4.6 Reversal of induction

Anyone who has stared in disbelief at their shrunken quadriceps after a full leg plaster cast has just been removed will appreciate the main imperative which drives the reversal

of induction. Just as astronauts lose the ability to walk if they spend enough time in weightless conditions without exercise, the cell will always husband valuable resources carefully and the vast increase in CYP transcription, translation and shipping to the SER is quickly curtailed in the absence of the inducer. In addition, the cell cannot afford the presence of a fully induced battery of functional CYPs without appropriate substrate, mainly because of the huge impact this biotransformational force would have on endogenous small molecules, such as hormones, seriously disrupting homeostasis. Finally, some CYPs, particularly CYP2E1, generate reactive species byproducts in the absence of substrate, so this could cause undesirable cellular oxidative stress if induction were not to be reversed.

As discussed in the sections above, the regulation of PXR, CAR and AhR all contain 'braking systems', which prevent CYP transcription, such as SHP. These systems act like the dead-man's handle on a train and re-assert themselves as soon as the inducer levels fall away, although this will prevent further CYP protein assembly, the large mass of induce functional CYPs in the SER must still be prevented from functioning, by degradation and recycling. As briefly mentioned in Chapter 3 (section 3.5.1), there are various systems which move finished CYP protein from the site of assembly to the SER. The CYPs must also undergo a form of quality control to ensure that they have not been misfolded. Once they reach the SER, they must then be anchored in the lipid alongside the REDOX partners to retain functionality. These processes are not fully understood, but when induction is reversed, the shipping and quality control abruptly ceases and the proteasome/ubiquitin system that controls CYP2E1 shreds other CYPs, such as CYP3A4. There is also evidence that the presence of various other factors, such as the chaperone proteins like BAP31 are required to maintain the CYPs in position in the SER. Once induction ceases, these factors contribute to the release the CYPs from their SER anchor and followed by ubiquitin labeling, they are shredded and their components re-used.

4.4.7 Cell transport systems and induction: P-glycoprotein

Purpose, structure and function

Many endogenous agents can enter and leave cells relatively easily through passive diffusion if they are reasonably lipophilic. The more charged agents need 'help' to pass across membranes and the OATPs (Chapter 2) and other transporters accomplish this. Although it is necessary for cells to pump in certain required nutrients, it is equally necessary to pump others out of one cell and into another, as part of the general circulation of endogenous agents, such as vitamins, amino acids sugars and proteins. A 'revolving door' system like the OATPs is adequate for agents moving with a concentration gradient. It is clearly much harder to pump molecules back out of a cell against a concentration gradient. This is a bit like ejecting a few rowdy fans from a stadium into a pressing throng of people trying to enter and obviously this requires energy input. The ATP-binding cassette transporters (ABC transporters) accomplish this and there are nearly fifty of them in three main sub-families. The major system which pumps endogenous substances and xenobiotics out of cells is known as P-glycoprotein (P-gp or Pgp), which is coded for by the MDR1 gene (found on chromosome 7 in man). P-gp is a 170 kDa trans membrane protein consisting

of two identical halves. Each half has six column-like segments that span the cell membrane. This is the actual pump structure, whilst two nucleotide binding areas are embedded below the membrane in the cytoplasm and they bind ATP, so they are the 'power' pack' of the pump.

Products of MDR genes such as P-gp possess a substrate specificity that is so wide as to be beyond promiscuity towards non-specificity; it has been described as 'fuzzy'. Some studies have suggested that substrates are likely to be lipophilic and contain a nitrogen atom that is positively charged at physiological pH ranges. In addition, substrate molecular weights are often greater than 400, have pKas greater than four, and the sum of their nitrogen and oxygen molecules are usually greater than or equal to eight. Non-substrates are low in nitrogen and oxygen, less than 400Mwt and a basic pKa of less than eight.

P-gp is part of the gut's barrier function to prevent the uncontrolled entrance of xenobiotics, as the transporter effectively works in tandem with the very high levels of CYP3A that are found in the gut (three times as much as in the liver in humans). If P-gp repeatedly pumps an agent out, it has more chance of meeting a CYP on its next entry. The system also has an element of insurance, as inhibitors of CYPs such as grapefruit juice do not necessarily always inhibit P-gp, so some residual barrier function remains as seven times as much P-gp is found in the apical areas of enterocytes compared with hepatocytes. The presence of P-gp can certainly retard drug absorption, without actually preventing it.

P-gp induction: clinical effects

P-gp is under the control of both PXR and CAR and potent inducers of CYP3A and CYP2C9 are well known to induce it. Rifampicin, St John's Wort and carbamazepine cause large increases in gut P-gp and often CYP3A substrates are also transported by P-gp, causing a combined effect of reduced cellular entry and accelerated clearance of drug molecules that do enter the gut. However, the clinical consequences of P-gp induction are not confined to CYP substrates. There are many drugs that are substrates of P-gp but are not metabolized by CYPs. Rifampicin can cause marked reductions in these substrate bioavailabilities through this mechanism without affecting their oxidative metabolism. The effects on digoxin are particularly serious, as it has such a narrow TI (0.5-3 ng/mL) and levels that exceed 3.5 ng/mL increase the risk of patient death. The therapeutic monitoring of this valuable but toxic drug has been revised to improve its safety and it is now recommended that the therapeutic window for this agent should be between 0.5-0.8 ng/mL. Clearly any drastic changes in bioavailability will make a strong impact on the patient, either through loss of efficacy or toxicity. Rifampicin is known to reduce the bioavailability of digoxin and St John's Wort's effects on cyclosporine (causing tissue rejection, see below) are at least partly due to its potent induction of P-gp through PXR. It is likely that these agents will also have the same impact on other anti-tissue rejection drugs such as tacrolimus. These agents are very difficult to manage clinically, as they can exert toxicity in all the major organs, including the transplanted organ or graft itself. Tacrolimus, for example, has a terminal half-life of the best part of four days and a plasma level of 30 ng/mL is considered the upper limit before toxicity ensues. Although cyclosporine has been reported to have a slight inductive effect on P-gp, some cellular studies suggest that

tacrolimus may decrease P-gp expression and function, so it may potentiate the effects of other drugs in terms of tissue penetration.

It has long been known that as many as 40 per cent of epileptics show resistance to drug therapy with AEDs. It has been proposed that P-gp or other ABC transporters 'protect' brain tissues from these drugs. It has been controversial as to whether some or all of the AEDs are P-gp substrates in the human brain. Recent work suggests that phenobarbitone, phenytoin may be P-gp substrates, lamotrigine and levetiracetam also, but not carbamazepine. Whilst results from *in vitro* work may be dependent on the type of assays used, it does appear that many of the AEDs could be P-gp substrates. If they are, then as many AEDs are inducers of CYPs, particularly CYP3A4, they should induce P-gp and MRPs in all tissues as well as the brain. This process should theoretically promote over time the exclusion of the AEDs from the very neural tissues they are designed to treat. This is extremely difficult to prove and is hard to investigate as patients are often taking many other drugs and have different ethnic backgrounds. Apparently, patients who had a very high number of seizures before treatment were more likely to have breakthrough seizures in the presence of an AED, so it is possible that ABC transporter-mediated issues may only be a component of the larger pharmacodynamic picture of drug resistant epilepsy.

P-gp and cancer

The P-gp system is found in virtually every species and is the subject of intense interest, as bacteria, protozoa and human cancerous cells all use it to protect themselves from potential toxic agents by detecting the toxin. This usually occurs through a nuclear receptor such as PXR, which induces P-gp to pump the drug out, be it an antibiotic or an alkylating agent. Indeed, the MDR1 gene that codes for P-gp actually stands for multi-drug resistance gene 1. If P-gp appears in sufficient quantity to clear the agent as fast as it enters, even potent cytotoxins will exert little or no effect, rather like wafting your hand through a flame quickly so it doesn't burn. Unfortunately, around half of all anti-cancer drugs are substrates for ABC-type transporters, which includes other pumps such as the MRPs (Chapter 6, section 6.11.2). It is possible that resistance to anticancer agents occurs through their binding to NRs such as PXR, as well as to other NR systems, although chemotherapeutic resistance seems to emerge more slowly than the timeframe of 2-4 weeks that the classical nuclear receptor induction process involves. It is possible that P-gp and its fellow pump systems are linked to other cellular receptor systems; this is because many anticancer drugs are not inducers of CYPs but do eventually induce local P-gp in the tumour target.

As discussed later in this chapter, cancer patients take large numbers of supplements and drugs related to the stress of their condition and this can have a serious impact on their therapy. St John's Wort accelerates the clearance of anticancer agents such as irinotecan and the tyrosine kinase inhibitor (TKI) imatinib and the Wort's induction of P-gp-mediated drug resistance at the cellular level may also be a critical factor in treatment outcome. Hence, not only do PXR inducers like St John's Wort accelerate drug oxidative metabolism, but their induction of P-gp is quite likely to blunt tumour penetration of what parent anti-cancer drug does enter the body and probably the metabolites also, thus effectively promoting resistance. P-gp induction's effect on pro-drugs metabolized by CYPs is likely to be more complex, but similarly deleterious.

4.5 Induction – general clinical aspects

From a clinical standpoint, important features of enzyme induction can be summarized:

- The process is relatively slow, i.e. usually days or even weeks.
- The potential changes in drug concentrations can be great enough to cause treatment failure.
- The induction process is usually, but not always, reversible over a similar time frame to its appearance, although reversal can be slower.
- Where a patient is stabilized on a high 'induced' drug dosage, if there is a treatment break of up to several days, drug accumulation and toxicity will occur.

The timescale of the induction process does largely depend on the potency of the inducer. Pentobarbitone causes a marked decrease in nortriptyline blood concentrations within only two days, doubling its clearance. Conversely, anecdotal evidence regarding teenagers suggests that induction of ethanol metabolism is a much more protracted process. Generally, the decline in drug levels caused by induction will lead to a commensurate loss of drug efficacy. Some of the most clinically relevant drug interactions caused by enzyme induction are described below.

4.5.1 Anti-epileptic agents

Drug combinations

In approximately one-third of cases of epilepsy, control of the condition can only be achieved with a combination of anti epileptic drugs (AEDs) which are also known as anticonvulsants. This combination therapy can lead to potential problems with the induction effects of carbamazepine (CYPs 2C9, 2C19, 3A4; Histories 1 and 2), phenytoin (CYPs 1A2, 3A4) and phenobarbitone (CYPs 1A2, 2C8, 3A4). In a combination of inducing anticonvulsants, co-administered compounds metabolized by these CYP enzymes will have their plasma concentrations significantly reduced. A good example of this is valproic acid, where plasma levels can be reduced by 80 per cent in the presence of phenobarbitone and by half with phenytoin and nearly 70 per cent with carbamazepine co-administration. Felbamate clearance is modestly accelerated by carbamazepine whilst among the second generation of AEDs, drugs such as topiramate and tiagabine are also cleared more rapidly in the presence of the AED inducers.

It is important to realize, however, that there are many alternative anticonvulsant drugs which are not inducers, such as valproate (an inhibitor of CYPs) lamotrigine, pregabalin, zonisamide, levetiracetam and topiramate. Gabapentin is also in this category and it is not actually metabolized at all and is cleared entirely renally as a parent drug. At the moment it is unlikely that these non-inducing AEDs will replace the inducing AEDs, which remain the main drugs of choice in epilepsy, despite their effects on other drugs. The newer agents are used both as first-line therapy and as substitutes for the inducing AEDs when the induction process presents an insurmountable clinical problem.

Drug withdrawal

It is generally stated that no AED, whether inducer or not, should be abruptly withdrawn. In regimens containing inducing AEDs, any changes or drug withdrawals can lead to acute problems related to the rapid reversal of the induction process (History 3). The remaining drug plasma levels might rise over the following few days as the inductive effect recedes and clinical signs of an intensification of the pharmacological effect will gradually become apparent. It is most desirable to anticipate this effect by tapering the dosage of the other drugs over days or weeks as appropriate. However, this is not such an easy process, as there is relatively little literature on how long it takes for the effects of standard inducers to fully wear off. There is some evidence that in some drugs it can take longer to disappear than the original onset time. It is better to taper the dose, or the patient might be subject to increased side effects, which they may or may not complain about. Overall, it is important that the drug levels remain within the therapeutic window and toxicity is avoided.

Epilepsy and brain tumours

The majority of brain tumours originate from malignant glial cells, known as gliomas. Although brain tumours account for less than 2 per cent of adult cancers, they are ten-fold more common in children. Between 50 and 70 per cent of patients with gliomas of various types suffer from epilepsy and studies have shown that use of the inducing AEDs present several clinical problems in this context. Antineoplastic drugs are a vital part of the therapy of gliomas and many of these drugs are cleared by the main inducible CYPs, so the inducing AEDs can accelerate the clearance of the anticancer drugs, reducing their effectiveness and in some cases change or accentuate their toxicity. The taxanes (paclitaxel and docetaxel) illustrate this point, as their clearance (CYP2C8) is increased by the inducing AEDs by about 1.5-2 fold. What is interesting, is that the usual adverse reactions caused by taxanes when given alone (gut toxicity and myelosuppression) change to peripheral neuropathy in the presence of inducing AEDs. This illustrates a re-routing of taxane metabolism and toxicity by induction. Some authors have recommended that either inducing AEDs should not be used with taxanes at all, or the anticancer agent's dosage should be increased by around half to ensure adequate therapeutic effect. During a weekly treatment cycle, docitaxel is generally administered after dexamethasone premedication to minimize fluid retention. Interestingly, as if the toxicity of the taxane was not bad enough, dexamethasone can have an amphetamine-like 'speeding' effect which may last several days. This is not uncommon, but it is not considered a major side effect. Although dexamethasone is also a CYP inducer, the benefit in the reduction of side-effects favours the use of the steroid.

Vinca alkaloid (vinblastine, vincristine, vindesine) clearance is increased by more than 60 per cent in the presence of inducing AEDs. Phenytoin increases the clearance of the camptothecin derivative irinotecan, which is a CYP3A4 substrate, by more than 50 per cent. Teniposide and etoposide are also CYP3A4 substrates; phenytoin and phenobarbitone can treble their clearances, requiring dosage increase to maintain antitumour effect. A clinical study has shown that teniposide's efficacy is actually poorer with inducing AEDs than without.

Other drug combinations

Anticonvulsants are co-administered with other CNS modulating drugs, such as antipsychotics, tricyclic antidepressants (TCAs), benzodiazepines and newer agents such as SSRIs. With respect to enzyme induction, anticonvulsants can greatly accelerate the clearance of antipsychotics like haloperidol and benzodiazepines such as midazolam, although temazepam clearance is not dependent on 3A4 and is not affected by inducers of this isoform. CYP3A4 inducers can also accelerate the clearance of some TCAs.

4.5.2 OTC (over the counter) herbal preparations

It has been estimated that nearly half of the US population has used herbal or complementary medicine in their lifetime and this rises to nearly 70 per cent of those suffering from life-threatening conditions such as HIV or cancer. Unfortunately, for many reasons, patients do not necessarily inform their medical practitioners of their use of these products. Although the clinical potency of many herbal remedies is at best arguable, the most popular, St John's Wort (*hypericum perforatum*), has been extensively clinically investigated and found to be effective in mild to moderate depression and anxiety. It contains the active components, hypericin, pseudohypericin and hyperforin, as well as several flavonoids, xanthones and many other chemicals. Of course, patients are usually unaware that hyperforin, (unlike the other constituents) is a powerful activator of human PXR, leading to induction of CYPs 3A4 and 2C9. Hyperforin itself undergoes extensive metabolism by CYP3A4 to hydroxylated products. It is safe to assume that St John's Wort will accelerate the clearance of any and all CYP3A4 and CYP2C9 substrates, which, as you know, involves the majority of prescription drugs. A 14-day course of St John's Wort and dosage of a total of 900 mg daily is capable of doubling alprazolam clearance. The herb has been shown to accelerate the metabolism of cyclosporine in renal transplant patients (at only 600 mg daily), potentially leaving them at risk of tissue rejection unless the immunosuppressant's dosage was increased by 60 per cent. This is also the case with the newer anti-rejection drugs tacrolimus and everolimus. Moreover, the inducing effect of the herb complicates the pharmacology of cyclosporine, as the increased production of metabolites is associated with greater nephrotoxicity, although the metabolites also exert an immunosuppressive effect. St Johns Wort also increases the clearances of amitriptyline and indinavir, although it does not increase the clearance of carbamazepine as this drug already maximizes its own induction. Confusingly, the herb has some inhibitory effects on CYPs *in vitro*, but this has not been so apparent *in vivo*. St John's Wort is also a potent inducer of the transporter P-glycoprotein (section 4.4.7) which is probably how it reduces digoxin levels by accelerating the rate it is pumped out of intestinal cells back into the gut.

Unsurprisingly, many patients who are undergoing a variety of conventional therapies are likely to become depressed and turn to herbal remedies such as St John's Wort for relief. A high proportion of cancer patients are known to use such remedies, as the processes of chemotherapy, radiotherapy and recovery from surgery can range from merely gruelling to unendurable.

There are several key clinical problems to summarize which may be encountered by the use of St John's Wort in particular:

- Many patients do not consider herbal remedies as 'real drugs' so they do not inform their medical practitioner they are taking or stopping them (History 4). In addition, patients often fully expect these remedies to be wholly beneficial without any side-effects.

- The onset of the inductive effect of St John's Wort is variable, with some studies suggesting it must be taken for weeks to see a full effect, whilst in contrast, its impact was demonstrated within three days in the case of cyclosporine co-administration in the renal transplant patients. This may be related to the hyperforin content of the extracts.

- The patient may abruptly terminate their self-medication if they feel better or encounter side-effects (History 4).

- Hyperforin, pseudohypericin and hypericin have long half lives (16-42 hours) so the timeframe of the diminution of the inductive effect once self-medication ceases may be unpredictable. Some studies have shown it can take up to three *weeks* for the effects to subside.

- Quality, purity and content of the active ingredients in herbal preparations can vary widely according to methods of preparation.

This final point is crucial: one study found a 62-fold difference between the hyperforin contents of various commercially available preparations. Clearly, some of these preparations contain so little hyperforin that they are unlikely to even show any pharmacological effect, besides any enzyme inductive actions.

Another problem with herbal preparations is that although many are available, few have undergone even preliminary evaluation for their effects on the clearance of drugs. Green tea (contains epigallocatechin gallate) and sleep promoting valerian extract have both so far shown no inductive effects on drug clearance, although garlic significantly reduced the bioavailability of the anti-HIV drug saquinavir, possibly through its effects on transporters. The effects of Echinacea are more complex. This herb has been used for centuries to combat the symptoms of colds and 'flu, although its efficacy in the prevention of common colds and its actions on the immune system are unproven. It may induce CYP1A2 and CYP2C9, although it has a mixed and unconfirmed effect on CYP3A4. As with many other remedies, such as ginger (a possible inducer *in vitro*) ginseng and kava-kava, the enzyme-inducing properties of most herbal preparations have not been substantiated or systematically investigated. A study has suggested that Gingko biloba extract maybe an inducer of CYP2C19, as it accelerated omeprazole clearance. One point important to emphasize, is that assuming various herbal remedies do contain active and potent substituents, there is virtually nothing known clinically about what effects mixing herbal remedies might have, in terms of pharmacology and toxicity. This area is unfortunately left for patients to discover for themselves. It has been reported that a combination of St John's Wort and kava-kava caused acute hepatitis in a 48-year-old female and although this possible interaction should be investigated further, it would seem to be reasonable to recommend to patients not to take these remedies simultaneously.

It is important that patients are asked if they have taken, or would consider taking, herbal remedies during a drug treatment regimen. For instance, this may be a problem where a

course of conventional antidepressants is embarked upon and the patient's symptoms do not improve quickly enough. Consequently they may understandably resort to assistance from an herb extract such as St John's Wort. Even if the patient is aware of the potential impact St John's Wort might have on their drug therapy, they should still feel confident in discussing this with their healthcare practitioner. From the practitioner's point of view, since this herb does have proven efficacy, it may be worthwhile considering accommodating its inducing effects in a drug regimen, if it is judged to be in the interests of the patient. In some cases, such as with statin therapy, a CYP3A4 substrate such as simvastatin could be substituted with pravastatin (cleared virtually unchanged), or rosuvastatin (<10 per cent cleared by CYP2C9). In other cases, the induced drug dosage could be increased to compensate for the inductive effect of St John's Wort.

4.5.3 Anticoagulant drugs

Atrial fibrillation (AF) promotes the formation of microemboli which increases a patient's risk of a stroke by five fold. As well as AF, those at risk of other blood clotting related conditions such as deep vein thrombosis (DVT) and valve replacement recipients can be protected with anticoagulants such as warfarin. Interestingly, in the UK it has been suggested that more than 30 per cent of patients who could benefit from warfarin do not receive the drug for various reasons. In practice, warfarin is monitored pharmacodynamically, that is to say its effect is monitored rather than its concentration. The measurement 'INR' is used which is the 'international normalized ratio', which effectively assumes that normal blood clotting time is taken as around 1, so for warfarin to anticoagulate enough to successfully treat AF, an INR of 2.5 is required. So through frequent clinical monitoring, the dose is adjusted to achieve an INR value of approximately 2–3. At the other end of the scale, those with mechanical mitral valve replacement require maintenance of an INR of 3–3.5. Warfarin is given as a mixture of two isomers, S and R. The S isomer is up to five-fold more potent an anticoagulant than the R isomer and the S is cleared by CYP2C9, whilst the R is metabolized by CYP1A2 and CYP2C19. CYP3A4 has a minor role for each isomer. Inducers of these enzymes will make a substantial reduction in the plasma levels of warfarin and after a lag period, its anticoagulant effects will recede (History 5). There is no substitute for checking INR to ensure that they remain within the therapeutic window. If an enzyme-inducing drug is withdrawn, there is the danger of accumulation of the anticoagulants, which will lead to haemorrhaging. It appears that most clinical problems with warfarin seem to occur as a result of the effect of inhibitors rather than inducers and some groups of patients on warfarin are more at risk than others from drug interactions, particularly those receiving cancer chemotherapy. When warfarin patients undergo minor surgical procedures, most authorities recommend they should continue to take the drug, as withdrawal's problems outweigh its benefits. Prior to major surgical procedures, 4–5 warfarin doses might be withheld, letting the INR fall to around 1.5. If an inducer is part of the regimen and this drug is stopped also, it is worth remembering that its inductive effect will wear off in a few days and dosage adjustments may have to be made when the full regimen is resumed. It is interesting to note that those with mechanical valves must be anticoagulated constantly and if warfarin is stopped for a major procedure, another agent such as heparin is used during hospitalization to ensure they do not develop life-threatening emboli.

4.5.4 Oral contraceptives/steroids

The CYP3A4 inducers including St John's Wort can accelerate the clearance of ethinyloestradiol; this is a particular concern with low-dose oral contraceptive preparations. Increasing the contraceptive dose, or a recommendation to use other methods of contraception, may negate this effect. Corticosteroids are potent inducers of CYP3A4 and CYP2B6 and they can cause the clearance of inducing AEDs such as phenytoin to be accelerated by a factor of nearly three. It is recommended that when inducing AEDs are used during steroid therapy they are closely monitored to ensure that their levels do not fall out of the therapeutic window. This effect also works the other way around, as the AEDs can cause the acceleration of exogenous and endogenous steroid clearance. In general, inducers of CYP3A4 will accelerate the clearance of corticosteroids.

4.5.5 Antiviral/antibiotic drugs

Of the newer anti-HIV antiviral compounds, ritonavir, nevirapine, indinavir and saquinavir are all metabolized by CYP3A4, so it is possible that inducers may affect their clearances *in vivo*. However, this situation is complicated by the fact that for example ritonavir is a potent inhibitor of CYP3A4 and induces its own metabolism. This induction effect means that at least 14 days' therapy is required before plasma levels stabilize. Potent inducers such as rifampicin do exert some effect on ritonavir plasma levels, but only to a relatively modest (~35 per cent) degree. It is believed that the observed acceleration of the clearance of drugs co-administered with protease inhibitors is most likely to be due to the induction of CYP2B6, 2C8/9 and to a lesser extent CYP2C19, as these CYPs are not inhibited by the proteases.

Of course any changes in the plasma levels of an antibiotic or antiviral agent can lead to subcurative drug concentrations and a possible selection of resistant variants of the infectious agent, so plasma levels should be closely monitored to ensure minimum inhibitory concentrations (MICs) are exceeded while toxicity is minimized. St John's Wort is known to cause indinavir levels to fall below the MIC. Certainly abruptly stopping and restarting inducing antibiotics such as rifampicin (Histories 6 and 7) will lead not only to resistance, but also to severe disruption of the clearances and efficacies of co-prescribed agents. Co-administered drug levels will climb above the therapeutic window until the inducing effect is re-established. Patient drug tolerance may be severely impaired during this period.

4.5.6 Anti-cancer drugs

As mentioned above, many antineoplastic agents are cleared or activated by CYP metabolism and changes in their plasma levels can have serious repercussions in terms of toxicity and therapeutic effects. The acceleration of taxane metabolism (CYP2C8) leads to their clearance of inactive metabolites and a loss of efficacy. Other antinoplastics, such as thiotepa are cleared by CYP2B6 and CYP3A4, but both parent and metabolite (tepa) are equally therapeutically effective, so dose modification in the presence of inducers is easier to manage clinically. The effects of induction on anti cancer 'pro-drugs' can be more complex. Cyclophosphamide and its sister compound ifosfamide are probably the most

widely used of this type. These agents are part of several cancer therapeutic regimens, for either primary or metastatic disease. Cyclophosphamide is activated to an active 4-hydroxy metabolite mainly by CYP2B6, CYP2C19, CYP3A4 and to a lesser extent, CYP2C9. The 4-hydroxy cyclophosphamide enters cells and then rearranges itself into a highly reactive phosphoramide mustard derivative, which is similar to the blister forming antipersonnel mustard agents used in the Great War (1914–1918). This metabolite indiscriminately kills any growing cells (malignant or non-maligant) by alkylating their DNA, leading to a therapeutic effect, but an appalling list of toxic effects, including hair loss, gut damage, nausea and vomiting, as well as cystitis, nephro/neurotoxicity and immunosuppression. Ifosfamide tends to be more neurotoxic than cyclophosphamide, mainly because it is much more subject to side-chain dechloroethylation than the cyclo derivative.

Cyclophosphamide and ifosfamide can induce their own metabolism within a treatment cycle of a few days and AED inducers such as carbamazepine accelerate this process markedly. With cyclophosphamide this leads to an increase in production of the 4-hydroxy metabolite by more than 70 per cent, leading to intolerable toxicity. As underlined in the previous section on epilepsy and brain tumours, it is recommended that non-inducing AEDs are used in patients taking cyclophosphamide and ifosfamide.

The role of CYPs in the metabolism and efficacy of the topoisomerase inhibitor irenotecan makes this agent vulnerable to the effects of enzyme induction. Irinotecan is used mainly in colon cancer and it is a prodrug, which is then hydrolyzed by carboxyesterases to its active metabolite SN-38, which is normally cleared by glucuronidation (Chapter 6, section 6.2.10) to the inactive SN-38G. Irinotecan is cleared by CYP3A4 to several metabolites and St John's Wort accelerates this process thus restricting availability of the parent drug for SN-38 production, which in turn causes SN-38 levels to fall by more than 40 per cent. Although this alleviates the side effects, sadly it also means that the drug loses its therapeutic effect and the herb should *not* be taken with irinotecan. It is important to note that any CYP3A4 inducer would cause this effect and some, such as phenobarbitone, also induce glucuronidation, so the parent and the active metabolite's clearances will both be accelerated, seriously eroding efficacy.

5 Cytochrome P450 Inhibition

5.1 Introduction

The previous chapter was mostly aimed at problems associated with drug failure due to enzyme induction. However, when drug clearance is slowed or even stopped for any reason, the consequences are more dangerous and occur much more rapidly compared with enzyme induction. Generally, the intended pharmacological effects of the drugs will be greatly intensified, leading to a clear manifestation of symptoms in the patient. In drugs with a wide TI, this may not be a problem and the effects of the drug accumulation will be reversible. In narrow TI drugs, the effects can be lethal in hours. In other cases, a drug may induce potentially serious unintended pharmacological effects that are only seen at high doses, considerably above the normal range. These effects, sometimes known as 'off target' pharmacological actions, may or may not have been seen in the initial pre-clinical (animal) toxicity testing of the drug. The following illustrative histories underline the effects of drug accumulation.

History 1

A previously healthy 29-year-old male used terfenadine twice daily for one year to treat allergic rhinitis. The patient drank grapefruit juice two to three times weekly. One day he consumed two glasses of juice, took his terfenadine dose, and then mowed his lawn; within one hour he became ill, collapsed and died. Although usually undetectable, post-mortem terfenadine and metabolite plasma levels were reported as 35 and 130 ng/mL respectively. These levels are within range of previously noted arrhythmogenic levels of terfenadine. The individual had no evidence of impaired hepatic function.

Analysis

The presence of grapefruit juice appears to have caused unusually high levels of the parent drug to be present in the patient's plasma. Hence some component of the juice prevented the clearance of the parent drug, leading to drug accumulation, which led to a fatal cardiac arrhythmia.

Human Drug Metabolism 2E, Michael D. Coleman

History 2

A 67-year-old male patient stabilized on warfarin began to drink cranberry juice twice daily in response to its reported benefits in recurrent kidney infections. Four days after starting to drink the juice, he suffered a fatal stroke. Post-mortem levels of warfarin were 40 per cent higher than previously sampled in this patient.

Analysis

That the patient had been stabilized on warfarin indicates that his clotting time was within acceptable limits and therefore the drug was being cleared at the same rate it was entering the patient's system. The onset of the consumption of cranberry juice coincided with marked accumulation of warfarin, rendering the patient highly vulnerable to haemorrhage, which occurred within the brain and led to death. It was apparent that the cranberry juice had prevented the clearance of warfarin to inactive metabolites.

History 3

A 64-year-old female with a history of depression was stabilized on amitriptyline, 150 mg/day, but without improvement in mood. Her GP added fluoxetine, 40 mg/day, and within three weeks, the patient's symptoms subsided, although one week later, she collapsed at home and was found in a coma by a relative. The patient recovered consciousness two days later and made a full recovery.

Analysis

The addition of fluoxetine to the regime was associated with accumulation of amitriptyline, which led to unconsciousness and could have been fatal had she not been discovered. The fluoxetine must have prevented the clearance of amitriptyline.

History 4

A 44-year-old female epileptic was stabilized on carbamazepine but on the advice of a friend started taking a liquorice preparation for stomach problems. Over a period of two days, she became gradually more sedated and confused, until she had difficulty standing up. She was admitted to hospital and recovered within three days.

Analysis

The liquorice extract was taken in considerable amounts and appears to have interfered with the clearance of carbamazepine, leading to drug accumulation and symptoms of toxicity.

History 5

A 55-year-old female stabilized on warfarin suffered from recurrent acid indigestion over the Christmas period and started to self-medicate with over-the-counter cimetidine on the advice of a relative. A few days later, while gardening, the patient noticed that a small cut bled profusely and did not appear to clot for a long period. The patient reported to a hospital accident and emergency room, where her INR was 3.3 compared to the usual 2.5. The hospital advised her to use an alternative anti-acid agent and her prothrombin time returned to normal over several days.

Analysis

The excessive anticoagulation was due to a reduction in the clearance of warfarin by cimetidine, which could be averted by the use of low-dose (<400 mg) ranitidine or famotidine, which are not usually associated with changes in warfarin pharmacokinetics. An acceptable proton-pump inhibitor would be lansoprazole, but not omeprazole.

Overall analysis

In these cases:

- The patient was already stabilized on a particular medicine, which suggests that the dosage and clearance were approximately balanced.
- The addition to the regime prevented clearance of the first drug, leading to accumulation and toxicity.
- The toxicity manifested as an intensification of the normal pharmacological response, again indicating that drug accumulation was responsible.
- The toxic responses occurred within hours rather than days, after the addition of the inhibitor drug.
- The toxicity manifests so quickly that death can occur before even the patient realizes what is happening.
- The toxic effects were rapidly reversible once the inhibiting drug was withdrawn.
- The effects can occur in response to the patient's decision to either self-medicate or change their diet routine, without consultation with medical staff, or the effect can occur after medical staff fail to be aware of the potential reaction.

5.2 Inhibition of metabolism – general aspects

In complete contrast to enzyme induction, drug inhibition is not usually a process where a logical adaptive response can be made by the patient's metabolism. The fact that some

inhibitors can impair CYP operation for as long as they are administered indicates that the patient's homeostatic systems are not equipped to detect the inhibition effect and cannot quickly respond to the change in the situation within the timescale – it is rather like suddenly blocking the exhaust pipe of a running engine – it will cough and then simply stop. Sometimes, another CYP or metabolizing system may be capable of clearing some of the accumulating drug at higher concentrations, or the kidneys may also eliminate some unchanged drug. That the lung can clear some volatile chemicals such as alcohols is exploited in road safety in the detection of drunk drivers. However, if the drug's main route of clearance is a particular CYP in the liver and clearance is mostly dependent on the liver, the resultant accumulation will occur relatively rapidly, followed by toxicity or even death.

It is clear that inhibition-based drug reactions are much more potentially clinically serious than induction effects, due to this short timescale and the speed that the patient's clinical situation can change, leading to irreversible damage (such as a stroke or heart attack) within hours of consuming the inhibitor. This is especially problematic in the light of the increasing prevalence of 'polypharmacy', where patients, such as the elderly, may be taking several pharmacologically active compounds at once.

Another factor is that the inhibitor may enter the regimen on the patient's initiative, through the desire to 'self-help', without informing their doctor. It is also possible that a mistake by a medical practitioner could lead to a potent inhibitor reducing the clearance of a potentially toxic drug. Errors such as these are probably more likely to occur, now that in many countries more than ten different healthcare professional groups have the power to prescribe.

Tissue homeostatic mechanisms in the liver and other tissues can respond to inhibition in certain circumstances, i.e. some form of adaptation to the situation can occur to restore clearance of the usual substrate. This depends on the type of inhibitor and the frequency of dosage and will be discussed later.

5.3 Mechanisms of inhibition

5.3.1 General aspects of inhibition

Enzymes and tissue/cell receptors share similar features. A receptor binds a molecule that then acts like a switch to trigger a cascade of molecules to instruct the cell to perform a function. The molecule must fit the receptor precisely and then trigger the cascade, like a key, which first enters a lock, then is successfully turned to open it. A key that fits and enters the lock, but does not turn it, not only fails to open the door but also prevents the correct key from being fitted. The lock is essentially 'inhibited'.

Although they are highly specialized, CYPs are enzymes like any other in the body and they are inhibited according to the same general principles as other enzymes. How tightly a chemical interacts with a CYP isoform is based on how powerful is the mutual attraction (affinity) between the chemical and the various areas of the active site of the enzyme.

In the case of CYPs and any given enzyme, affinity must be strong enough to ensure the substrate is bound for sufficient time to process it to a product. The quicker this process occurs, the faster the 'turnover' of the enzyme and the more efficient it is. It is useful to try to visualize a CYP isoform, or any other human enzyme for that matter, as a three-

dimensional machine tool, perhaps like a spot welding machine. The enzyme cycles hundreds of times a second. If any single aspect of substrate binding or processing (oxidation or reduction), followed by product release is prevented, the sequential nature of these events means that the enzyme stops functioning. Another analogy might be an automatic paper stapler in a photocopier. Whatever analogy you might use, it is useful to try to visualize enzymes as dynamic micro machines. Broadly, inhibitors of CYPs may frustrate the enzymes' operating processes in two main ways, with varying impact on drug clearance and the individual enzyme 'health' and survival. At high concentrations, many inhibitors might block several CYP subfamilies, but at lower concentrations, they show more selectivity and their potency in blocking individual isoforms can be measured. Inhibition itself can occur through four main processes: competitive, non-competitive, uncompetitive and mechanism-based. Which type of inhibition occurs with various drugs can depend on many factors, such as drug concentration and the characteristics of a particular CYP isoform. Many drugs can act as competitive inhibitors with one CYP and non-competitive with others. Studies with inhibitors of drug metabolism are carried out *in vitro* with human CYPs, either in human liver or in expressed enzyme systems (see Appendix A). These studies do not always reflect what will happen when the drugs are used in patients, but are a reasonable starting point to predict whether a new drug might interfere with the metabolism of another.

5.3.2 Competitive inhibition

This is the simplest form of inhibition, where the substrate (drug) and the inhibitor are very similar in structure and have similar affinities for the same place, i.e. the CYP active site (Figure 5.1). A CYP substrate is normally processed to a different molecule, that is,

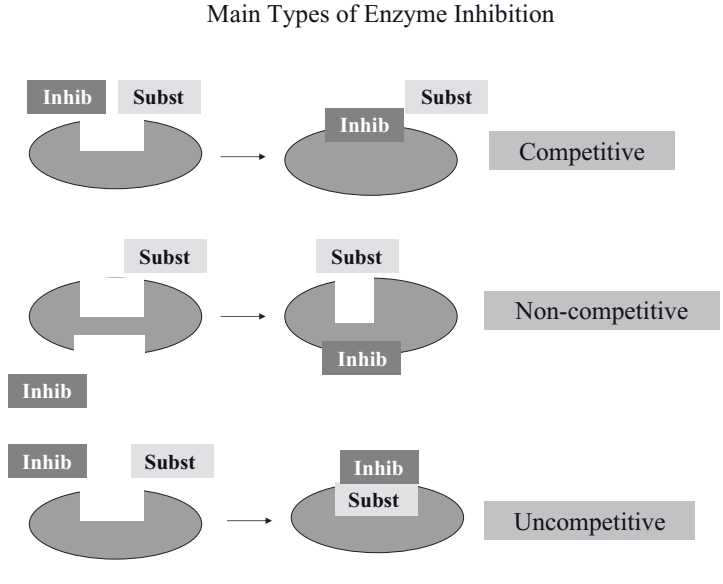

Figure 5.1 Main types of enzyme inhibition that apply to CYP isoforms.

a metabolite, which then has a much reduced affinity for an active site and is more water-soluble, so it diffuses elsewhere. A competitive inhibitor of a CYP isoform is usually not a substrate and acts like a similar key to the correct key for a doorlock; it may enter and leave the lock freely but does not operate it. As it is not processed into a product, it does not leave the vicinity of the CYP and binds and detaches continually. The CYP might be unable to metabolize the inhibitor, due to particular features of the molecule that might prevent oxidation, but promote binding to the active site. This form of inhibition is common in CYPs and is governed by the law of mass action, which states that the rate of a reaction (in this case enzyme binding) is governed by the concentration of the participants. So for CYP metabolism, whichever agent, drug or inhibitor, is in the greatest concentration, then this will occupy the active site. At low inhibitor concentrations more drug can be added to overcome the inhibitory effects. However, as drug levels must be increased to overcome the inhibitor, this effectively means that the drug's affinity falls for the site (K_m increases) in the presence of the inhibitor. Enzymes are often subject to this process of competitive inhibition because it is usually part of the endogenous feedback control mechanism on product formation. This generally involves enzymes that use cellular energy, or are at the junction of several biosynthetic pathways. When high levels of product are formed, these inhibit the substrate, so limiting the enzyme's 'turnover', i.e. when the desired product level is reached. This is rather like a thermostat in a heating system, which automatically maintains a preset temperature irrespective of outside temperatures. This is seen in the regulation of vital endogenous molecules like NADPH and glutathione (GSH) and the process avoids unnecessary use of cellular energy. Although the enzyme is temporarily disabled, it is undamaged and has not cycled or used any reducing power. Mathematically, if a Lineweaver–Burk double reciprocal plot is made of competitive inhibition, the K_m (inverse of the affinity) changes, but the V_{max} does not; in other words, the enzyme will still run at a maximum rate if enough substrate is used, but affinity falls off.

A new drug might be evaluated as a possible inhibitor of a given CYP isoform; if the inhibition of the known CYP substrate yields a Lineweaver–Burk plot as described above, then the new drug is a competitive inhibitor of that CYP and it is likely that the inhibitor is binding the CYP at its active site. There are several examples of competitive inhibitors of CYP isoforms. Indeed, if two drugs of similar affinities are cleared by the same isoform, then competitive inhibition can occur. The major clinically relevant group of competitive inhibitors includes the azole antifungal agents.

Azoles

It is not surprising that these agents are potent human P450 inhibitors, as a great deal of money, time and effort was put into designing them to inhibit fungal CYPs. They prevent the fungal synthesis of ergosterol, by blocking lanosterol alpha-C_{14}-demethylase, so causing the substrates (14-alpha-methylsterols) to accumulate and this disrupts fungal membranes. Unfortunately, as mentioned in Chapter 2, since all living system CYPs originate from a common bacterial source, inhibition of azole compounds also occurs in human CYPs. Interestingly, this is relatively specific; ketoconazole was initially the most commonly used azole agent and this is a potent competitive inhibitor of CYP3A4, as well as a number of other sex steroid-handling CYPs. This meant that the drug was quite toxic,

as it caused a significant fall in testosterone levels in blood, which could lead to feminization of males. This could be seen as the appearance of breasts (gynaecomastia), loss of spermatozoa production and impotence. The female menstrual cycle was also disrupted. These effects, coupled with other toxicity, such as GI tract irritation, nausea, vomiting and occasional severe liver toxicity, propelled the continuing development of these agents to less toxic azoles, which would be more potent therapeutically, but with less human CYP impact. These appeared in the 1990s, in the form of itraconazole and fluconazole, which were followed by the third-generation triazoles, such as voriconazole and posaconazole, which were much more effective than ketoconazole as antifungals *in vitro*, although they do still inhibit CYPs.

Clinically, there is plenty of evidence that azoles inhibit the metabolism of other CYP3A substrates. Fluconazole has caused a patient on simvastatin to develop rhabodomyolysis, which is an uncommon but potentially fatal condition associated with statins. This effect only occurs at high systemic levels of statin and was due to the inhibition of clearance by the azole. Peak plasma concentrations of the CYP3A4 substrate felodipine were increased eightfold and the area under the curve (AUC, or the amount of drug in the plasma) six-fold by the presence of itraconazole. Fluconazole has been shown to increase the half-life of omeprazole by threefold and it's AUC by a similar value. Other CYP3A4 substrates, such as midazolam, terfenadine and lovastatin, show similar effects with this azole. Clearly, the impact of the inhibition on the pharmacological effects of these drugs is very strong, with significant potentiation of their particular effects. It is interesting that although the inhibitory action of these drugs is simple and reversible, the clinical effect of this process on other drug effects can potentially be extremely serious. However, in a matter of hours after the withdrawal of the azole, the inhibiting effect is lost and substrate clearance resumes. There appears to be no way that the liver CYP nuclear 'management system' which is seen operating so successfully with enzyme inducers, can overcome the effects of a drug such as ketoconazole when the agent is taken for a long period of time, although anti-fungals are usually taken for either single topical doses, or relatively short courses and are stopped after the infection is eradicated. The serious disruption of steroid metabolism (gynaecomastia again) testifies to the potential problem posed by longer exposure to these drugs. The inhibition appears to be stable for as long as the drug is administered. This has led to attempts to use inhibitors such as ketoconazole and cimetidine to deliberately block the clearance of certain drugs, of which more later. Voriconazole is cleared by CYP2C19 and CYP3A4 and thus is subject to effects linked to polymorphisms of CYP2C19 (Chapter 7, secton 7.2.3)) and ironically its clearance is significantly reduced by CYP3A4 inhibitors, such as ritonavir.

Azoles and immunosuppressants

Although fluconazole is effective against the common fungal pathogen *Candida albicans*, it is no use against the loathsome mould species *Aspergillus* and *Cryptococcus* which often infect immunosuppressed transplant patients. The third generation azoles were mainly developed to destroy these moulds, so fluconazole therapy is sometimes switched in immunosuppressed patients to voriconazole where fungal breakthrough occurs. Unfortunately, *in vitro* human liver microsome studies have revealed voriconazole to be capable of blocking CYP2B6 and CYP2C9, CYP2C19 and CYP3A at single figure micromolar concentrations, whilst its effects on CYP2C8, CYP2A6, CYP1A2 and CYP2D6

are relatively weak. This unfortunately translates to the clinical situation, where voriconazole is a potent inhibitor of the metabolism of the newer anti-rejection drugs (tacrolimus, everolimus and sirolimus). Indeed, when voriconazole replaces fluconazole, it has been shown to be a more potent inhibitor of tacrolimus clearance than its predecessor. It is already recommended that the tacrolimus dose should be reduced by a third when voriconazole is used, although a further reduction of around 20 per cent may be necessary when existing fluconazole therapy is replaced by voriconazole. This situation can be complex, depending on whether the azoles are used orally or intravenously. These effects have been ascribed to the high levels of CYP3A4 found in the gut (more than 70 per cent of total CYPs) compared with the generally lower CYP3A hepatic levels (~30 per cent). All azoles, including others such as miconazole and clotrimazole, are generally competitive inhibitors, due to their lone pair of electrons on the azole nitrogen, which temporarily binds to CYP haem groups. This is mostly borne out by *in vitro* studies with voriconazole, as its most potent inhibitory effects on CYP2B6 and CYP3A are competitive, although there is an element of non-competitive inhibition at slightly higher concentrations. As well as voriconazole's effects, its sister agent posaconazole is also capable of increasing sirolimus peak concentrations and AUC by nearly nine fold, which is likely to cause severe toxicity to a transplanted kidney.

The use of azoles with immunosuppressant drugs is particularly problematic and it has been recommended that a highly individualized patient approach should be taken due to the complexity of the situation and the risks involved. This should involve close therapeutic monitoring to minimize side effects so retaining good patient morale and compliance. In addition, adequate azole must be present to eliminate the fungal infection quickly and of course it is vital that the organ or graft is not endangered during the treatment of the infection. In contrast, many cancer patients develop fungal infections and the effects of azole antifungals on the clearance and activation of antineoplastic agents does not appear to be well documented. Given that several anticancer agents rely on CYPs for either activation or clearance, it is reasonable to believe that azoles may interfere with these processes.

5.3.3 Non-competitive inhibition

Non-competitive inhibition does not involve the inhibitor and substrate competing for the same active site (Figure 5.1). In non-competitive inhibition, there is another site involved, known as the allosteric site, which is distant from the active site. Once a ligand binds this allosteric site, the conformation of the active site is automatically changed and it becomes less likely to bind the substrate and product formation tails off. This process of allosteric binding is another example of the endogenous control of product formation, perhaps by another product/substrate from a related or similar pathway. The Lineweaver–Burk plot will show a fall-off in V_{max} (enzyme cannot run at maximal rate) but K_m does not change, that is, the affinity of the substrate for the active site is unchanged.

It has been demonstrated experimentally that many drugs are non-competitive inhibitors of CYP isoforms. This means that the inhibitor is not binding at the active site and must exert some allosteric effect elsewhere. As knowledge of the active sites of many CYPs is still incomplete, we are still not fully aware as to exactly where these allosteric sites are and where they figure in the control of CYPs. In Chapter 3 (section 3.6.3), it was discussed

that CYP3A4 had more than one site available for binding and that various substrates could influence the binding of other substrates, probably connected with hormone metabolism. This potentially provides an hour-by-hour modulation of CYP activity, which is of course necessary during steroidal control of reproductive processes. It is likely that non-competitive inhibition is a result of drugs fitting these allosteric sites within most CYP isoforms and influencing binding of substrates to the main catalytic site. There are several examples of non-competitive inhibitors of CYPs. St John's Wort extract (hyperforin) is an inhibitor of CYP2D6 *in vitro*, although this does not translate to the clinical situation. Omeprazole and lansoprazole are non-competitive CYP3A4 inhibitors *in vitro*.

5.3.4 Uncompetitive inhibition

This is an unusual form of inhibition, where the inhibitor binds only to the enzyme/substrate complex (Figure 5.1). This has the effect of stimulating enzyme/substrate complex formation so increasing affinity (fall in K_m), although the enzyme/substrate/inhibitor complex is non-functional, so the V_{max} falls. This appears to be a relatively rare form of inhibition of human CYPs by therapeutic drugs, although the NSAID meloxicam is capable of uncompetitively inhibiting quinidine *in vitro*, it is not likely to be a significant clinical interaction. Some dietary agents contain inhibitors such as the flavonoid tangeretin, which is found in citrous fruits. Tangeritin is an uncompetitive inhibitor of CYP3A4 in human liver microsomes.

5.3.5 Mechanism-based inhibitors

This type of inhibition is outside the normal classification as outlined with competitive, non-competitive and uncompetitive inhibitions. Mechanism-based inhibition generally involves the same initial steps as a competitive inhibitor, but then the CYP catalytic cycle proceeds, reducing power is consumed and a metabolite is formed, which then occupies the P450 active site for a far longer period than the usual substrate would (Figure 5.2). To extend the 'machine gun' analogy of a CYP, mechanism-based inhibition is like failure to extract a spent cartridge. Mechanism-based inhibitors could occupy an allosteric site in a CYP and thus act as non-competitive inhibitors; macrolide antibiotics are sometimes classed as non-competitive inhibitors even though they are mechanism-based. The nearest mechanical analogy to a mechanism-based inhibitor would be the incorrect key turning fully in the lock and not opening, followed by difficult extraction of the key, or even the key breaking off in the lock. This form of inhibition can range from delayed product release, all the way to a violently reactive species-mediated covalent binding of a metabolite, which effectively destroys the active site and terminates the enzyme's activity. There are degrees of mechanism-based inhibition and moderately potent inhibitors such as the macrolides (like erythromycin and clarithomycin) are eventually removed from the CYP active site, but do not usually damage the enzyme. However, highly potent mechanism-based inhibitors such as the contents of grapefruit juice damage the enzyme to a degree that it is non-functional. This latter process is often termed 'suicide' inhibition. Clinically, a competitive inhibitor should wear off after just one or two half-lives, i.e. a few hours to a day or so, depending on a number of factors (inhibitor and substrate dosage, etc). The

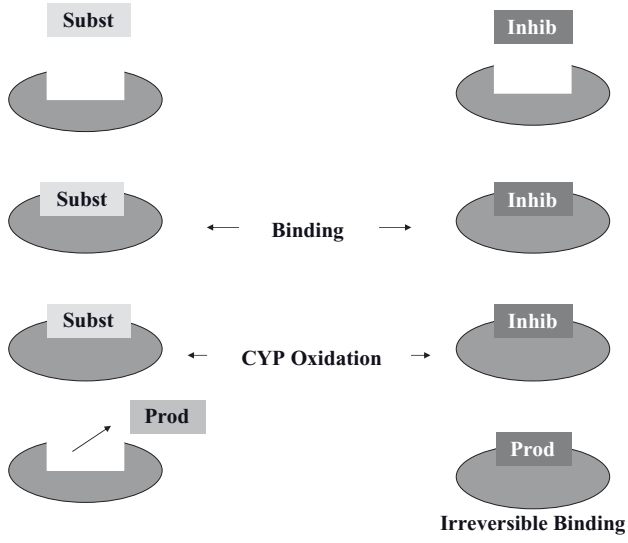

Figure 5.2 Scheme of normal substrate CYP binding (left) and mechanism-based inhibitor on the right, which results in the irreversible binding of product and inactivation of the CYP isoform

most extreme form of mechanism-based inhibition, such as grapefruit juice, norfluoxetine or MDMA-mediated 'suicide' inhibition, destroys the enzyme from one dose of inhibitor and this takes several days to resolve.

The effects of mechanism-based inhibition can be shown very clearly *in vitro*, where the potency of the inhibition is much greater when the CYP enzymes are incubated with NADPH and the compound prior to the addition of the usual substrate. This enables the enzymes to use the reducing power to run the catalytic cycle, which forms the reactive metabolite, which starts disabling the enzyme. The longer this process goes on, the more enzyme is disabled, so the inhibition becomes more potent over time. V_{max} falls and affinity decreases; then obviously the inhibition cannot be overcome by more substrate, as the law of mass action cannot apply because the inhibitor will be already covalently bound to key areas of the CYP active site. If the substrate is present in reasonably high concentrations prior to the appearance of the inhibitor, the substrate can protect the enzyme, although if the inhibitor continues to be present in adequate concentration, this protective effect will eventually be lost. This idea was exploited with neostigmine to protect military personnel against nerve agents. *In vivo*, mechanism-dependent inhibition lasts for days as previously mentioned, although the inhibition is clinically reversible, but as far as the individual enzyme is concerned, irreversible. This is because the clinical effect is consistent with the time taken for more P450 enzyme to be resynthesized to replace the inactivated enzyme. Obviously this will only be clinically reversible if the inhibitor was only dosed once, or over a short period. Mechanism-based inhibition is often summarized as follows:

- The inhibition becomes stronger over time.
- Inhibition does not progress without co-factors (NADPH).
- Presence of substrate slows the rate of inhibition by protecting the CYP.

- After inhibition, intact enzyme cannot be detected by analytical techniques (irreversibly inactivated).

Although there are examples of suicide inhibitors with other CYPs (such as ticlopidine with CYP2C19 and CYP2B6) CYP3A4 tends to be particularly susceptible to mechanism-based inhibition and although there are many different structures that can cause this effect, agents possessing a tertiary amine, acetylene or furan group are more likely to inhibit in this way. There are many clinically important non-competitive and mechanism-based 'suicide' inhibitors, which vary in the intensity of their inhibition. These include the macrolides (erythromycin, clarithromycin, oleandomycin), HIV protease inhibitors (ritonavir, indinavir, saquinavir, nelfinavir), the SSRIs (e.g. fluoxetine), anti cancer agents (tamoxifen and irinotecan) some antihypertensives (diltiazem, verapamil) and finally grapefruit juices. Unfortunately, all these compounds are metabolized by, and eventually inhibit, our major CYP, CYP3A4, whilst the illicit amphetamine derivative MDMA irreversibly blocks CYP2D6 (Appendix B). There is also evidence that the partially withdrawn second generation antidepressant nefazodone (section 5.3.8) is probably a mechanism-based CYP3A inhibitor, as its effect increases over time and it shows non-linear kinetics consistent with autoinhibition of CYP3A. If a prospective therapeutic agent is found early in drug development to be a potent mechanism-based CYP3A4 inhibitor *in vitro*, its days are generally numbered, unless it is virtually the only prospect available for a life-threatening disorder.

5.3.6 Mechanism-based inhibitors: grapefruit juice

Although patients have been heroically consuming grapefruit juice for their health for decades, it took until the late 1980s before its effects on drug clearance were noted and several more years before it was realized that there could be a major problem with drug interactions (History 1). This led to the regulatory authorities to remove terfenadine from the list of OTC medicines in the UK. The most noteworthy feature of the effect of grapefruit juice is its potency from a single 'dose' which coincides with a typical single breakfast intake of the juice, say around 200–300 ml. Studies with CYP3A substrates such as midazolam have shown that it can take up to three days before the effects wear off, which is consistent with the synthesis of new enzyme. The most interesting aspect is that grapefruit juice was thought not to inhibit hepatic CYP3A4, but gut CYP3A. It is useful to clarify this point. In the mid 1990s it was shown that grapefruit juice did not appear to inhibit the clearance of intravenously dosed drugs, although it would inhibit when the drug was orally dosed. More recently it has been established that *in vitro*, there is no difference in the inhibitory effects of other agents such as azoles (fluconazole and ketoconazole) on CYP3A4 from human gut or liver. Therefore, grapefruit juice should be technically capable of blocking hepatic CYP3A4. However, when modest amounts 200–300 mls of average strength juice are consumed, the combination of its irreversible CYP binding and a high gut CYP3A expression, means that virtually none of the inhibitor physically reaches the liver. Human volunteer studies have shown that if the dosage of grapefruit juice is very high, enough inhibitor escapes the gut CYP binding to block hepatic CYP3A4.

It might at first appear unusual that an inhibitor of gut wall metabolism would have such a devastating effect on systemic levels of a drug. However, there are a number of drugs that are subject to a very high gut wall component to their 'first-pass' metabolism

(or pre-systemic metabolism); these include midazolam, terfenadine, lovastatin, simvastatin and astemizole. Their gut CYP clearance is so high that if the juice inhibits it, the concentration reaching the liver can increase six- or sevenfold. If the liver normally only extracts a relatively minor proportion of the parent agent, then plasma levels of such drugs increase dramatically towards toxicity (see History 1). If the inhibited drug is fairly polar and is an OATP1A2 substrate, this toxic effect may be partly alleviated by grapefruit juice-mediated inhibition of this uptake (influx) pump (section 5.4.1), so reducing intestinal drug uptake to the portal circulation.

As has been mentioned, the inhibitor effects of grapefruit juice in high first-pass drugs is particularly clinically relevant as it can occur after one exposure of the juice. Obviously, the higher the pre-systemic metabolism of a drug (low bioavailability) the greater effect the juice is going to show. One interesting characteristic of the grapefruit juice effect is that the plasma half-lives of the drugs do not change, as the liver carries on metabolizing what drug it can take up, provided the grapefruit juice 'dose' is modest.

To summarize, drugs that should not be used with grapefruit juice are ones which:

- undergo high pre-systemic (enteric) metabolism;
- are metabolized by CYP3A;
- are narrow TI drugs;
- are agents with lethal off-target (unexpected and not related to their main use) pharmacological effects.

Here are some examples of the variable risk of grapefruit juice.

*Drugs that should **not** be taken with grapefruit juice:*
Terfenadine, statins (simvastatin, cerivastatin (withdrawn, 2001), atorvastatin, lovastatin), amiodarone, astemiszole, buspirone, indinavir, sildinafil, pimozide, cilostazol, etoposide, saquinavir, sirolimus, tacrolimus and cyclosporine.

Drugs that may be problematic with grapefruit juice:
Benzodiazepines (midazolam, triazolam, diazepam), cyclosporine, nifedipine, nisoldipine, synthetic opiates (methadone, dextromethorphan), macrolide antibiotics (erythromycin), carbamazepine, quinine, sertraline, azole antifungals (itraconazole), losartan and some steroids (prednisolone).

There are several drugs where others in their chemical class are inhibited by grapefruit juice but they are unaffected:

Fluvastatin, pravastatin, rosuvastatin and loratadine.

As to the precise component of grapefruit juice that is responsible for these effects, there are several agents that have been evaluated. The juice contains large numbers of flavonoids, which include naringin, a weak CYP inhibitor, which can be metabolized by gut bacteria to naringenin, which is a more potent inhibitor. However, the bergamottins (6′7′dihydroxybergamottin and bergamottin itself), a group of furanocoumarins, are among the most potent CYP inhibitors in the juice. The 6′7′dihydroxy derivative is much more

potent *in vitro* than bergamottin itself. Bergamottins are also found in Seville orange juice, which can exert similar effects to grapefruit juice. It appears that however the juice is prepared, either as concentrate, canned, as segments, or the fruit itself, there is no escape as the inhibitory effects still occur. It has also been found that a further furanocoumarin, epoxybergamottin, which is present in grapefruit peel, is also a CYP3A4 inhibitor. Recent studies have shown that grapefruit juice plus peel is a more potent inhibitor of CYP3A than without, suggesting that the furanocoumarin levels are higher in the peel than the rest of the fruit. It is also possible that epoxybergamottin from the peel undergoes hydrolysis to the most potent CYP3A4 inhibitor in the juice, 6′, 7′-dihydroxybergamottin. A number of other citrus fruits have flavonoid inhibitors also, such as the pomelo, which is a precursor of the grapefruit.

5.3.7 Mechanism-based inhibitors: cranberry juice

Cranberry juice has gained popularity due to its effectiveness in preventing urinary tract infections. It is said to achieve this by disrupting the ability of bacteria to adhere to epithelial cells and it also may have some bactericidal action. The disadvantage is that the concentrated juice is really very acidic and rather indigestible – after drinking it you might wonder if it could dissolve coins instead of just cleaning them. Not surprisingly, several glasses a day of the stuff are required to exert its effects, so to make it more tolerable it is often mixed with other juices or sweetened.

Although cranberry juice has been known for some time as an inhibitor of warfarin metabolism, studies aimed at exploring the effect with other drugs at first did not show any interaction with the CYP3A substrates cyclosporine or midazolam. These studies only looked at one brand of the juice and so a later more systematic report tested several brands *in vitro* for their effects on various CYPs prior to conducting a volunteer study. It was found that a concentrated juice increased the AUC of midazolam by around 30 per cent in the volunteers, whilst the blends with other juices and products were much less potent. The effect did not change the half-life of the drug, suggesting that gut metabolism of the drug was effectively inhibited by what transpired to be a lipophilic agent or agents within the cranberry juice. Given that midazolam is a CYP3A substrate and warfarin is cleared mainly by CYP2C9 and CYP1A2, it is possible that cranberry juice contains either multiple inhibitors of CYPs or a single very potent broad spectrum inhibitor.

Other fruit juices

There are hundreds of fruit preparations available that have been specifically marketed for their ‘superfood’ antioxidant capacities, such as purple grape, pomegranate, blueberry and acai juices. Many are not only extremely palatable, but also some studies have shown benefits in various conditions such as prostate cancer. Clearly many people will drink them in considerable quantities and gain benefits from their consumption, although little or nothing is known of their capacities to influence drug metabolism. As they all contain large numbers of diverse phenolics and are pharmacologically active, they should be consumed with some caution during drug therapy. It is likely that if there is a problem in this area, it will, of course, be discovered the hard way by some unlucky patients.

5.3.8 Mechanism-based inhibitors: SSRIs

Depression: clinical context

The TCAs and MAO inhibitors (MAOIs) are considered the first generation of antidepressants and these agents have many disadvantages. TCAs have narrow therapeutic indices, causing CNS and cardiotoxicity, alongside their unpleasant atropine-like (constipation, dry mouth) effects. MAOI use is problematic for pharmacodynamic reasons, such as the risk of serotonin syndrome when given with SSRIs and some other agents. In addition, MAOIs cause massive hypertension upon ingestion of tyramine-rich foods. As a response to these problems, a second generation of antidepressants appeared after the late 1980s. These broadly include the serotonin reuptake inhibitors (SSRIs), the serotonin and noadrenaline uptake inhibitors (SNRIs), as well as several other miscellaneous drugs. Of these drugs, the market penetration of SSRIs has been extraordinarily effective, as to date eight out of ten prescribed antidepressants are SSRIs. Some reports have argued that depression may be as great a world health burden as cardiovascular disease or HIV. Indeed, it has been estimated that 8–10 per cent of the world's population are either suffering from depression or will suffer from it in the future. Interestingly, there are two women for every man being treated for this condition.

Since the SSRIs replaced the TCAs as the first line drugs for depression, it is clear that they are undoubtedly safer than TCAs and MAOIs in terms of the dangers of overdose, which is a major advance. In 2002, deaths related to single drug overdoses for all TCAs were recorded as 35 per million prescriptions, compared with figures of 20 for MAOIs and only 1.6 for SSRIs. The SSRIs and other second line agents such as flupentixol are by far the safest antidepressant drugs, which is extremely important in the context of depressive illness.

Clinically, SSRIs are also much better tolerated than the TCAs, although the second generation drugs are no better than TCAs in terms of efficacy, as around 40 per cent of patients do not respond to both drug classes. As with the TCAs, it still takes around 5–7 weeks for a patient to fully respond to an SSRI, although it is generally reckoned that if there is no sign of a response in the first 14 days, chances are that even subsequent dose increases in that drug will be unsuccessful. It is often the case that failure of response and/or intolerance to one SSRI may be remedied by another SSRI. This has been demonstrated by a study which showed that more than 60 per cent of patient nonresponders to fluoxetine did respond to citalopram. However, in severe depression, SSRIs are less effective than the most effective TCA, amitriptyline. Clearly, the therapy of depression needs to progress further, as around a fifth of patients do not respond within two years to drug therapy with the consequential increased risk of suicide. The SSRIs, however, have been linked with a wide spectrum of physical and psychological side-effects and some, particularly paroxetine, have been associated with increased suicide risk, especially in the young. Although they have been wildly successful financially, on balance, their net benefit to humanity remains controversial.

The second generation antidepressants are mainly cleared by oxidative metabolism and some are metabolized to potent CYP inhibitors. As they are the current first line drugs for depression, adding them to an existing regimen of several drugs requires some thought as to the consequences of their considerable inhibitory effects. There are now six major SSRIs used in clinical practice: fluoxetine, paroxetine, fluvoxamine, citalopram,

escitalopram and sertraline. Although they can be potent *in vitro* inhibitors of CYPs such as CYP2D6, CYP1A2, CYP2C19 and CYP3A4, their *in vivo* effects are not always as potent. The major documented interactions for this widely used class of drugs are discussed below.

Fluoxetine and paroxetine

Fluoxetine (Prozac) is N-demethylated to S-norfluoxetine by CYP2D6, although there is some contribution from CYP2C9, CYP2C19 and even CYP3A4. The parent and its metabolite are cleared only fairly slowly and their half-lives can be as long as two weeks, as this is probably linked with inhibition of its own metabolism. Although CYP2D6 is inhibited most strongly, they can also have a less intense effect on CYP2C9 and CYP219. Regarding paroxetine (Paxil or Seroxat), the parent drug, rather than its metabolites, is the potent CYP2D6 inhibitor, although it does not seem to affect other CYPs. As these drugs are mechanistic 'suicide' inhibitors, their inhibitory effects can pose major therapeutic problems as they can last several weeks, depending on the patient. It is to be expected that these drugs will affect the clearance of the main classes of drugs mostly cleared by CYP2D6 (antipsychotics, TCAs, opiates, some beta-blockers and antiarrhythmics), although other agents cleared by the other main CYPs (CYP3A4, CYP2C9) may also be affected.

Antipsychotics: first generation drugs such as perphenazine (and to a lesser extent haloperidol), as well as newer drugs such as risperidone can have their AUCs increased several fold, leading to intensification of the classical antipsychotic side-effects such as tardive dyskinesias, akathisias and Parkinsonian symptoms. Fluoxetine appears to have a much more potent and better documented inhibition of the atypical antipsychotic clozapine, compared with paroxetine. Clozapine is termed 'atypical' as it is effective in many patients who do not respond to other antipsychotics, although it can cause fatal agranulocytosis and prescribing it is actually conditional on receipt by the dispenser of a normal white blood cell count (Chapter 8, section 8.5.6).

TCAs: three- to sixfold increases in plasma levels of desipramine have been documented and both fluoxetine and paroxetine are well known as potent inhibitors of TCAs (history 3).

AEDs: at higher doses, fluoxetine has caused carbamazepine toxicity, probably through inhibition of CYP3A4. The drug also has a potent inhibitory effect on phenytoin clearance, leading to a several fold increase in plasma levels of the AED and severe toxicity. It is likely that this occurs through CYP2C9 inhibition. Paroxetine has not been shown to cause this effect in a clinical trial.

Cardiovascular agents: fluoxetine is known to inhibit CYP2C9-mediated S-warfarin clearance as it causes an increase in the INR. Fluoxetine and paroxetine can also inhibit the clearance of some beta-blockers such as pindolol, metoprolol, propranolol and carvedilol, increasing AUCs by more than 75 per cent. Interestingly, pindolol actually augments the efficacy of SSRIs in some clinical studies. Fluoxetine, and possibly paroxetine may reduce the clearance of the antiarrhythmic propafenone.

Perhexiline: the clearance of the anti-anginal agent perhexiline is known to be strongly inhibited by paroxetine and this is a particular problem as perhexiline's half-life can exceed three weeks in some patients, so if any CYP2D6 inhibitor enters an established regimen

containing perhexiline, the concentrations of this drug could stay at toxic levels for many days, particularly in the elderly. Perhexiline metabolism is particularly subject to CYP2D6 polymorphisms (Chapter 7, section 7.2.3).

Opiates: fluoxetine and paroxetine can strongly impact their efficacy by preventing the CYP2D6-mediated demethylation required to convert opiate prodrugs, such as codeine, dihydrocodeine and tramadol (Chapter 7, section 7.2.3) to morphine or morphine-like derivatives.

Fluvoxamine

Although clinically fluvoxamine only has minor inhibitory effects on CYP2D6, it is cleared by this isoform as well as CYP1A2. It is, however, a potent inhibitor of CYP1A2 and CYP2C19, with less effect on CYP2C9 and CYP3A4.

Antipsychotics: whilst fluvoxamine can double plasma concentrations of olanzapine, it can cause four fold increases in the first generation agents and it can show an even greater inhibition on the atypical antipsychotic clozapine. This effect is caused by fluvoxamine's inhibitory effects on CYP1A2, CYPC19 and CYP3A4.

TCAs: interestingly, fluvoxamine's inhibitory action on CYP2C19 means that the demethylation of some TCAs is markedly inhibited (amitriptyline clomipramine and imipramine), whereas desipramine (which is desmethylimipramine) desmethylclomipramine and nortryptyline are not affected by fluvoxamine.

Cardiovascular agents: in an elderly patient established on warfarin, fluvoxamine nearly doubled the INR in four weeks. Fluvoxamine can increase warfarin levels by 65 per cent in patient studies, probably through inhibition of CYP1A2 (clears R-warfarin) and possibly an effect on CYP2C9 (clears S-warfarin). The SSRI has a similar potent effect on propranolol levels, causing several fold increases in drug levels by inhibition of CYP1A2 and CYP2C19.

Citalopram, escitalopram and sertraline

Citalopram is actually a racemic mixture of an active S-isomer and an inactive R-isomer, so the pure S-isomer was marketed cunningly in 2003 as escitalopram. Citalopram escitalopram and sertraline are much less potent CYP inhibitors than the other SSRIs and are consequently less likely to impact the clearance of other drugs. This makes them relatively straightforward to fit into existing multidrug regimens, particularly in the elderly and chronically ill. All three agents are relatively weak CYP2D6 inhibitors, although they can still increase the plasma levels of desipramine by 20–70 per cent, although this is much less likely to be translated into a serious intensification of side-effects as seen with more potent CYP2D6 inhibitors in this drug class. These three drugs do not appear to make much significant impact on the clearance of the older or newer antipsychotics, although at higher doses sertraline is likely to show more detectable inhibitory effects than the other two drugs. As the R-isomer of citalopram is thought to be a more potent inhibitor of CYPs than the S-isomer, it is probable that escitalopram is the weakest CYP inhibitor of the SSRIs and is the easiest to insert into existing clinical regimens.

Venlafaxine, duloxetine and reboxetine

If patients do not respond to SSRIs, the next class of drugs considered is usually the SNRIs, as they are safer than TCAs and MAOIs. Unfortunately, the complication in this context is the surprisingly high lethality in overdose of venlafaxine (13.2 fatalities per million prescriptions in 2002). This figure is actually uncomfortably much closer to the TCAs than the SSRIs, so its use is discouraged in severely depressed potentially suicidal patients, who are very likely to use their medication to attempt to end their lives. Venlafaxine may cause cardiovascular problems as well as convulsions in overdose. It is effectively a prodrug, being O-demethylated to O-desmethylvenlafaxine by CYP2D6. In its favour it is considered a low-level inhibitor of CYP2D6 and has a minor effect (20 per cent reduction) on the clearance of TCAs such as desipramine and CYP2D6 substrates in general.

Duloxetine is a newer SNRI, which is considered effective and easier to withdraw when compared with venlafaxine. The drug is cleared to non-active metabolites by CYP2D6 and CYP1A2. However, the CYP2D6 inhibiting effects of duloxetine are considered to be more potent than citalopram, escitalopram and sertaline, but less severe than the 'hard-core' inhibitors fluoxetine and paroxetine. Therefore duloxetine does have significant impact on the clearance of the major classes of drugs cleared by CYP2D6 (antipsychotics, TCAs, opiates, some beta-blockers and antiarrhythmics) and should be used with caution with these agents. Reboxetine is cleared by CYP3A4 and there is no CYP2D6 involvement in its metabolism. It is not thought to affect any CYP-mediated drug clearances.

Miscellaneous antidepressants

Other newer antidepressants either antagonize serotonin's effects and/or as well as inhibiting other biogenic amines' reuptake. Of these agents, nefazodone is the most problematic in terms of its practical usage. Nefazodone had some advantages over other agents, in that it had a low level of side effects, caused fewer sleep problems than the SSRIs and was effective in agitation and anxiety, which can accompany depression. However it is activated to a potent quinone-imine mechanism based inhibitor of CYP3A4 which makes it very difficult to use therapeutically, as the majority of prescribed agents are CYP3A4 substrates. Inhibition of many CYP3A4 substrates can lead to a form of fatal cardiotoxicity known as torsades des points (see section 5.5.2). The final nail in nefazodone's 'coffin' was its propensity to cause hepatobiliary toxicity, which leads to cholestasis and at least 20 fatal liver failures have been attributable to the drug, which has been withdrawn in most countries. Trazodone has a similar pharmacodynamic profile to nefazodone and it is a useful sedative which improves sleeplessness. However, it is structurally similar to nefazodone and perhaps not surprisingly it too has a reputation for causing hepatotoxicity, probably because CYP3A4 clears it to a reactive quinine-imine intermediate.

Of the other antidepressants, bupropion (known previously as amfebutamone) rejoices under the rather arch trade name of 'Wellbutrin'. It has also been marketed successfully as 'Zyban' as an aid to smoking cessation, due to the drug's ability to reduce nicotine dependence and cravings. Although it is structurally dissimilar to the SSRIs and is cleared by CYP2B6, it can exert some inhibitory effects on CYP2D6, doubling peak desipramine concentrations. Mirtazapine is another second line antidepressant, although its side-effects (causes patients to be sedated and put on weight) make it a less popular option. This drug

is cleared by CYP2D6, CYP3A4 and CYP1A2, but has been shown to have minimal inhibitory effects on CYP2D6 substrates. The antipsychotic flupentixol is used as an antidepressant and this is not thought to inhibit any of the main CYPs.

5.3.9 OTC herbal remedy inhibitors

Herbal preparations contain some mechanism-based inhibitors of various CYP isoforms and these are capable of making a similar impact on the clearance of prescribed drugs as grapefruit juice. These preparations are often spontaneously adopted by patients on the recommendation of a friend or after reading some form of publicity. Although patients should tell their doctors and healthcare workers they are taking these substances, they often do not. This is probably because they do not feel that it is relevant or important.

Capsaicin

Found in various hot peppers and used as flavourings in spicy foodstuffs. Pepper extracts have been used medicinally to treat many conditions from diabetes to inflammatory diseases. However, capsaicin is oxidized by CYP2E1 to reactive metabolites such as epoxides and phenoxy radicals that irreversibly inhibit the CYP isoform.

Liquorice extract

Contains an isoflavan known as glabridin, which is a potent mechanism-based inhibitor of CYP3A4, although it can competitively inhibit CYP2C9 (History 4). Extracts have been used in the South Pacific islands for many years for a wide variety of applications.

Goldenseal extract

Extract of goldenseal (*Hydrastis canadensis)* is capable of around a 50 per cent inhibition of CYP2D6 activity in clinical studies. This herb contains two alkaloids, berberine and hydrastine, although the former agent is thought to be the most potent inhibitor. It is strongly recommended that goldenseal should not be taken with CYP2D6 substrates, which includes antipsychotics, opiates, some antiarrhythmics and beta blockers, as well as illegal substrates (MDMA). Goldenseal has been used against infections for centuries by North American Indian tribes, although there is little hard evidence that it is beneficial. What is also important is that the herb should not be used by lactating and/or pregnant women, as berberine can cause uterine contractions and may also be toxic to infants.

Other possible herbal inhibitors

Among other popular herbal preparations, Gingko biloba has been shown to significantly inhibit CYP3A4-mediated nifedipine clearance, although most of the rest of the herbs

evaluated clinically so far appear not be inhibitors of CYP2D6 or any of the other major CYPs. These include milk thistle, kava-kava, black cohosh, echinacea and saw-palmetto. Kava-kava has been associated with liver damage, from mild toxicity, all the way to liver failure, requiring transplant.

5.4 Cell transport systems and inhibition

5.4.1 Uptake (Influx) transporters: OATPs

The main hepatic and gut uptake solute carriers (SLCs), or transporters, are known as organic anion transporting peptides, or OATPs. As outlined in Chapter 2, section 2.6.2, they operate a facilitated diffusion, swapping cellular molecules such as glutathione for extracellular endogenous anions and some predominantly polar drugs. In the context of this chapter, 'inhibition' particularly of CYPs has so far translated into accumulation and subsequent drug toxicity. However, in the complex process of absorption of some polar drugs which are too charged to just diffuse across cell membranes, inhibition of these influx transporters can cause two possible opposing outcomes, depending on whether the SLC inhibited is in the gut or the liver. If it is in the gut, then inhibition of the OATPs can effectively prevent systemic exposure, causing drug levels to disappear out of the therapeutic window. If the inhibition is in the liver, then the organ cannot take the agents out of the portal circulation to metabolize them, so systemic exposure increases dramatically.

Gut transporter inhibition

OATP1A2 is found at high levels on the luminal (inside, facing the gut contents) surface of the human small intestine and it is responsible for the facilitated diffusion of polar drugs such as fexofenadine. This agent is the active metabolite of terfenadine, which was withdrawn from OTC status due to the interaction with grapefruit juice. Rather ironically, grapefruit juice has been found to be a very potent inhibitor of OATP1A2, which is one of the major routes of intestinal uptake of fexofenadine. In effect, only 300 ml of grapefruit juice can reduce fexofenadine absorption by more than 60 per cent. This is caused by naringin, which as mentioned previously is not a strong inhibitor of CYPs, but it is an even more potent inhibitor of OATP1A2, than its fellow flavonoid glycoside hesperidin, which is found in orange juices. Naringin does not account for all the OATP1A2 inhibitory effects yielded by grapefruit juice and it is likely that many other juices contain inhibitors which could be as effective in impacting drug absorption. The specificity of this effect is remarkable, as fexofenadine is also a substrate for the efflux pump P-glycoprotein (see next section) and grapefruit juice is without effect on this transporter. Grapefruit and orange juice mediated inhibition of OATP1A2 lasts more than two hours after juice ingestion, so its persistence is capable of ensuring that on regular juice consumption, drug absorption can be severely and consistently impaired in daily life. There are several known drug substrates of OATP1A2 whose absorption could be reduced by grapefruit juice; these include steroid and thyroid hormones, ouabain and the anticancer agent methotrexate. It is likely that more OATP1A2 drug substrates will be discovered in the future. The OATPs are polymorphic

so this variability in expression in different individuals has the potential for significant prescribing problems in certain patient populations (Chapter 7, section 7.2.10).

Hepatic transporter inhibition

In the past it was often assumed that changes in the systemic exposure of a drug in the presence of another agent were simply caused by inhibition or induction of CYPs or some other enzyme. However, more detailed examination of some drug interactions revealed the role of inhibition of drug SLCs like the OATPs on systemic exposure of an agent. For example, a single dose of rifampicin neither inhibits CYPs nor induces them over a few hours, although it will increase atorvastatin levels by *sevenfold*. Similarly, rosuvastatin is not significantly cleared by CYPs, but is usually excreted mostly unchanged in faeces. The presence of cyclosporine increases the statin's plasma concentrations sevenfold. These two interactions are due to direct inhibition of hepatic OATP1B1 by rifampicin and cyclosporine, which both act to prevent hepatic uptake of the statins, so they cannot be metabolized. They bypass the liver and their bioavailability increases vastly, rendering the patient vulnerable to concentration-related statin toxicity, such as rhabdomyolysis. As mentioned in Chapter 4 (section 4.1), this condition results in mass breakdown of muscle fibres which can lead to renal failure. Indeed, clinically, the bioavailability of cerivastatin can be increased sixfold by concurrent administration of gemfibrozil and more than 50 deaths from renal failure linked to rhabdomyolysis led to the withdrawal of this statin in 2001. This particular interaction was due to a combination of gemfibrozil-mediated inhibition of OATP1B1 and CYP2C8 and exactly the same mechanism applies with gemfibrozil on co-administered repaglinide. Cyclosporine has a similar impact on the plasma levels of atorvastatin and simvastatin, again related to a combination of CYP3A4 as well as OATP1B1 inhibition.

5.4.2 Efflux transporters: P-glycoprotein (P-gp)

P-gp and systemic drug absorption and distribution

As P-gp has such a broad list of substrates, some lists of inhibitors are even longer than that of the substrates. Although there is growing awareness of the clinical problems posed by P-gp inhibition on drug bioavailability and toxicity, until recently it was very difficult to generalize and predict which classes of drug might be inhibitors of P-gp. For example, atorvastatin can inhibit P-gp *in vivo*, whilst pravastatin cannot. There are dozens of drugs which are known inhibitors of P-gp; among the most potent are verapamil, cyclosporine, ketoconazole, erythromycin, atorvastatin, mibefradil (withdrawn in 1998), spironolactone, doxorubicin, amiodarone, quinine and quinidine. Some P-gp inhibitors are also CYP3A inhibitors (erythromycin and ketoconazole), so they are capable of causing a marked impact on first pass of a P-gp substrate and especially a P-gp and CYP3A substrate. This has the effect of increasing the bioavailability of this category of drug. The anti-rejection agent tacrolimus has a narrow TI, so small changes in its bioavailability may have serious clinical effects. The causes of changes in first pass are not always easy to separate: tacrolimus is a CYP3A and P-gp substrate and blood levels increase in the presence of pomelo juice, probably mainly due to CYP3A inhibition rather than an effect on P-gp.

As detailed in the previous chapter (section 4.4.7) one of the most investigated P-gp substrates is digoxin, which is of relevance due to its narrow TI, toxicity and high likelihood of being prescribed with several inhibitors of P-gp in older patients. Many clinical studies have established that digoxin levels can rise by up to 60 per cent in the presence of multiple P-gp inhibitors such as verapamil, amiodarone and atorvastatin. This is particularly serious as the 3.5 ng/mL toxic plasma level can be easily exceeded. Digoxin is fortunately an extensively therapeutically monitored drug, but even this is complicated by the interference caused in digoxin immunoassays by P-gp inhibitors like spironolactone. This can show falsely low digoxin values, which of course could be life-threatening. It is important that P-gp-related problems should be considered during the addition of digoxin to existing complex regimes, or the addition of drugs to digoxin-containing regimes.

It has also become apparent that drug distribution to various tissues is strongly dependent on efflux and influx systems and despite adequate absorption, drugs may still completely fail to enter target tissues as either P-gp ejects them or a drug may penetrate tissues it does not normally enter in the presence of a P-gp inhibitor. Although opiates can curtail excessive gut motility and fluid production such as in diarrhoea, their central effects are normally too severe to use in this context. Loperamide does not penetrate the brain, as P-gp ejects it very efficiently, but it is effective at the level of the gut. In the presence of P-gp inhibitors like quinidine, it can reach the medulla and cause depression in respiration. As discussed in Chapter 4, section 4.4.7, the penetration of AEDs, antipsychotics and antidepressants into the CNS is almost certainly dependent on P-gp and other transporters. There is even evidence in animals that verapamil can increase CNS levels of antidepressants, so the role of P-gp in drug distribution and tissue penetration remains to be fully explored.

P-gp and cancer: therapeutic inhibition

As mentioned in the previous chapter (section 4.4.7), around half of all anti-cancer drugs are substrates for ABC-type transporters, hence, as long as a drug keeps entering a tumour cell, the PXR-mediated P-gp induction process will respond aggressively by pumping it out and blunting therapeutic effectiveness. Trying to reverse resistance to anticancer agents with existing P-gp inhibiting drugs is potentially a very cost and time-effective therapeutic strategy. Several drugs such as verapamil, the calcium channel antagonist and P-gp inhibitor were evaluated to this purpose. Unfortunately, as P-gp is so non-specific and ubiquitous, to date, no drug has been judged to be a selective, safe and predictable enough P-gp antagonist for clinical use by regulatory authorities. Clinical evaluation of some promising agents such as tariquidar has been beset with difficulties such as halted clinical trials, despite its good safety profile. However, a 2009 study showed the drug to be effective in increasing vinorelbine retention in target tissues, which underlines the potential of this approach.

There are several other agents under consideration for development as P-gp inhibitors. Among the most promising are the tyrosine kinase inhibitors (TKIs). These drugs were developed against chronic myeloid leukaemia (CML) and made a huge positive impact on survival figures. A whole series of TKIs, including imatinib, are potent P-gp inhibitors, partly because their pharmacodynamic mode of action is disruption of specific aspects of ATP metabolism, which powers the P-gp system. One of the most promising of these agents is nilotinib, which is a potent competitive inhibitor of P-gp pumps and does not affect their expression. The mode of its inhibition suggests that its effect should be controllable with

dose, rather than an irreversible inhibitor which would be much harder to deal with therapeutically. Possibly the only shadow on the use of TKIs, is that some CML patients have developed resistance to the earlier drugs such as imatinib and it is possible that P-gp in tumour cells may also develop resistance to the inhibition effect over time.

Overall, the contribution of the multi-factorial complexity of pre-systemic metabolism is still being researched and it is often difficult to establish what contribution cellular transport systems make to bioavailability. Indeed, it is emerging that one of the reasons for the very wide variety of drug bioavailability in modern medicine could be the sheer number of possible inhibitors and substrates that exist for P-gp in the diet, such as a number of natural products like the flavonols, which can be as potent as cyclosporine or verapamil as P-gp inhibitors. Natural dietary inhibitors have advantages in their general lack of toxicity, but the basic problem of a lack of predictability in their effects on P-gp substrates remains.

Since no two people's diets are identical, the impact of P-gp modulation on drug absorption could be simply too complex to unravel. Efflux transporter systems are discussed again under the heading of Phase III of metabolism, where MDR-type transporters remove conjugated metabolites from the cell using efflux pumps of similar structure and function to P-gp (Chapter 6, section 6.11).

5.5 Major clinical consequences of inhibition of drug clearance

5.5.1 Introduction

Although CYP inhibition can be competitive, non-competitive, uncompetitive or mechanism dependent, in clinical practice the main concern is how rapidly the inhibitor causes drug levels to climb towards toxicity and whether the toxic effects can be treated before serious injury or death results. As has been mentioned already, there are a number of major clinical conditions caused by inhibition of drug clearance that can overtake even healthy individuals in a matter of hours. These effects can be just a more intense version of the drug's usual pharmacodynamic effects which is dose related and reasonably predictable. If the drug is designed to lower blood pressure, then an accumulation will reduce it to dangerous levels. However, most drugs at higher concentrations can exert those unintended 'off target' pharmacodynamic effects as mentioned previously.

The speed at which these problems can be manifested cannot be overemphasized. Unlike induction processes, which take days, unintended pharmacological effects caused by drug accumulation happen within hours of regime change. Clearly, the best option is prevention:

- Firstly, by ensuring that healthcare professionals do not make mistakes; if these do occur, someone should immediately 'pick up the ball' and ensure that the mistake is not translated to a potentially fatal prescription that could be handed to a patient. As so many different categories of healthcare professional can now prescribe medicines, there may be more opportunity for breakdowns in communication, or alternatively, more prescribers should hopefully mean more vigilance.

- Secondly, the patient must be informed about the dangers of some drugs in combination with inhibitors. This should prevent patient intake of both dietary inhibitors and over-the-counter/herbal preparations that could block the metabolism of prescribed drugs. Even today, some patients are washing down their statins with grapefruit or other juices.

5.5.2 Torsades des pointes (TdP)

Mechanism of TdP

Probably the most feared off-target effect is torsade des points, which literally translated means 'twisting of the points' and often abbreviated to 'TdP'. This is the rather chaotic-looking and often lethal manifestation of ventricular tachycardia on an electrocardiogram (ECG), which can result from exposure to high concentrations of a worryingly large group of drugs. Although all the processes that lead to TdP are not fully understood, it is worthwhile revisiting some cardiac physiology to place it in context.

The heart performs two vital functions simultaneously; one is to convey deoxygenated blood to the lungs and the other is to distribute the reoxygenated blood to the body. To accomplish this, the heart has evolved into a coordinated dual pump. The right atrium receives deoxygenated blood from the periphery and sends it via the right ventricle to the lungs for re-oxygenation. The left atrium receives oxygenated blood from the lungs and propels it to the left ventricle, which pumps the blood back to the periphery. The heart valves act to ensure blood flow is unidirectional. During ECG analysis, the electrical control of the process is described with the letters PQRST.

The heart's pacemaker, the sinoatrial (SA) node, begins each pumping cycle by stimulating both the atria as well as the atrioventricular (AV) node. As the right atrium is closer to the SA node it depolarizes first. On the ECG, this is the 'P' wave (atrial depolarization). The AV node delays firing the ventricles until the atria have filled them with blood. The 'PR' interval is the time between start of atrial depolarization and the beginning of the next sequence, which is the large spike on the ECG called the 'QRS' complex, which is the depolarization of the ventricles firing. This is the real 'power stroke' of the heart, particularly the 'R', the largest wave, which represents the main mass of heart ventricular muscle contracting. Next, the 'ST' segment occurs and is flat, representing the beginning of the repolarization of the ventricles ready for the next contraction. The final 'T' wave is the completion of ventricular repolarization. The cellular key to the whole repolarization, or myocardial recovery process, is dependent on the delayed rectifier potassium current, known as I_{kr}. This is when potassium ions flow out of the myocardial cells through channels whose entrance is coded for by a gene known as hERG, KCNH2 or $K_v11.1$. Unfortunately, these hERG or I_{kr} channels contain a large entrance area, known as the vestibule, which is vulnerable to blockade by a very wide range of drugs. If the potassium current is slowed by this blockade, the 'QT interval', that is, the period elapsed between ventricular depolarization and repolarization will exceed 0.45–0.5 of a second. This effectively means that the heart's recovery time or rest period, goes on too long. However, the expression 'long QT interval' is an oversimplification.

In the ventricles, there are three main types of myocardial cell; the endocardial, epicardial and 'M' cells and they don't all repolarize at the same rate. The fastest repolarizers, the

epicardial cells, are at the peak of the T wave, whilst the slowest are the M cells. Indeed, the M cells are very prone to prolonging their repolarization. Because the myocardial cells have differential rates of repolarization, the extended nature of repolarization is termed 'transmural dispersion of repolarization' or TDR. Hence, if a drug blocks hERG/ I_{kr} in the M cells this causes an increase in TDR, which promotes early afterdepolarizations (EADs), which are abnormal depolarizations which are a sign of myocardial instability. These EADs promote the process of TdP, which is characterized by polymorphic ventricular tachycardia, arrhythmia and eventually fibrillation. Within a few minutes of the patient becoming aware of their irregular heartbeat, they are likely to faint (syncope). If TdP is drug-induced, recovery of heart rhythm is likely to depend on the potency and concentration of the hERG-blockading agent. Ideally, administration of intravenous magnesium sulphate, potassium ions and a beta-blocker should help the heart re-assert its rhythm and of course the causative agent should be withdrawn. If total cardiac disorganization and no detectable QRS complex is present, rapid defibrillation is necessary (History 1) or death will ensue.

TdP: causes and prevention

There are ten types of genetic abnormalities which lead to long QT syndrome, of which type 2 is related to abnormal hERG channels. These inherited conditions are surprisingly common (1 : 2000) and they are the most likely cause of sudden death in previously healthy young people. Women are at greater risk of TdP due to oestrogenic-effects on the QT interval and the majority of fatalities caused by terfenadine TdP were female. The aftermath of pregnancy (stress, sleeplessness, high oestrogens) can severely aggravate type 2 long QT syndrome, with individuals sometimes suffering multiple episodes of TdP in the weeks after birth. Obviously drugs which block hERG channels are strongly contraindicated in these individuals. Myocardial infarction is also a cause of long QT syndrome. Interestingly, some drugs, such as amiodarone and pentobarbitone can extend the QT interval but actually reduce the TDR. These drugs do not tend to cause TdP, so underscoring the importance of TDR in the development of TdP.

Unfortunately, more than 50 drugs are known to cause TdP. Among the most commonly listed include sotalol, procainamide, disopyramide, pimozide, the anti-migraines sumatriptan, naratriptan and zolmitriptan, (cisapride was withdrawn in 2000 for this reason); some SSRIs, such as fluoxetine, paroxetine and sertraline; antipsychotics, like chlorpromazine, haloperidol and risperidone; non-sedative antihistamines such as terfenadine and astemizole (withdrawn), several TCAs, the macrolides erythromycin and clarithomycin; the immunosuppressive tacrolimus and several azole antifungals. With some fluoroquinolones, the potency of hERG blockade varies markedly. Sparfloxacin, gatifloxacin, moxifloxacin and levofloxacin are potent enough to cause clinical effects, whilst ciprofloxacin and ofloxacin are not a problem. Obviously any inhibitor that prevents clearance of these agents could precipitate QT interval prolongation. This has been found to be the case with a number of CYP3A4 substrates, including pimozide, cisapride, terfenadine and astemizole. Clearly it is important to avoid any possibility of triggering QT interval problems; for example, terfenadine can be replaced with its active metabolite, fexofenadine (carboxyterfenadine).

It is now required as early as possible in drug development to test whether a new prospective therapeutic drug has an effect on cloned human hERG channels in several *in vitro*

assays. This has led to wastage of candidate compounds of between 40 and 70 per cent as so many agents appear to block the channels in the assays. Whether all these compounds really would cause TdP clinically is not known. A great deal of effort is being made to devise more realistic test systems which will ultimately provide accurate clinical predictions of the TdP TI for each new agent in comparison with positive controls, such as moxifloxicin or sparfloxacin.

5.5.3 Sedative effects

The risk of sedation is obviously less of a problem in the home, rather than perhaps operating heavy machinery with razor-sharp rotating blades. The co-administration of inhibitors with drugs such as the benzodiazepines and others such as buspirone can potentiate their sedative effects markedly. Benzodiazepines in general have their disadvantages, as they can induce dependence and withdrawal problems (sometimes euphemistically called 'discontinuation syndrome'). However they remain very useful for short-term treatment of anxiety. Clinically, depression is often inextricably linked with anxiety disorders and although SSRIs are the first choice for depressive disorders, their characteristic delayed onset applies to anxiety as well as depression, so benzodiazepines are co-administered because they treat the anxiety immediately. As the SSRI starts to improve the depression as well as the anxiety, the benzodiazepine is gradually withdrawn. Unfortunately, given the effects of SSRIs on CYPs, this can sometimes retard the clearance of the benzodiazepine, leading to intensification of their major side effects. Fluvoxamine presents the most severe interaction, as it causes sedation and impairment of psychomotor activity with most of the benzodiazepines, such as diazepam (Valium) and alprazolam (Xanax), increasing their plasma levels by up to 70 per cent. It is recommended that less than half the usual dose of these benzodiazepines should be used with fluvoxamine, or one of the benzodiazepines cleared by glucuronidation should be used (such as lorazepam). Fluoxetine retards alprazolam's clearance and intensifies its side-effects, whereas sertaline, paroxetine and citalopram do not appear to be a problem.

The CYP3A4 substrate midazolam is used as a sedative in intensive care units and particularly in children (Chapter 7, section 7.3.2) and there is already a large variation in individual clearances, so any inhibition of the metabolism of this drug may cause excessive sedation. The azole inhibitors, in the expected order of severity, can seriously retard midazolam clearance: ketoconazole > itraconazole > fluconazole/voriconazole, as can any CYP3A4 inhibitor. Co-administration of SSRIs and other CYP3A4 substrates may cause accumulation, such as with carbamazepine (History 4). In addition to sedation, the effects of high plasma levels of carbamazepine can lead to mental confusion, ataxia (staggering gait) and even unconsciousness.

5.5.4 Muscle damage (rhabdomyolysis)

This is when striated muscle disintegrates and the released myoglobin enters the blood and then the urine, eventually leading to renal failure. Blunt force trauma, some infections, burns, electric shock, ischaemia, or severe exercise usually cause it. However, it can also occur in response to exposure of some drugs and chemicals. Heroin and solvent abusers

can develop it, but it can occur in response to statin treatment. The use of cerivastatin has been curtailed as rhabdomyolysis has occurred when the drug was used with gemfibrozil. It seems that if statin plasma levels rise to high levels, creatine kinase levels are elevated in plasma and this can lead to rhabdomyolysis. The statins have become increasingly important as their cholesterol-lowering effects are a valuable component of the general effort to reduce ischaemic cardiovascular disease. They are seen as quite safe drugs and some statins are now available OTC at relatively low doses. However, simvastatin, lovastatin, atorvastatin and cerivastatin are CYP3A4 substrates and are vulnerable to elevation of plasma levels in the presence of potent CYP3A4 inhibitors. This is a particular concern with inhibitors such as grapefruit juice, the azoles and erythromycin. If statin therapy must be continued in the presence of a 3A4 inhibitor, it would be wise to use those which are cleared by other CYPs, such as fluvastatin (CYP2C9) or pravastatin. Patients taking immunosuppressants and those with renal problems are more prone to develop rhabdomyolysis than others and are at particular risk. It is safest to avoid the interaction by substituting non-inhibiting drugs or statins cleared by other CYPs.

5.5.5 Excessive hypotension

As you will, of course, no doubt remember from your pharmacology studies, there are several different drug options in the management of hypertension. This is fortunate, as this condition is very common, sometimes does not respond to therapy and deteriorates with age. This means that antihypertensives of various types are prescribed in vast amounts to older patients, usually for many years. These include CYP3A4 substrates, such as the dihydropyridines (nifedipine, felodipine, nicardipine, and nimodipine). These calcium channel blockers very useful and well tolerated. Nicardipine is more selective for heart vessels, while nimodipine is more effective in cerebral vessels. However, the most common problem with them is excessive vasodilatation that can lead to postural hypotension, dizziness, and headache. They can work too well, to the point where blood pressure is insufficient to force blood through diseased coronary arteries and they can cause reflex tachycardia; these effects can make some forms of angina worse.

Obviously any marked changes in the clearance of these potent drugs could lead to potentially major deleterious changes in cardiovascular function. It is easy to see how the azoles and the macrolide inhibitors could cause severe cardiovascular problems due to non-clearance of dihydropyridines. Their high pre-systemic metabolism means that grapefruit juice would have a particularly potent and possibly life-threatening effect. Interestingly, the lack of gut metabolism of amlodipine makes this agent less susceptible to grapefruit juice interactions. Any antihypertensive agent that is a CYP3A4 substrate will be liable to cause excessive reductions in blood pressure if they accumulate in the presence of an inhibitor. This is a particularly problematic effect in the case of suicide inhibitors like grapefruit juice and norfluoxetine.

5.5.6 Ergotism

Until the advent of the highly effective group of triptan 5HT agonists, severe migraine sufferers were faced with the prospect of using ergotamine tartrate or suffering the

extremely unpleasant pain that this syndrome can inflict. As a migraine sufferer myself, my one experience with ergotamine in 1980 led to a painful effect as if there were wires tightening inside my calf muscles. This took two days to wear off and I still suffered the headache. Ergotamine can also cause severe neural derangement known as 'St Anthony's fire'. This sometimes affected people who ate bread that had been made with mouldy flour because it contained a considerable dose of ergot alkaloids. Ergotamine is cleared by CYP3A4, so the effects of any inhibitor of this isoform on ergot clearance would lead to an extremely grim series of peripheral and CNS symptoms. Fortunately, the triptans (sumatriptan, naratriptan, zolmatriptan, etc.) are the mainstay of acute migraine treatment and the ergotism problem with CYP3A4 inhibitors should now be very rare.

5.5.7 Excessive anticoagulation

As discussed in Chapter 4, warfarin remains the most commonly used agent for the treatment of a number of conditions where thrombosis is at high risk, such as those with replacement heart valves, atrial fibrillation and deep venous thrombosis. Inhibition of CYP2C9-mediated S-isomer clearance has more impact on warfarin's pharmacological effect than effects on the other CYPs, although they do show some impact. Cimetidine, an inhibitor of 1A2 and 2C19 (History 5), is not recommended for concurrent therapy with warfarin, although it is available OTC and thus there is potential for a moderate increase in prothrombin time. Potent CYP2C9 inhibitors, such as some azoles and SSRIs, can cause a major increase in warfarin's half-life and thus dangerously magnify its anticoagulating effects. A number of other drugs can also partially inhibit warfarin metabolism and lead to increases in prothrombin time; these include amiodarone, trimethoprim, isoniazid, sulphamethoxazole (in the old 'bactrim' combination with trimethoprim), sulfinpyrazone, propafenone, metronidazole (inhibits the S-isomer), some statins and disulfiram.

Warfarin acts by antagonizing the effects of vitamin K, which is necessary for the formation of several clotting factors. A therapeutic dose basically knocks out around half the usual formation of these factors. It is worth noting that changes to warfarin clearance can take some time to be reflected in changes in prothrombin time. This is because the effect of the drug depends on the rate of removal of blood clotting factors that had already been formed before the drug took effect. From an initial dose, it can take up to a day and a half before any change in INR occurs. The drug already has a long half-life (1–3 days) so when a drug increases warfarin's clearance, it will take perhaps 1–2 days before an effect is seen in terms of prothrombin time and this effect will persist, again for some days. The most serious effects of excessive coagulation are GI tract bleeding and intracranial haemorrhage, both of which can be fatal.

5.6 Use of inhibitors for positive clinical intervention

5.6.1 Introduction

The effects of inhibitors on the concentrations of CYP substrates can be so dramatic that it has occurred to a number of scientists and clinicians to explore various strategies to exploit this effect to provide some form of benefit to the patient. This can take the form

of preventing the formation of a toxic metabolite, to modulate hormone levels in cancer chemotherapy, or even to reduce the cost of prescribing an expensive drug. The key factor in this approach is whether the increased burden and risk to the patient of taking another drug in what is effectively an unlicensed application is really beneficial to the patient. As you can imagine, these applications have met with varying levels of success and acceptance and are discussed below.

5.6.2 The use of inhibitors to arrest hormone-dependent tumours

This is by far the most successful clinical application of inhibitors of CYP-mediated metabolism, although the development of these drugs has taken nearly 30 years. Most breast cancers are hormone dependent for their progression, so two treatment strategies can be pursued, one to blockade the receptors (tamoxifen) and the other to prevent the oestrogens being formed from androgenic precursors, such as 4-hydroxyandrostenedione. Unfortunately, the 'ATAC' Trial showed no benefit in combining the two strategies. The main distinguishing characteristic of an oestrogenic molecule to an oestrogen receptor is the aromatized 'A' ring and the androgenic precursor is subject to a series of CYP19 'aromatase'-mediated reactions, rather like a spot-welding robot in car factories, which result in the aromatic 'A' ring; the oestrogen is completed by CYP17 that also alters the substituents on the D (far right-hand) ring of the steroid. The first aromatase inhibitor to be useful clinically was aminoglutethimide, which blocked oestrogen formation in peripheral tissues. However, this drug was quite toxic and resulted in haematological problems and potentially lethal agranulocytosis (loss of all neutrophils, Chapter 8, section 8.5.4).

There was interest in the 1970s that ketoconazole had the potential to treat some breast cancers, although it was not pursued due to its hepatotoxicity. A great deal of research into aromatase led to the development of anastrazole (Arimidex®), exemestane (Aromasin®), and letrozole (Femara®). These agents are vastly more potent than aminoglutethimide, although the latter agent is still used in Cushing's syndrome and to control adrenal hormone formation in post-menopausal women. Anastrazole is highly effective in abolishing oestrogen formation, although it does show the side effects expected from loss of oestrogen, such as loss of body strength, nausea and hot flushes. However, it is superior to tamoxifen in hormone sensitive early breast cancer and more anastrazole-treated patients remained disease-free for up to four years, showing that its advantages over tamoxifen are long-lasting. The aromatase inhibitors have been applied to gynaecological cancers, such as relapsed ovarian cancer and endometrial cancers with mixed responses. They have shown some efficacy in rarer conditions such as endometrial stromal sarcoma and clinical research is ongoing in this area. Anastrazole has also been applied successfully to other hormone dependent conditions, such as persistent pain from endometriosis.

5.6.3 The use of inhibitors to reduce toxic metabolite formation

There are a number of P450-mediated metabolic reactions which result in short-lived, highly unstable and exceedingly toxic products which are capable of severe toxicity. Some of these agents will be described in detail in Chapter 8. The capacity of CYPs to form

potentially toxic metabolites is usually rooted in the oxidation of a metabolized molecule, which, according to its structure, may become highly unstable. Some metabolites are so reactive they destroy the enzyme, which has been seen in 'suicide' inhibitors mentioned earlier. Slightly less reactive metabolites might enter the rest of the hepatocyte and react with protein structures resulting in change in function and eventual necrosis or apoptosis, depending on the rapidity of formation and the reactivity.

Paracetamol-mediated hepatic necrosis

The best example of this is the metabolism of paracetamol in overdose to reactive quinone-imine derivatives. The resulting damage leads to necrosis of the liver. This process is covered in more detail in Chapter 8 (section 8.4.2). It was shown in the 1980s that various inhibitors could slow or prevent the metabolism of paracetamol to its reactive metabolites and several animal studies were carried out to show that this could work clinically. However, this approach never became a clinical reality. Acutely, patients presenting before liver damage was sufficient to cause necrosis could be saved with glutathione (GSH) precursor supplements, such as N-acetylcysteine. After liver damage was too severe, then it would either be time for a transplant or a funeral. Considering a preventative approach, including an inhibitor in paracetamol tablets could potentially prevent the formation of the toxic metabolite without affecting clearance that much, as 95 per cent of paracetamol clearance is accomplished by sulphation and glucuronidation. However, the main inhibitors of CYP2E1 are mostly sulphur-containing agents and inhibit other enzymes such as alcohol dehydrogenase and aldehyde dehydrogenase. It would not be practical to include a CYP2E1 inhibitor in paracetamol tablets because as soon as the patient drank any alcohol, they would be violently ill (see 'Use of inhibition in alcoholism' below). So the use of a CYP inhibitor to prevent paracetamol-mediated hepatic necrosis is a dead end.

However, another route of inhibition may in the future have some therapeutic value. A small proportion of paracetamol is cleared to a reactive cytotoxic metabolite (NAPQI: Chapter 8, section 8.4.2) and this can be detoxified by GSH, either directly, or through catalysis by the cytosolic glutathione-S-transferases (GSTs). Studies in knockout mice have shown that in animals where GST pi has been deleted they sustain less hepatoxicity than those with intact enzyme. This seems unexpected, as the enzyme should be necessary to catalyze NAPQI clearance to a benign mercapturate. In both knockout and control animals, GSH levels were depleted, so thiol consumption during detoxification occurs. So it is possible that GST pi depletes GSH in a process that is not relevant to NAPQI clearance. Subsequent studies associated low GST pi levels found in female mice with much improved resistance to paracetamol-induced hepatic necrosis compared with males. Although there does not appear to be a sex-bias to paracetamol-mediated necrosis in man, there is already strong interest in developing GST inhibitors as part of efforts to prevent GST-catalyzed detoxification of anti-cancer agents, as GSTs can be hugely overexpressed in cancer cells (see next chapter, section 6.5.3). The ability of GST inhibitors quinine and ethacrynic acid to restore tumour cell sensitivity to doxorubicin in cell lines shows that this approach has potential. Whatever the role of GST in paracetamol-induced necrosis, in the long term, new more specific GST inhibitors might prevent hepatotoxicity in the later stages of liver damage in paracetamol overdose and in combination with N-acetyl cysteine, the standard antidote, may rescue those previously doomed to liver failure.

Dapsone-mediated methaemoglobin formation

The sulphone drug dapsone is used in leprosy therapy in the Third World, but is also a useful anti-inflammatory agent in conditions which feature the infiltration of activated neutrophils, such as the skin condition dermatitis herpetiformis (DH). The drug is very effective and will suppress DH symptoms, such as the intense pruritus and skin eruptions within hours of dosage. This brings rapid relief from a condition that can make patients' lives intolerable. However, the drug causes methaemoglobin formation, which is due to the CYP2C9-mediated oxidation of the drug to a hydroxylamine (Chapter 8, Figure 8.3). This particular hydroxylamine is a relatively poor substrate for glucuronyl transferase and the greater the drug dose, the more hydroxylamine escapes conjugation, enters the circulation and oxidizes haemoglobin to methaemoglobin, which cannot carry oxygen. The more methaemoglobin formed as a percentage of total haemoglobin, the more tissue anoxia occurs; symptoms range from a headache/hangover-like effects at sub 10 per cent levels to hospitalization (nausea, tiredness and breathing problems) at 20 per cent. The standard daily dosage in leprosy of around 100 mg of dapsone usually leads to around the 5–8 per cent level of methaemoglobin and it is just about tolerable to most patients, in the light of the alternative of the progression of the disease. However, with DH, the dosage varies wildly from patient to patient. Some can be fully controlled on 25 mg per week, whilst others must take 400 plus mg of dapsone daily and the condition is only partially suppressed. At this dosage, the patient's quality of life is much diminished by the drug and the only reason to persist with treatment might be a lack of effect of the only other drug alternative (sulphapyridine). Even moderately effective drug therapy with high side effects is better than the recurrence of the disease symptoms.

In rat studies in the late 1980s a number of potential inhibitors were tested for their ability to retard or arrest dapsone-dependent methaemoglobin formation. Piperonyl butoxide, an insecticide and broad CYP inhibitor, was effective, as was cimetidine, although ketaconazole and methimizole were not. Although it was known then that animal and human CYPs were not the same, the main families of CYPs were still being unravelled. These animal studies were reinforced by *in vitro* work with human liver microsomes, which again showed that cimetidine could be effective. This work led to volunteer studies that showed that cimetidine on a single dose would reduce hydroxylamine formation. Multiple dose studies in animals were also promising and a clinical study in DH patients finally showed that the hydroxylamine formation could be reduced, but not abolished, although methaemoglobin formation fell by nearly 30 per cent and the drug retained its clinical effects and improved patient tolerance. Subsequent studies underscored the possibilities of using cimetidine in patients who could only respond to high dapsone doses and would normally have had to endure considerable methaemoglobin formation. This method has reached clinical practice in some areas.

Interestingly, it was clear that the rat was a poor model for man, in that cimetidine was far more effective as an inhibitor in the rat. It would probably have been undesirable, though, to use too potent an inhibitor long term, as endogenous CYP functions would have been severely affected. As a coda to this work, in 2003 the antioxidant dihydrolipoic acid (formed from lipoic acid in human erythrocytes *in vivo*) was found to partially block the reaction between the hydroxylamine and oxyhaemoglobin *in vitro*. Hence, a study could one day be designed to use lipoic acid and cimetidine in combination to make an even

larger reduction in methaemoglobin formation in patients on high-dosage dapsone, without completely blocking the CYPs.

5.6.4 Use of inhibition in alcoholism

The effects of alcoholism are covered in more detail in Chapter 7, section 7.7. Among the treatments for alcoholism is the use of the potent inhibitor of aldehyde dehydrogenase and CYP2E1, known as disulfiram (antabuse). This compound is taken by the alcoholic to help the abstinence process. In the alcoholic, ethanol is cleared mainly by alcohol dehydrogenase to acetaldehyde, which is cleared by aldehyde dehydrogenase to acetic acid and water. If alcohol is imbibed during antabuse treatment, the clearance of ethanol to acetaldehyde occurs, but the process stops there and acetaldehyde accumulates, causing a severe effect that includes flushing, nausea, vomiting and sweating. Even small amounts of alcohol will show this effect, such as in accidental consumption. There are many medicinal and hygiene-based products, ranging from cough mixtures to mouthwashes that can contain from 5–20 per cent ethanol, so patient awareness is valuable in this context.

5.7 Summary

Inhibition of drug clearance has the greatest clinical impact on a patient's well-being, in terms of the rapidity of the effect and its severity. This is particularly important when the patient or healthcare professional is for a time unaware that a potent inhibitor has been consumed. Currently, in the light of the numbers of potent dietary and OTC inhibitors available to the patient, it is as important to educate the patient in the dangers of inhibition of narrow TI drugs as it is to educate the healthcare professional.

6 Conjugation and Transport Processes

6.1 Introduction

You might recall from Chapters 1 and 2 that drugs and many endogenous chemicals, such as steroids, are essentially oil-soluble (lipophilic) agents that exploit their lipophilicity and stability to carry out their biological function, crossing membranes, binding specific carrier molecules and finally entering cells to bind to the appropriate receptors. This lipophilicity and stability also means that they are very hard to control. In this context, control entails the termination of biological function and subsequent removal from the body. With steroid hormones, for example, the ability to modulate synthesis and destruction allows the exertion of exceedingly fine control over the structural and biochemical changes they promote. As radical chemistry was involved in their assembly, body clearance of such stable molecules means that radical chemistry must also be applied to change these oil-soluble agents to water solubility.

So the objectives of metabolizing systems could be summed up thus:

- To terminate the pharmacological effect of the molecule.
- Make the molecule so water-soluble that it cannot escape clearance, preferably by more than one route to absolutely guarantee its removal.

These objectives could be accomplished by:

- Changing the molecular shape so it no longer binds to its receptors.
- Changing the molecular lipophilicity to hydrophilicity to ensure high water solubility.
- Making the molecule larger and heavier, so it can be eliminated in bile as well as urine.
- Efflux pump systems, which ensure that a highly water-soluble metabolite actually leaves the cell to enter the bloodstream, before it is excreted in bile and urine.

The CYP system ensures that virtually all lipophilic (and many hydrophilic) molecules can be oxidized and made at least slightly more water-soluble. However, many hydroxylated metabolites are not water-soluble enough to ensure that they remain in urine when

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filtered by the kidney and not be reabsorbed into the surrounding lipophilic tissue of the collecting tubes. So CYP-mediated metabolism can increase hydrophilicity, but it does not always increase it enough and it certainly does not make the molecule any bigger and heavier, indeed, sometimes the molecule becomes lighter as alkyl groups are removed during O, N and S-dealkylations. CYP-mediated metabolism does not always alter the pharmacological effects of the drug either; in the case of the benzodiazepines and other drugs, metabolites do exert a great deal of pharmacological effect.

However, CYPs do perform two essential tasks: the initial destabilization of the molecule, creating a 'handle' on it. A crude analogy would be to liken a stable lipophilic molecule to a solid block of steel, and the CYP would be the high-speed drill that bores a hole in it, so that a hook or bolt could be attached. CYPs also 'unmask' groups that could be more reactive for further metabolism. The best examples of this would be the various dealkylation reactions. These reveal groups such as amines, hydroxyl and sulphides that can undergo more metabolism to make the molecule heavier, a different shape and more water-soluble.

This CYP-mediated preparation can make the molecule vulnerable to the attachment of a very water-soluble and plentiful agent to the drug or steroid, which accomplishes the objectives of metabolism. This is achieved through the attachment of a modified glucose molecule (glucuronidation), or a soluble salt such as a sulphate (sulphation) to the prepared site. Both adducts usually make the drug into a stable, heavier and water-soluble ex-drug.

Some oxidative metabolites are more than soluble enough to be found in urine and some conjugated metabolites do not require any prior oxidation 'preparation' before they are conjugated. However, with many drugs, their stability and lipophilicity mean that their clearance must take more than one metabolic operation to make them water-soluble.

A final problem is created by the formation of highly water-soluble metabolites, in that they can be too hydrophilic to easily leave the cell. The control of this process is sometimes termed Phase III of metabolism. In this case, a series of molecule pump systems have evolved, or efflux transporters, which provide a powered gradient to encourage the egress of these molecules into the interstitial cell fluid. Once out of the cell, there is no way back for such hydrophilic molecules and from the blood they are filtered by the kidneys into urine.

6.2 Glucuronidation

6.2.1 UGTs

Glucuronidation is the largest capacity conjugative clearance system in man and is accomplished by a set of enzymes known as UDP (uridine diphosphate) glucuronosyl transferases, or UGTs. These are found in many tissues, but the greatest concentration is in the liver. Their main homeostatic roles are in the control of bilirubin, bile acid and steroid hormone conjugation, which facilitates clearance into bile and urine. These functions are particularly important, as bilirubin and bile acids are toxic if they accumulate and of course failure to clear steroid hormones results in loss of homeostatic control of several processes.

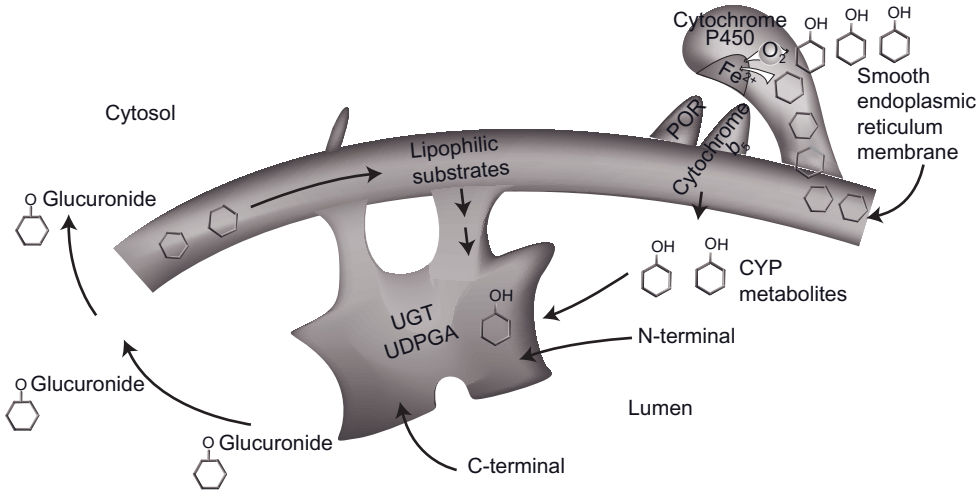


Figure 6.1 Scheme showing the approximate position of glucuronyl transferases (UGTs) in the smooth endoplasmic reticulum in relation to that of CYPs

The active site of a UGT is positioned inside the lumen of the endoplasmic reticulum (SER) membrane (Figure 6.1) and like several other enzymes (it seems to me, anyway) is vaguely butterfly-shaped, with a large C-terminal 'wing' which binds the co-factor (UDPGA, see below). The C-terminal area is attached to an anchor protein structure that runs through the SER membrane. There is a small 'tail' section that sticks out into the cytoplasm. The other 'wing' of the butterfly is the N-terminal section, which binds the substrate. This section also has a portion of its structure embedded in the SER membrane, which is thought to act like a 'scoop' or access channel (rather like the CYPs) to facilitate the entry of lipophilic substrates which 'flow' through the SER membranes. Effectively, UGTs are SER, transmembrane, luminal enzymes. Why they are luminal is not clear, but it does place them in close proximity to the CYP isoforms and also ensures that they meet lipophilic agents directly themselves, so it is not always necessary for the CYPs to oxidize the drug prior to glucuronidation (Figure 6.1). These enzymes utilize and activate glucose and convert a huge number of chemicals to beta-D-glucopyranosiduronic acids, or glucuronides, by a nucleophilic substitution reaction. Interestingly, human UGTs do not function as rapidly as those of other animals, such as dogs.

6.2.2 UGT mode of operation

All UGT systems process their substrates in the same basic way (Figure 6.2). The enzyme's objective is to place a polar glucose derivative in the correct position on the substrate and glucose itself is not reactive enough to achieve this unless it is activated in some way. So the first stage takes place in the cytoplasm away from the SER. This is the preparation of glucose to make it thermodynamically easy for the enzyme to catalyze its reaction with

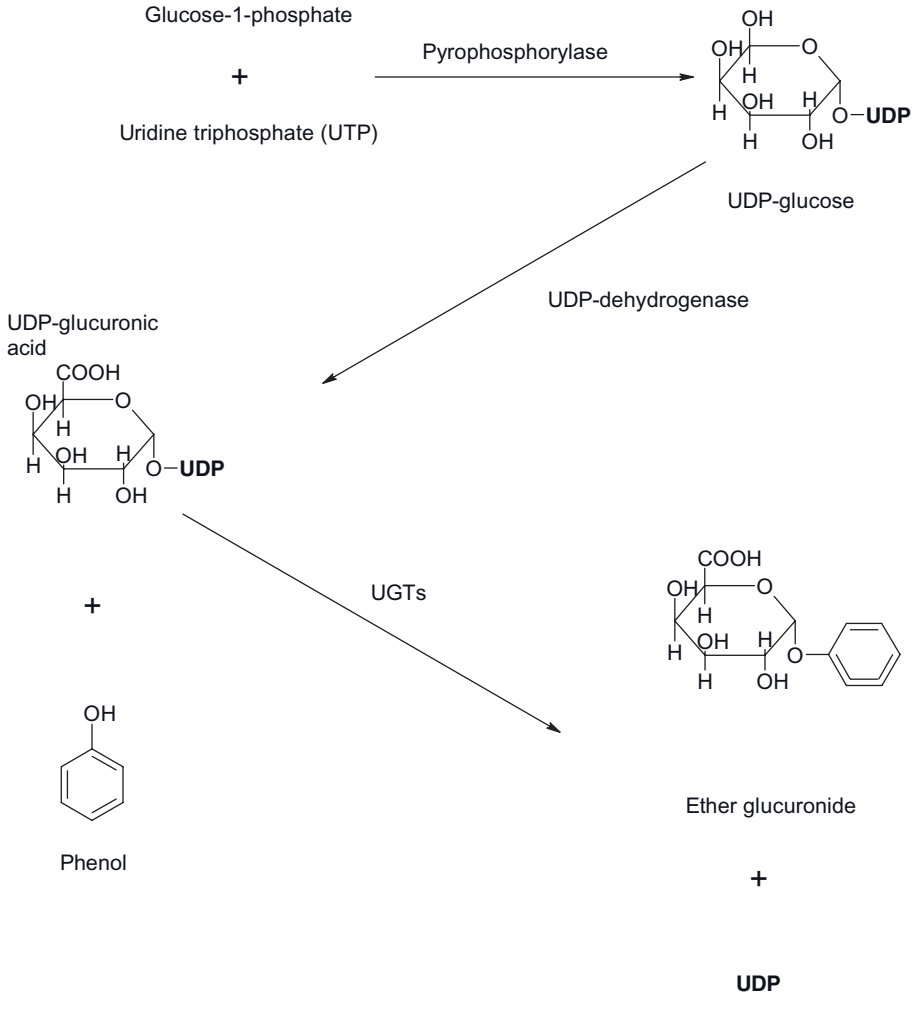


Figure 6.2 Process scheme for the preparation of glucose to UDP glucuronic acid, the co-factor for UGT-mediated formation of an ether glucuronide of phenol

the substrate. Glucose-1-phosphate is reacted with uridine triphosphate (UTP) to eventually form uridine diphosphate-glucuronic acid (UDPGA), which is then pumped into the lumen of the SER by transmembrane pumps called nucleotide sugar transporters (NSTs). These pumps are similar to OATPs, in that they are an automatic 'revolving door' system which does not require ATP and without them the extremely water soluble UDPGA would not be able to penetrate the lipophilic SER membrane. The UDPGA enters and UDP-N-acetylglucosamine is pumped out into the cytoplasm. UGTs use nucleophilic attack to gluconidate substrates ranging from thiols, primary and secondary amines, carboxyls and hydroxyls such as a phenol, which is shown in the basic UGT reaction:

$$\text{Phenol} + \text{UDPGA} \rightarrow \text{Phenolic glucuronide} + \text{UDP}$$

This reaction is actually reversible, but the SER system has evolved to ensure that it cannot reverse, as OATP-like anion transporters sweep the glucuronides out of the SER into the cytoplasm extremely rapidly. The UDP is also pumped out and recycled. There are some β-glucuronidases in the ER, which are intended to convert hormone glucuronides back to the parent drug as part of their shipment around cells, but it is thought that loss of the main production of SER glucuronides to β-glucuronidases is likely to be trivial given the amounts of glucuronides which are formed and how quickly they are ejected from the SER.

6.2.3 UGT isoforms

Although these enzymes had been documented for many years, little detailed information about them was available until cDNA cloning technology allowed the expression of large quantities of the enzymes, so enabling the study of the different UGT isoforms. It emerged that in the same way different families of CYPs had evolved to metabolize broad groups of substrates, the same system applies to UGTs. Of the human UGT cDNAs which have been cloned and sequenced so far, 16 correspond to functional UGTs and they are all structurally similar. The UGTs share a common 'backbone' with specific isoforms differing from each other in their N-amino termini. They have been extensively studied by expressing the human enzymes in various cellular heterologous systems. These are systems where human genes have been inserted into different bacterial and eukaryotic cell systems and the enzymes expressed in large enough amounts to study specificity and catalytic activity (Appendix A).

There are two main families of human UGT enzymes: UGT1A and UGT2B. UGT1A subfamily is coded for by just one gene that is located on chromosome 2, whilst the UGT2B subfamily is coded for by several genes on chromosome 4. Concerning human liver and gut, some important UGT1A and UGT2B isoforms and some of their substrates are summarized below.

UGT1A hepatic family

UGT1A1 (hepatic)

This vital isoform clears bilirubin, oestrogenic endogenous steroids, thyroxine (T4) as well as ethinyloestrodiol (not androgens or any other steroid molecules). Other xenobiotics processed include paracetamol, gemfibrozil, some statins (atorvastatin), metabolites of buprenorphine and statins, plus large aromatic carcinogenic hydrocarbons such as benzopyrenes. Anti-cancer agents such as etoposide and SN-38, the active metabolite of irinotecan, are also cleared by this variant of UGT.

UGT1A3 (hepatic)

Bile acids, thyroxine (T4) tertiary amines, flavonoids, some statins and phenolic compounds.

UGT1A4 (hepatic)

Bile acids. aromatic amines, trifluoperazine, trycyclic antidepressants, such as imipramine, midazolam (N-glucuronidated) and lamotrigine

UGT1A6 (hepatic)

Small, planar, phenolic chemicals, such as naphthols and paracetamol.

UGT1A9 (hepatic)

Both small and bulky phenolic chemicals, coumarins, paracetamol, flavones, amines and the candidate anti-leukaemia drug SAHA (Suberoylanilide hydroxamic acid)

UGT2B family

UGT2B4 (hepatic/extra-hepatic)

Bile acids, several xenobiotics, such as catalyzing the formation of an O-glucuronide from the CYP3A4 product hydroxymidazolam.

UGT2B7 (hepatic)

Bile acids. catechol oestrogens, morphine and naproxen, hydroxymidazolam, gemfibrozil, AZT and many other drugs.

UGT2B15 (hepatic)

Phenols, flavonoids, some sex steroids, oxazepam and probably the MDMA oxidative metabolite HMMA (4-hydroxy-3-methoxymethamphetamine).

UGT2B17 (hepatic/extra-hepatic)

Xenobiotics such as SAHA, as well as endobiotic compounds.

Gastro-intestinal tract UGTs

UGT1A10

Small and bulky aglycones.

UGT1A7

Endogenous ligands.

UGT1A8

Small and bulky phenolic chemicals and SAHA.

6.2.4 UGTs and bilirubin

As with all biotransforming enzymes, the endogenous functions of UGTs are part of cellular 'housekeeping'. Of the 16 human UGTs the most important is UGT1A1, which plays a key homeostatic role in bilirubin processing. Once erythrocytes have reached the end of their lifespan, they are dismantled and the major waste product is the iron-containing haem from haemoglobin. The haem is converted to biliverdin by haem oxidase, followed by conversion to bilirubin by a reductase. Bilirubin is very lipophilic and can enter the brain to cause neurotoxicity, so it cannot be allowed to accumulate. But it does react rapidly with reactive oxygen species, forming biliverdin, which is then re-reduced to bilirubin. Indeed, bilirubin is such a good antioxidant that those who have relatively high (but non-neurotoxic) plasma levels have a lower risk of developing heart disease and even cancer. So the balance of bilirubin plasma levels and excretion into bile is crucial and the main function of UGT1A1 is to process this 'waste' product. UGTs actually reside in the SER rather like torch (flashlight) batteries in packs of two (dimers) and four (tetramers). The advantage in this is that UGT dimers tend to form bilirubin monogluconides, but the UGT tetramers can concentrate the bilirubin monoglucuronide and form bilirubin diglucuronides, which are hydrophilic enough to be cleared in bile after they are pumped out of the SER by the transporters.

As it is the only UGT capable of handling bilirubin, impairment of UGT1A1 expression can have life-threatening consequences. The most severe condition is Crigler–Najjar syndrome, classed as CN-1 and CN-2. CN-1 is fatal (without a liver transplant) due to non-expression of UGT1A1, because of a fault in the UGT1A1 promoter system. CN-2 is a less dangerous version with reduced UGT1A1 expression. A milder polymorphism of UGT1A1 is Gilbert's syndrome, which is another promoter defect (UGT1A1*28) that restricts expression of UGT1A1 to less than 30 per cent of normal. The condition is particularly highlighted by an inability to clear major drug glucuronidation substrates like paracetamol. This is predominantly found in those of African ancestry, as well as other populations to varying degrees (Chapter 7, section 7.2.7). Any polymorphic impairment in UGT1A1 predisposes a patient to gut toxicity from the main metabolite of irinotecan, SN-38, which is normally cleared to a glucuronide by this isoform. UGT expression levels differ widely, so conferring different degrees of bilirubin-mediated antioxidant protection, as well as vulnerability to both bilirubin and xenobiotic-mediated toxicity.

6.2.5 UGTs and bile acids

Several UGTs (UGT1A3, UGT1A4, UGT2B4. UGT2B7) are responsible for the clearance of around a third of bile acids. These amphipathic (detergent-like) molecules facilitate the absorption of fats, various fat-soluble vitamins and cholesterol. They are actually made from cholesterol and their conservation is normally extremely efficient, as extensive recycling means daily losses are less than 5 per cent. However, due to their hydrophobicity and detergent effects they can be hepatotoxic, probably by damaging membrane integrity. Bile acids partly regulate bile flow, so if routes of clearance such as glucuronidation were to be impaired, accumulation would occur, which can lead to cholestasis, or shutdown of bile flow. Although there are many different routes to cholestasis, ranging from liver malignancies to biliary cirrhosis, it can cause serious liver damage and organ failure. In

addition, cholestasis is often associated with severe chronic pruritis (itching) which can drive patients almost to suicide and impacts quality of life as much as intractable pain. This is believed to be linked with bile salts as well as bilirubin and the itching can be controlled by cholestyramine, which sequesters the bile salts but is associated with severe constipation and is not always effective. Rifampicin has been used as a second line treatment as it induces the bile salts CYP3A4-mediated 6α-hydroxylation and glucuronidation simultaneously, so promoting systemic clearance. Some reports have found that some patients developed hepatotoxicity with rifampicin, although others found that relatively short courses of 150 mg/twice daily were effective and well tolerated.

6.2.6 Role of glucuronidation in drug clearance

With many xenobiotics, glucuronidation appears to happen after some form of oxidative metabolism; however, there are a number of drugs that are cleared virtually entirely by direct glucuronidation, without any prior oxidative metabolism. These include lorazapam, oxazepam, temazepam and morphine (Appendix B); the latter narcotic is cleared to a 3- and a 6-glucuronide. Glucuronidation is the major route of clearance for a vast array of chemicals: these include endogenous substances such as steroids, bilirubin, bile acids, fatty acids, retinoids and prostaglandins, as well as environmental pollutants, dietary constituents and of course drugs. Chemically, phenolics, carboxylic acids, hydroxylamines, amines, opioids and exogenous steroids can all be conjugated with glucuronic acid by these enzymes. The products are extremely water-soluble and provided that they are stable, they ensure that the agent is cleared into urine or bile. In general, glucuronides are so radically different in shape and water solubility from their parent drugs that they do not exert any pharmacological effect. The most often quoted exception to this rule is morphine-6-glucuronide, which is more potent than morphine. UGTs have a seriously high capacity and are practically unsaturable; the first-generation anti-HIV agent AZT is a good example of this. This toxic compound was cleared entirely by UGTs and such is the capacity of these pathways that the drug had to be taken several times daily to remain in the therapeutic window. With the exception of UGT1A1 and bilirubin, there is arguably a larger overlap in many of the UGT specificities compared with those of the CYPs, so even if several UGTs were poorly expressed the glucuronidation of a drug may still occur in quantities sufficient to clear the agent from the body.

6.2.7 Types of glucuronides formed

There are several options for UGTs to insert a glucuronic acid group into a molecule. The ether glucuronide as formed from simple phenols (Figure 6.2) is one option, as is an ester glucuronide, which would appear when chemicals similar to benzoic acid are glucuronidated (Figure 6.3). S-glucuronides can also be formed. Glucuronidation of amines is more complex and interesting from a toxicological perspective. Aromatic amines can either be glucuronidated directly on the nitrogen of the amine to form an N-glucuronide (Figure 6.4), or the amine can first be oxidized to form a hydroxylamine, where the glucuronide can be attached to either the nitrogen or the oxygen of the hydroxylamine to form an N-glucuronide or an O-glucuronide.

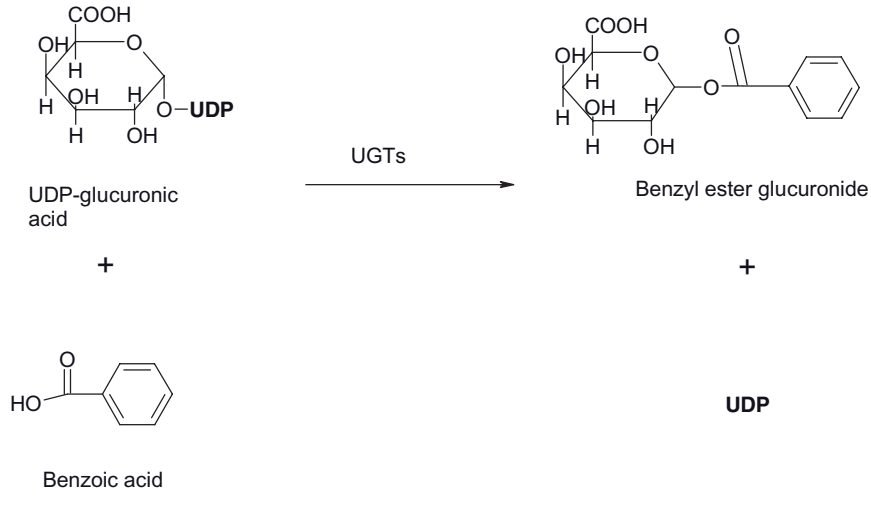


Figure 6.3 Formation of an ester glucuronide from benzoic acid

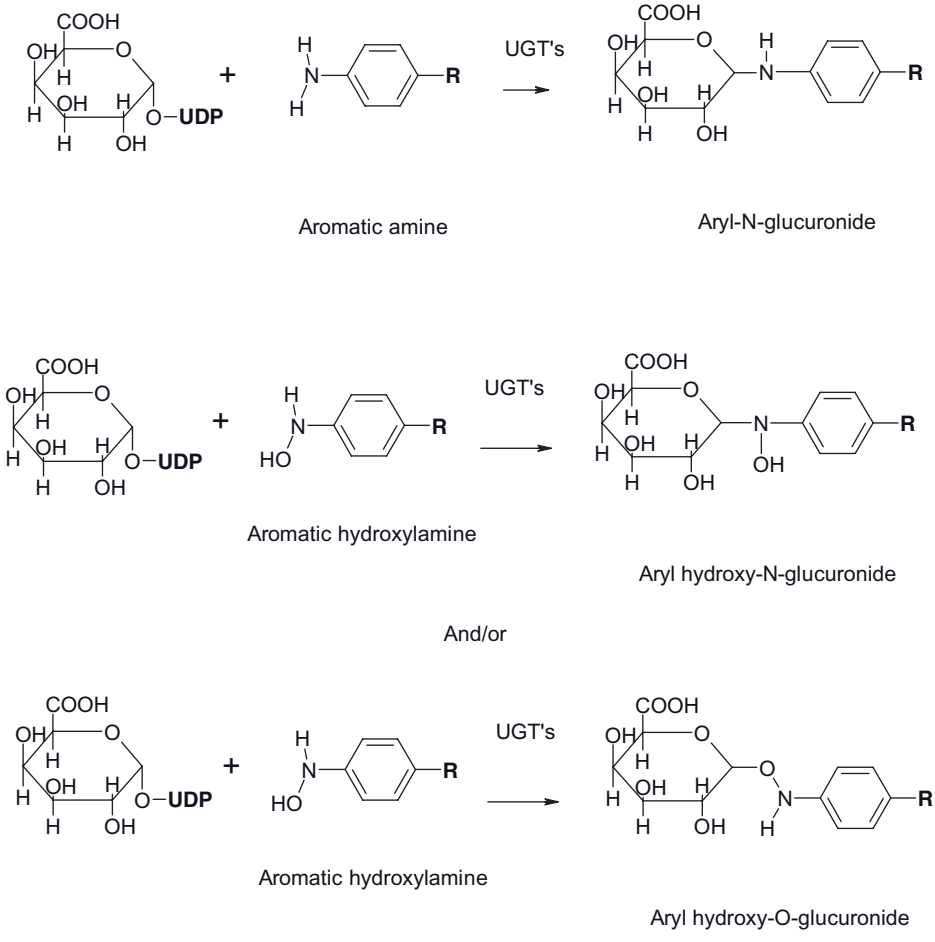


Figure 6.4 Scheme for glucuronidation of aromatic amines and hydroxylamines

With most aromatic amines, the N-glucuronide is formed directly from the amine and oxidative hydroxylation is unnecessary, although the N-hydroxy (hydroxylamine) metabolites can also be glucuronidated. The question is whether this would occur on the nitrogen (N-glucuronide) or the oxygen of the hydroxylamine (O-glucuronide).

The sulphone, dapsone, is an interesting example of the conjugative fate of an aromatic amine. In volunteers, the parent drug could be monoacetylated, which could then be N-hydroxylated along with the parent drug (CYP2C9) to form acetylated and non-acetylated hydroxylamines. These hydroxylamines were then predominantly glucuronidated, as around a third of the dose is recovered in 48 hours as glucuronides. Acid hydrolysis with β-glucuronidase (which splits the glucuronic acid off the drug or metabolite at acid pH) left intact dapsone hydroxylamine as the major metabolite. Irrespective of the position of the glucuronide, the hydroxylamine was just stable enough to survive after the glucuronic acid was hydrolyzed away. With dapsone, oxidation to the hydroxylamine is so rapid that there is probably little opportunity for the parent amine to be directly conjugated to form an N-glucuronide. Subsequent studies with benzidine, a carcinogen that is N-hydroxylated and glucuronidated, suggested that the O-glucuronide formed from the hydroxylamine is less stable to hydrolysis compared with N-glucuronides.

The stability and position of glucuronides on an amine are crucial issues with regard to the potential carcinogenicity. N-glucuronides in general are very susceptible to acid hydrolysis, which can occur in urine made acid by a diet rich in meat and dairy products. Human urine also contains β-glucuronidases, which operate most efficiently at acid pH. Those who have a predominantly vegetarian diet have more alkaline urine and little hydrolysis will occur. It appears that the acid hydrolysis of N-glucuronides of aromatic amines like benzidine leads to liberation of the parent amine and possibly the hydroxylamines in the urine. What occurs next is still under debate, but the released parent drug and other oxidative metabolites can lead to bladder cancer, which occurs in many workers exposed to benzidine-like compounds in various dye industries, now sited in Third World countries. This is discussed in more detail in Chapter 8 (Figure 8.11).

6.2.8 Control of UGTs

Hopefully you will have read and understood the chapter on the induction of CYP isoforms, which outlined the sophisticated expression control system which has evolved to both coarse and fine-tune CYP isoform expression according to substrate ‘load’. So it is entirely logical that there should be a similar system for the control of glucuronidation, given the capacity, importance and flexibility of this conjugative system. Interestingly, it has been known for many years that UGT1A1 glucuronidation is responsive to substrate ‘load’. Around half of newborns can be jaundiced due to bilirubin accumulation, which usually clears in a few days. If it does not, bilirubin-induced brain damage (kernicterus) may result. It was reasoned that a UGT inducer like phenobarbitone would accelerate bilirubin clearance and the approach works, although it requires at least ten 100 mg doses of the drug towards the end of pregnancy to induce UGT1A1 sufficiently to reduce the incidence of jaundice. Other UGT1A1 inducers could be used, which are non-sedating, but the general clinical approach is to treat jaundiced babies with phototherapy and blood transfusions *in extremis*. Rifampicin is also capable of inducing UGTs; however, this again is not a suitable agonist for this purpose due to the side

effects of this agent, the least of which is to colour every bodily secretion orange. Cases of Gilbert's syndrome and CN-2 can be treated with phenobarbitone to prevent hyperbilirubinaemia.

Receptor-mediated regulation of glucuronidation

If a student were to be asked which nuclear receptor (NR) systems controlled glucuronidation and they were to reply 'all of them', this answer might sound rather facetious, but it would not be far from the truth. CYP induction usually helps assert the cell's programming to retain control of lipophilic chemicals which might cause intermediate to long term derangement of function (Chapter 2). In contrast, there are many endogenous agents cleared by UGTs that are rapidly toxic if they accumulate, so it is imperative for the organism's immediate health that the UGT genes respond quickly to changes in a very wide range of chemical structures. Hence, multiple NRs are involved in the control of individual UGT isoforms.

Clinical experience has also shown that a range of drugs can also induce UGT expression. Aside from phenobarbitone and rifampicin, 3-Methyl cholanthrene and oltipraz can also induce UGT1A1 in human hepatocytes. UGT1A6 and UGT1A9 are inducible through agonists of CYP1A1 (dioxins and β-naphthoflavone). The clearance of the beta-blocker labetolol is induced in pregnant women and this is caused by progesterone-mediated induction of UGT1A1, but interestingly, UGT2B7 is unaffected by the hormone.

UGT1A1 (Figure 6.5) is probably the most intensively studied UGT isoform and as you might expect with an enzyme induced by phenobarbitone, the start of the gene contains a PBREM (phenobarbitone responsive enhancer module). However, UGT induction can

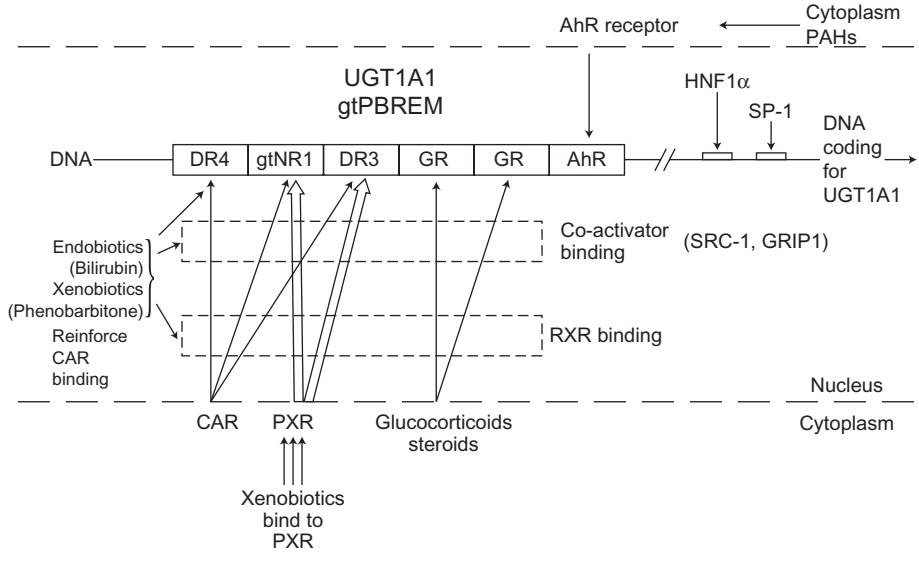


Figure 6.5 Control of UGT1A1 expression: the multi-site gtPBREM allows the isoform to respond to many receptor systems and a variety of endogenous, dietary and therapeutic stimuli

occur in response to so many different agents, that its enhancer module is quite unlike that of CYP genes; it is called the gtPBREM (Figure 6.5) and contains six receptor binding sites: a DR4 site that responds to CAR; a gtNR1 and a DR3 site, both of which bind CAR and PXR; two glucocorticoid receptor GR binding sites and finally a site which binds AhR. Upstream from gtPBREM, there are two more promoter sites that bind the transcription factors HNF1α and SP-1. CAR and PXR appear to operate in the same way that they do with the CYPs; PXR binds the ligand in the cytoplasm, such as rifampicin and migrates to the nucleus to seek the heterodimer partner RXR. CAR operates continuously and its complexing with RXR is tightened by the presence of the ligand. In general, after the NRs recruit RXR they lock onto the DNA response elements with their co-activators such as SRC-1 and GRIP1. With gtPBREM, HNF1α must also bind at the upstream site before maximal induction can occur. The cross-talk between the various NRs and HNF1α is still being studied. gtPBREM is activated by other NRs such as GR in response to dexamethasone and other steroids, as well as AhR binding polycyclics. HNF1α is involved with the regulation of the expression of UGTs1A3-5 and UGTs1A8-10.

Bile acid elimination by UGTs is controlled by CAR and PXR, as well as by the farnesoid X-receptor and the vitamin D receptor. UGT1A3's bile acid regulation is controlled by other NRs such as the liver X-receptor (LXR) and peroxisome proliferator-activated receptor α (PPAR α). The fibrate class of drugs act as agonists on this latter receptor and that is part of how they reduce plasma lipid levels. Again, these NR seek the heterodimer partner RXR to bind DNA response elements.

Regarding the role of NRs in neonatal jaundice, several major metabolic adjustments must be made in the few days after birth. These include the replacement of foetal haemoglobin with the adult version, which involves increased erythrocyte turnover. The resultant increase in bilirubin occurs when neonatal UGT capacity is less than 10 per cent of that of adults. It is thought that the loss of the mother's glucose supply triggers increases in glucocorticoids, which feedback on GR and CAR, which serve to induce UGT1A1. The presence of bilirubin in cells is also capable of promoting CAR activity; hence, a great deal of UGT capacity needs to develop in a short space of time. Therefore, it is not surprising that such a high proportion of neonates are jaundiced. That the jaundice improves within a few days in the vast majority of babies suggests that the NR-mediated induction and development of UGT capacity does function adequately.

Remarkably, PXR, CAR and FXR are also part of the process whereby the liver can sense whether its own metabolic capacity and physical size is sufficient to respond to homeostatic demands. Hence, alongside various growth factors, the NRs facilitate the amazing process whereby the liver regenerates itself after areas of the organ are removed or damaged.

As CYPs, UGTs, other biotransforming systems and efflux transporters are meeting the same xenobiotic or endobiotic stimuli in different tissues and degrees of exposure, it is logical that the HNF, NR and AhR receptor systems integrate and coordinate their responses. It is also likely that specialized systems intended to respond to electrophiles and other reactive species, such as the Nrf2/ARE system (see section on GSTs) also may influence the UGTs. These multi-receptor mechanisms enable levels of induction to be customized for individual tissues to deal with different chemical threats. Essentially, according to diet, chemical and drug exposure, each individual will possess a unique expression array of UGTs and CYPs which will be constantly fine-tuned throughout life.

6.2.9 Enterohepatic recirculation

This term usually refers to the recycling of bile salts during digestion, but can also be used to describe an apparent rise in blood drug levels hours after a single dose has been given. Since another dose has not been given, it is rather paradoxical to see a rise in blood levels just as the drug is gradually being eliminated from the system. This effect starts with clearance to a conjugate, usually a glucuronide, but it can also involve sulphates and amino acids. The conjugate is then excreted into bile and may be unstable when it reaches the gut. The conjugate could be chemically unstable, or is hydrolyzed by gut bacteria; either way, free drug is then reabsorbed. This effect can extend the half-life of a drug and make its plasma levels variable and difficult to predict. In addition, it can expose the gut to levels of the parent drug which may be much more toxic than the conjugate (irinotecan and SN-38; see 6.2.10 and Chapter 7, section 7.2.7). This effect is seen in NSAIDS and contributes to their gut toxicity. Opiates, antiparasitics such as ivermectin, many dietary antioxidant flavones and other phenolics are also enterohepatically recirculated. It can occur in steroids, although it is not thought to be clinically relevant in the cases of norethisterone and gestodene. Antibiotic administration can accelerate the clearance of enterohepatically recirculated drugs by killing the gut flora responsible for the hydrolysis of the conjugates.

6.2.10 UGT inhibition

It has emerged that there are many drugs capable of inhibiting UGTs with varying potency and clinically these effects have been manifested in derangement of hepatic homeostasis, such as hyperbilirubinaemia and even cholestasis during therapy with a considerable range of drugs. The major UGT inhibitors appear to be the protease inhibitors, which are not metabolized by UGTs. Indinavir, an older agent, acts as competitive inhibitor of UGT1A1, UGT1A3 and UGT1A7. This translates into hyperbilirubinaemia in HIV patients, although saquinavir is less likely to do this. The problem is most pronounced in those with marginal UGT capacity, such as those with inherited defects in UGT expression, like CN-2 and Gilbert's syndrome. There are complex combinations of UGT deficiencies so the risk of hyperbilirubinaemia occurs in HIV patients treated with protease inhibitors can exceed proportion of the population with Gilbert's syndrome. This can translate to more than 10 per cent of Caucasians being at risk from bilirubin problems with UGT inhibitors. Unfortunately, atazanavir has caused hyperbilirubinaemia in nearly 40 per cent of HIV patients, suggesting that it is a much more potent inhibitor than others in its class and it can cause clinically relevant inhibition in those with normal UGT capacity. The situation is compounded by the potent inhibition of OATP and other transporters caused by the protease inhibitors. So in some individuals treated with these drugs, the combination of inadequate UGT expression, impaired bilirubin uptake into hepatocytes as well as inhibition of those UGTs that are expressed, leads to severe hyperbilirubinaemia. Of course things would become even worse if the transporters (MRPs, see later) that pump bilirubin out of the hepatocytes into the bile were to be compromised by genetic factors or inhibition.

There are several other drugs that are also UGT1A inhibitors, such as ketoconazole, sulfinpyrazone and amitriptyline, whilst the NSAID mefenamic acid is a UGT2B7 inhibitor. In the case of patients on complex regimes such as those with HIV, there is

unfortunately ample scope for UGT inhibition impacting the clearance of other drugs in the regime. In addition to effects seen with the protease inhibitors, any inhibition of the glucuronidation of SN-38, the active metabolite of irinotecan will result in neutropenia and gut toxicity. There are other, less intense UGT inhibitory effects that are more like mild competition for the enzyme's active site in nature, rather than the potent inhibition caused by the protease inhibitors. Some fibrates have mild inhibitory effects on some statin glucoronidation, with an approximately 1–4 to 1.6-fold change in AUC of atorvastatin in the presence of gemfibrozil, although there was no detectable effect with fenofibrate.

An emerging issue is the contribution of dietary inhibitors of UGTs. Some curcuminoids have been shown to be broad inhibitors of CYPs as well as UGTs at micromolar levels, such as curcumin, demethoxy and bisdemethoxycurcumin. This may become clinically relevant if the dietary agent or herbal extract is taken in quantity for their pharmacological effects. The curcuminoids are antioxidants and have received attention for their possible benefit in Alzheimer's disease. As discussed in the previous chapter concerning other herbal preparations, it is likely that serious interactions are likely to be discovered when unfortunate patients are immortalized in case reports.

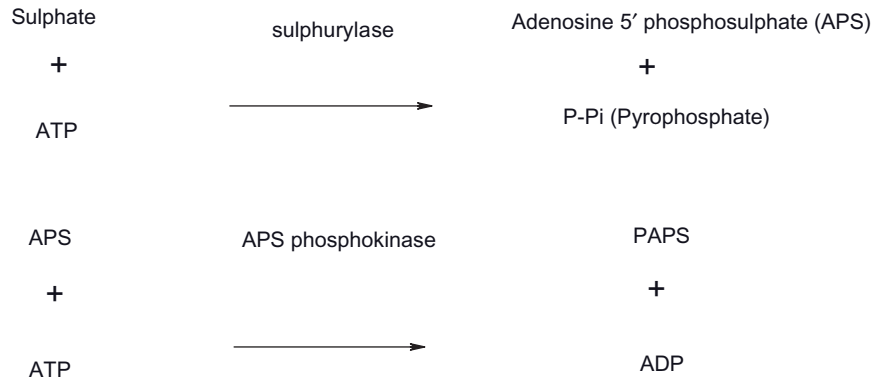
6.3 Sulphonation

6.3.1 Introduction

Sulphonation is accomplished by a set of enzyme systems known as sulphotransferases (SULTs) and they are found in most tissues to varying degrees of activity. The major sites of activity are the liver, small intestine, main intestine and colon, although they are also found in the brain and the placenta. The main detoxifying isoforms are cytosolic enzymes, but there are membrane bound forms which are located in the cytosolic Golgi bodies and these forms are engaged in biosynthetic cellular housekeeping tasks rather than biotransformation. Rather than directly attach the sulphate molecule to the xenobiotic, they require sulphurylase + APS phosphokinase to manipulate the sulphate into a sulphonate ($SO\text{-}_3$) form that is thermodynamically easiest for the enzyme to attach to the xenobiotic (Figure 6.6). The first step is to 'load' the sulphate into adenosine 5′phosphosulphate.

The general aim of sulphonation is to make the substrate more water-soluble and usually less active pharmacologically. Sulphonated molecules are more readily eliminated in bile and urine. All the SULTs are virtually identical structurally in the area of the enzyme where they bind the co-factor, although there are obviously considerable differences in the substrate binding sites, which confer on the different enzymes the ability to bind groups of similar substrates in the same manner as CYP and UGT enzymes.

Although sulphotransferases do not have the vast capacity that the UGT systems possess, they are capable of forming large quantities of sulphate metabolites in a relatively short timescale. Again, our knowledge of this metabolizing system has benefited greatly from heterologous expression systems. However, the full range of the endogenous and xenobiotic-metabolizing roles of SULTs remains to be uncovered, as these enzymes are extremely important in many metabolic roles, particularly steroid metabolism in different tissues, including the brain. It has been estimated that more than half the circulating steroids in the body are sulphonated. The SULTs are also important in bile salt processing, with the majority of sulphonated bile acids (more than 70 per cent) cleared in urine. They



The co-factor known as PAPS (3′-phosphoadenosine-5′-phosphosulphate) acts as the final sulphate carrier.

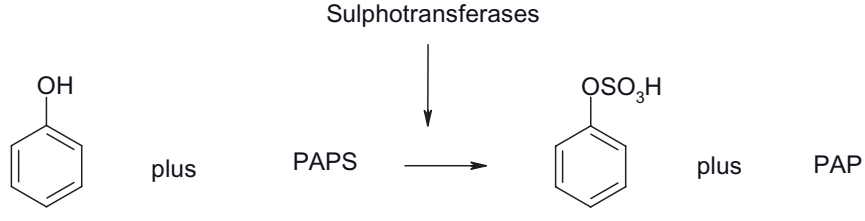


Figure 6.6 Basic reactions of sulphotransferases

are especially active at the beginning of life as they are found in high levels during foetal development. This is closely linked with the SULTs role in thyroxine metabolism. Their roles regarding xenobiotic metabolism seem to be contradictory, with many SULTs involved in the activation of carcinogens, as well as the detoxication of other reactive species. All SULTs are subject to genetic polymorphisms, with a high degree of individual variation in their expression and catalytic activities (Chapter 7 section 7.2.8); this is currently under investigation, particularly from the standpoint of individual risk factors for carcinogenesis. There is evidence that SULT enzyme profiles in breast tumours are significantly different from normal tissue; tumours express high levels of the extra-hepatic SULT1C1, but no SULT1A2 at all.

Regarding classification of the superfamily of SULTs, it is assumed that 47 per cent amino acid sequence homology is indicative of same family members and 60 per cent homology for subfamily members. To date, there are 47 mammalian SULT isoforms so far discovered, which are derived from ten human sulphotransferase gene families; there are five detoxifying cytosolic families, with SULTs1 and 2 appearing to be the most important.

SULT1 family

This is the major group of human sulphotransferases, which contains four subfamilies (1A, 1B, 1C and 1E) and a considerable number of isoforms, including 1A3, 1B1, 1C1, 1C2,

and the oestrogen sulpho-transferase 1E1. 1A3 (not found in the liver) as well as 1E1 are potent sulphators of xenobiotic oestrogens and are inhibited by oestrone and quercetin. However, the most important isoform from the xenobiotic metabolism point of view is the phenol and aryl amine sulphating sulphotransferase SULT1A1.

SULT2 family

These enzymes are hydroxysteroid sulphotransferases and there are only two subfamilies, SULT2A and SULT2B, with SULT2A1 and SULT2B1 being the best understood. There are a large number of individual enzymes which sulphonate a variety of sex hormones and hydroxysteroids. SULTB1 metabolizes sex steroids and predictably is found in the placenta, uterus and prostate and is thought to be heavily involved in the regulation of androgen levels.

6.3.2 SULTs1A1-3

The genes which code for SULTs 1A1, 1A2 and 1A3 are found very close together on chromosome 16. SULT1A1 is found in large quantities in the liver, whilst SULT1A3 is found mainly in the foetal liver, gut and lung where it forms sulphonates from catecholamines. Toxicologically and from the perspective of drug clearance, SULT1A1 has become more and more important as its role in the clearance and activation of xenobiotic molecules has been unravelled. The polymorphic variants of this isoform (designated SULT1A1*1-3; Chapter 7, section 7.2.8) are found mainly in the liver and gut, but also the lung, skin and brain. SULT1A1 isoforms are capable of metabolizing a wide variety of hydrophobic molecules and how they achieve this has become much clearer with the crystallization of the enzyme, showing its full structure.

This enzyme, being found in the aqueous cytosol, has been crystallized (SULT1A1*2). The isoform is rather clam shaped, possessing a binding site for the co-factor PAPS as well as an L-shaped hydrophobic binding pocket which can accommodate two substrate molecules. To illustrate the subtle differences in these isoforms in terms of structure that lead to huge differences in function, SULT1A3 shares 7 out of 10 aromatic residues in its substrate binding site with SULT1A1, but it has much narrower specificity, only really metabolizing catecholamines like dopamine to sulphonates. The ten aromatic residues in SULT1A1's substrate binding pocket allow it to metabolize extremely hydrophobic substrates. As with the CYPs, the crystal structure does yield detailed knowledge of the enzyme's layout, but not always its function: SULT1A1 will process small entities such as paranitrophenol and much larger molecules such as thyroid hormones, although it is harder for the isoform to sulphate oestrogens. From the crystal structure, the oestrogens do not appear to fit the active site as well as the other molecules. However, the limitations of crystal structures were discussed in Chapter 3; they are just a frozen snapshot of the enzyme and it does not show how it actually operates; in the same way it is difficult to see today how King Charles II was such a successful womanizer from his later portraits. That the enzymes will sulphonate such a variety of compounds, from phenols, to aromatic amines, polycyclic aromatics as well as sex steroids, shows that they can bind practically any shape molecule, from flexible ring structures and planar aromatics to what is termed

'extended fused ring systems', such as steroid-related molecules. It is likely that SULT active sites alter their conformations considerably to process molecules such as steroids. This feature could involve changes in the enzyme's internal dimensions which occur to accommodate the structural characteristics of the potential substrate, thus 'moulding' itself around it. This process was discussed in Chapter 3 (section 3.4.3) with the main CYPs such as CYP3A4 and such plasticity confers tremendous flexibility in substrate specificity.

6.3.3 Control of SULT enzymes

Since the first edition of this book, it seems that the study of the control of SULT enzymes is still very much in its infancy and knowledge of the role of NRs and AhR in human SULT expression has progressed in animals but not really in humans. This is partly due to the fact that rodent SULT profiles are quite different to ours, such as their single member of the SULT1A family, compared with our three. SULTs are highly gender specific in rats, but apparently not in humans. Many studies have been carried out in rodents, which have produced rather contradictory results which often show suppressing roles of the NRs and AhR rather than induction. As SULTs are part of the system for the clearance of thyroid hormones, which are closely controlled in concentration, it is possible that CAR is involved in their regulation; elements on SULT2A1 genes have been shown to respond to CAR in human cellular systems. Other work shows that SULT1A1 is inducible by dexamethasone through PXR effects in animal systems, but there was no effect in human hepatocytes. The SULT1A1 gene promoter contains areas which bind SP-1, a transcription factor which is also part of the control of UGT1A1. SULT1A1 and SULT1A2 have similar promoter systems, whilst SULT1A3 appears different, suggesting that SULT gene promoter regions are relatively specific to related groups of isoforms. It seems that whilst SULTs in general are not as responsive to inducers as CYPs and UGTs, their basal expression is much higher, although interindividual expression does vary considerably and this may have severe toxicological consequences, in terms of xenobiotic toxicity and carcinogenicity. There is also some evidence that diet is a strong influence on individual SULT profiles. SULT enzymes can also be inhibited by a number of different chemical agents, such as those that resemble substrates like chlorinated derivatives of dinitrophenol. However, antioxidants like quercetin and curcumin are also effective inhibitors, as are a considerable number of flavonoids such as hesperetin and eriodictyol. These latter agents are extremely potent inhibitors of the SULT1A family and relatively modest levels in the diet would exert protective effects against SULT1A1 metabolites, as this isoform can promote the formation of reactive and highly unstable metabolites that are potentially carcinogenic.

6.4 The GSH system

6.4.1 Introduction

One of the main problems with the oxidation of various molecules by CYP enzymes is that they are often destabilized and sometimes form highly reactive products. The analogy used previously in this book was that of a child given a hammer and told to hit anything metallic hard. CYPs occasionally form metabolites so reactive that they immediately

destroy the enzyme by reacting with it, changing its structure and, therefore, its function. Sometimes, this kind of damage can be self-limiting because the reactive species formed destroys the CYPs, so no more reactive species are formed until several days later, while more enzyme is assembled. Meanwhile, the chemical substrate itself is not a problem unless it is oxidized and it might well be cleared through other routes in the meantime.

The most dangerous forms of reactive species are those that evade UGTs and SULT enzymes, or are inadvertently created by conjugation processes. These species escape into the cytosol and even into the nucleus, where potentially carcinogenic events may result. At the very least, such reactive species might react with cytosol and membrane protein structures, which eventually results in such high cellular damage that the cell necroses and disintegrates, or enters an apoptotic state (Chapter 8, section 8.6.1).

CYPs are not the only source of reactive species generated within cells. Around 75 per cent of our food intake is directed at maintaining our body temperature and a great deal of energy must be liberated from the food to accomplish this. Cells derive the vast majority of their energy through oxidative phosphorylation and this takes place in the 'engine rooms' of the cell, the mitochondria. With a car or any fuel-burning machine, aside from the achievement of the desired function, energy is also wasted in the form of heat and toxic exhaust gases. In cells almost all the oxygen we breathe is consumed in oxidative phosphorylation, forming ATP, heat and reactive oxidant species in the mitochondria that could cause severe damage to the structure and function of the cell if they were allowed to escape. So all cells, particularly hepatocytes, have evolved a separate system to accommodate such reactive toxic products and this is based on a three amino acid (cysteine, glycine and glutamate) thiol known as glutathione, or GSH. Thiols in general are extremely effective at reducing and thus 'quenching' highly reactive, electrophilic species. GSH has a very high redox potential of –0.33, and donates electrons to reactive species. During this process it loses a proton and forms a glutathionyl radical (GS•), which is capable of causing oxidant damage itself, but more usually undergoes a complex series of reactions resulting in GSSG. This is now stable and is 'spent' and by itself it is useless in controlling reactive species.

To say that GSH can act as a cellular 'fire extinguisher' is an imperfect analogy, as it implies that GSH is only useful in emergencies. In fact, if cells are depleted of GSH by blocking its synthesis (by using buthionine sulphoxime), cell death follows and the organism itself will die in a few days, due to uncontrolled activity of endogenous radicals. A better analogy for GSH would be the oil in a car engine. Without oil, the engine will essentially weld itself together and seize. To stretch the analogy somewhat, a combination of a good oil quality and pressure means that a well-designed car engine will last almost indefinitely. If GSH levels are not maintained in the cell over a long period of time, the cell wears out more quickly; for example, diabetic complications and HIV infection are linked with poor GSH maintenance. In fact, among its many functions, the GSH system acts like a cellular 'battery charger', recharging the oxidized spent versions of other antioxidants such as ascorbic acid and vitamin E.

6.4.2 GSH system maintenance

In the same way a central heating system maintains a set temperature, GSH levels are 'thermostatically' maintained in different subcellular 'pools' at preset levels. The ratio

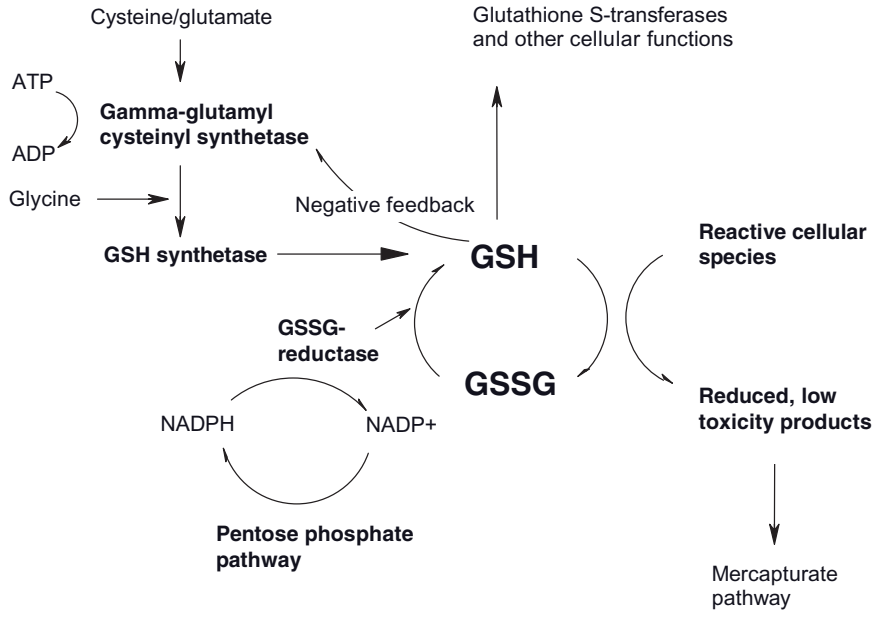


Figure 6.7 The GSH maintenance system in man

between GSH and GSSG will be very high in mitochondria, to ensure protection against reactive species, whilst the ratio is lower in other less radical-threatened areas of the cell such as the rough and smooth endoplasmic reticula. Overall, the hepatocyte maintains GSH at a very high (8–10 mM) intracellular level, whilst normal erythrocytes hold it at around 1–2 mM. Plasma levels are usually only in the micromolar range. Intracellular GSH concentrations are maintained by two methods: firstly, by a two-stage ATP-dependent direct synthesis (γ-glutamylcysteinyl synthetase and glutathione synthetase), and secondly, by recycling the GSSG to GSH by the operation of GSSG reductase, which consumes cellular reducing power (NADPH; Figure 6.7). This system is so efficient that at any one time, 98 per cent is GSH and less than 2 per cent will be GSSG. The cell can completely restock its GSH level from nothing in less than 10 minutes. GSH levels are maintained through allosteric negative feedback mechanisms, where high GSH concentrations inhibit GSSG-reductase and the GSH synthetic enzymes. GSH maintenance can only be frustrated by a lack of raw materials (a sulphur containing amino acid, methionine or cysteine) or lack of reducing power. The cell will often succeed in maintaining high GSH levels even under severe attack by oxidative species. It is not usually necessary for GSH to leave a cell and it cannot cross membranes without a specific transporter such as MRP-1 or SLCs like the OATPs. Transporters actively pump GSSG out of cells in times of oxidative stress to prevent it reacting with other cellular thiols. GSSG egress is actually a good indicator of oxidative stress in a cell. When GSH quenches a reactive species it sometimes written as a GS-conjugate, which undergoes further processing to emerge as a mercapturate, which is excreted in urine (Figure 6.8).

Currently, over 30 essential cellular functions have been found for GSH, and the GSH system is so highly evolved that it does not solely rely on GSH spontaneously reacting

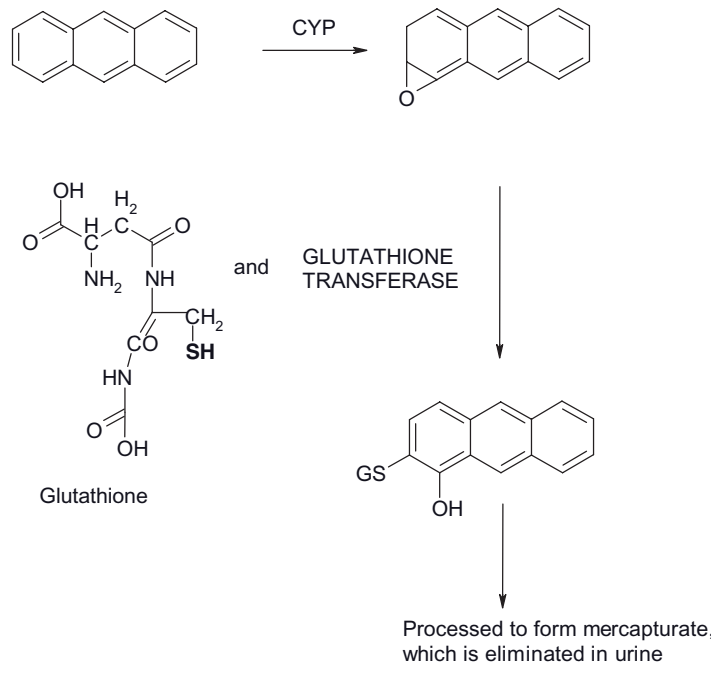


Figure 6.8 A typical GST catalyzed reaction of GSH with a potentially reactive xenobiotic. The hydrophilic GSH molecule substantially increases the water solubility and molecular weight of the aromatic and will also detoxify it and ensure it will be transported out of the cell. It is unlikely anyone would make you learn the structure of GSH, but it is useful to look at it and appreciate that it is highly water-soluble

with dangerous species. There are several enzymes that promote and catalyze the reaction of GSH with potential toxins to ensure that reactive species are actively dealt with, rather than just passive GSH-mediated reduction. Probably the most important from the standpoint of drug metabolism are the GSH-S-transferases.

6.5 Glutathione S-transferases

6.5.1 Structure and location

Forming around 5 per cent of total cellular protein, the glutathione transferases, or S-transferases as they are usually known (GSTs), are the key cellular defence against electrophilic agents formed from endogenous or xenobiotic oxidative metabolism. Although GSH is capable of reacting directly with reactive species, the GSTs could be likened to using a fire hose instead of hand extinguisher. These enzymes only exist functionally as 'twins' or roughly 50,000 molecular weight homodimers, or sometimes heterodimers. These consist of two monomers, each of which has an 'H' site which is on the carboxy-terminal and binds the hydrophobic electrophile. These H sites differ according to the GST isoform and confer their abilities to bind their particular range

of substrates. The GSH 'G' site (the amino-terminal end) does not vary and is actually only fully assembled when the two monomers join to form the dimer. Rather than go for the usual butterfly or clam shape, to get some idea of how they look like in 3-D, its time to use your hands again. If the upper area of the palm of each hand corresponds to the G sites and the base of the palms are the H sites, then join your hands together as if in prayer and then rotate one hand through 180 degrees whilst holding the other hand still. There is some evidence that the H site undergoes some considerable conformational change to accommodate different substrate structures, which echoes CYP3A4's flexibility (Chapter 3).

Almost all the GSTs are found in the cytosol, although some are associated with the endoplasmic reticulum and mitochondria. The hydrophobic GSTs are structurally different from the cytosolic GSTs and they metabolize leukotrienes and prostaglandins and are known by the acronym MAPEG. Aside from the detoxification roles of cytosolic GSTs, they can even repair damaged proteins, which have been S-thiolated. The major classes of mammalian enzymes are Alpha, Mu, Pi, Theta and Omega, although these enzymes are found right across the animal and plant kingdoms. In humans, the presence of mercapturates in urine (Figure 6.8) is usually a reasonably good indication of the formation of a reactive species somewhere in the hepatic handling of a xenobiotic agent.

6.5.2 Mode of operation

The GSTs firstly bind GSH at the G site and the pKa in the immediate environment is lowered from 9.2 to 6.2–6.6, which promotes the removal of the proton (the hydrogen ion) forming the reactive anionic thiolate radical (GS•), which is a strong nucleophile that immediately attacks the electrophilic substrate and the resultant thioether can be rearranged through further metabolism (mercapturate pathway) to form mercapturates, which are stable and non-toxic. It is still not exactly known how the enzyme manages this process and some theories suggest that a tyrosine residue holds the GSH and draws the proton away (tyrosine assisted proton transfer). The tyrosine may have a crucial role to play, as if it is substituted or removed catalysis is lost. More recently it has been proposed that the enzyme uses the glutamyl gamma carboxylate (GGC) group in the GSH itself together with a water molecule to remove the proton to form the thiolate nucleophile (water-assisted proton transfer). Indeed, the enzyme conspicuously will not operate with GSH analogues or indeed any other ligand instead of GSH and this exquisite degree of specificity could be accounted for by the necessity for the GGC structure in GSH for catalysis.

6.5.3 GST classes

The GSTs are found in humans in several major classes. Generally, 60 per cent amino acid sequence homology is required for an isoform to be assigned to a particular class. The classes contain several subfamilies, with around 90 per cent common sequence homology. These enzymes are polymorphic (Chapter 7, section 7.2.9)) and their individual expression ranges from complete absence in some isoforms to overabundance as a response to anticancer therapy.

GST Alpha class

GSTA1-1 is an important representative of the A class, which is found in high quantity in the liver and kidney and to a lesser extent other tissues (intestine, lung, testis). Hepatic GSTA1-1 accounts for 1 per cent of cytosolic protein, providing considerable protection against electrophiles. Finding GSTA1-1 in the blood is a clear sign of liver damage and is a more sensitive marker for monitoring the progress of liver toxicity, as it is more closely associated with the liver than aspartate aminotransferase (AST) or alanine aminotransferase (ALT) and is more rapidly eliminated, so a more up-to-date picture of liver pathology is available. The substrate-binding site of the Alpha GSTs is most efficient at processing small hydrophobic molecules. Of the other human GSTA isoforms, GSTA2-2 is also abundant in the liver, whilst GSTA3-3 is found in the adrenal glands, placenta, as well as the testes and ovaries. GSTA4 can be inhibited by ethacrynic acid, lipid hydroperoxides as well as 4-hydroxyalkenals (products of lipid breakdown). These enzymes are also capable of carrying out GSH peroxidase activities.

GST Mu class

The representative Mu class GSTM1-1 has a larger more open active site than the alpha GSTs and it contains a deeper binding cleft than the GSTP variants. GSTM1-1 is found in high levels in the liver, as well as the brain, testis, kidney and lung and will oxidize many bulkier electrophilic agents, such as 1-chloro-2; 4-dinitrobenzene (CDNB); aflatoxin B1-epoxides; and trans-4-phenyl-3 buten-2-one and benzpyrene diols.

GST Pi class

GSTP1-1 is widespread and is the main GST in erythrocytes, although it is not a hepatic GST. It is overexpressed in tumour cells by as much as 200 per cent. It will process a variety of toxicologically dangerous agents as well as endogenous species; these include CDNB, acrolein, adenine, propenal, benzyl isothiocyanate and 4-vinylpyridine. Aside from their functions in xenobiotic metabolism, GST Pis and the Mu class GSTs appear to regulate a mitogen-activated protein (MAP) kinase pathway that is part of the apoptosis control system. The role of GSTP1 is especially troublesome in the induction of resistance to alkylating agents in cancer chemotherapy. Part of this process is the upregulation of GSH formation, but it also appears that GSTP1 and other isoforms in the series defend the tumour cells by direct detoxification as well as by blocking apoptosis through their effects on MAP kinase.

GST Theta class

These enzymes differ from the other GSTs as they do not use the tyrosine residue to catalyze the reaction between the substrate and GSH. Serine accomplishes this activity in the GST-T isoform and it is likely that the site is capable of some structural rearrangement that assists in the catalytic process. This GST is associated with the metabolism of envi-

ronmental and industrial carcinogens, including planar polycyclic aromatic hydrocarbons, halomethanes, dihalomethanes and ethylene oxide. Interestingly, GST-T in erythrocytes is identical to the hepatic version, so methyl bromide or ethylene oxide turnover by the enzyme in sampled erythrocytes is used to determine if an individual expresses this isoform.

GST Omega class

This class of enzymes processes CDNB, 7-chloro-4-nitrobenzo-2-oxa-1, 3-diazole, *p*-nitrophenyl acetate particularly effectively and are found in most tissues. These isoforms are thought to be responsible for protein repair, where thiol adducts are trimmed off cytosolic structures as the enzyme acts as a thiol transferase. It has a very large and open hydrophobic binding site, which allows it to bind polypeptide chains. This isoform is also involved in preventing cellular apoptosis by blocking calcium ion mobilization from intracellular stores.

GSTs and drug action

GSTs tend to be viewed exclusively as detoxifying enzymes, although they do appear to have a role in the activation of azathioprine, an immunosuppressive drug which is also used in childhood leukaemias. This agent is a pro-drug, as it is converted to 6-mercaptopurine (6-MP), which is processed further to toxic 6-thioguanine nucleotides (6-TGN) that block purine synthesis. GSTA1-1, GSTA2-2 and GSTM1-1 can form 6-MP and their role has not been fully explored. Therapeutically, 6-MP must be formed in quantities sufficient to control the cancer, but not to cause severe toxicity. A balance between 6-MP formation and its destruction by TMPT (Chapter 7, section 7.2.6) was thought to be the key to successful therapy with azathioprine. TMPT is polymorphic and was believed to be the main factor in whether efficacy or toxicity was achieved. However, it appears that as the GSTs are also polymorphic and interindividual variability is so wide, they may be much more influential in 6-MP formation than was previously believed and it remains to be elucidated whether GST expression is a major player in the success or failure of azathioprine therapy.

GST therapeutic inhibition

The upregulation of GST is a serious problem within cancer therapeutics and resistance to a range of drugs including melphalan and doxorubicin is linked with GST detoxification. Much research has been directed at inhibitors of GST isoforms to reverse or even prevent the development of resistance to anti-neoplastic agents. Unfortunately this strategy has not been successful in the clinic, partly due to lack of selectivity and also the GSTs are so ubiquitous and toxicity has resulted. Known inhibitors such as ethacrynic acid inhibit other conjugation systems and have worked in cellular models but have been unsuccessful clinically. However, there are several lines of investigation proceedings studies with GSH analogues such as TER 199 have led to a patent (until 2015) as a GSTP1 inhibitor, although its application is to protect bone marrow from toxicity during chemotherapy. It is also

apparent that many dietary flavonoids are also potent GSTP1 inhibitors, but they also inhibit other systems like SULTs. The most recent direction has been towards designing highly specific F2 GSH peptide fragment inhibitors as the basis for progress towards therapeutically effective and selective inhibitors of tumour GSTs.

6.5.4 Control of GSTs: role of Nrf2/ARE system

GST expression is often already high in cancer cells even before they are treated with cytotoxics, although expression can be induced by the presence of alkylating agents and various other reactive species. It is likely, but not completely established, that these vital cellular defence enzymes are at least partly coordinated with CYPs, UGTs, SULT enzymes and the efflux transporters. Human systems are so efficient in the biotransformation and detoxification of endogenous and xenobiotic agents that it is clear that such coordination in practice usually nullifies the risks of cell damage inherent in CYP oxidation so the system works, but it is not clear how GSTs are controlled in this context. Although the nuclear receptor series of CAR, PXR, and so on, are well documented in their control of CYPs, UGTs and the efflux transporters, there is little evidence so far that they influence the reactive species specialist enzymes, the GSTs. So if reactive species are not suitable for the PXR/CAR systems, then how do the GSTs genes mount their response to reactive species? In effect, they exploit the very thing that makes electrophiles and other unstable species different and more dangerous than stable NR substrates, that is, their reactivity. In mammalian GST genes, AREs or antioxidant response elements, have been found, which are sometimes termed electrophile response elements (EpREs; Figure 6.9). These

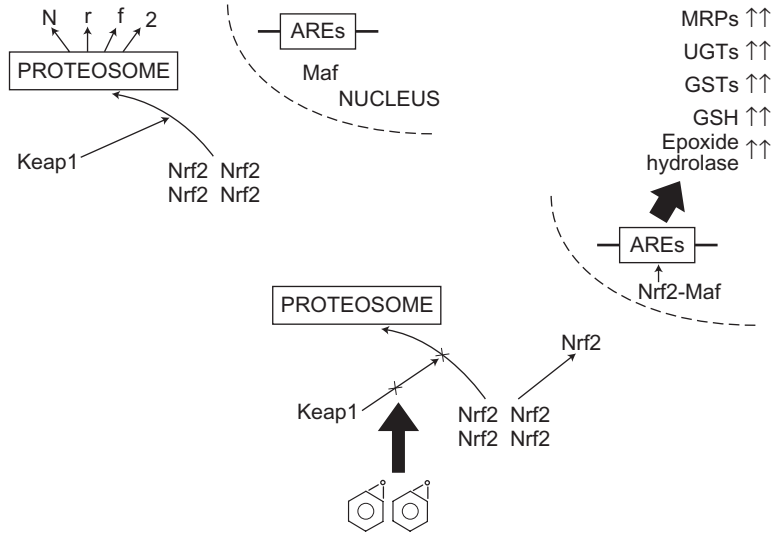


Figure 6.9 The Nrf2 system is intended to respond to electrophiles, which prevent Keap1 from guiding Nrf2 towards proteosomal destruction and allow surplus Nrf2 to bind Maf transcription activators. The complex binds DNA antioxidant response elements (AREs) so expressing a range of detoxification enzymes, such as the GSTs, epoxide hydrolases, UGTs and GSH synthesis

DNA elements initiate GST transcription post binding with Nrf2 (nuclear factor erythroid 2 045-related factor 2). The Nrf2 system works a little like CYP2E1 (Chapter 4, section 4.4), in that it is usually locked up with Keap1 protein (kelch-like ECH-associated protein 1, obviously) which specifically attaches Nrf2 to the actin cell skeleton in the cytoplasm, which means it is ubiquitinized and destroyed quickly by the proteosome. It was first thought that the reactivity of toxic electrophiles could break the Keap1 lock-up and the freed Nrf2 heads for the nucleus whilst recruiting some Maf (transcription activator) proteins along the way towards eventual binding the DNA AREs. However, some dietary agents such as sulforaphane can activate the Nrf2 system, but they do this without actually breaking all the bonds between Nrf2 and Keap1. So it has been proposed that electrophiles prevent Keap1 from shepherding Nrf2 to the proteasome, so all the Keap1 is then bound to Nrf2, which is in constant production and when Keap1 is fully occupied, the excess Nrf2 gets through to the AREs in the nucleus and activates the GSTs (Figure 6.9). Since the GSTs are nothing without GSH, Nrf2 also upregulates GSH synthesis through a binding site on γ-glutamyl cysteinyl synthetase to keep pace with increased demand for detoxification by overexpressed GSTs. It is believed that this Nrf2 system is one of the common links between GSTs, some UGTs, the epoxide hydrolases as well as the efflux transporters (MRPs see below), so providing a coordinated response to various electrophilic agents and toxins, particularly anti-cancer drugs.

It would be expected from an evolutionary standpoint that organisms which possess an unusually high capacity to respond to a xenobiotic toxic threat which is far in excess of endogenous molecules will thrive at the expense of organisms which cannot neutralize the reactive species and clear them from their cells. This appears to be the case in humans, as there is considerable variation in the phenotype of these enzymes, conferring a wide defence for our species from environmental toxicity often at the expense of the individual.

6.6 Epoxide hydrolases

6.6.1 Types of epoxide hydrolases (EHs)

Epoxide hydrolases are important multifunctional enzymes for both the deactivation and activation of reactive species; in addition, they have a number of equally important endogenous functions. In the same way as CYPs, higher organisms have ‘appropriated’ epoxide hydrolases from bacteria, with little real change in structure and function. Indeed, there is 75 per cent similarity across various mammals as well as man in the amino acid composition of these enzymes. From the drug/toxin metabolism standpoint, their main function is to convert any potentially reactive epoxide, formed by CYPs, to a diol (also known as dihydrodiols), which is usually less reactive, more water-soluble and more likely to be cleared by GSTs. There are two main variants of epoxide hydrolases:

- Microsomal epoxide hydrolase (mEH, or EPHX1) exists as two ‘Types’. *Type I* is ideally positioned in the hepatic endoplasmic reticulum to ‘intercept’ epoxides of polycyclic aromatics (like styrene 7,8 epoxide), or drugs (carbamazepine 10,11 epoxide; Figure 6.10) formed by CYPs and make them into diols. *Type II* mEH is found in the hepatocyte plasma membrane, where it controls the uptake of bile

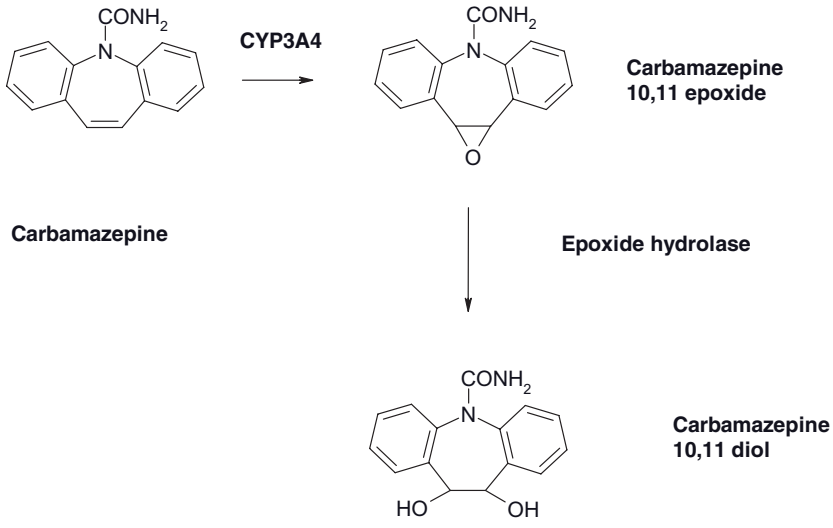


Figure 6.10 A typical microsomal epoxide hydrolase reaction: formation of carbamazepine 10,11 diol, which is thought to have anticonvulsant activity

acids in the liver in association with a taurocholate binding protein; this system is involved in a number of endogenous processes, such as the control of cholesterol metabolism.

- Soluble epoxide hydrolase (sEH, or EPHX2) also forms diols from many endogenous and exogenous epoxides. Unlike mEH, it has been found in most tissues as well as the liver, lung and kidney, as it is often closely associated with CYP enzymes such as CYP2C8 and 9. EPHX2 appears to regulate many pathways related to endogenous systems such as fatty acid and leukotriene epoxide metabolism and is involved in blood pressure regulation and inflammatory responses. Epoxide hydrolases are regulated by the Nrf2 system and they respond to electrophilic epoxides, such as those generated from PAHs or drugs such as carbamazepine.

6.6.2 EH mechanisms of action

The mechanisms of action of these enzymes have been described (Figure 6.11), partly on an experimental basis and partly on the crystallization of sEH. It appears that although the enzyme has a low turnover number, it manages to catalyze the formation of diols extremely quickly, using tyrosine residues as proton donors and the carboxy group of an aspartate residue, which forms an intermediate enzyme–substrate ester, which then slowly hydrolyzes to give the diol. This latter step is rate-limiting, although it is likely the enzyme accelerates the process of hydrolysis, as ester bonds are usually stable.

Epoxide hydrolases are thought to be responsible for the formation of diol-epoxides of PAHs, which are potent carcinogens. There are slow and fast versions of these enzymes (Chapter 7, section 7.2.4) and there is considerable human variation in their phenotypes.

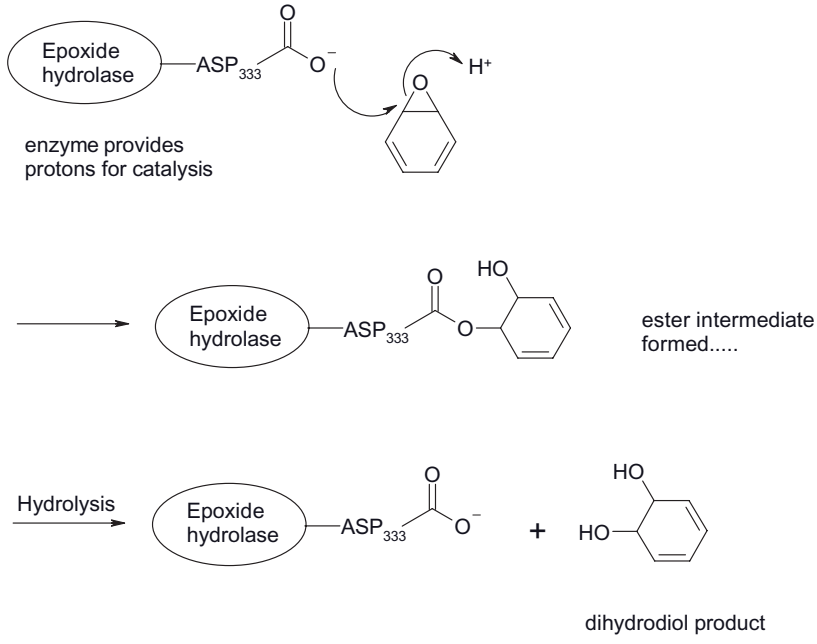


Figure 6.11 Mechanism of action of epoxide hydrolase in the conversion of a typical CYP-formed epoxide to a dihydrodiol

6.7 Acetylation

By now, you will hopefully be familiar with the central idea that biotransformation increases water solubility at the expense of lipid solubility. Acetylation is generally classified as a Phase II process, although it appears to be rather contradictory as acetylated metabolites are less water-soluble than the parent drug; indeed, this often makes acetylated metabolites difficult to eliminate in urine. *In extremis*, acetylated metabolites of some old sulphonamides were so poorly water-soluble that they crystallized (painfully) in the patient's kidneys. You may be wondering what acetyltransferases are actually for, since they do not at first appear to contribute positively to the clearance of their substrates. There are a number of acetyltransferase gene families found in most cells and they have a large number of functions connected with cell homeostasis; for example, histone acetyl transferases (HATs) regulate DNA transcription through activating histone proteins by acetylating them, whilst some hormones such as melatonin are regulated through acetyltransferases. Although most metabolic reactions are to some extent reversible, acetylation is much more reversible than most. Indeed, there are many of these enzymes that are predominantly deacetylases, as well as acetylases. Many acetylated molecules can act as 'on' and 'off' switches in the regulation of the functions of nuclear receptor systems. The acetyl transferases relevant to human drug metabolism include two families of genes which are expressed, N-acetyl transferase 1 (NAT-1) and N-acetyltransferase 2 (NAT-2). These enzymes use acetyl Co enzyme A as a co-factor to acetylate their substrates by using what

is known as a double displacement (ping-pong) mechanism. There are two sequential steps to the reaction: firstly, the acetyl group is moved from acetyl CoA to form an acetylated enzyme intermediate, then the substrate is acetylated and CoA is released. Iodacetate and N-ethylmaleide are irreversible inhibitors of the process, whilst reversible inhibitors (salicylamide) are similar in structure to the substrates. NAT-1 is found in many tissues, particularly in the colon, but also in erythrocytes. NAT-1 expression was thought to be fairly constant through human populations, but recent studies have shown that there is at least a two-fold difference in some populations and it is believed that NAT-1 may be inducible in response to certain xenobiotic and endogenous substrates. It prefers to process substrates such as para-aminobenzoic and para-aminosalicyclic acids and is not generally associated with the acetylation of drugs. The consequences of genetic variation in acetyltransferases are discussed in Chapter 7 (section 7.2.5).

6.8 Methylation

As with acetylation, adding lipophilic methyl groups to various drugs that decrease water solubility does not appear very logical in the general context of conjugative metabolism. However, as with acetylation, there are a huge number of cytosolic methylases that are responsible for many stages in DNA regulation and other cellular housekeeping tasks. S-adenosyl methionine (SAM) is used as a carrier for the methyl group. SAM is made from ATP and L-methionine. SAM-dependent methyltransferases methylate RNA and DNA, many proteins, polysaccharides, lipids and many other molecules. N, O and S-methyltransferases can be found in human systems.

N-methyltransferases can methylate various histamine-related compounds and amines. In the brain and the liver, catechol-O-methyl transferase (COMT) O-methylates the phenolic groups of a number of catecholamine neurotransmitters, including adrenaline and noradrenaline, although it also methylates dopamine; tolcapone (unfortunately a hepatoxin) was developed to inhibit the enzyme to increase dopamine levels in the brain in Parkinsonism. This drug was withdrawn in 1998 but re-introduced on a restricted basis in 2004. Methyl transferases can also methylate adrenaline, thiouracil, histamine and pyridine derivatives.

6.9 Esterases/amidases

There are a number of drugs that possess ester or amide linkages and these are vulnerable to the activity of esterases and amidases. Esterases are sometimes known as carboxylesterases and can overlap in activity with amidases (Figure 6.12). These enzymes clear procaine, acetyl salicylate and chloramphenicol, although genetic polymorphisms can prolong the clinical effects of a number of neuromuscular blocking drugs (succinylcholine, atracurium mivacurium) that are hydrolyzed by butyrylcholinesterase (BChe; Chapter 7, section 7.2.4). There are many esterase enzymes in various tissues and the plasma and they are responsible for the rapid clearance of cocaine and heroin (Appendix B). The liver and kidney enzymes can hydrolyze the drugs listed above as well as several organophosphate and carbamate insecticides, as well as some herbicides. All the esterases, which

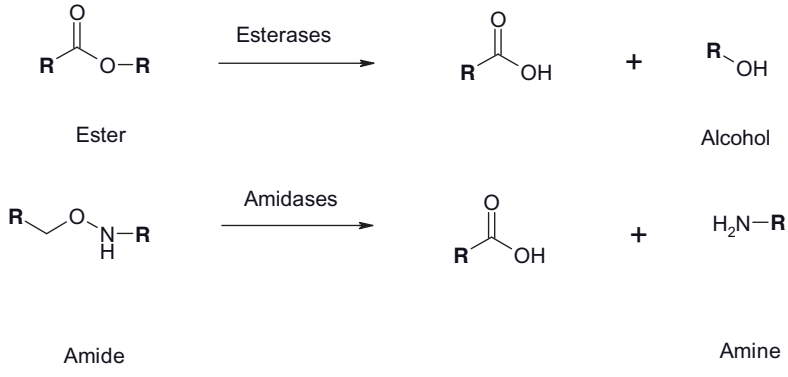


Figure 6.12 Esterase and amidase reaction sequences

include neural acetylcholinesterase and butylcholinesterases, operate in a fairly similar manner, using an anionic and esteratic site to bind the substrate and a serine hydroxyl group to catalyze the hydrolysis of the ester linkage. Once an amidase has hydrolyzed an amide, if an aromatic amine is released, the subsequent oxidative N-hydroxylation of the amine can cause systemic toxicity.

6.10 Amino acid conjugation (glycine or glutamate)

This is a comparatively minor but specialized route of conjugative clearance generally involving glycine and occasionally glutamate. Glycine conjugation is the main route of clearance of salicylate, and a number of small aromatic alcohols and carboxylic acids can also act as substrates for the enzymes that catalyze the process, acyl-CoA synthetase and N-acyltransferase. The enzymes are found in the liver and kidney, but it appears that in the case of salicylate glycine conjugation the kidney is the main clearance organ. The amino acid is attached to the drug through an amide bond and can depend on cellular glycine supplies. Small organic acids like benzoic acid, a food preservative, are also cleared to hippuric acid derivatives through glycine conjugation. This is of relevance in the biological monitoring of toluene which is cleared by CYP2E1 to benzyl alcohol and thence via glycine conjugation to hippuric acid. It has been shown that industrial monitoring of toluene exposure is compromised by benzoic acid from several sources such as blueberries, grapes and apples and even green tea and coffee.

6.11 Phase III transport processes

6.11.1 Introduction

Obviously once xenobiotics have been converted into low-toxicity, higher-molecular-weight and high-water-solubility metabolites by the combination of CYPs, UGTs, SULTs

and GSTs, this appears at first sight to be 'mission accomplished'. However, these conjugates must be transported against a concentration gradient out of the cell into the interstitial space between cells. Then they will enter the capillary system and thence to the main bloodstream and filtration by the kidneys. The biggest hurdle is the transport out of the cell, which is a tall order, as once a highly water-soluble entity has been created, it will effectively be 'ion-trapped' in the cell, as the cell membrane is highly lipophilic and is an effective barrier to the exit as well as entry of most hydrophilic molecules. In addition, failure to remove the hydrophilic products of conjugation reactions can lead to:

- toxicity of conjugates to various cell components;
- hydrolysis of conjugates back to the original reactive species;
- inhibition of conjugating enzymes.

If the cell can manage to transport them out, then they should be excreted in urine or bile and detoxification can proceed at a maximal rate. Processes such as enterohepatic recirculation complicate this situation, where conjugates are hydrolyzed and parent drug appears in the gut for reabsorption and transport by the efflux pump systems.

Consequently, an impressive array of multi-purpose membrane bound transport carrier systems has evolved which can actively remove hydrophilic metabolites and many other low molecular weight drugs and toxins from cells. The relatively recent (1990s) term of Phase III metabolism has been applied to the study of this essential arm of the detoxification process.

6.11.2 Efflux transporters

The main thrust of research into efflux transporters has been directed at the ABC-type transporters, of which there are 48 genes that code of a variety of ATP-powered pumps. P-glycoprotein (P-gp, Chapters 4 and 5) BCRP (breast cancer resistance protein) and multi-drug resistance associated proteins (MRP1-3, also known as MDRs) are the best known and studied. Aside from the role of P-gp in gut drug efflux, these transporters are partly responsible for the resistance of cancer cells to chemotherapy and indeed, generic MDRs, or multi-drug resistance transporter proteins, are also used as mentioned previously throughout the bacterial and protozoan world to evade the effects of antibiotics. Whilst P-gp is intended to pump out relatively bulky lipophilic agents (orally administered drugs especially, the main endogenous role of clearing charged, water-soluble conjugates lies with MRPs 1–3.

MRP1–5 (ABCC1-3, GS-X pumps)

So far, ten MRP genes have been identified and their enzyme products have been known by a number of names, including ABCC, GS-X and MOAT (multi-specific canalicular organic anion transporter) pumps. They have approximate molecular weights of around

190,000 and they only show about a 15 per cent resemblance to P-gp, so they are fundamentally different in their substrates. MRPs usually pump out molecules of toxicological significance, like GSH conjugates, steroid glucuronides and sulphated metabolites. They are found in most tissues, such as the liver, lung, testis and skin, and they are thought to act in a coordinated manner with GST enzymes. MRP1 may have evolved to remove GSH-related and other conjugates from cells (and is particularly important in the lung), whilst MRP2 is mainly found in the liver, gut, kidney and lung and is responsible for pumping bilirubin glucuronides into the bile in humans. A condition known as Dubin Johnson syndrome is associated with non-expression of MRP2 and patients cannot clear bilirubin conjugates efficiently. MRP1 and MRP 2 can both apparently use GSH in a co-transport capacity to clear non-conjugated and conjugated agents like methotrexate, vincristine, conjugated nitrosamines and (MRP-2), cisplatin.

MRPs are controlled by the nuclear receptors PXR, CAR and FXR and MRP2 is particularly well documented in its PXR–mediated expression. MRP1 is usually associated with substrates like GS conjugates of etoposide and melphalan, as well as aflatoxin B1 metabolites and several tobacco-related nitrosamine glucuronides. It is thought that MRP1 and 2 have multi-point binding pockets which enable them to bind the hydrophobic and polar areas of conjugated metabolites simultaneously at several points on the substrate, although MRP2 has a lower affinity for conjugates than MRP1.

MRP3 is much closer in structure to MRP1 than MRP2, but does not transport GSH and is a much less efficient glucuronide transporter than MRP1. MRP3 is also known as D-MOAT and is induced by phenobarbitone and the carcinogen 2-acetylaminofluorene. Interestingly, MRP3 can pump bile acids and is strongly induced during cholestasis, where it acts to try to clear the backlog of bile acids and conjugates by forcing them into the blood, as they will not be cleared through bile. This has a detoxifying action and the agents have some chance of being cleared in urine. MRP4 (was MOAT-C) and MRP5 are organic anion transporters and they export various pyrimidine bases as well as methotrexate and they are linked to resistance to the anti-cancer agent 6-mercaptopurine. Inhibiting MRPs to improve the effectiveness of anticancer agents is difficult, as many other transporters, as well as the other ABC transporters could be blocked also, leading to widespread toxicity. There is some evidence that in experimental studies with doxorubicin, the flavonoid naringenin may be a useful and reasonably specific ABC transporter inhibitor, as it blocks MRPs, but not P-gp at non-cytotoxic levels.

DNP-SG ATPase and other pump systems

This pump system was one of the earlier ones to be identified and this was named as it was shown first to be stimulated by *S*-(2,4-dinitrophenyl)glutathione (DNP-SG) and ATP was consumed. This broad specificity pump system is found in most tissues and it is unusual in that it will transport both anionic (e.g. glutathione-related conjugated metabolites) and cationic (doxorubicin) substrates. Dubin-Johnson patients who cannot express MRP2 have residual anionic efflux ability that is thought to occur through the operation of DNP-SG ATPase. There are a number of other non-ATP dependent pump systems which work in concert with the MRP series of efflux pumps, such as RLIP76, a non-ABC, GTPase-activating protein which transports GSH-related and other conjugation products.

6.12 Biotransformation-integration of processes

As has been mentioned already, it is clear that the whole process of detection, metabolism and elimination of endobiotic and xenobiotic agents is minutely coordinated and is responsive to changes in load in individual tissues. The CYPs, UGTs, MRPs and P-gp are all tightly regulated through the NR system of PXR, CAR, FXE, PPAR α, LXR etc, as well as the AhR receptor system. Some enzyme/pump processes are closely linked, such as CYP3A4 and P-gp, as inducers powerfully increase both systems capacity. The reactive species protection 'arm' of biotransformation is also controlled through a separate but almost certainly 'cross-talking' Nrf2/Keap1 system which coordinates not only the interception of reactive species by GSTs, but also the supply of their GSH substrate, UGTs and the MRPs. This latter coordination is particularly relevant in resistance to cancer chemotherapy and happens because overexpression of any one entity alone cannot rid the cell of the toxin. If the GSTs or the UGTs alone were upregulated, their products would soon negatively feedback on their activity and they would also run out of co-factors. This is particularly true of GSH, as not only is it the only GST co-factor/substrate, it is also necessary for the activity of MRPs 1 and 2 (but not MRP3) as they use it to extrude the conjugates from the cell. The upregulation of GSH synthesis probably by Nrf2 requires more ATP which must come from upregulation in mitochondrial production. The MRPs, GSH production and GST/UGT activity must be induced in concert. Whilst much of the integration and coordination of detoxification processes remains to be uncovered, the mere

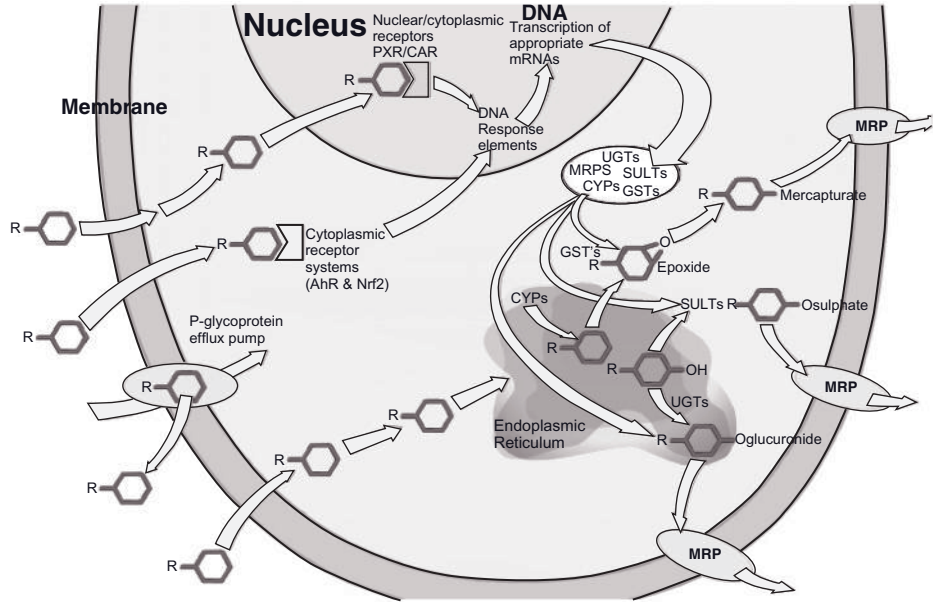


Figure 6.13 A scheme showing some major biotransformational systems operating in concert for the detection and elimination of xenobiotic molecules such as aromatic hydrocarbons

fact that at the time of writing Keith Richards is still with us testifies to their resilience and effectiveness.

Figure 6.13 shows a scheme that tries to sum up the main aspects of how biotransformation is coordinated alongside the Phase III systems of efflux proteins such as the MRPs. This system operates to some extent in virtually every tissue in the body, as well as the main areas of metabolism, such as the liver, gut, lung kidney and gonads.

7 Factors Affecting Drug Metabolism

7.1 Introduction

As you are may be aware, drugs are initially tested at the preclinical stage in animal populations which are usually inbred and display little variation from animal to animal. Data from animal studies in one country are usually comparable with that of another, provided the animal species and strain are the same. This provides a consistent picture of the basic pharmacological and toxicological actions of a candidate drug in a living organism, although controversy still rages over the value of this picture. Currently, animal data, combined with human tissue studies, are intended to give some approximation as to how the drug might affect humans. Unfortunately, you might also be aware that some drugs reach the clinic only to be withdrawn or have severe strictures placed on their usage, based on some form of toxicity or unintended pharmacological effect. Although it has been obvious since animal testing began that there would be large differences in the way a drug might perform in man compared with animal species, perhaps in the last 15–20 years it is clear just how vast and ultimately costly these differences can be. Unfortunately, there is no experimental model yet designed that can not only consider human biochemistry and physiology, but also the effects of age, smoking, legal and illegal drug usage, gender, diet, environment, disease and finally genetic variation. Indeed, many clinical studies have revealed enormous differences in drug clearance and pharmacological effect even in age, sex and ethnically matched individuals. In effect, this means that the first year or so of a drug's clinical life is a vast, but monitored experiment, involving hundreds of thousands of patients and there is no guarantee of success. This chapter discusses some of the factors that can influence drug metabolism and their impact on the achievement of our goal of maintaining drug levels within the patient's therapeutic window.

7.2 Genetic polymorphisms

7.2.1 Introduction

Apparently, cheetahs are all genetically identical and can receive skin grafts from any other cheetah like an identical twin could in humans. The same goes for elephant seals. Environmental and hunting pressures shrank the species to such small numbers that only one genetic variant now exists. The long-term survival of this type of population is

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unlikely, as genetic diversity is vital to ensure that no one toxin, bacteria or virus can eliminate all the population. In practice, genetic diversity is manifested in differences in single DNA nucleotides and/or whole genes that code for particular proteins. This results in a proportion of a population which expresses a protein that is different in structure and function to the majority. These differences may manifest as a substitution of a single amino acid with another, or a whole amino acid sequence may be different. These are termed 'polymorphisms' and their study is termed 'pharmacogenetics' or 'pharmacogenomics'. A polymorphism is a genetic variant that appears in at least 1 per cent or more of a population. The range of effect is wide: the polymorphic protein may simply not be as efficient as the majority, or it may be completely non-functional. If you recall earlier chapters where enzyme structures have been described, you might remember (hopefully) that a single amino acid in a precise position is often vital for the enzyme to function. The most common polymorphisms seem to be due to a change in one nucleotide, which means that the gene now specifies a different amino acid in a critical position. These are called SNPs or single nucleotide polymorphisms and the resultant enzyme is structurally and often functionally different from the wild-type.

Although occasionally, polymorphisms can mean increased enzyme activity, they usually have a deleterious impact on enzyme performance, ranging from no enzyme appearing at all, all the way to the formation of incomplete isoforms due to arcane faults in mRNA expression. In Chapter 4 you saw that control of gene expression itself required a complex series of promotors and co-activators, all of which have to possess the exact structural and physicochemical properties to fulfill their function. Even if the area of the gene that codes for an enzyme itself is potentially functional, defects in expression regulation will impair functional translation and transcription. If you take into account the myriad systems which 'quality control', chaperone and position various enzymes, it is little short of miraculous that there are relatively so few serious genetic failures. Again, this is the result of two billion years of evolutionary 'research and development'.

Polymorphisms arise due to mutations, but persist in human populations due to factors that may involve some advantage of the milder forms of the polymorphism (heterozygotes). If a faulty enzyme's endogenous function can be shouldered by other systems, then even homozygotes for the polymorphism may never be aware of their genetic make-up, as they suffer no symptoms. Glucose-6-phosphate dehydrogenase deficiency (G-6-PD) in Afro-Caribbeans is only a problem if the individual takes certain antimalarials (primaquine or dapsone, Chapter 8), which generate toxic species that overwhelm the G-6-PD individual's compromised oxidant defence mechanisms. Other defects may not affect some tissues, but may affect risk elsewhere. In Chapter 6, Gilbert's syndrome does not lead to hepatic injury, but improves resistance to carcinogenicity. If only one enzyme can clear a particular therapeutic drug, a polymorphism in that enzyme may cause extremes of either drug failure or drug toxicity. Of course any given drug response is based partly on the sensitivity of the appropriate receptor as well as the amount of drug in the system. Although both these factors are subject to polymorphisms, the following sections concentrate on polymorphic effects on the mechanisms that govern drug clearance. Most biotransformational polymorphisms that might potentially cause a problem clinically are due to an inability of those with defective enzymes to remove the drug from the system. Drug failure can occur if the agent is administered as a pro-drug and requires some metabolic conversion to an active metabolite. Drug accumulation can lead to unpleasant side effects and loss of patient tolerance for the agent.

Polymorphisms: terminology

A polymorphic form of a CYP is usually written with a * and a number for each allelic variant, or translated version of the gene. The wild-type allele, or the major form expressed in the population is often termed *1, as in CYP2B6*1, for example. Regarding the polymorphic forms, they might contain one or more SNPs in the same allele. For example, CYP2B6 has eight other significant allelic variants besides the wild-type; among these variants, CYP2B6*4 has just one SNP, whilst CYP2B6*6 possesses two SNPs. The scientific literature often refers to SNPs either by the particular codon and nucleotide substitution and/or by the actual amino acid which is substituted in the finished enzyme. So for the CYP2B6*4 variant the SNP will be termed A785G or A>G 785. This means that a wild-type adenine base has been replaced by a guanine in this SNP in codon 785 on the gene. On the enzyme, at amino acid 262, a wild-type lysine (DNA codon AAA or AAG) is now an arginine (codon AGA or AGG). The respective single letter codes for lysine and arginine are 'K' and 'R', so this SNP is also known as 'K262R'. Often a polymorphism will be rather confusingly referred to by either of these identifications. So for the dual SNP CYP2B6*6 variant, it has a G516T (or Q172H; glutamine replaced by histidine) as well as the A785G (K262R) described above. There are also variants of CYP2B6 that have three SNPs, although these are extremely rare.

Most population studies of human polymorphisms list the allelic frequency, that is, how many of an ethnic group contain the alleles in question. For CYP2B6, about 50 per cent of Caucasians have the wild-type allele, but only 28 per cent have the CYP2B6*6 allele. This does not mean that 50 per cent of Caucasians actually express wild type enzyme. The actual haplotypes in the population, that is, which individuals express which combinations of alleles, are not the same as the population allelic frequency. In a real population, a considerably smaller number, in the case of CYP2B6, about 30 per cent of individuals are homozygous for the wild type (CYP2B6*1*1). These individuals are usually classed as extensive metabolizers (EMs). There will be various combinations of the wild-type and other defective alleles, such as *1/*6, (about 30 per cent of the population) and *1/*5 (about 10 per cent). These heterozygotes might be classed as intermediate metabolizers or IMs, as the enzyme is slightly impaired but serviceable. Then there are various combinations of defective versions of the gene, such as *5/*6 or homozygous *6/*6 which might be only 7–8 per cent of the population. These individuals are usually termed poor metabolizers (PMs).

If an SNP or a combination of SNPs is a fairly mild defect in the enzyme when it is homozygously expressed, then the heterozygotes will show little impairment and the polymorphism may be clinically irrelevant. With other SNPs, the enzyme produced may be completely non-functional. Homozygotes will be virtually unable to clear the drug and heterozygotes will show impairment also. There are also smaller populations of UMs, or ultra rapid metabolizers which may have a feature of their enzyme which either makes it super efficient or expressed in abnormally high amounts. All these figures are very approximate and vary widely for each enzyme.

Sometimes SNPs in different enzyme families are inherited together, where the individuals might express two different defective CYPs (or other enzyme) at once. This combination may only come to light with a particular drug which relies on both CYPs for clearance and is also seen with UGT1A1 and 1A7 (section 7.2.7).

Genotype and phenotype

In practical terms, it is important to distinguish between genotyping and phenotyping patients for a particular enzyme isoform. Genotyping uses the patient's DNA to determine whether they are homozygous or heterozygous for particular alleles. This will show that for only a few alleles, there are likely to be dozens of haplotype combinations contributing to the full spectrum of drugs clearance. Phenotyping usually involves administering a single probe drug for a particular enzyme and measuring clearance and comparing it with data from other patients. As you can imagine, what should be predicted from the genotypic tests is not necessarily true phenotypically for all substrates of that enzyme and *vice versa*, as there are so many confounding factors, such as age, gender, weight, alternative routes of clearance and other variables, known and unknown. Phenotyping will group patients in very broad EMs, IMs or PM categories, but will be unable to distinguish between heterozygous and homozygous EMs. Although genotyping may be very helpful in dosage estimation in the initiation of therapy, there is no substitute for the normal process of therapeutic monitoring, which is effectively phenotyping the individual in the real world in terms of maximizing response and minimizing toxicity.

Indeed, you may see the term 'phenocopying', which essentially means that if an inhibitor blocks the clearance of another drug it effectively reduces a genotyped EM to the status of a phenotypic 'PM' for that drug. If the drug inhibits its own metabolism, it is sometimes called 'autophenocopying'. In effect, a whole patient population, including the EMs and UMs can be 'converted' to phenotypic PMs by phenocopying or autophenocopying. This is less likely to occur after single dose expression than during 'real world' chronic administration, as many drugs inhibit their own clearance after a few days, such as SSRIs and some protease inhibitors.

7.2.2 Clinical implications

From the sections below it is clear that there is a vast amount of genetic variation across humanity in terms of biotransformational capability and so the idea that in therapeutics, 'one size fits all' is not only outdated, but fabulously naïve. You may be curious as to why customization of drug therapy to the individual's needs is not already routine. Unfortunately, detecting and responding successfully to human biotransformational polymorphisms has proved to be extremely problematic. In terms of polymorphism detection, this area is a classic illustration of how the exploration of the human genome with powerful molecular biological tools may unearth many apparently marked polymorphic defects that may not necessarily translate into a measurable clinical impact in terms of efficacy and toxicity. In reality, many more scientists have the opportunity to discover and publish such polymorphisms *in vitro*, than there are clinical scientists, resources and indeed cooperative volunteers or patients in sufficient quantity to determine practical clinical relevance. Indeed, clinical studies of polymorphism relevance perhaps need three essential components: sufficient numbers to convince in terms of statistical relevance, detection of pharmacokinetic changes and importantly, how those changes affect drug pharmacodynamics. Many studies cover one or other of these three essentials, but not always all three.

Thankfully, the majority of relatively modern drugs have wide therapeutic indices, so it is emerging that polymorphisms may well cause marked increases in plasma levels

in PMs, but they are rarely enough to cause serious off-target effects and frank toxicity. In the case of pro-drugs, it is often apparent that even though production of the most potent metabolite is lacking in PMs, the drug retains enough clinical effect for the polymorphism to have only academic interest and a minor change in dosage optimizes treatment.

However, our overriding objective is patient safety plus optimum therapeutic effect and drug designers are now very much aware that in some drug categories, certain combinations of factors makes the likelihood of a particular polymorphism exert strong impact on treatment outcome and drug tolerance in some groups of individuals. These factors are as follows:

- *Narrow therapeutic index (TI):* aside from the greater likelihood of a toxic event occurring in a narrow TI drug, the nature of the toxicity and the drug's target organ are crucial. For instance, off-target cardiotoxicity such as TdP (Chapter 5, section 5.5.2) is far more dangerous over a short timescale than most other organ-directed toxicities.

- *Single pathway mediated clearance:* if only one enzyme is responsible for the clearance of an agent, inhibition of this pathway has much more impact than a drug with multiple pathways of elimination.

- *Background variability of pharmacodynamic response:* in the case of off-target responses, in effect, the TI in one patient may be considerably narrower than another and it may not be possible to predict or anticipate this.

- *Delay in toxic response to high drug levels:* this is most marked in a drug which may have a complex mode of action and the onset of deleterious effects is hard to predict, such as with warfarin.

If all four of these factors are present, then it is likely to be worthwhile considering some form of pharmacogenomic testing, if there is an available test for the isoform in question. Two more factors may make the test process more cost-effective in terms of clinical time and materials.

- *Drug levels equate directly with toxicity:* if the relationship between concentration and effect are consistent and predictable, then once a polymorphism is identified in an individual, dose escalation can be better informed.

- *Long term therapy:* saving a patient from potentially damaging toxicity over many months or years more than justifies the considerable effort and expense of polymorphism determination. This may be particularly applicable to instances such as the controversial relationship between sustained high neurileptic drug levels and the development of tardive dyskinesias in schizophrenic patients.

The following sections discuss the detection of major polymorphisms in human biotransformation and reviews current appraisals of their clinical relevance. After these sections, the process of response to these perceived clinical problems will also be discussed.

7.2.3 Genetic polymorphisms in CYP systems

CYP1A1

CYP1A1 is not a constitutively expressed hepatic isoform and it is not really relevant to drug clearance, but its greatest significance is as the inducible producer of potentially carcinogenic species from PAHs, particularly in the lungs in smokers. Indeed, high levels of this isoform are often seen in tissues affected by cancers, such as the lung and breast. The enzyme has evolved as a protection against PAH-like molecules, activating them into epoxides or other reactive species, which are then conjugated by adjacent conjugative systems to non-reactive excretable products. Why this system is linked to many cancers may be due to a variety of reasons, such as the large inter-individual variation in the degree of induction (Chapter 4, section 4.2) of CYP1A1, as well as polymorphisms in the isoform itself. From Chapter 4, it can be recalled that there are several steps in the AhR induction of this CYP and the proteins used in these steps are all vulnerable to genetic mutations. There are four currently described polymorphic forms of CYP1A1, of which CYP1A1*2 and CYP1A1*3 are the most studied. These differ only in one amino acid from the other forms and it has been suggested that this makes them easier to induce so forming more reactive species, which in turn increases cancer risk. This is very difficult to prove experimentally and many more studies need to be done to substantiate this.

CYP1A2

CYP1A2 specializes in xenobiotic polycyclic aromatic amines, as well as oestrogen metabolism. A considerable amount of liver CYP content has been identified as this isoform (approximately 10 per cent) and several drug classes are cleared at least partly by this CYP, such as the ‘R’ isomer of warfarin, some antipsychotics like clozapine and olanzapine, as well as caffeine, theophylline and two major antidepressants, fluvoxamine and duloxetine. This list accounts for around 9 per cent of prescribed drugs. So far, two variants have been found; one called CYP1A2*1C in Japanese people that may be defective, as it shows compromised caffeine metabolism, whilst another (CYP1A2*1F) is found in some European smokers and is of higher activity than the wild-type CYP1A2. It is not clear whether either of these polymorphisms have much relevance in drug clearance, as little research has been carried out in this area. Other polymorphisms of this CYP are too rare to be of clinical relevance.

CYP1B1

This CYP is strongly inducible and oxidizes many precarcinogens, such as PAHs, heterocyclic amines aflatoxins and other environmental toxins. It is also the most efficient oxidizer of oestrogens and is linked with hormone-dependent cancers. Overall, interest has centred on its role in predisposition to carcinogenesis, particularly in tobacco users and its toxicological significance is yet to be fully realized. Several polymorphisms have been investigated, including CYP1B1*2, which has two SNPs and may cause increased expression compared with wild-type. CYP1B1*3 has the wild-type leucine changed to a

valine at 432 and some studies have indicated that this increases the activity of the enzyme, as the valine is close to the haem active site of the isoform. CYP1B1*3 is also thought to be more easily induced than wild-type. The CYP1B1*4 is degraded faster than the other forms and may be protective as the variant's half-life is too short to make large amounts of reactive species. Research on the influence of these variants on predisposition to cancer can be conflicting, with CYP1B1*3 shown to increase risk of breast and other cancers, but be protective in 4-aminophenol-linked DNA adduct formation in smokers, whilst the leucine wild-type showed higher adduct formation. Alcohol intake, plus CYP1B1*2 may increase the carcinogenicity of smoking in terms of head and neck cancers and the risk of possessing *3 is many fold higher than wild-type in lung cancer predisposition. The *2 variant is more common in Indians than Caucasians. Both *2 and *3 show high risk for oral cancer with those who chew tobacco. CYP1B1 status may also be linked with endometrial cancer and drug resistance during tamoxifen therapy, as it forms a weak oestrogenic agonist from the active metabolite of the drug. Overall, the role of CYP1B1 in cancer risk is subject to myriad other factors and it may be some time before genotyping may predict risks for specific populations and cancers with any degree of certainty.

CYP2A6

The major interest in this CYP is its role in the metabolism of tobacco-related chemicals. There are some polymorphisms associated with the isoform, with at least two mutant types where there are deletions of the gene, meaning that no CYP2A6 is expressed at all. These genes are of significance regarding the risks of lung cancer from smoking. CYP2A6 is known to clear several nitrosamines such as NNK to reactive species which are linked to malignancy in lung tissue in smoking. At first examination, it would appear that the absence of CYP2A6 should reduce the risk of lung malignancy by a considerable degree. In Japanese smokers it was found that the platelet-activating factor antagonist SM-12502 is a good CYP2A6 probe substrate and using this drug has shown that potentially up to 20 per cent of the Japanese population possess the allele CYP2A6*4C, where the entire enzyme is absent. In Caucasians, the version of CYP2A6 where the isoform is absent is known as CYP2A6*2, which is only found in 1–3 per cent of the population. Epidemiological studies show that Japanese smokers with the *4C variant are less likely to develop lung cancer, whilst in Caucasians, rates of lung cancer are much higher, possibly because their CYP2A6 absence allele is so much more rare than the Japanese version. However, the situation is complicated by the role of CYP2A6 in nicotine metabolism. From Chapter 3 (section 3.6.2), you might recall that CYP2A6 is part of the process of nicotine clearance and if the enzyme is blocked by an inhibitor, or if it is absent, then nicotine's half-life in such individuals is significantly extended and so the drug craving is said to be less intense and the addiction easier to break. It is also likely that CYP2A6-deleted individuals do not smoke nearly as much as those with the wild-type isoform. These are the occasional smokers who can limit their tobacco consumption to a few (probably someone else's) cigarettes per day, so their net toxic tobacco exposure is much lower than the wild-type individuals which may also be part of the reason why they have lower rates of lung cancer. These observations may shed light on how Japanese longevity rates are amongst the highest in the world, yet so many of the population are smokers. Indeed, people of Asian (Japanese, Chinese and other races) descent are more resistant to cigarette-induced lung

cancer than Caucasians. Patients who cannot give up smoking sometimes cite their grandfather or uncle who smoked vast amounts of industrial strength double-black shag tobacco and lived to 101, when they of course died of natural causes. This is most likely to have some basis in fact if their uncle or grandfather was Japanese.

CYP2B6

Although CYP2B6 comprises a lower proportion of total hepatic CYPs than CYP1A, (approximately 5–10 per cent) it has assumed increasing importance in view of its high inducibility and very high baseline variability in expression. Although relatively few drugs are cleared by this CYP, several are quite toxic in high concentrations and many are inducers of this isoform also. Cyclophosphamide, efavirenz, rifampicin, nevirapine and carbamazepine are substrates and inducers, whilst methadone, ketamine, MDMA (ecstasy), pethidine and propofol are also substrates. Recent work has shown that there are large numbers of polymorphisms of CYP2B6, but only two or three really significant variants. The most important variant as mentioned in section 7.2.1, is CYP2B6*6 which contains two SNPs, meaning that two incorrect amino acids are coded for; these are designated G516T (Q172H) and A785G (K262R). The G516T fault causes defective CYP2B6*6 expression, leading to a short mRNA which of course codes for a short protein so individuals with this variant have only around a quarter of the wild-type's CYP2B6 catalytic activity. The CYP2B6 variant with just the G516T SNP alone is rare, but is designated CYP2B6*9, whilst CYP2B6 with just the A785G SNP is called CYP2B6*4 when it rarely appears as an allele on its own. The CYP2B6*6 variant (which has both SNPs) is common and it appears in more than 60 per cent of Papua New Guineans, 40 per cent of African Americans, Africans, Chinese, Hispanics and South Indians, about 28 per cent of Caucasians, and 17 per cent of Japanese. There is another variant which is more common than first thought, called CYP2B6*18 (also known as T983C), which is very poorly functional and is found in as much as 10 per cent of African populations, but it has not been found in Caucasians.

The clinical impact of CYP2B6 polymorphisms is an emerging area, but it is clear that efavirenz clearance is retarded sufficiently in CYP2B6*6 individuals to yield plasma levels that were nearly three-fold higher than wild-type individuals. This caused CNS problems such as changes in mood, headaches and fatigue. This problem was significantly alleviated when the Japanese HIV patients were genotyped for CYP2B6*6 and their doses were reduced; the disease was treated and CNS toxicity was greatly reduced. The same genotyping approach should be effective for another anti-HIV agent nivirapine. Whilst in total contrast, patients expressing the CYP2B6 G516T SNP actually cleared cyclophosphamide to its 4-hydroxy active metabolite more rapidly than wild-type individuals did. With bupropion, it is more controversial and some authorities feel that it is likely that its anti-smoking effects are not affected by CYP2B6 polymorphisms. With methadone, its two isomers have different efficacy and toxicity profiles. The R isomer is more potent as an opiate and less dependent on CYP2B6 than the S, which is more likely to block hERG channels and cause TdP. What seems to happen in CYP2B6*6 patients is that they have twice the S methadone levels than wild-type individuals who are more at risk of long QT syndrome leading to TdP and sudden death. It appears that there is less effect on the drug's efficacy. It is apparent that the impact of the G516T SNP varies

according to the drug involved and it underlines that polymorphisms are not necessarily always a 'defect'; they are, as they have evolved to be, just 'different', to ensure that the population does not become too homogeneous and therefore vulnerable to a single negative factor.

CYP2C8

Approximately 6 per cent of commonly prescribed drugs are cleared by this CYP in some form, although only a few agents rely on it as their main route of clearance. These are usually stated to be the antineoplastic agent taxol (paclitaxel) and two antidiabetic agents, rosiglitazone and repaglinide. Aside from the wild-type (*1) there are four major polymorphic forms (CYP2C8*2, *3, *4 and *5. In those of African ancestry, the *3 variant is very low in frequency, but the *2 allele is found at levels of up to 20 per cent. The *5 is found at a very low frequency in Japanese and the isoform is useless as most of the enzyme's structure is missing.

The *3 and *4 variants are found in Caucasians (the 2* is absent in this group) whilst *3 contains two SNPs R139K and K399R. The *3 variant is linked with CYP2C9*2 and CYP2C9*3 polymorphisms (see next section) and studies with the 'R' isomer of ibuprofen, a safe and useful probe for CYP2C8, have shown a four-fold increase in drug half-life in homozygous *3 variant individuals. Given that the 3* allele frequency is 13–23 per cent in Caucasians, as well as the high frequency of the *2 variant in Africans, it is interesting that an impact on paclitaxel clearance has not been established already. Although the effect has been shown *in vitro*, it has not been shown conclusively yet that the antineoplastic drug's clearance is retarded enough to cause side effects due to drug accumulation. Highly toxic drugs such as the taxols are used under close supervision in a hospital or clinic, so even if the CYP2C8*3/*3 (homozygous) variant was to show marked impact on a Caucasian patient in their first treatment day of a chemotherapeutic cycle, dose modulation would swiftly follow resulting in efficient customizing of treatment to that individual. Alternatively, any impact of CYP2C8 polymorphisms on a relatively casually used over the counter (OTC) drug such as ibuprofen's clinical effects are probably not really likely to be relevant clinically either, given the drug's good safety profile.

The CYP2C8*3 variant has been associated with sub-clinically effective repaglinide concentrations, although this could be partly due to OATP1B1 transport rather than a CYP effect. The effects of the *3 variant on the glitazone drugs are unproven, but given that gemfibrozil is such a potent inhibitor of rosiglitazone and pioglitazone clearances, it is clear that CYP2C8 has a major role in these drugs' oxidative metabolism, so it is possible that future studies may uncover a clinically relevant effect in CYP2C8 polymorphic individuals.

CYP2C9

Around 30 per cent of hepatic CYP content is accounted for by this isoform. Indeed, polymorphisms in this CYP are likely to be very significant in terms of clinical impact, as not only is it responsible for approximately 17 per cent of commonly prescribed drugs, but many of these agents have narrow TIs and can be lethally toxic in high concentrations.

There are two major variants, each with their unique SNP; the CYP2C9*2 shows a mild reduction in activity and the allele is found in about 20 per cent of Caucasians, 7 per cent of African Americans and Hispanics and is absent in Africans. The second more severely compromised variant (CYP2C9*3) has a catalytic capability that is less than 10 per cent of the wild-type and is present in 12 per cent of Caucasians, but is rare in other ethnic groups. Fortunately, it is only a major clinical problem in homozygous individuals (CYP2C9*3/*3), or around 0.5 per cent of the Caucasian population. There are co-heterozygotes who have both the *2 and *3 variants and homozygotes of the *2 variant. Although nearly a third of Caucasians have a defective CYP2C9 allele and will have some impairment in CYP2C9 performance, in practical terms, perhaps only 2–2.5 per cent of Caucasian populations are likely to have severely impaired CYP2C9 to some degree and this proportion is much smaller for the other major ethnic groups.

At first glance the proportion of patients with severe deficiencies of CYP2C9 seems small. However, if it is assumed that the Caucasian population of Europe and the USA is around 600 million, it is conceivable that that around 3 million Caucasians are CYP2C9*3/*3 variants. There is plenty of evidence that this variant is very much at risk from drug toxicity. The major class of drugs cleared by CYP2C9 where toxicity is particularly important is the coumarin anticoagulants. As you might remember from earlier in this book, about 8/10ths of the S-isomer of warfarin is cleared by CYP2C9 and this isomer supplies about 70 per cent of the drug's clinical effect. CYP3A4 and CYP1A2 clear the R-isomer. In wild-type individuals, R-isomer plasma levels are about twice those of the S-isomer. The *2 and *3 variants cause significant change in this ratio, with S-isomer plasma levels rising according to the severity of the polymorphism. At the extreme end of the spectrum, CYP2C9*3/*3 individuals can be fully anticoagulated on 10 times less warfarin than wild-type patients. Regarding other coumarin drugs, acenocoumarol is active through its R-isomer rather than its rapidly CYP2C9-cleared S-isomer. It too is markedly affected by CYP2C9*3 variants, with a 20–30 per cent reduced dose needed for even heterozyogotes and down to 60–70 per cent reduction for homozygotes. A less marked dosage may be required for CYP2C9*3/*3 individuals with phenprocoumon, as CYP2C9 has a much smaller role with this anticoagulant.

However, there are other factors involved in coumarin clinical response, such as age, weight, sex and particularly the status of the VKORC1 gene, which codes for the vitamin K epoxide reductase that is the coumarin therapeutic target. Even considering all these variables, such as the CYP, the target enzyme age, sex etc, still, the whole of the patient variability seen with warfarin cannot be entirely accounted for. Fortunately, there are several reasons why the effect of CYP2C9 polymorphisms is virtually attenuated in the clinical setting. As mentioned in Chapter 5, warfarin patients are stabilized not by dose but by clinical effect, the INR. In clinical practice, if patients over-respond from standard doses because they cannot clear the drug due to a CYP2C9*3 defect, it just takes longer for the clinic (around three months longer) to find a dose that will maintain their INR in the required area. Those against genetic testing for warfarin might argue about the cost, lack of training and awareness, as well as the myriad factors mentioned above which result in even wild-type CYP2C9 patients being stabilized on low drug doses. Those in favour of genetic testing with warfarin would argue that initial evaluation of genotype would prevent episodes of overmedication and bleeding in the first phase of therapy, which could be severe and even fatal in CYP2C9*3/*3 patients. In the final analysis, the vast clinical experience of the use of INR probably means that warfarin therapy would probably not

necessarily benefit from genetic testing, just some added caution is required during initial dosing given awareness of the prevalence of defective CYP2C9.

Among the other drug classes cleared by CYP2C9 include the non-steroidal anti-inflammatories (NSAIDs; diclofenac, ibuprofen, naproxen), the hypoglycaemic sulphonyl ureas such as tolbutamide and glipizide, as well as the angiotensin II blockers losartan and irbesartan and a number of other drugs such as dapsone, some sulphonamides, amitriptyline, some SSRIs, phenytoin and tamoxifen. Of these agents, the NSAIDs can cause gastrointestinal bleeding at high concentrations and although there is little evidence for any CYP2C9 polymorphic effects on diclofenac, ibuprofen is interesting as its R-isomer is a good probe for CYP2C8 and the S-isomer by CYP2C9. As CYP2C8*3 is linked with CYP2C9*3 and CYP2C9*2, if a patient was to be CYP2C9*3/*3 and CYP2C9*3/*3, that is, a double homozygote for each CYP, then their clearance of the NSAID would be less than 10 per cent of the wild-type. In practice, most variants with the CYP2C8 and CYP2C9 alleles have about twice the blood concentrations of wild-types. This may not necessarily be a problem in most patients for the period of time this drug is normally used. The association between gastric bleeding and other NSAIDs and CYP2C9 status is not believed to be particularly strong. Although there have been reports of hypoglycaemia due to the accumulation in sulphonyl ureas, there do not appear to be any significant problems with the angiotensin II blockers.

The clinical picture related to phenytoin is well documented and there is a strong relationship between CYP2C9 status and drug plasma levels. Several studies have shown that if drug doses for wild-type individuals were regarded as 100 per cent, then CYP2C9*1/*3 variants would require two-thirds, whilst CYP2C9*2/*3 variants would need about half this dose. If the *2 or *3 individuals are given the same doses as the wild-types, plasma levels can be up to three times higher in the polymorphic individuals. There are several unpleasant side-effects seen with phenytoin which has a well documented low TI and its kinetics are not linear, which means that there is little direct relationship between dose and plasma levels. The toxicity of phenytoin includes CNS-based and cutaneous problems and these are significantly worse in polymorphic individuals. However, in practice, these problems can be avoided by dose reduction when the patient is seen to be experiencing adverse effects. Although the drug is toxic, it is likely to be in use for many years yet and there is plenty of experience in manipulating its dosage to accommodate its potent induction properties. In general, the most relevant and severe effects with CYP2C9 are likely to be with the rare homozygous CYP2C9*3/*3 and the lack of enzyme activity is so marked that it would seem that it would be fairly ‘visible’ clinically through exaggerated pharmacological response and toxicity early in the treatment process.

CYP2C19

First detected more than thirty years ago as S-mephenytoin hydroxylase, CYP2C19 has emerged as important in the clearance of several classes of exceptionally widely used drugs, such as the proton pump inhibitors (PPIs), the phenytoin and S-mephenytoin, as well as several barbiturates, benzodiazepines, SSRIs and the antimalarial proguanil. The isoform has 90 per cent of its amino acid structure in common with CYP2C9, but it has very different properties. Seven variants have been found to date and three are likely to

be significant clinically. Of these, two code for completely non-functional enzyme (CYP2C19*2 and CYP2C19*3), which leads to virtually zero activity in homozygotes, whilst one variant (CYP2C19*17) causes ultra rapid metabolism. This latter version's promoter is thought to be faulty, causing very high levels of isoform production, rather like a throttle stuck on full. The allele for *2 is the commonest, found in 15 per cent of Caucasians, 17 per cent African Americans and 30 per cent of Chinese. The *3 variant is rare in most ethnic groups, with frequencies in Caucasians of practically zero, whilst 0.4 per cent of African-Americans and 5 per cent Chinese; by contrast, as many as 25 per cent of Koreans have this allele. The ultra fast metabolizing *17 allele is found in about 18 per cent of Swedish and Ethiopians, but only 4 per cent of Chinese.

There is some evidence that benzodiazepines are cleared more slowly in Chinese/Japanese populations due to CYP2C19*2 variants, but CYP3A4 is involved in their metabolism also, so the effect is not as emphatic as with the PPIs. With the SSRIs sertraline, citalopram, and fluoxetine levels can increase by about 1.5 fold, although this is not thought to be really clinically significant, again as other CYPs are involved with the oxidation of these drugs. There are minor effects on phenobarbitone clearance which are also not thought to be significant. Although phenytoin is partly cleared by CYP2C19, there may only be a problem with *2 or *3 variants when these individuals also have CYP2C9 polymorphisms. The antimalarial pro-drug proguanil must be cleared to its active metabolite cycloguanil and it would be expected that PMs would form insufficient levels of the active agent to destroy the *Plasmodia*; however, this has not been proven clinically. This problem may be masked as antimalarials are always used in combination with an agent which has a different mode of action. So proguanil is often used with chloroquine in areas where this combination is effective and resistance has not developed. The azole voriconazole shows up to 6 times higher plasma levels in CYP2C19 polymorphic individuals and although this is not thought to be a problem from the toxicity standpoint, it will probably improve the therapeutic effect. Unfortunately, it may also magnify its CYP inhibitory properties and cause a more potent inhibition of CYP3A4 substrates given concurrently.

CYP2C19, PPIs and clopidogrel

A major concern in CYP2C19 polymorphisms is in the therapy of peptic ulcers. Until the discovery that most ulcers were actually caused by the bacterium *Helicobactor pylori*, this condition was treated by years by the extremely commercially lucrative use of gastric acid suppressives like cimetidine and ranitidine. Now the ulcers can be eradicated in a week or so by ruthlessly clubbing the bacteria to death with antibiotic combinations, usually any two of amoxicillin, clarithomycin and metronidazole. A PPI is used to suppress the acid formation to let the ulcer heal and also to improve the efficacy of the antibiotics. It appears that this rationale is sound, as CYP2C19 deficient individuals have better treatment outcomes than EMs as the drugs (omeprazole, lansoprazole and pantoprazole) are cleared more slowly and they build more pharmacodynamically effective concentrations. Indeed, up to 15 times more drug is found in PMs compared with EMs, although fortunately, the PPIs in general are safe and well tolerated drugs. With omeprazole, drug persistence in EMs is improved by its tendency to inhibit its own CYP2C19-mediated metabolism, which does not happen with the other drugs. It has been shown that a four-fold increase in dosage of lansoprazole in EMs vastly improves the therapeutic effect. Studies have shown that virtually all PMs are cured in a week of therapy, whilst cure rates range from 60 to 70%

with EMs. It has been suggested that if PMs could be identified, they could be given just the PPI and a single antibiotic, whilst the EMs would be given the triple combination of two antibiotics and a PPI. The ultra-rapid CYP2C19*17 homozygotes may well need much higher doses and other antibacterials such as bismuth compounds, although further work will need to be done to confirm this.

Finally, the presence of EM CYP2C19 status is necessary for an adequate clinical response to the thienopyridine anti-platelet drug clopidogrel. In a study in Koreans, nearly third of patients were resistant to the drug and this was linked with the CYP2C19*3 allele. This is unusual, as the drug is a potent inhibitor of CYP2C19. The weakness of clopidogrel is that only 15% of the parent is converted to the active agent, so there is a relatively narrow corridor of efficacy which is easily blocked by either a polymorphism or a CYP2C19 inhibitor. Indeed, there are conflicting reports on the degree to which PPI omeprazole reduces the clinical efficacy of clopidogrel, although the consensus seems to be that this does not actually affect overall treatment outcome. It is likely that this effect is mostly caused by omeprazole's pronounced ability to inhibit CYP2C19 and reduce clopidogrel's metabolism to its pharmacodynamically active species. The other PPIs, lansoprazole and pantoprazole appear to have a much less potent effect on clopidogrel's efficacy and some authors have recommended the replacement of omeprazole with either of the other two agents in patients taking clopidogrel alongside longer term anti-ulcer therapy, such as those who are undergoing intensive care. This seriously ill group of patients can develop 'stress ulcers', which are not related to infection. The major risk factors for this type of ulcer include mechanical ventilation for more than two days, nasogastric intubation, major organ failure, head injury or sepsis. These individuals can be prophylactically treated with PPIs for in excess of a couple of months, so it has been recommended that pantoprazole or even another class of anti-ulcer drug be used, such as the slightly less effective ranitidine, to minimize the impact on clopidogrel's efficacy. Certainly, individuals who have low or very low CYP2C19 capacity will only marginally respond to clopidogrel anyway and a PPI would probably completely eliminate what little platelet response they exhibited. In those cases, ranitidine appears to be the safest and indeed, the cheapest option.

However, the latest thienopyridine, prasugrel, shares clopidogrel's mode of action, but the new drug's activation is through carboxyesterases followed by a number of CYPs, with no one isoform dominant. Prasugrel's efficacy is not as affected by PPIs, or CYP2C19 polymorphisms and inhibition as much as that of clopidogrel; although it has shown a higher tendency towards bleeding episodes in clinical trial in comparison with clopidogrel. Although this was not translated into increased mortality in the new drug, it will be interesting to see whether prasugrel proves safe enough in the long term to supersede clopidogrel. Hence, the issue of clopidogrel's vulnerability is a situation where it is potentially easier to wait for the next generation of drugs rather than screen for the defective CYP2C19*3 genotype. This may be a useful strategy in a lucrative and fast-moving clinical area such as cardiovascular disease, but not in areas where drug development is minimal, such as with antimalarials and cutaneous disease.

CYP2D6

The endogenous function of CYP2D6 is not entirely clear and it is very unusual amongst CYPs, in that it is resolutely non-inducible, but it was noticed in the 1970s that the clearance of some (now obsolete) drugs (sparteine and debrisoquine), was greatly retarded in a small

proportion of Caucasian populations. The enzyme is still sometimes termed 'debrisoquine hydroxylase', or 'spartein/debrisoquine hydroxylase'. Subsequent research has shown that 2D6 is subject to a variety of defects that include one-nucleotide deletions as in SNPs or two nucleotide deletions, or even the complete deletion of the gene, so the CYP2D6 protein does not appear at all (null allele). Almost all of the 25 per cent or so of Caucasians carry the CYP2D6*4 allele and around 1 per cent have it in Chinese/Japanese populations. In African-Americans *4 is found at around 5–10 per cent levels. This allele has a SNP which so deranges mRNA splicing, that in homozygotes no enzyme is formed. The *5 allele is similarly useless as the gene is deleted and is found in around 3–5 per cent of most populations. Nearly half of Chinese people have CYP2D6*10, which codes for unstable enzyme which is less active than wild-type but still functional. The *10 variant is found at less than 1 per cent frequency in Caucasians. The *10 variant is so common in Chinese, that CYP2D6 substrates are eliminated more slowly compared with all other races. About 17 per cent of Africans have another allele that forms poorly active enzyme known as CYP2D6*17 which is absent in other races. Potentially, in Europe, there could be more than 30 million poor metabolizers of 2D6-cleared drugs and maybe 10–15 million in the USA.

The CYP2D6 gene is unusual in that it is prone to amplifying itself many times in some populations and this has the effect of producing so much of the isoform (up to 13 versions) that the net effect is very similar to receptor mediated induction seen in other CYPs. Unfortunately, this multiplying effect cannot be switched off and is present for life. The UM phenotype is not fully resolved, as some UMs display very high clearance of CYP2D6 substrates and they are not subject to the gene amplifying effect. CYP2D6 UMs are found in nearly a third of Ethiopians and some Arab races, as well as Swedish (1 per cent) and Spanish (7 per cent) populations. The UM variant has been explained as an adaptation to the presence of toxic plant alkaloids in local foods, partly as CYP2D6 tends to specialize in such compounds.

The implications of CYP2D6 polymorphisms in terms of the vast range of metabolic capability between the PMs and the UMs, the considerable numbers of human populations affected and the variety of drugs that rely on the CYP for either clearance or activation, has meant that this isoform has been studied extensively over the past 25 years.

CYP2D6 and antipsychotics

As if the problems of schizophrenics were not enough, the drugs available to control the symptoms are well known for their extensive and unpleasant side effects. Difficulties in drug tolerance during lifelong therapy are likely to contribute to the high suicide rate amongst schizophrenics. However, the relevance of CYP2D6 polymorphisms to tolerance of individual antipsychotics is disputed, with some studies showing increases in drug levels and consequent adverse reactions and others much less so. It is thought that the most affected antipsychotics are perphenazine, thioridazine, risperidone and possibly haloperidol, as these agents have some CYP2D6 element in their clearance, which is often very complex. Other atypical agents such as clozapine are not cleared by this isoform. However, the severity and permanence of many of the adverse reactions associated with antipsychotics, as well as the paramount importance in facilitating patient tolerance to avert extreme behaviour makes even marginal increases in toxicity related to phenotype an important issue. Among the most difficult to control and distressing effects are tardive dyskinesias

(TDs) which are involuntary movements of the lips, tongue, face, arms and legs, which begin to occur during the first year or so of treatment with some of these drugs. TDs are part of a series of involuntary disorders known as 'extra-pyramidal effects'. After around 10 years or so on antipsychotic drugs, more than half the patients will have developed permanent TDs. The causes of TDs and related dyskinesias could be related to neurological damage caused by the drugs and/or their metabolites. TDs can be present without the drugs, although the drugs may make them significantly worse. Some studies have shown patients with CYP2D6 polymorphisms are much more prone to TD symptoms to the point that the drugs cannot be tolerated, and interestingly, patients who are heterozygous for the polymorphism also are more likely to develop TDs. There does not appear to be any evidence that CYP2D6 UM status impairs therapy through excessively rapid drug clearance. This may be due in part to clinical experience in dealing with initial lack of suppression of symptoms by dose adjustment, as the symptoms of the condition are so severe that dosage in this context is better tailored to the individual than other areas of healthcare.

CYP2D6 and tricyclic antidepressants (TCAs)

These drugs were the mainstay of treatment for depression until the advent of the SSRIs and newer mixed-function agents discussed in Chapter 5. TCAs are seen as essentially superseded drugs, although they are still used in a number of complaints aside from depression, such as intractable pain. They were difficult to use and dangerous from a number of perspectives, not least because with antidepressive agents in general, the onset of therapeutic effect is so slow (six weeks or more) and their atropine-like side effects (dry mouth, constipation, etc.) make them poorly tolerated. If the patient did not respond to these drugs, then the patient might choose to overdose (Chapter 5, section 5.3.8). This, combined with a narrow TI, led to many TCA fatalities. Inability to clear these drugs due to a patient's status as a 'poor metabolizer' would provide the twin problem of a high level of the atropinic side effects, combined with an even narrower TI in that patient and a risk of death from a modest overdose. However, in reality scientific and medical hard evidence is lacking to show that the TCAs are a severe problem in CYP2D6 polymorphisms. One group in the Netherlands searched for studies linking nortriptyline with CYP2D6 polymorphisms and they only found nine articles that involved less than 200 patients in total. Many studies have shown that the clearance of various TCAs is retarded in PMs and accelerated in UMs, however, only a few studies have shown PM-mediated increased toxicity or EM-mediated treatment failure and clearly more work is necessary to confirm these effects. Among the difficulties encountered in assessing the impact of polymorphisms on TCAs, is that they are high clearance drugs and most of their metabolites are formed in some quantity and are usually just as active as the parent drugs. For example, nortriptyline is formed from amitriptyline, whilst desipramine is formed from imipramine. In general, the TCAs parent drugs can show a 3–5 fold increase in PM plasma levels and EMs can show accelerated metabolism at a similar rate. Experimental studies have not always looked for the manifestation of these levels in terms of the usual side effects seen with this class of drugs, such as dizziness, drowsiness and excessive atropine-like effects. It is highly likely that patients with CYP2D6 PM alleles do experience them and may well not complain about them. Although EMs have been shown to suffer treatment failures, this effect is probably less common than with other classes of drugs due to the therapeutic

activity of the TCA metabolites and this problem is hopefully fixed on a day-to-day basis by incremental dose optimization.

CYP2D6 and Beta-blockers

Although some are entirely renally cleared, like atenolol, the major beta-blockers cleared to some degree by CYP2D6 (and CYP2C19) are timolol, propanolol, pindolol, carvedilol and metoprolol. Their metabolism can be complex as many are chiral, in that they have R and S isomers which are differentially metabolized and often the S-isomer is much more potent as a beta-blocker. There is some indication that accumulation of these drugs in PMs can accentuate their side effects, which include bronchospasm, heart problems, headaches, sexual dysfunction, nightmares, depression, problems with glucose metabolism and general tiredness. Much of this is linked to excessive beta-blockade. It has been suggested that metoprolol should be used at 75 per cent of normal dosage with CYP2D6 PMs.

CYP2D6 and SSRIs

The metabolism of SSRIs is complex, as they are extensively oxidatively metabolized by CYP2D6, leading to saturation and inhibition. They are also cleared by other isoforms such as CYP3A4 and CYP2C19. Fluoxetine, paroxetine and fluvoxamine have the largest CYPD6 component in their clearance and although some marked increases in plasma levels have been shown in single doses in PMs, after several weeks of drug exposure, the differences between fast and slower metabolizers disappear. This is probably due to CYP saturation and inhibition. Clinically, it may mean that PMs might suffer from more initial adverse effects, which might necessitate dose adjustment. However, as soon as this is achieved they should tolerate the drug over several months in a similar fashion to EMs. Citalopram and sertraline are cleared by other CYPs and will not be affected by CYP2D6 status. Again, there is enormous clinical experience with these drugs and plenty of choice if a patient cannot live with a particular drug. Occasionally, combinations of SSRIs and TCAs are still used which can cause severe drug accumulation in ‘wild-type’ individuals, so the effects could be quite severe in CYP2D6 PMs.

CYP2D6 and antiarrhythmics

Flecainide, mexiletine and encainide have potent effects on cardiac electrophysiology and these are sensitive to dosage, so high concentrations of these agents may promote arrhythmias rather than reducing them. To date, any adverse effects in CYP2D6 PMs have mainly been seen in mexiletine-treated rather than flecainide-treated patients, with light-headedness and nausea being reported in CYP2D6 PMs.

CYP2D6 and opiate analgesics

These include agents such as codeine, dihydrocodeine, oxycodone, fentanyl, pethidine (meperidine) tramadol and propoxyphene. Many of these drugs are activated to a morphine

derivative and CYP2D6 status is an important influence on their potency as analgesics. Codeine and dihydrocodeine are pro-drugs, as they are methylated versions of morphine. Codeine is less potent but more commonly used than the dihydro derivative and usually only about a tenth of the codeine dose needs to be O-demethylated by CYP2D6 to liberate morphine to act analgesically. In PMs, codeine and to some extent dihydrocodeine both show much less efficacy, although it has been suggested that codeine-6-glucuronide may have some analgesic effects in CYP2D6 PMs. Oxycodone is normally converted to oxymorphone partly by CYP2D6 and this drug's efficacy is also impaired in poor metabolizers.

Tramadol is employed in many contexts as it is much less toxic than morphine, although it should be used with caution with SSRIs and other seratoninergic agents, as it can cause serotonin syndrome with these drugs. CYP2D6 forms O-demethyl tramadol (often called M1) that has a 200-fold stronger opioid action compared with the parent drug. Tramadol is also N-demethylated (metabolite M2) by CYP3A4 and CYP2B6 may also be involved. M1 is mainly glucuronidated and to some extent sulphated. In homozygous PMs, around 75 per cent less M1 is formed compared with the EMs and there is of course less opioid effect. However, there are other factors that to some extent preserve tramadol's efficacy in PMs. The formation of M1 is rather drawn out even in heterozygotes and in PMs its half-life is more than three-fold longer than in the EMs. In addition, PM parent drug levels are nearly double those of EMs and tramadol itself has other effects such as on noradrenaline uptake which contribute to analgesia. In neonates also, M1 clearance is delayed by a lack of glucuronidation, so UGT status will also impact M1's residence time. All this goes some way to explain why in the real world, whatever your CYP2D6 genotype tramadol will have some analgesic action, but it will be more potent in EMs.

Fentanyl is a highly potent opiate that is predominantly cleared by N-dealkylation by CYPs 3A4 and 2D6 to norfentanyl and other metabolites. Poor metabolizers may be in danger of an intensification of the opiate effects from a relatively low dose. In general, CYP2D6 EMs have been reported to suffer from excessive opiate side effects due to higher conversion of opiate pro-drugs to morphine-like derivatives.

CYP2D6 and antiemetics

Ondansetron, dolasetron and tropisetron are 5-HT-3-receptor antagonist antiemetics which are also CYP2D6 substrates. Clinical studies have shown that CYP2D6 UMs clear the drugs so quickly from a standard dose that efficacy falls and breakthrough nausea and vomiting occurs. This effect is more pronounced in tropisetron than the other drugs due to its greater reliance on CYP2D6. Interestingly, in one study with ondansetron, up to half of UMs suffered treatment failure, whilst 92 per cent of the PMs showed good efficacy. Fortunately, the drugs are well tolerated so higher levels in PMs do not seem to be a problem. The UM variant is thought to be too rare (perhaps 1 in 50) for it to be practical to genotype patients to detect it.

CYP2D6 status and TdP-mediated sudden death

The list of drugs that lengthen the QT interval (Chapter 5, section 5.5.2) is ever increasing and some of these drugs are CYP2D6 substrates. Many of these agents are taken for many

years and may be part of complex and fluid drug regimes. Although some groups of patients, such as females and those with genetic variants of hERG channels, are more susceptible to TdP than the rest of the population, there are other factors to consider which complicate the issue. Whilst many agents can cause long QT syndrome, this does not necessarily lead to TdP. As with any pharmacological effect, susceptibility to long-QT syndrome is linked to the potency of the drug's blockade of the potassium channels, as well as to local drug concentration. The danger of sustained hERG-inhibitory levels could be accentuated by CYP inhibition as well as CYP status or a combination of the two. It is also problematic to identify whether a sudden death is linked to drug-induced TdP, as heart disease is already extremely prevalent in Western countries and very large numbers die from heart failure every day. However, it is now established that some drug/patient combinations lead to sudden death in the absence of any accompanying heart disease. Schizophrenics stabilized on the older antipsychotics such as thioridazine, haloperidol and chloropromazine are twice as likely to die of TdP-induced cardiac failure compared with non-drug users. The immediate conclusion might be that CYP2D6 PMs would be more likely to suffer TdP episodes with these drugs and the use of newer atypical agents would reduce this risk. However, a 2009 study revealed that the risk of suffering a fatal episode of TdP is dose related and no different with the newer atypical drugs (olanzapine, clozapine quetiapine and risperidone) compared with the older drugs, even though only risperidone is a CYP2D6 substrate. Hence, other factors, such clearance by different CYPs, as well as the underlying cardiotoxicity of antipsychotics are also present.

CYP2D6 and other drugs

Venlafaxine is a noradenaline and serotonin reuptake inhibitor and is often a second-line treatment in depression after SSRIs have failed to help. It is intermediate in safety between the TCAs and the SSRIs (Chapter 5, section 5.3.8) and the anticholinergic effects seen with the TCAs are not seen with this drug. Unfortunately when it was first used efficacy was not impressive and side effects were high (restlessness and nausea). This may be because it was usually tried after SSRIs had failed and fluoxetine is a potent 2D6 inhibitor through its norfluoxetine metabolites. This effect, a combination of the slow clearance of the SSRI and the long-acting inhibiting effect, can last for weeks. Venlafaxine is oxidized by CYP2D6 to two major active metabolites, O-desmethyl venlafaxine (ODV) and N-desmethyl venlafaxine (NDV). The SSRI effect may have impacted on the efficacy of venlafaxine, whilst retarding its clearance and increasing the side effects. This suggests that a longer 'washout' period is required before venlafaxine is started after SSRIs have been abandoned, thus unfortunately necessitating a gap in therapy that must be endured by the patient.

The clinical response to the oestrogen receptor blocker tamoxifen has always been complex and variable and it is now believed that CYP2D6 status is specifically relevant to outcomes with this drug. Tamoxifen is cleared by UGTs and FMOs in competition with several CYPs, but there are two equipotent main oestrogen receptor inhibiting metabolites, which are formed by CYP2D6. The most significant is 4-hydroxy-N-desmethyltamoxifen, which is found in the largest amounts in plasma, although the 4-hydroxy derivative is also present. CYP2D6 PMs show less clinical response than EMs due to lack of formation of these metabolites. You would think that using CYP2D6 inhibitors with tamoxifen would make a significant impact on its efficacy, but in the US paroxetine and fluoxetine are used

to treat the hot flush side effects of the drug. This approach would seem to be inappropriate and is notably not used in some other countries. Unfortunately, it is apparent that worldwide, a significant proportion of women treated with tamoxifen are co-prescribed fluoxetine or paroxetine, when other SSRIs of less CYP2D6 inhibitory potency would be more logical. The role of SULTs in tamoxifen response is discussed in the later section on SULT polymorphisms.

The antianginal agent perhexiline (Chapter 5, section 5.3.8.) is a known CYP2D6 substrate and its toxicity, particularly neuropathy and hepatotoxicity, led to its withdrawal in many countries, with the exception of Australia and New Zealand. It is now recognized that CYP2D6 status is usually predictive of perhexiline toxicity and that this CYP is virtually the only route of clearance of perhexiline. It has been suggested that this drug is a worthwhile candidate for CYP2D6 genotype testing alongside careful therapeutic monitoring to exploit its high clinical value in angina therapy.

CYP2E1

The major interest in this CYP is toxicological rather than pharmaceutical, as apart from chlorzoxazone and a crucial fraction of paracetamol clearance (Chapter 8, section 8.4.3), very few drugs are cleared by CYP2E1. Instead, a number of features make it probably the most toxicologically relevant CYP. Firstly, as well as the liver, it is found in many 'barrier' tissues, such as the lung, gut, oesophagus, which encounter pollutants such as PAHs, nitrosamines, ethanol, organic solvents and small carcinogenic heterocyclics. Secondly, the CYP is rapidly inducible (Chapter 4, section 4.4.4), found in many sensitive areas of the cell and its background variation in expression is very high-up to 50-fold in human livers. Thirdly, the CYP not only activates many of these compounds to reactive species, it also churns out reactive oxygen species when not occupied by substrate, which is probably a major source of hepatic damage in alcoholism (section 7.7.6). Hence, this CYP forms potential toxins and carcinogens in tissues which already have high cellular turnover and thus its polymorphic expression has led it to be linked with a variety of disease states, from alcoholism to a number of cancers.

In general, the higher the expression and inducibility of CYP2E1, the greater likelihood exists of it forming sustained amounts of reactive species in the presence of a xenobiotic. There are variants of the CYP which are more highly expressed than the wild type, which itself doubles the risk of nasopharyngeal cancer in smokers and causes low birth weights in the children of smokers as well as increasing the risk of oesophageal cancer by sevenfold. Wild-type expression varies across the different races. Those of Japanese descent show around 30–40 per cent less CYP2E1 activity compared with Caucasians. Of the main polymorphisms, homozygosity for Dra1 (T>A 7668, known as *6) is linked with increased risk of alcoholism and peripheral neuropathy and is found in around 8 per cent of Caucasians. The neuropathy is caused by CYP2E1-mediated formation of 2,5, hexanedione from n-hexane, now banned in manufacturing (Chapter 3, section 3.11.3). There are two other well documented variants known as PstI and RsaI, which are sometimes linked in an allele known as *5B, which is found in only about 2 per cent of Caucasians, but is much more common in Eastern races and Mexicans. The *5B homozygotes are linked with non-alcoholic steatohepatitis (NASH). RsaI, known as *5, is an SNP in the HNF1α binding site, which is thought to reduce inducibility and activity; this CYP is linked with

lower lung and bladder cancer in Eastern races, but others have linked it to higher risks for colon and rectal cancer in smokers, but not in non-smokers. There is another more common variant, *7B, at around 7 per cent of Caucasians, which is linked with higher rates of lung cancer. As with CYP1B1, due to large variation and all the complicating factors seen in human studies, many reports trying to link variants of CYP2E to diseases have produced conflicting data and the precise risk factors for different human populations are still some way off.

CYP3A

Since the CYP3A group (chromosome 7) metabolize around half of all drugs, intensive efforts have been made to account for variation in the metabolism of CYP3A substrates, which can be up to ten-fold in terms of drug clearances and up to 90-fold in liver protein expression. This is partly due to the responsiveness of the CYP to all the inducers and inhibitors that enter human systems. It appears that CYP3A drug clearance can be resolved mostly in terms of the sum of CYP3A4 and 3A5 isoforms, although a CYP3A7 and a CYP3A43 (very low levels of expression) have also been found.

Regarding CYP3A4, it does appear to be remarkably well conserved across humanity and no major variants in its structure have been found in levels high enough (>1 per cent) to be classed as a significant polymorph. The PXR binding elements in the regulatory area of the gene do not appear to vary much either, although there is a fault in the promoter of CYP3A4 in some individuals, who possess a CYP3A4*1B, which has been suggested to lead to reduced CYP output in response to hormone stimulus. This is said to lead to greater hormone levels that might promote neoplasms, such as in prostate cancer. Other rarer variants of CYP3A4 include CYP3A4*2 and *3, as well as *4–6, found in Chinese individuals. It is likely that the full extent of the variation in CYP3A4 is still to be discovered in other aspects of its regulation.

While it is thought that CYP3A4 is not subject to an obvious major polymorphism, CYP3A5 definitely is. CYP3A5*1 is the functional version, whilst a single nucleotide polymorphism causes the misreading of the CYP3A5 gene leading to the formation of shortened and catalytically inadequate CYP3A5*3. Essentially, *3/*3 individuals form no serviceable CYP3A5. Functional CYP3A5 is found in around 20 per cent of Caucasians, half of Chinese/Japanese, 70 per cent of Hispanics and more than 80 per cent of African Americans. In these populations, or high expressors of CYP3A5, in practice it means that it constitutes about half of their CYP3A.

Although this looks radical, it makes little difference to the clearance of most CYP3A substrates with one or two exceptions. Tacrolimus appears to be cleared predominantly by CYP3A5, as *3*3 individuals appear to require lower doses than *1 individuals. This makes relatively little difference clinically, as these drugs are so closely monitored due to the severity of the consequences of drug accumulation (graft toxicity) or disappearance (graft rejection). There is some evidence that CYP3A5 makes a substantial contribution to statin clearance, with *3*3 individuals showing higher plasma levels of atorvastatin. CYP3A5 may also have a considerable role in indinavir and saquinavir clearance, but this is not fully established. Predisposition to neoplasms linked with hormone metabolism is thought to be most likely associated with polymorphisms in CYP3A5, such as the *3 variant, rather than CYP3A4.

7.2.4 Genetic polymorphisms in non-conjugative systems

Flavin mono-oxygenases (FMOs)

As outlined in Chapter 3, the FMOs role in drug clearance has only recently been appreciated and some of the features of these enzymes, such as their lack of induction, has drawn the attention of drug designers who wish to exploit their more predictable clearance of certain drugs is part of novel clinical regimens. As alluded to in Chapter 3 (section 3.8.1), it is thought that FMOs are not inducible because, perhaps like CYP2D6, there is a great deal of genetic variation in their expression 'built in' to humans. The milder defects such as reduction in enzyme activity appear to be mostly SNPs, whilst frame-shifts (which lead to short mRNAs) and deletions account for inactive short enzyme or complete absence of inactive enzyme. Of the five main isoforms, the least variable (FMO-4) has 30 variants and the most possess 57 (FMO-2). However, the full significance of this variation in terms of drug clearance is not fully understood. FMO-3 is the major isoform in human liver and is the most studied in terms of its expression in human populations. Although attempts have been made to look at various drug substrates as markers for expression (clozapine, S-nicotine), the main problem is that there are many other enzymes (such as CYPs) which are much more efficient clearers of these drugs.

Ironically, in a field that requires complex DNA array technology to detect elusive polymorphisms all that is necessary to detect clinically relevant FMO-3 deficiency is a sense of smell. Although all of FMO-3's main homeostatic tasks have not been elucidated, from a personal perspective, its most important function is to clear trimethylamine (TMA: which gives off a fishy smell) to the odour-free TMA N-oxide. In wild-type individuals (about 99 per cent of Caucasians), FMO-3 clears around 90–95 per cent of the TMA. A clearance level of 60–90 per cent is classed as mild fish-odour syndrome (trimethylaminuria, TMAU) and clearance levels of below 60 per cent are associated with the most severe syndrome. In studies with FMO-3 so far, around ten alleles are linked with decreased activity, seven form inactive protein and two variants have increased activity and the rest are 'wild-type'. Around 20–25 per cent of Caucasians have at least one or more defective alleles (such as E308G or E158K) with around 2–5 per cent homozygous for at least one duff allele. There are various complex heterozygotic combinations of the different defective alleles that lead to a range of TMAU and possibly also problems with other pathways. Although there is interest in exploiting FMO-3 in drugs such as itopride (Chapter 3, section 3.8.1) and pro-drugs which may be better absorbed as amidoximes, clearly those with TMAU of varying degrees will present a problem therapeutically, although at least it does not require sophisticated and expensive equipment to detect severe TMAU in the clinic.

Dihydropyrimidine dehydrogenase (DPD) and 5-fluorouracil

The antineoplastic agent 5-fluorouracil (5-FU) is still used for solid tumours of the head, neck and breast sometimes in combination with cyclophosphamide and methotrexate. However, 5-FU is more than capable of killing the patient on its own, as it can cause severe gut and neurotoxicity, as well as bone marrow damage. The reason it can be so toxic and difficult to use is the striking effect of the polymorphism that affects DPD, which

is 5-FU's main route of clearance. Those with wild-type DPD show an average 15 min half-life, with 80 per cent cleared to inactive metabolites and rest is removed unchanged renally. Conversion of only 1–2 per cent of the parent to the active anti-metabolites is necessary to inhibit tumour growth. Those with DPD*2 allele (about 1–2 per cent of Caucasians) take the best part of three days to clear the drug and of course the tumours are often overexpressing protective enzymes (such as DPD) so the DPD deficient individuals not only endure high toxicity, but the drug often does not work either. The genotype test is not particularly accurate as it measures the DPD in the blood mononuclear leucocytes that is variable and not necessarily related to the hepatic version that clears the drug. The problem can be alleviated by the use of DPD inhibitors such as eniluracil, which mean that 5-FU disposition is much easier to control in the patient.

Butyrylcholinesterase polymorphisms

This polymorphism was first seen around 60 years ago, when patients under anaesthesia who were treated with non-depolarizing neuromuscular blocker suxamethonium. This type of drug is still used to paralyze skeletal muscle during surgical procedures and it usually works in seconds and is cleared in a few minutes, making its pharmacological effect extremely controllable. The drug is cleared and the patient should resume a normal breathing pattern in 5 minutes or so. In some patients, this did not happen and their intercostal muscle paralysis might take more than half an hour to resolve. The condition was called scoline apnoea and it was found that drugs such as suxamethonium were cleared by butyrylcholinesterase (BChE) that was subject to polymorphisms. BChE is a 340 KiloDalton tetramer that is similar to acetylcholinesterase that has the crucial task of hydrolyzing acetylcholine in the neuromuscular junction and in autonomic nervous system ganglia. BChE is also responsible for the metabolism of cocaine and heroin (Appendix B). The wild-type BChE is known as BChE UU and is found in 96 per cent of Caucasians. A further 4 per cent have alleles that are defective and show some minor reduction in enzyme activity. The real problem lies with the 1 : 3500 individuals who have virtually zero BChE activity (such as BChE AA or SS). This problem was really marked in individuals with mivacurium, which was introduced in 1992, but is now not used in the US and several other countries. This agent's effects should resolve in half an hour but sometimes took up to eight hours. Although the effect is marked, scoline apnoea is so rare that it is probably not very cost effective to test every patient for it. It is treatable, as the patient is ventilated until the drug is eventually cleared. As drugs such as cocaine and heroin are used in such quantity, it is difficult to determine how much BChE polymorphisms contribute to their toxicity and fatal overdoses. It is probable that large numbers of individuals are affected.

As BChE is a target for nerve agents such as sarin, soman and VX and it is under investigation as a possible antidote to nerve agent poisoning. The idea is that large amounts of transgenically produced human BChE are infused into the poisoned individual and the BChE binds and removes the nerve agent from the plasma. Although it is an interesting idea and the threat of terrorist usage of nerve agents remains, some of these toxins, such as VX are so potent and rapid in onset that this approach is questionable. There is also interest in using a longer acting BChE to remove cocaine from plasma in cases of toxicity and possibly even modulating or reducing the addictive effects of the drug.

Epoxide hydrolases

Most of the research into polymorphisms of EPHX1 (microsomal epoxide hydrolase) and EPHX2 (soluble epoxide hydrolase) have centred on the link between these enzymes and various disease states. Several studies have tried to link the risks of carcinogenicity EPHX1, usually in relation to the activation of various aromatic hydrocarbons, although the results have often been mixed. Wild-type EPHX1 is sometimes known as Y113/139H, with a tyrosine at position 113 and a histidine at 139. There are two main substitution polymorphisms; a 'slow' variant, which operates at 50 per cent of wild-type capacity and is an exon 3 EPHX1 Tyr to His at amino acid 113 (usually called EPHX1 Y113H, or 337T>C on the gene) and a 25 per cent faster than wild-type version, known as EPHX1 His to Arg H139R (416 A>G). There are also individuals with both alleles and more than 29 other SNPs have been found. About half of Japanese populations have the slow variant and just under 15 per cent have the fast version. The enzyme variants can be inconsistent in their clearance of different epoxide substrates, which may explain the variability in studies aimed at linking variants to disease predisposition. Fast enzyme protects against 1,3 butadiene carcinogenicity (Chapter 8, section 8.6.4), whilst the slow isoform is now associated with increased risk of oesophageal cancer, as it is thought that reactive species from, say, tobacco, are not cleared quickly enough. Whilst the fast version confers reduced carcinogenic risk as it clears the epoxides formed by CYPs and other enzyme systems to diols more quickly, thus acting to protect tissue. Confusingly, other studies have indicated that EPHX1 His113His variants are at lower risk of lung cancer than other individuals, but higher risk of chronic obstructive pulmonary disease and emphysema. The risks conferred by the different variants probably reflect the complex relationship between detoxification of epoxides to diols, as well as the toxification reaction where reactive epoxides are formed from diols. Interestingly, there is much more to discover about EPHX1, as recent work has shown that it has more than one promoter; an E1 close to the gene and an E1-b promoter which is quite distant from the gene that may be the major controller of enzyme expression. It seems that the E1-b promoter is polymorphic and may confer even more variability on the enzyme's expression than was first believed.

EPHX1 is part of the clearance of phenytoin and carbamazepine epoxides and there is also some evidence that polymorphisms may also influence warfarin metabolism in Chinese individuals as well as the disposition of carbamazepine in Japanese patients. Carbamazepine is cleared by CYP3A4 to its pharmacologically active 10,11 epoxide, which is also responsible for some of its side-effects, which are related to concentration and lead to vomiting and ataxia. Recent studies have suggested that the presence of the two EPHX1 variants Y113H and H139R have such a strong bearing on carbamazepine 10, 11-epoxide clearance to the inactive diol, that genotyping would be useful in dosage estimation.

7.2.5 Conjugative polymorphisms: acetylation

Acetylation was the first polymorphism to be investigated and was based on the observation that the antitubercular drug isoniazid caused a different level of neural toxicity in Japanese compared with US patients. Although there are two N-acetyltransferase isoforms, NAT-1 and 2, NAT-2 (found mostly in the liver) is relevant to drug polymorphisms. It acetylates drugs such as isoniazid, dapsone and the sulphonamides. Historically, as with

most polymorphisms, three distinct groups have been described, that is, fast, intermediate and slow acetylators. The highest frequencies of fast acetylators are in Asian countries, particularly Japan, with over 90 per cent of the population. Frequencies in the Indian subcontinent are much lower, at around 30 per cent; with European populations around 40 per cent are fast/intermediate acetylators. Interestingly, only about 5 per cent of fast acetylators are wild-type homozygotes (NAT2*4*4) the wild-type, so the vast majority of fast acetylators are heterozygotes.

This polymorphism has dramatic effects on the plasma levels of the parent form of acetylated drugs. With fast acetylators, perhaps only 20 per cent of drug-related material in the plasma will be parent drug and therefore clinically effective (assuming metabolites are not active). This situation could lead to potential treatment failure. In contrast, in the slow acetylators, more than 80 per cent of drug-related material in the blood will be parent drug and these levels may be so high as to approach toxicity in some individuals.

Acetylation and sulphonamides

In a study in patients taking sulphasalazine (sulphapyridine and 5-aminosalicylate linked by an azo bond) for ulcerative colitis, parent drug (sulphapyridine) plasma levels were between three- and fourfold higher in the slow acetylators compared with the fast acetylators, whilst the acetylated drug was found in approximately three-fold higher levels in the fast acetylators compared with the slow.

Despite the potential problems caused by wide disparities in plasma concentrations between populations and individuals, sulphonamides were cheap, effective and were used in vast amounts as broad-spectrum antibacterials for over 50 years. In fast acetylators, providing there was enough drug in the plasma to suppress bacterial growth or exert an anti-inflammatory effect, efficacy would be adequate. However, in some individuals, it is likely that sustained low plasma levels of the drug contributed to the selection of resistant bacterial populations that reduced drug effectiveness. Resistance was offset by the use of sulphonamides in combination with 2,4 diamino-pyrimidines (pyrimethamine and trimethoprim) which are synergistic in effect, as the drugs attack the bacterial/protozoan DNA synthesis process in two places, thus requiring much lower plasma concentrations to be effective. Currently, a combination of sulphamethoxazole with trimethoprim (SMX and TMP) remains effective in treating *Pneumocystis jiroveci*-induced pneumonia in HIV-positive individuals who have T-cell counts below 200.

Sulphonamides and their combinations also fell out of favour for broad-spectrum antibacterial usage, because of a relatively high rate of adverse reactions, which included some gruesome conditions such as Stevens–Johnson syndrome (Chapter 8, section 8.5.1). Indeed, more than half of HIV patients taking the SMX/TMP combination suffer from milder adverse reactions such as various rashes, which can abolish patient tolerance of the drugs. These reactions are not connected with the parent drug or the acetylated metabolites, which are not cytotoxic on their own. Unfortunately, all aromatic amine-based drugs such as sulphonamides and sulphones and other substrates of NAT-2 are also subject to extensive CYP-mediated (usually CYP2C9) oxidative metabolism. These metabolites are the main route of clearance of these agents and they are mostly hydroxylamines, which are usually eliminated as conjugated sulphates or glucuronides. However, enough of the cytotoxic hydroxylamines escape conjugation to be the cause of the adverse reactions associated with these drugs.

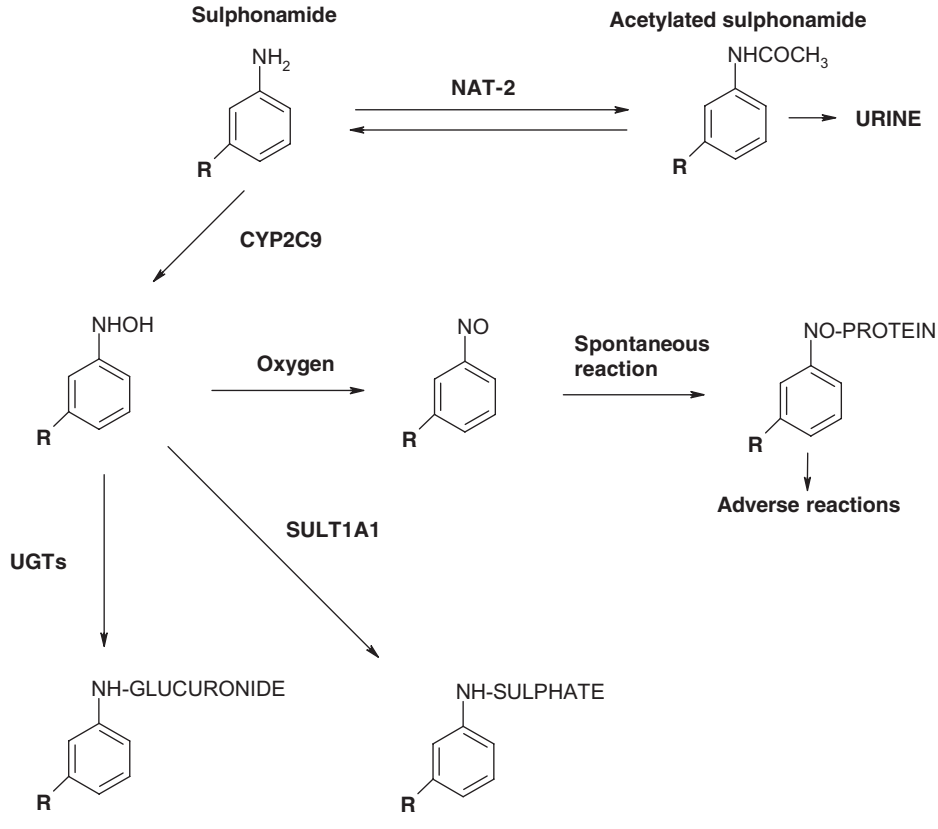

Figure 7.1 Basic scheme for sulphonamide metabolism and how adverse reactions are related to metabolism

So you can see that NAT enzymes could be seen as detoxification pathways for aromatic amine-related drugs; this is because once an amine has been N-acetylated, then there should be less opportunity for it to be oxidized by the CYPs to hydroxylamines (Figure 7.1). This would only apply if the acetylated derivative was excreted into urine and did not undergo further oxidative metabolism. With sulphapyridine, a modest amount (approximately 20 per cent) of the dose was found in urine as the acetylated derivative, so providing some support for other studies which suggested that in drugs which only possess one aromatic amine (sulphonamides), the frequencies of adverse reactions related to the oxidative metabolites were lower in fast acetylators and correspondingly higher in slow acetylators. So it appears that the slow acetylators are more vulnerable than fast acetylators to toxicity from the oxidative route of metabolism.

Sulphones

However, in drugs with two aromatic amine moieties, such as dapsone, this protective effect does not apply, as diacetylation is a minor pathway in dapsone metabolism and only a few per cent of the dose is found in urine as the monoacetylated derivative and trace

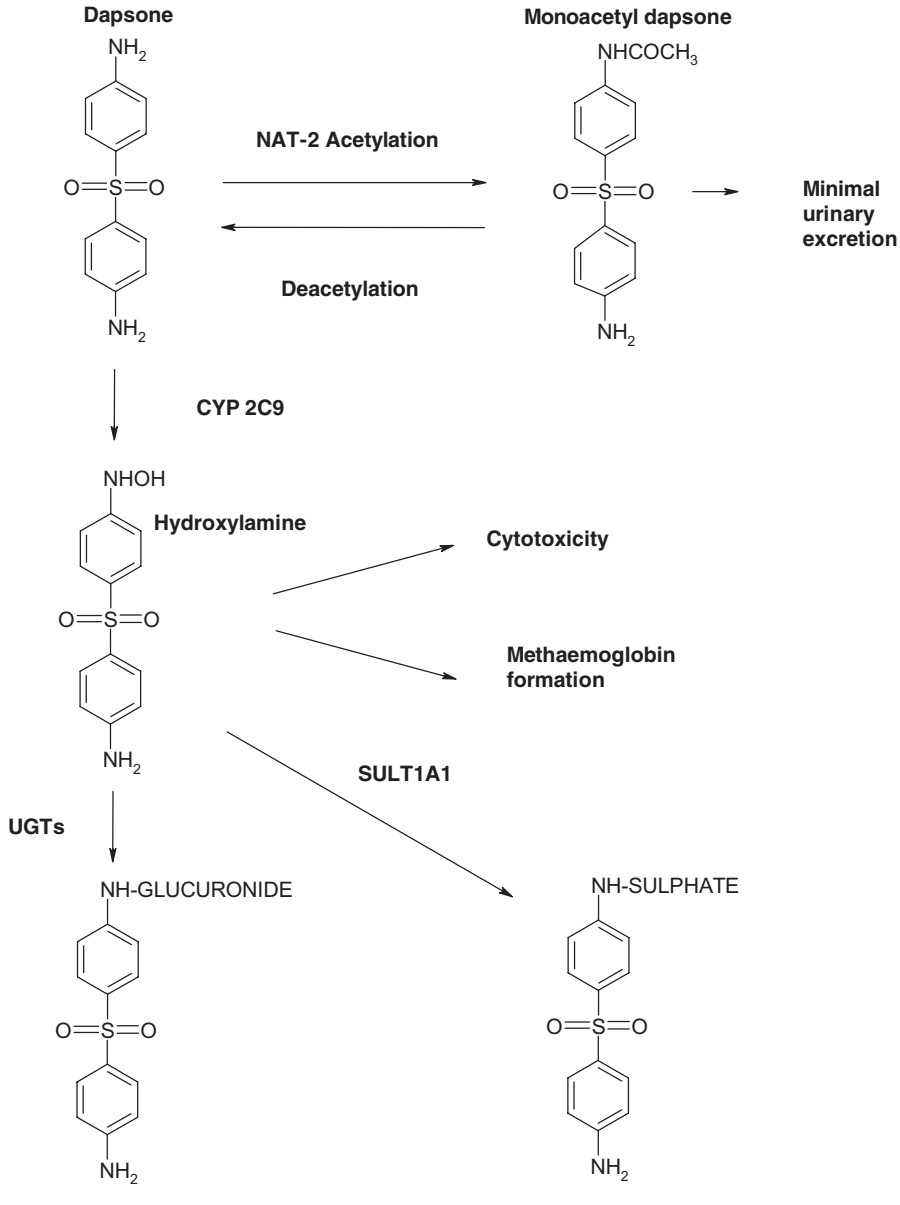

Figure 7.2 The role of acetylation in dapsone metabolism

amounts of the highly lipophilic diacetylated sulphone (Figure 7.2). Monoacetylated dapsone is much more lipophilic than the parent drug, so essentially, acetylation can delay dapsone clearance, by 'holding it up' in the acetylation/deacetylation pathway. N-hydroxylation is the only effective way of making dapsone less lipophilic and even the free amine group of acetylated dapsone is N-hydroxylated. The hydroxylamines are eliminated as N-glucuronides and sulphates of monoacetyl and dapsone hydroxylamines. So

hydroxylamines are formed regardless of the acetylator phenotype of the individual, even though plasma parent drug levels are higher in slow compared with fast acetylators. The toxic oxidative metabolism of dapsone will be discussed in Chapter 8 (section 8.2.5).

Isoniazid metabolism

Isoniazid (INH) has been the cornerstone of the therapy of tuberculosis for over 50 years. Resistance has only emerged relatively slowly due to other drugs being used in combination with INH. This drug has a unique mechanism of action that only operates in *Mycobacteria tuberculosis* and its closely related pathogens. INH remains effective in many areas, but it is toxic and if tuberculosis were a major disease of the developed world, it certainly would have been superseded. Unfortunately, no new antituberculosis drug has been introduced since the 1960s.

INH's tendency to cause peripheral neurotoxicity can be pretty much removed by taking pyridoxine (Vitamin B6). However, its major clinical drawback is significant elevation of liver transaminase enzymes (AST, ALT) in many patients. Around a fifth of patients show perhaps 3–5 fold increases in AST and ALT over the first 4–8 weeks of treatment, which often settles as the liver adjusts and probably upregulates GSTs and GSH levels to compensate. It is obviously not desirable to withdraw the drug if it can be helped. Once the enzymes rise to the 300–500 range, (around 10-fold normal) more worrying hepatotoxicity may be developing which may progress to liver failure in 1–2 per cent of individuals if the drug is not stopped immediately. Liver problems are sometimes more associated with females than males and older patients are more susceptible also.

INH is a substrate for NAT-2 and it is believed that slow acetylators are most at risk from INH hepatotoxicity; it has been suggested that CYP-mediated metabolism is linked with the toxicity. NAT-2 forms N-acetylisoniazid that can hydrolyze to form acetylhydrazine, which is a potent nucleophile in its own right and does not need any more metabolism to be cytotoxic. It is thought that fast acetylators can eventually form greater quantities of diacetylhydrazine, which is not toxic, whilst the slow acetylator livers must contend with acetylhydrazine for much longer periods of time. It might be expected that fast acetylators would initially form more acetylhydrazine and then be more susceptible to toxicity, than the slow acetylators, but this is not borne out by clinical studies (Figure 7.3). Slow acetylation appears to be associated with greater risk of liver toxicity and up to three-fold higher plasma INH levels, although parent drug levels are not associated with the risk of liver damage. However, any concurrent therapy such as rifampicin (or heavy ethanol use) predisposes to hepatotoxicity. This suggests that perhaps several CYPs may be involved with conversion of either the parent drug or acetylhydrazine derivatives to more reactive necrotic species. This would account for the protective effect of acetylation, which essentially promotes the formation of a relatively reactive but containable metabolite (acetylhydrazine) on the way to a safe one (diacetylhydrazine), so restricting entry of the parent drug and acetylhydrazine itself into oxidative metabolism. Interestingly, rifampicin is extensively deacetylated (that wonderful orange metabolite) and it may increase the risk of liver damage by competing for the NAT2 and leaving more drug to be oxidized by other pathways, as well as its other inductive and disruptive effects on liver function. There is no evidence that fast acetylators are disadvantaged in terms of INH's efficacy.

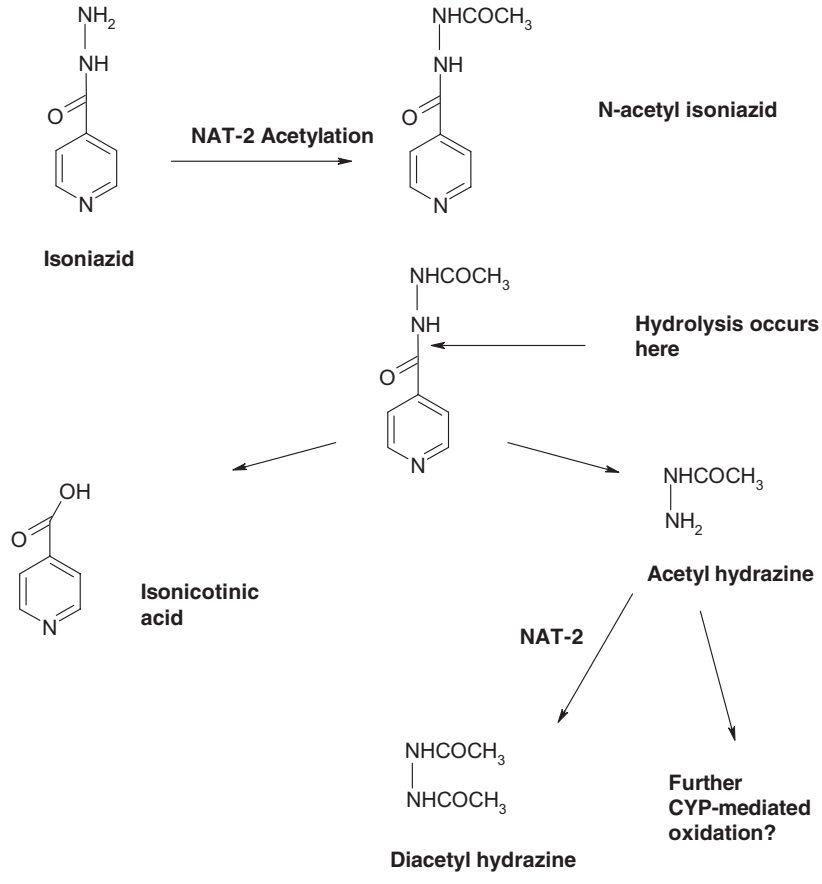

Figure 7.3 Acetylation and isoniazid metabolism

Toxicological significance of acetylation

To complicate this situation further, some acetylated metabolites can undergo further oxidation themselves to form highly reactive cytotoxic and carcinogenic species. Indeed, in recent years, acetylation has become intensively studied almost entirely due to its role in the carcinogenic activation of aromatic amines and this is discussed briefly in Chapter 8 (section 8.6.4). Perhaps the best context to see acetylation is a homeostatic process that unfortunately xenobiotics do enter and undergo metabolism, often leading to outcomes that are at best rather equivocal.

7.2.6 Conjugative polymorphisms: methylation

A particularly dangerous polymorphism clinically was identified in the 1980s for one of the methyltransferases. The endogenous role of *S*-methylating thiopurine *S*-methyltransferase (TPMT) is not that clear, but it is capable of *S*-methylating thiopurine derivatives like mercaptopurine and azathioprine. These drugs are antimetabolites that mimic purine to

disorder DNA metabolism in malignant cells. They are effective in some childhood leukaemias and are activated to 6-thioguanine nucleotides by an enzyme called HGPRT. TPMT is the main route of clearance of these toxic 6-thioguanines and this polymorphism can greatly distort the clinical behaviour of this drug in the patients. Those who are poor metabolizers and have low TPMT expression can suffer from lethal myelosuppression and abnormally high levels of the active 6-thioguanine nucleotides. The polymorphism is due to a single nucleotide change that leads to two alterations in the amino acid sequence of the enzyme and changes to its cellular survival time. As with CYPs and acetylation, in Caucasians, there are PMs (<1 per cent) IMs (10 per cent) and the rest (EMs) are wild-type. There are several variants, TPMT*2, *3A, *3B and *3C. Homozygous PMs are TPMT*3A/*3A. Heterozygotes (IMs) have less than half the activity of the wild-type (TPMT*1*1). A study revealed that around 2 per cent of the EMs are ultra-rapid metabolizers, with more than double the activity of the EMs. The frequency of homozygotes has been estimated worldwide to around 1 : 300 although it is virtually absent in Chinese/Japanese. The faster metabolizers clear the thiopurines too quickly and reduce their cytotoxic efficacy, leading effectively to drug failure. Drug failure in the context of a progressive and life-threatening disease is obviously extremely serious. A test was developed for TPMT expression based on the fortunate fact (in common with GST-theta and NAT-1) that enzyme expression in red cells is linked with systemic expression. This means that the patients are tested for TPMT expression prior to therapy which reduces the risk of life-threatening toxicity.

TPMT: genotyping or activity measurement

TPMT highlights the genotyping/phenotyping issue mentioned earlier in the management of patients with polymorphisms. Genotyping will reveal the level of TPMT expression that should be expected in the otherwise healthy patient. However, there are many factors which impact day-to-day TPMT expression during thiopurine therapy. TPMT activity is inhibited by salicylates, which are used concurrently with thiopurine drugs; TPMT activity varies widely among patients and thiopurine pressure induces the enzyme's activity. Hence, what might be predicted from a genotype test may bear little resemblance to how the enzyme is performing on a particular day in a treatment cycle. So clinically, it is preferred to test actual TPMT activity. Even so, despite the testing, the drugs (like all antineoplastic agents) still cause toxicity in the form of bone marrow suppression in wild-type individuals so there is no substitute for caring and conscientious clinical monitoring. Recently a complicating factor has emerged, where the supply of 6-mercaptopurine (6-MP) formed from azathioprine is linked with GST activity (Chapter 6, section 6.5.3). Ultimately, clinical response and toxicity is likely to depend on the balance of 6-MP production and TMPT destruction of the thioguanines and in the future GST activity may also need to be evaluated as well as that of TMPT.

7.2.7 Conjugative polymorphisms: UGT1A1/UGT1A7

The worst and probably rarest (1 in 10^6 children) UGT polymorphism is Crigler-Najjar syndrome variant CN-1, with complete deletion of UGT1A1, resulting in no enzyme and death from bilirubin damage a couple of years after birth without liver transplantation. CN-2 expresses some enzyme and will respond to phenobarbitone induction to push UGT1A1

levels to around 20 per cent of normal. The commonest UGT polymorphisms in the general population and the pharmacologically relevant ones are caused by promoter faults.

Perhaps half of all gene expression is promoted by a final 'switch' which is termed a TATA box, a series of precisely coded repeating thymine/adenines which in UGT1A1*1/*1 (wild-type) is actually six TAs followed by a TAA sequence. This switch is thrown by the binding of a TATA box binding protein that is the last step in the initiation of transcription. In the UGT1A1*28 allele seen in Gilbert's syndrome, there are seven TAs instead of 6, which is known as an insertion polymorphism. This makes a large dent in the efficiency of the basal promotion of the expression of the gene as it runs at only 30 per cent of wild-type (*1*1). The system still responds to xenobiotic induction, however (Chapter 6, section 6.2.8). Up to a quarter of those of African origin are UGT1A1*28/*28 (homozygotic) for the commonest variant of UGT polymorphism and between 5 and 15 per cent of Caucasians, although the trait is rarer in other races. As mentioned earlier, this variant predisposes to several conditions including susceptibility to some cancers linked with exposure to environmental toxins such as PAHs as well as hormone-dependent cancers. Genetic variation in human UGTs has been reported to be as high as 100-fold. The strong bearing that UGT1A1*28 allele has on SN-38 toxicity (gut toxicity and neutropenia) during irinotecan therapy (Chapter 6, section 6.2.10) is now recognized in the literature included with the drug packaging. However, it is worthwhile noting that clinical studies are, as ever, conflicting in this area and although recommendations from the FDA suggest dose reductions may be appropriate in *28 individuals, it is also apparent that higher SN-38 levels can mean improved therapeutic response in these patients.

Significantly, many *28/*28 individuals do not suffer from SN-38 toxicity and it was discovered that other UGTs may have a role in SN-38 clearance. UGT1A7 is found in quantity in the gut so it can intercept SN-38 and it is catalytically five times more efficient at forming SN-38 glucuronides compared with UGT1A1. It is thought that due to its position and its selectivity for SN-38, UGT1A7 essentially compensates for UGT1A1*28 in most patients. However, not surprisingly, UGT1A7 is subject to at least three SNPs leading to the formation of inefficient enzyme, the commonest of which is termed UGT1A7/W208R. To complicate things further, UGT1A7 has a promoter TATA box fault in some individuals called UGT1A7-57T/G, which cuts basal expression by 70 per cent.

Overall, it appears that if you suffer from Gilbert's syndrome, you not only have the faulty UGT1A1*28, but you are 75 per cent likely to express UGT1A7-57T/G which can also be linked with UGT1A7/W208R. So you can see that toxicity due to any drug that is glucuronidated will probably occur in individuals who have poor expression of two major UGTs and at least one catalytically faulty isoform. Clearly, just searching for UGT1A1*28 is not enough to prevent SN-38 toxicity.

As with other potent and potentially toxic drugs used in a hospital context, normal therapeutic monitoring should already customize drug dosage to the patient and clinical experience with irinotecan is extensive, although the more knowledge we have in predicting vulnerability to toxicity due to this drug the better.

7.2.8 Conjugative polymorphisms: sulphonation

Understanding of sulphonation and its roles in endogenous as well as xenobiotic metabolism is not as advanced compared with that of CYPs; however, the role of SULTs in the

activation of carcinogens is becoming more apparent. One of the major influences on SULT activity is their polymorphic nature; in the case of one of the most important toxicologically relevant SULTs, SULT1A1, this isoform exists as three variants, SULT1A1*1 (wild-type), SULT1A1*2 and SULT1A1*3. The *1 variant allele is found in the majority of Caucasians (around 65 per cent), whilst the *2 variant differs only in the exchange of one amino acid for another. This single amino acid change has profound effects on the stability and catalytic activity of the isoform. The *2 variant is found in approximately 32 per cent of Caucasians and catalytically faulty, although the amino acid substitutions are not close to the active site, they do reduce activity somehow. SULT1A1*2 was originally thought to be unstable, but essentially it disappears six times faster than the wild-type does because it fails cellular quality control-the proteosome (remember from CYP2E1) destroys the enzyme very rapidly. The *2 variant is also less subject to substrate inhibition, which may have consequences regarding the general feedback metabolic control of the *2 variant. About 9 in 10 Chinese people have the *1 allele and about 8 per cent have allele are *2. About half of African-Americans have *1 and a third have *2. Interestingly, there is a *3 which is rare in most races but accounts for more than 22 per cent of African Americans. There is also considerable variation in SULT2A1 and SULT2B1, which are the major hydroxysteroid sulphators in the body, which may have implications for sex steroid and cholesterol handling.

The relevance of SULTs to drug clearance is still emerging, but there seems to be some impact of SULT1A1 on tamoxifen clinical effectiveness. In the previous section on CYP2D6, you will recall that there are two main hydroxylated active metabolites. Clearly, the drug's effectiveness depends on the residence time of these agents in plasma and tissues. SULT1A1 is responsible for clearing these metabolites to sulphates and possession of SULT1A1*2 alleles prolong the active metabolites' residence time and improve their effectiveness. SULTS also show a similar effect to CYP2D6 where they express multiple copies of the enzymes, which can also increase activity. Studies showing the catalytic capabilities of SULT1A1 proteins with various flavonoids and oestrogens show that *1 is the most efficient sulphator, whilst the 3 version found mostly in African Americans is the next most efficient and is far ahead of *2.

So from the cancer-risk viewpoint, a highly active SULT1A1*1 is usually an advantage in that it usually removes reactive species rapidly as stable sulphates. With some agents it is problematic as certain carcinogens such as acetylfluorene are indirectly activated to reactive species by SULTs. In addition, protective dietary flavonoids like quercetin, chrysin and genistein are also rapidly cleared by SULT1A1*1, so there is a combination of production of toxins and loss of protective dietary agents. In terms of carcinogenesis risk, SULT1A1*2 could be a liability as potentially damaging substrates such as electrophilic toxins cannot be cleared rapidly. However, in some circumstances the *2 allele can be rather protective as it is comparatively useless at activating carcinogens, it can't really remove tamoxifen's active metabolites and it also allows protective agents remain in tissues for longer periods. The combinations are endless and so it is often extremely difficult to predict risks of carcinogenicity for individuals and toxin exposures.

7.2.9 Other Conjugative polymorphisms

GSTs are polymorphic and much research has been directed at linking increased predisposition to cytotoxicity and carcinogenicity with defective GST phenotypes. Active

wild-type GSTMu-1 is found in around 60 per cent of Caucasians, but a non-functional version of the isoform is found in the remainder. This is caused by a gene deletion that also occurs in GST-Theta isoforms. GST-M1 null (non-functional alleles) can predispose to risks of prostate abnormalities and GST Pi is subject to several SNPs and many attempts have been made to link these SNPs with the consequences of failure to detoxify reactive species, such as the risk of lung cancer. Regarding the effects of dietary and smoking habits, linking GST expression and increased DNA damage is problematic partly because other detoxifying enzymes such as epoxide hydrolase (mEH) might be operating and masking the effects on the test cell system. mEH itself is subject to a polymorphism, with a fast and slow variant of the enzyme. Carcinogenesis may be due to a complex mix of factors, where different enzyme expression and activities may combine with particular reactive species from specific parent xenobiotics that lead to DNA damage only in certain individuals. Resolving specific risk factors may be extremely difficult in such circumstances.

However, in cancer chemotherapy, there is evidence that the presence of GST-M1 and GST-T1 null (non-functional) alleles predisposes children to a six-fold higher level of adverse events usually seen with antineoplastic drugs, such as bone marrow damage, nephrotoxicity and neurotoxicity. In addition, GSTP1 polymorphisms are linked with increased peripheral neuropathy in docetaxel therapy, which is thought to be due to oxidative stress caused by failure of GST null variants to clear reactive species. Such vulnerability to the toxicity of cytotoxic drugs is compounded by the existence of double null GST alleles, where GST-M1 and GST-T1 are absent in the same patient. In such instances, the tumour may well protect itself through a number of mechanisms, but the patient will be subject to increased oxidative stress and risk of mortality. In the future of cancer chemotherapy, it is highly likely that genotyping of certain GST isoforms would significantly reduce toxicity and improve patient drug tolerance, hopefully without impact on therapeutic effect.

7.2.10 Transporter polymorphisms

The importance of the solute carriers, as well as the ATP-binding cassette transporters has been realized over the last decade or so and of course differential expression of these systems in various populations is likely to be part of the variation in drug clearance seen in human populations. In terms of hepatic drug entry and efflux, there are various polymorphisms in the OATP solute carriers, as well as the ATP powered MRPs, PgP and BCRP. To date, little is known of the direct clinical relevance of transporter polymorphisms in terms of drug uptake and metabolite efflux and their relationship with plasma concentration. There is some evidence that more than 40 per cent of those of Chinese extraction express a particular variant of BCRP (421C>A), which causes increased plasma levels of rosuvastatin, whilst this variant is rare in Caucasians and those of African descent. There are other highly expressed individual variants of P-gp (>40 per cent) that are found in the major ethnic groups, although there seems to be less variation in MRP-1 and 3, with more in MRPs 2, 4 and 6. Further studies will establish the clinical relevance of these polymorphisms in drug uptake and distribution, as well as excretion of polar metabolites.

7.2.11 Polymorphism detection: clinical and practical issues

The previous sections have highlighted the issues of method, knowledge and cost which have influenced the drive to make the detection of patient polymorphisms part of clinical

practice. In many cases the limitations of the drug development process are mirrored in our search for polymorphic population frequencies. A new drug may enter the market after use in a few thousand individuals and in the case of Vioxx, within three or four years more than 100 million patients may have used it. Similarly, assumptions about the genetic content of populations of hundreds of millions are made after studies with a few hundred patients. Clearly, in global potential markets of billions of patients, far more extensive and better designed clinical studies must be commissioned and financed to avoid making far-reaching and possibly harmful assumptions on polymorphic frequencies. There are also important issues regarding the completeness of our knowledge of even thoroughly investigated polymorphisms. This has been highlighted with the toxicity of SN-38 and the 6-thiopurine analogues.

It is also important to establish whether genotyping tests would offer real advantages in the context of current therapeutic drug monitoring. In many cases, the most potent and toxic agents, such as anti-cancer agents, antipsychotics, anticoagulants and digoxin are already used with a high level of close clinical supervision, which involves directly measurable clinical responses and adverse reactions. It could be argued that it is already well known that predisposition to potentially lethal toxicity such as TdP in antipsychotics, or strokes with warfarin are concentration related and thus cautious escalation in dosage would normally prevent dangerous accumulation even in polymorphic individuals. However, some polymorphisms do have such an impact on drug disposition that treatment failure or overwhelming toxicity could occur so rapidly in some individuals that even cautious dosing may be inadequate to protect the patient from the consequences of their abnormality. It could be argued that if the genetic tests like the 'Amplichip' CYP DNA microarray test systems became sufficiently cheap and convenient, they could be incorporated into routine blood analysis. This would reveal patients at severe risk of very steep and rapid drug accumulation or failure to form an active metabolite. Hence, optimization of treatment could be achieved more quickly and efficiently in terms of clinical time and manpower and the tests might become cost-effective. This may well be very useful with drugs such as perhexiline.

With many other drugs, before this could happen, it is likely that future genotyping must actively consider pharmacodynamics as well as biotransformation. This 'multityping' approach has been advocated with warfarin, where not only CYP2C9 status is important, but also that of VKORC1. With antipsychotics, CYP2D6 activity might be assessed alongside hERG channel gene status. Tamoxifen's efficacy would be improved with oestrogen receptor genotype, CYP2D6 and SULT status determination. This approach may be hugely beneficial in anticancer therapy, where multiple biotransformational processes, such as GSTs, UGTs SULTs and epoxide hydrolase activities should all be considered alongside the neoplasm's susceptibility to the chosen agent. Ideally, the development of such combination DNA test arrays could transform the efficacy and tolerability of antineoplastic therapy and markedly improve survival rates, as well as preventing long term developmental and fertility damage in children and young adults. It will also be necessary with such detailed information to provide prescribing healthcare professionals with reliable dosage guidelines to interpret the data to inform day-to-day clinical decision-making. In addition, the experience with irinotecan, suggests that official guidelines are much more meaningful when we are in full possession of all the relevant pathways and their impact on the drug. With the current emerging state of our knowledge with many drugs, this is all some way in the future.

In terms of drug development, modern molecular biological techniques have now allowed pharmaceutical companies to use many more human cellular systems to determine with confidence most of the biotransformational pathways to which a drug may be subject

and which may govern clinical response and toxicity thresholds. Although the primary driving force behind drug development remains efficacy, the use of such systems will also accelerate the development of new agents that neatly circumvent common polymorphisms such as prasugrel and eniluracil.

7.3 Effects of age on drug metabolism

The effects of age on drug clearance and metabolism have been known since the 1950s, but they have been extensively investigated in the last 20 or so years. It is now generally accepted that at the extremes of life, neonatal and geriatric, drug clearance can be significantly different from the rest of humanity. In general, neonates, i.e. those less than four weeks old, cannot clear certain agents due to immaturity of drug metabolizing systems. Those over retirement age cannot clear the drugs due to loss of efficiency in their metabolizing systems. Either way, the net result can be toxicity due to drug accumulation.

7.3.1 The elderly

Interestingly, although memory and information processing speeds decline with age, intelligence apparently does not. Hence, elderly patients may not always remember instructions related to their medicines or conditions, but it should be noted that they are likely to understand the explanation when they are reminded. Unfortunately many other aspects of the human body decline fairly precipitously beyond the age of around 70 and the ability to clear drugs is no exception. Most elderly people are taking not just one, but sometimes rather complex combinations of drugs, which is termed 'polypharmacy'. Some have estimated that a high proportion of 70-year-olds are taking as many as eight or more drugs daily. It seems that the inability of older people to clear drugs is not necessarily related to the efficacy of their CYP-mediated oxidations, which are often not much different from that of younger individuals. Studies with the major CYPs *in vitro* have revealed that CYP2D6 is unaffected by age, as are most other CYPs, with the exception of CYP1A2, which does decline in activity in the elderly. This was shown in a study with the flecainide, which is mainly cleared by CYP2D6 with some CYP1A2 component. An age-related decline in flecainide clearance was linked with CYP2D6 PMs, as in those individuals a greater proportion of flecainide was processed by CYP1A2. In general, there is little significant change in the inducibility in most CYPs, or in the capability of conjugation systems *in vitro*.

It has been suggested that elderly livers do not supply enough oxygen to the CYPs *in vivo*, which was known as the 'oxygen limitation theory'. This, however, is rather academic, as the major limitations on drug clearance in the elderly are due to other more pressing physiological factors. There are significant changes in the liver itself, as it decreases in mass and its blood flow is reduced as we age. This occurs at the rate of around 0.5–1.5 per cent per year, so by the time we hit 60–70, we may have up to a 40 per cent decline in liver blood flow compared with a 30-year-old.

Other factors include gradual decline in renal function, increased fat deposits and reduction in gut blood flow, which affects absorption. This is most noticeable in drugs that have a high 'intrinsic clearance' (Chapter 1, section 1.4.2), in that their clearance is directly related to blood flow; propranolol is a good example, with a 50 per cent reduction in

clearance in the elderly. Phenytoin is a low intrinsic clearance drug and is less affected by age. The problem arises that the drug's bioavailability increases due to lack of first-pass clearance; this means that from a standard dose, blood levels can be considerably higher than would be expected in a 40-year-old. This can be a serious problem in drugs with a narrow TI, such as antiarrhythmics. In addition, average doses of warfarin required to provide therapeutic anticoagulation in the elderly are less than half those required for younger people. The person's lifelong smoking and drinking habits, as well as older individuals' sometimes erratic diet also complicate this situation. Among the drugs cleared more slowly in older people are antipsychotics, paracetamol, antidepressants, benzodiazepines, warfarin, beta-blockers and indomethicin. Some authors have drawn the distinction between 'fit' and 'frail' elderly. In the fit elderly their mortality and morbidity after elective surgery is often no different to the general population, which signifies a high degree of robustness, however, several studies have shown the frail elderly to have much more impaired drug clearance than the fit elderly, usually because of multiple pathologies such as hip fractures and organ failure.

Nearly 20 per cent of elderly people are believed to be clinically depressed and SSRIs are now the first-line treatment for this condition. It is recommended that the dosages for several of these drugs should be reduced in older patients. Fluoxetine and paroxetine therapy inhibits CYP2D6 and other CYPs, so sustained reductions in the clearance of co-administered agents will occur. Obviously, the inhibitory effects of SSRIs on CYP2D6 and to a lesser extent on the CYP3A series suggest that extreme caution should be exercised when prescribing these drugs to the 'polypharmaceutical' elderly. Overall, the population of fit elderly is increasing and life expectancies continue to increase in the developed world. Although drug clearance is impaired in this group generally, once therapy is optimized in the fit elderly, the situation should be stable in many cases into their 80s provided no major pathologies develop.

7.3.2 Neonates

Babies are classed as neonates when they are less than four weeks old, although in some reports, older babies are sometimes included as neonates. What research that has been done in neonates has revealed that CYP activities are less than half and perhaps as little as one-third of adult activity. This is reflected in the inability of neonates (full term and premature) to clear many drugs that rely on the major CYPs for their clearance. Often, drug half-lives can be up to 10 times longer than those of adults. There is even a marked difference between premature neonates and full-term babies; on average, the premature neonates clear drugs less than half as quickly as full-term neonates. This is a combination of poor renal clearance and poor CYP oxidative performance. Predominantly, renally cleared drugs such as cimetidine, vancomycin and ampicillin are cleared at less than one-third the rate of adults. This is partly due to immaturity of kidney function and may also be related to fluid intake that can be variable if the babies are not parentally fed. Interestingly, there is little difference between renal drug clearance of premature and full-term neonates. There are other complicating factors regarding drug behaviour in neonates, such as much higher drug absorption in the immature intestinal tract (less than two weeks old) compared with adults. In very premature neonates, skin permeability can be up to 100 times that of adults. So from many respects, premature neonates, neonates and children less than 6 months old are

distinct in their drug handling in many more respects besides just body mass. Finally, in spite of the clear need to formulate dosing guidelines in neonatal drug therapy, there are many obvious obstacles to assessing drug clearance in neonates experimentally, such as parental permission, ethical considerations and limitations on sample volume and frequency. Hence, many published studies produce data from babies who are under intensive treatment and may be seriously ill and not necessarily representative of neonates in general.

Oxidative metabolism in neonates

Regarding CYP-mediated oxidative drug clearance, foetal liver contains a number of unique CYP enzymes, such as CYP3A7; however, this CYP clears 'standard' 3A4 substrates such as carbamazepine much more slowly than mature 3A4 and it is likely that foetal CYP enzymes are sufficient for the clearance of endogenous substances such as steroids, but are not really intended to clear many xenobiotics, as the maternal liver should deal with them. Neonate levels of CYP2C series (10–20 per cent of adults), CYP2E1 (10 per cent) and especially CYP1A2 are very low, which in the latter case is borne out by nine-fold slower caffeine clearance seen in premature neonates compared with adults. Other agents such as midazolam and nifedipine are cleared less than half as efficiently in premature neonates as full-term babies, whilst some studies have shown foetal livers not to be capable of metabolizing some CYP3A substrates such as cisapride at all. Indeed, CYP3A levels are only around 5 per cent of adult capacity at birth. At birth CYP3A7 and FMO-1 levels peak and then decrease rapidly. FMO-3 levels start to rise as FMO-1 is switched off fairly abruptly after birth. In general, CYP expression in neonates exhibits a much greater variation compared with adults.

However, the difference between adult and neonate drug clearance is rapidly eroded 2–6 months in age. In the first few months of life CYP activities increase extremely quickly, to the point that drug clearance can be faster in toddlers than in adults, as the children possess matured CYPs and renal sufficiency as well as higher hepatic blood flow. By the age of 12 months or so, CYP3A7 is still the main isoform, but it continues to decline. CYP2E1 levels are several fold higher than neonates and CYP3A4 levels are nearly at adult levels.

The use of midazolam in the sedation of neonates in intensive care units illustrates the difficulties in balancing dose and effect. Although this drug is preferred as its half-life is short and so can provide brief periods of sedation, doses used are often too high, leading to excessive sedation. There is wide variability between neonates of similar ages in the clearance of the drug – indeed half-life determinations vary from 5 to more than 12 hours. Midazolam is a useful CYP3A4 probe drug and it has been suggested that its clearance could monitor this isoform's expression in neonates and young children. In favour of this approach, midazolam is a poor substrate for the major neonatal isoform CYP3A7, but other CYPs may also contribute to the clearance of the drug in neonates. Overall, doses used for neonates must be considerably lower than those used for older babies and extreme caution must be used in the face of such variability and fragility.

Conjugative metabolism in neonates

It has long been known that with glucuronidation, neonates and term babies exhibit low UGT expression. This is clear in the relatively high incidence of jaundice in newborns,

which is due to immaturity of UGT-mediated bilirubin clearance (Chapter 6, section 6.2.8). Indeed, in babies who are not jaundiced, their low UGT capability can be revealed by drug administration; in the 1950s, the use of chloramphenicol for infections in babies led to 'grey baby syndrome' where the drug was cleared to a hydroxylamine (probably by a CYP2C variant) which would be glucuronidated in adults, but in children the poor UGT capability led to the metabolite escaping the liver and causing methaemoglobin formation. Chloral hydrate was once used as a sedative in neonates; however, it is cleared to trichloroethanol, which is not only poorly glucuronidated by neonates but also competes with bilirubin for this pathway and jaundice can be exacerbated. In neonates with Gilbert's syndrome, jaundice is likely to be more pronounced than unaffected individuals and recent work has revealed that some neonates with Gilbert's syndrome have even higher levels of bilirubin as they are glucose-6-phosphate dehydrogenase deficient (G-6-PD) also. This is most likely to occur in those of African ancestry and is due to increased haem processing caused by G-6-PD that overloads a very low capacity system. Neonates and young children with Gilberts and G-6-PD will exhibit extremely long half-lives in drugs cleared by glucuronidation such as lorazepam, morphine, AZT and propofol. Indeed, normal premature neonates show greater than four-fold slower glucuronidation of such drugs compared with adults.

In general, glucuronidation systems mature more slowly than other biotransforming systems according to current knowledge. In studies in human foetuses at 20 weeks' gestation, there was no evidence of any expression of UGTs. At 6 months old, UGT1A1, 3, 4, 6 and 9, as well as 2B4, 7, 10 and 15 were expressed, although activities were much lower than adults. Indeed, in children around toddling age (1–2 years), their clearance of ibuprofen, amitriptyline and oestrone can be more than ten-fold lower than that of adults. This is particularly significant for the use of morphine in neonates and young children as this agent is cleared by glucuronidation, as is the active M1 metabolite of tramadol. Fortunately with tramadol, very little of the M1 is formed in many neonates. With paracetamol, clearance in neonates and babies is just over half that of older children. Although the drug is cleared mostly by glucuronidation in adults, in neonates it is eliminated mostly as a sulphate, as SULTs appear to be expressed a reasonably high levels. Interestingly, paracetamol is not as toxic to neonates and children as it is to adults, mainly because their CYP2E1 is of lower activity and only low levels of the hepatotoxic quinoneimines are formed. Those that do appear are detoxified by the glutathione system, which is more active in neonates than adults. Some reports have gone as far as to suggest that neonatal paracetamol overdose is not a major concern.

Overall, drug clearance in premature neonates can be 10 times less than adults and they possess little ability to glucuronidate drugs. Term neonates also have immature oxidative and conjugative systems, but by 2–3 years old the system is virtually adult, although variable in capacity.

Drug clearance in older children

Although CYP and conjugative systems are maturing rapidly after the toddler stage (2–3 years), children up to the age of 12 often display marked changes in drug clearance related to adults. This can be related to factors such as renal clearance and increased metabolic rate, as well as higher food intake related to body weight. Fluvoxamine is used in children

aged 6–12 for the treatment of obsessive-compulsive disorder (OCD) and it is cleared more slowly than in adults and it is recommended that doses should be reduced, especially in girls. Methylphenidate is cleared at less than half the rate of adolescence in children of 6–12 years. Alternatively, some agents are often cleared more rapidly in children aged 6–12, such as benzepril, remifentanil, buspirone and gabapentin. On a weight basis the dosage of the NSAID etodolac must be doubled in children. It is also important to remember that young children are a 'work in progress' and are growing rapidly; so therapeutic drugs can impact on this process. Fluoxetine is used in children to treat OCD and major depression and there is evidence that it actually slows their growth and weight gain. By contrast, atomoxetine, can cause PMs to gain more weight than EMs without any effects on height. There are also many pharmacodynamic drug effects that are unique to children, such as the paradoxical effects of some stimulants in calming hyperactivity and gabapentin's tendency to cause aggression and hostility. In all respects besides those pertaining to drug metabolism, children are not just miniature adults.

7.4 Effects of diet on drug metabolism

The old saying 'you are what you eat' still has resonance and diet can influence the clearance of drugs as much as general health. Some of the possible diets that are available in Western countries can be more toxic than many drugs and Morgan Spurlock's documentary 'Super Size Me' did induce some well-known purveyors of fast food to pay some attention to the composition of their products. Food contains a vast array of chemicals, such as antioxidants, plant toxins, preservatives, polyphenols, polycyclic aromatics and various other environmental pollutants. It is clear from the preceding chapters that the profile of our biotransformational systems has been designed to respond to the pattern of our xenobiotic intake and since most of our dietary habits vary relatively little over our lives, our metabolizing systems adapt to the xenobiotic stimuli in our food intake also. Chapter 5's descriptions (section 5.3.6) of the effects of various fruit juices underlines that many of the compounds absorbed from our diet are as potent as drugs in terms of their impact on CYP expression and catalytic activity.

7.4.1 Polyphenols

Thousands of polyphenols are found in plants, vegetables, fruit, as well as tea, coffee, wine and fruit juices. These molecules have specific uses in the plants, either as antioxidants to extend the 'shelf-life' of fruit on the plant to maximize the attraction to insects and birds to spread seeds, or as toxins to ensure a hideous lingering death for any animal that has the nerve to eat the plant. These compounds are a broad classification and can include phenolic acids, stilbenes, flavonoids and lignans. Each subclass, such as the flavonoids, has a wide array of related agents (flavonols, isoflavones, etc.). The drastic effects of the polyphenols such as naringenin and bergamottin from grapefruit juice on biotransformational activity have already been discussed in Chapter 5 (section 5.3.6). Flavonoids such as quercetin and fisetin are excellent substrates for COMT, so competitively inhibiting the metabolism of endogenous catecholamine and catechol oestrogens. Quercetin and other polyphenols are found in various foods such as soy (genestein) and they are potent

inhibitors of SULT1A1 which sulphate endogenous oestrogens, so potentiating the effects of oestrogens in the body. Many of these flavonoids and isoflavonoids are manufactured and sold as cancer preventative agents; however, it is more likely that their elevation of oestrogen levels may have the opposite effect in the long term.

It is also likely that various polyphenols influence other endogenous substrates of sulphotransferases, such as thyroid hormones and various catecholamines. It is gradually becoming apparent that polyphenols can induce UGTs, indeed; it would be surprising if they did not. Certainly drug-efflux transporters are modulated by polyphenolic isoflavones. This effect has been seen with P-glycoprotein and MRP1–3. The flavonoid chrysin, found in honey, is a potent inducer of UGT1A1. Overall, it is likely that there are a large number of polyphenols that are potent modulators of CYPs and conjugative enzymes.

7.4.2 Barbecued meat

In the UK, the weather effectively prevents the excessive consumption of so-called char-broiled or barbecued meat. However, many fast-food outlets claim that their burgers are 'flame-broiled', so it is possible through some modest effort to maintain a high consumption of barbecued meat in the UK. However, this exposes the consumer to a combination of polycyclic aromatics, nitrosoamines and hetercyclic aromatic amines which all induce CYP1A2 and CYP1A1. If consumption is heavy enough, the clearance of drugs such as amitriptyline, clozapine, fluvoxamine, mexiletine, haloperidol, naproxen, olanzapine and paracetamol will be increased significantly. GSTs and UGTs can also be induced, so that the carcinogenic effects of the induction of these CYPs can be offset to some extent. An even more effective way to offset the effects of induction is to consume considerable amounts of vegetables with cooked meats. These contain a variety of inhibitors of these CYPs, so reducing the risk of activating the polycyclics and ameliorating the inducing effect on any prescription drugs that might be washed down with the barbecue.

7.4.3 Cruciferous vegetables (sprouts, cabbage, broccoli, cauliflower)

It is now scientifically accepted that a diet containing plenty of vegetables can stave off many cancers. However, it seems that the cruciferous vegetables may the most effective anti-cancer agents in our diets. It is also clear that sprouts and other cruciferous vegetables can be a less than popular choice in many diets. Indeed, a former US President (George H.W. Bush) was once heard to refuse to eat broccoli. However, this type of vegetable can be highly beneficial; they contain a broad array of beneficial polyphenols, glucosinolates and isothiocyanates, especially the young shoots. If you would like to avoid malignant disease in later life, it is apparent that you need to consume these vegetables regularly from an early age. The most studied is probably broccoli, which has been bred selectively since antiquity. Its major glucosinolate is glucuraphanin, which is converted enzymatically to sulforaphane when broccoli products are crushed or otherwise manhandled. Sulforaphane has been intensively studied and it appears that it mimics a toxic electrophile as it activates the Nrf2 system (Chapter 6, section 6.5.4) to induce detoxification enzymes such as the GSTs and epoxide hydrolases, as well as many other systems in the gut and liver. In addition, sulforaphane boosts GSH levels in cells partly by reacting with cellular pools of GSH

and freeing the synthesis system from its allosteric inhibition and partly as Nrf2 controls GSH synthesis (Chapter 6, section 6.4.2). The net effect is to hoover up reactive species by upregulating protective systems whilst providing them with sufficient co-factors like GSH. Incredibly, sulforaphane also downregulates CYP3A4 which then reduces steroid formation, so protecting against breast cancer and it even causes malignant cells to enter apoptosis so it can disrupt the process of carcinogenesis. It is also anti-inflammatory and has cardiovascular benefits. To obtain this apparently impressive package you just need to eat around 200–300 g of broccoli daily and within 1–2 weeks the benefits kick in. It is, of course, not quite that simple. Although many studies have shown that around five servings of cruciferous vegetables weekly can reduce the risk of bladder, prostate and colon cancers, some reports have shown no effect and others increased tumour rates. On balance, it is probably a good long-term plan to eat some broccoli regularly, maybe around the recommended five servings per week level and no more.

It would be expected that sulforaphane would be extracted from broccoli and immediately marketed as a cancer protective. However, there is evidence from animal studies that the whole broccoli is probably better than the sulforaphane alone, as broccoli, rather like petrol (gasoline), is a 'team' effort. There is a variety of beneficial agents of varying physicochemical properties in different proportions in the complete vegetable probably for a good reason. Although broccoli itself is a hybrid, cruciferous vegetables in general may well have evolved to attract and retain the health of animal populations in order to spread their seeds reliably over wider areas. Essentially, as scientists, we should occasionally ignore our 'reductionist' tendencies that drive us to always look for one specific chemical that might be a 'miracle' cure. The taste of a fine wine is not something that can be created in a laboratory, so we should probably accept that this is an area where several million years of research and development in the plant is often superior to our fitful efforts. Having said this, some 'superbroccoli' variants have been bred possessing much higher yields of isothiocyanates, which may be valuable as part of future cancer-aware diets.

From the perspective of drug metabolism, broccoli and its sister vegetables also can induce CYP1A2, but you need to eat about 500 g/day to get that effect. It appears unlikely that many would consume that amount of cruciferous vegetables per day and it is probably not necessary. Drugs cleared by CYP1A2 include clozepine, caffeine and theophylline and their clearance is accelerated by regular cruciferous vegetable consumption. As sulforaphane does not activate the AhR system, it was found that other agents released from crucifers induce the CYP, such as indole-3-carbinol. Crucifers also induce CYP1A1 and this CYP is associated with the production of reactive species and possibly carcinogenicity and it has been difficult to delineate between crucifers' inductive oxidative and conjugative effects. Some studies have seen upregulation of both pathways, with increased levels of glucuronides/mercapturates found in urine. So even if more reactive species are formed, the conjugative systems will attenuate them and the presence of an enlarged capacity of detoxification enzymes is ready to deal with other xenobiotics. It has been suggested that sustained induction of CYP1A2 for example, may be beneficial in women, as through 2-hydroxylation it increases oestrogen clearance to anti-oestrogenic metabolites, so acting to protect against oestrogen-driven tumours. Women with breast cancers have been found to have a lower frequency of 2-hydroxylation related to 16α hydroxylation, which forms oestrogenic metabolites and is catalyzed by CYP3A4. Whilst indole-3-carbinol and its other inducers increase CYP levels, sulforaphane downregulates and inhibits others such

as CYP2E1 and other known formers of reactive species. Experimentally in human cellular systems, sulforaphane can bind to PXR to achieve its downregulating effects on CYP3A4 and probably other CYPs also. Overall, the broccoli and cruciferous vegetable effect requires more research to fully unravel.

It is likely that a balanced diet where crucifers are eaten with other vegetables means that you don't absolutely have to eat exactly 200 g daily as long as you do eat some when it is in season. This way you can enjoy the benefits of upregulated GSTs alongside a minimized CYP induction effect. Certainly the smell of boiled cauliflower in your house could well erode your popularity amongst your friends and broccoli haters will pull faces and probably not dine with you. However, your life-long cruciferous vegetable consumption will ensure that you will still be around in old age to remember how you annoyed them all. Perhaps if it was revealed that Keith Richards is an avid broccoli consumer, the popularity of this valuable vegetable could be significantly boosted.

7.4.4 Other vegetable effects on metabolism

If you think sprouts taste bad, try watercress. However, the taste 'grows' on you, and it contains phenethyl isothiocyanate; this compound blocks CYP1A2 and 2E1, and inhibits the activation of a number of tobacco-related carcinogens, so again, eat it quickly and live longer. Glucobrassicin, found in sprouts, also inhibits these CYP isoforms. Some studies have been carried out with raw garlic. It would be expected that consumption of sulphides related to garlic and onions would affect CYP2E1 or 1A2, however it had no visible effect, although N-acetyltransferase was stimulated. Consumption of vegetables leads to an alkaline, rather than acid urine and this is beneficial in maintaining the stability of a number of conjugated metabolites of aromatic amines and preventing their degradation to the parent metabolite in the bladder, an organ not exactly renowned for its detoxification capability.

7.4.5 Caffeine

Moderate caffeine (1,3,7,trimethylxanthine) consumption is a good idea which also makes getting out of bed worthwhile, particularly if you grind your own fair-trade beans and brew good quality coffee, rather than the instant version, which is an abomination. Caffeine is a mild stimulant that enhances alertness and performance and it is found in a vast array of products. These range from various teas and carbonated cola drinks through to some cold cures, chocolate and specialized vile-tasting stimulant drinks aimed at young people. It is also available pharmaceutically over the counter in tablet form. In order of caffeine dosage, the stimulant tablets can be 200 mg or more, whilst a strong brewed cup (about 200 mL) of good coffee around 100 mg, whilst an equivalent volume of tea, made from black loose tea leaves, will contain perhaps around 50 mg, slightly less than that of instant coffee. Many caffeinated drinks yield around 35–40 mg per can and are unsuitable for young children as the drug affects appetite as well as acting as a stimulant. Almost caffeine's entire metabolism is by CYP1A2 and it appears to act as a competitive inhibitor of substrates that are cleared by this CYP. An average consumption of caffeine could be taken to be around 3–4 cups of tea or coffee per day, which is in the range of 250–400 mg per day, or about 6 mg/kg body weight. This intake is not considered performance

enhancing and results in less than the International Olympic Committee limit of about 12 mg/litre excreted in urine. If a patient is stabilized on a CYP1A2 substrate (fluvoxamine, warfarin and clozapine) then this habitual level of consumption of caffeine should not make much practical difference to other CYP2A2 drug clearances as most of us are creatures of dietary habit. However, if the patient binges on caffeine or stops it altogether, changes in the CYP1A2 substrate clearance will occur. This is more likely to register in terms of adverse reactions in a narrow TI drug such as clozapine and patients should be made aware of this possible effect. The situation is more complex in smokers, where CYP1A2 is strongly induced and they will require perhaps 2–3 times more caffeine than non-smokers. Of course there is huge variation in caffeine metabolism and women clear caffeine more slowly than men due to lower CYP1A2 activity (see section 7.5).

7.4.6 Diet – general effects

It is clear that diet can substantially modulate biotransformation – the consumption of high levels of cooked meat induces CYPs 1A1/2, but prior established consumption of cruciferous vegetables and watercress, for example, can negate the carcinogenic effects of the meats. This is why in the Mediterranean, the local diet is sufficiently rich in stimulators of detoxification pathways and inhibitors of oxidative metabolism, that it is possible to eat what you like, drink everything in sight and smoke pounds of shockingly pungent tobacco and live to be 116 years old. As to the effects on prescription drugs, this is more difficult to measure reliably, although abrupt changes in a person's diet may significantly alter the clearance of drugs and lead to loss of efficacy or toxicity.

7.5 Gender effects

For many years, there was a very large scientific literature on the sex differences between rats and other animals in their clearance of drugs. In humans, this was less well investigated; however, a number of differences have been found, although it is not often taken into account clinically. In hindsight, it should be glaringly obvious that the highly sophisticated control system for menstrual steroid metabolism, one of the *raisons d'etre* of human biotransformation, should indicate that women are likely to clear drugs differently from men and that drugs which modulate metabolism are more likely to lead to adverse effects on female metabolism than male.

In general, experimental or 'probe' drugs such as antipyrine, which are used to study the activities of a number of CYPs, are metabolized more quickly by women than men. This is allowing for differences in weight, fat distribution (body mass index) and volume of distribution, but not necessarily mood. This effect was most pronounced at the time of ovulation and the luteal phase of menstruation. Indeed, pre-menopausal women can clear several substrates (theophylline, prednisolone and anticonvulsants) more quickly than men. There are some contradictions, such as with CYP1A2, where women demethylate caffeine more slowly than men. Indeed, studies with human liver microsomes and hepatocytes have shown that CYP1A2 and CYP2E1 are markedly more active in men, but there were few other significant differences, apart from CYP3A4 which in some studies has been shown to be twice as active in women compared with men. CYP2B6 and

CYP2A6 are also more active in women, although there is thought not to be any differences with CYP2D6 and the CYP2C series.

Many CYP3A4 substrates such as erythromycin are cleared more rapidly in women than men. It appears that CYP expression is linked to growth hormone (GH) and about the same amount is secreted over 24 hours in both sexes. In animals the pattern of release of the hormone is crucial to the effects on the CYPs; in females, GH is secreted in small but more or less continuous pulses, while males secrete large pulses, then periods of no secretion. The system is thought to be similar in humans.

In female epileptics, phenytoin metabolism is significantly accelerated just before the beginning of their period. This can cause the drug levels to fall out of the therapeutic window. The effect can be so pronounced that carbamazepine must be used instead. Anticonvulsant metabolism can be accelerated in pregnancy also. This suggests that some CYPs (2C9/19) could be more sensitive to female sex-hormone cycles than others (CYP3A4), although this could be a reflection of the much greater expression of CYP3A4 compared with other CYPs. Little is known of the effects of the menopause and hormone replacement, where steroid metabolism changes dramatically. It is highly likely that these events could have profound effects on female drug clearance. There are other marked differences which are not fully explained: fluoroquinolones such as ciprofloxacin are cleared significantly more slowly in women, so that peak drug levels are more than a third higher than those of males and females suffer more adverse effects from these drugs compared with men.

It is also apparent that females in general are more susceptible to drug adverse reactions than males, especially hepatotoxic effects. This may be a reflection of their increased formation of reactive or toxic metabolites and less robust detoxification, although there are also pharmacodynamic differences in drug sensitivity. Women are much more likely to develop torsades des points, or dangerous lengthening of the QT interval (Chapter 5, section 5.5.2). This is because endogenous oestrogens lengthen the interval anyway and it does not take a great deal of a QT-lengthening drug to cause a severe and rapid effect in women; indeed, many more females died from terfenadine-induced torsades des pointes than males, before the drug was withdrawn from OTC status.

7.6 Smoking

Smoking tobacco still has a very strong hold on humanity, although its well-documented tendency to prematurely end the lives of its adherents and smoking bans in public and workplaces have facilitated its decline in most sections of Western societies except perhaps cautiously rebellious teenagers. Not surprisingly, the tobacco manufacturers have exploited the general aspirational tendencies of Third World economies to market smoking as highly attractive and this has resulted in expanding tobacco consumption worldwide. As far back as the 1860s, it has been known that cigarettes are toxic (there is a tirade against the dangers of cigarettes in Dostoevsky's *Crime and Punishment*), so it is frankly bizarre for anyone today to be unaware of tobacco's toxicity. Although incomprehensible to non-smokers, it has been said to be easier to give up heroin than cigarettes. Many cigarette manufacturers' manipulate the design and effect of their product to improve the volatility of nicotine during pyrolysis. This, among other features, effectively makes a cigarette a sophisticated, consistent and efficient nicotine delivery system, which is essentially a

pharmaceutical product. Therefore cigarettes should be subject to the rigorous testing a new drug must complete, although this is somewhat unlikely to transpire. Having said this, in 2009 the FDA did take some control of cigarette additives (flavourings/menthol) which might promote the habit in those cautious young tearaways. Tobacco smoke contains around 4000 chemicals, of which around 45–50 are carcinogens. Among the few proven human bladder carcinogens, β-naphthylamine and 4-aminobiphenyl are present in tobacco smoke, which make smoking the primary cause of bladder cancer worldwide especially in males. Most of the aromatic hydrocarbon carcinogens induce CYP1A1 and CYP1A2 as well as GSTs Mu and Alpha particularly in the lungs. CYP1A1 contributes little to the clearance of drugs as it is extrahepatic and is subject to polymorphisms, so it is not inducible in all Caucasians.

The impact of smoking on drug clearance is relatively narrow and is confined to CYP1A2, but this CYP is significant clinically. CYP1A2 is inducible to a high extent in about 45 per cent of Caucasians and is a component in the clearance of a number of drugs such as theophylline, fluvoxamine, warfarin and the atypical antipsychotics clozapine and olanzapine. There are two interesting clinical issues with smoking; the first is that certain patient groups, particularly the mentally ill like schizophrenics, tend to be very heavy smokers. Secondly, smokers can stop rather unpredictably, due to health warnings or just their own initiative. If they stop smoking after they have been established on a regime that includes a CYP1A2 substrate, then chances are the drug plasma levels will climb over the 2–4 weeks needed for the inductive effect to subside. Hence, the smoker may encounter significant side effects and as a few weeks have passed, the problem will not necessarily be attributed to the smoking cessation. Of course, stopping smoking is notoriously difficult and the smoker may have a CYP1A2 drug regime adjusted to reflect their non-smoker status, only to start smoking again. The majority of confirmed smokers tend to return to their previous level of nicotine dosage, so the drug may then fall out of the therapeutic window due to induction over a few weeks. Fluvoxamine clearance is doubled in smokers and it is thought that smokers' clearance of the 'R' isomer of warfarin may be slightly higher, although this is not yet fully investigated. There have been reports where smokers have stopped and their INRs have increased. With clozapine, it is reckoned that smokers require about 1.5 times the dose of non-smokers, whilst with olanzapine induction may still take place, it has a wider TI than clozapine so there is less risk of toxicity with this drug when smokers stop the habit abruptly. CYP2E1 is inducible by a number of small heterocyclic carcinogens found in tobacco smoke and is induced in smokers. As mentioned in Chapter 3 (section 3.6.2) and earlier in this chapter (section 7.2.3), the role of CYP2A6 in nicotine metabolism has received much attention and lower expression of this CYP can mean it is easier to control or stop tobacco use.

7.7 Effects of ethanol on drug metabolism

7.7.1 Context of ethanol usage

Around 10 per cent of men in the UK regularly exceed the healthy limits of ethanol intake and probably half of those are fully dependent on ethanol. Ethanol is basically a sedative and its ability at modest doses to relax inhibitions in a generally pleasant manner and its easy availability has made it humanity's primary source of chemical succour. Of course

it is famously addictive and increasing proportions of women and adolescents are also becoming dependent on a high ethanol intake. It has an unfortunate tendency to promote risk-taking whilst impairing reflexes and physical awareness. This translates to ethanol's central role in more than half of all violent crime and fatal road accidents; the drug also makes many city and town centres (in the UK) extremely hazardous places at night. It is not often appreciated that children are very sensitive to ethanol, as it causes hypoglycaemia particularly in toddlers and intoxication in young children at very small doses. There is more than enough ethanol in a modest helping of homemade sherry trifle to cause ataxia and vomiting in a toddler. Tragically, it is thought that ethanol has a hand in more than 40 per cent of accidental deaths in children such as falls or drownings. Nevertheless, as ethanol is the major legalized mind-altering substance, the vast majority of the population drinks regularly and the ethanol industry is exceedingly keen to bring to our attention more imaginative, palatable and profitable ways to consume it, although less effort has gone into an effective hangover cure. Since ethanol use is all pervading in most societies, whether it is legal or not, it is important to consider its effects on real-world prescription drug usage.

The question as to whether ethanol intake can affect the clearance and efficacy of prescribed drugs does rather depend on patient honesty – the difference between what a patient tells a doctor he drinks and what he actually consumes can be considerable. The range of ethanol consumption and the accompanied self-delusion is correspondingly wide, from a couple of glasses of beer per week, to bottles of spirits per day. Since drinking habits are built up gradually, many patients are genuinely unaware that they are exceeding the limit for what is generally considered healthy, i.e. approximately two spirit measures worth per day. Around 10 spirit measures per day, or 5 pints of reasonable strength beer, is well into dependence. The limits for women are about two-thirds of those values.

7.7.2 Ethanol metabolism

The main route of metabolism is cytosolic alcohol dehydrogenase (ADH), which is found in many other tissues such as the stomach and gut, besides the liver. ADH oxidizes ethanol to acetaldehyde, which is extremely toxic. ADH is under the control of the AhR and possibly the Nrf2 systems and it uses NAD as a co-factor to remove the hydrogen atoms that are abstracted from the ethanol to form NADH, which then dissociates from the enzyme. Around 90 per cent of ethanol is cleared by the ADH/ALDH system, with the remainder oxidized by CYP2E1, which has a 4–6 fold lower affinity for the drug. The genes that code for ADH are found on chromosome 21 and there are five classes, (ADH1-5) in man. Each class has separate allelic variants: ADH1A ADH1B and ADH1C are found in the liver, whilst ADH3, ADH4 and ADH1C are found in the gut. Nobody has any idea what ADH5 is for, or what it does, but it is very unstable.

The acetaldehyde formed by ADH is dealt with by aldehyde dehydrogenase ALDH, which is sourced on a variety of chromosomes. The two most relevant forms are cytosolic ALDH1A1 and mitochondrial ALDH2, which actually function as tetramers, unlike ADH. Rather cunningly, both ALDH variants are fearsomely efficient at processing acetaldehyde-in the case of ALDH2, it is several hundred fold more efficient at clearing acetaldehyde than ADH1A*1 is at producing it. You might say that this adaptation signals a healthy evolutionary respect for the systemic toxicity of acetaldehyde. ALDH and ADH are

polymorphic and as we all know through experience, the variation in the ability to clear ethanol in humans is enormous, of which, more later.

Ethanol is primarily an inducer of CYP2E1, although CYP1A1 and CYP3A are also affected. The degree of induction will of course be dependent on the patient's usual consumption, but CYP2E1 does not appear to contribute that much to ethanol clearance even in very heavy cirrhotic drinkers. The toxicological consequences of its induction are much more relevant to drug clearance and hepatotoxicity than ethanol oxidation. Interestingly, although ethanol can be so destructive to the liver, this organ is not the only route of elimination; some investigators have reported more than as much as half of ethanol is cleared extrahepatically even in alcoholics.

7.7.3 Ethanol and inhibitors of ALDH

Patients often believe that they should not drink when given antibiotics. This is only true for antibiotics that block ALDH, which as mentioned above, normally clears acetaldehyde formed by ADH. Inhibition of acetaldehyde clearance causes a severe flushing/vomiting/sweating and nausea effect which is exceedingly unpleasant. There is a surprising list of ALDH inhibiting drugs such as metronidazole, cefoperazone, cefamandole, griseofulvin, chloramphenicol, nitrofurantoin and sulphamethoxazole. Other agents, which can be inhibitory, include isoniazid and sulphonyl ureas. Antabuse, or disulfiram, is intended to block ALDH, so exploiting acetaldehyde toxicity to help the alcoholic stop drinking (Chapter 5, section 5.6.4). Given that ethanol clearance is polymorphic, if you have been paying attention you will see that this is a form of phenocopying (section 7.2.1).

Tuberculosis patients should definitely not drink when on isoniazid, as induced CYP2E1 converts this drug to several reactive species. Isoniazid (INH) is not that well tolerated in otherwise healthy individuals and it does cause high liver enzymes in heavy drinkers and can lead to severe liver damage in those cases. If the patient can stop drinking, then their CYP2E1 induction will fall and their problem with INH should diminish. Of course, many who contract tuberculosis in Western countries are at the margins of society and are highly likely to be very serious drinkers indeed. So they are more likely to sustain liver damage due to the INH/ethanol problem, which means they stop the INH, and the disease reignites, this time resistant to the drug.

7.7.4 Mild ethanol usage and drug clearance

ADH has no bearing on drug clearances, although CYP2E1 is responsible for the clearance of a number of anaesthetics, paracetamol, isoniazid and chlorzoxazone. In non-drinkers, in the absence of ethanol, or in occasional drinkers, ADH metabolizes most of the ethanol and CYP2E1 induction is likely to be modest Consequently, in occasional drinkers, clearance of CYP2E1 substrates is not likely to be affected by their ethanol intake. When ethanol is consumed in considerable quantities (a good night, but not to the point of being taken home in a supermarket trolley), then CYP2E1 substrates will have to compete with the ethanol for the enzyme, their half-lives may be lengthened and their CNS effects pronounced. This effect will be combined with the standard intoxicating effects of ethanol. So a single moderate drinking session is likely to change the pharmacokinetics of prescribed

CYP2E1 substrates. Moderate drinking can also reduce the first pass of tricyclic antidepressants but even small doses of ethanol can affect warfarin metabolism, reducing first pass and leading to excessive bleeding. Warfarin patients should not drink ethanol at all.

7.7.5 Heavy ethanol usage and drug clearance

For those chronically dependent on ethanol their CYP2E1 levels can be ten-fold higher than non-drinkers and they would clear CYP2E1 substrates extremely quickly if they chose to be sober for a period of time. This may lead to the accumulation of metabolites of the substrates. It is apparent that alcoholics who are sober can suffer paracetamol (acetaminophen)-induced liver toxicity at overdoses of around half that of non-drinkers, which is due to CYP2E1 induction. However, it is less than likely that the overdose would be washed down by anything other than a heroic amount of ethanol, so in reality, the situation is complicated by the state of induction of the CYP and the strong competitive effect of the ethanol preventing the CYP-mediated formation of the hepatotoxic metabolite (Chapter 8, section 8.4.3). It may be that as long as the alcoholic keeps drinking, this may well have a protective effect against fatal liver injury from paracetamol overdose. Worryingly, liver failure has been reported in relatively moderate drinkers after a few days of the recommended dose of paracetamol (<4 g/daily), when they have stopped drinking (whilst in hospital for example) and have needed pain relief. Interestingly, their paracetamol plasma levels were well short of the usual toxic range. The mechanisms of the alcoholic's sensitivity to paracetamol hepatotoxicity are not fully understood. It is likely to be a combination of low thiol levels, caused by a combination of poor diet and general health, as well as the constant drain of detoxifying acetaldehyde and other drink-related toxins. This level of organ stress probably leaves the chronic drinker exquisitely vulnerable to the necrosis caused by the paracetamol metabolites, particularly if alcohol consumption is interrupted for a significant time, which is neither sufficient for the CYP2E1 induction to subside, nor for the liver to recover.

When alcoholics are 'maintenance' drinking, that is, enough ethanol to be able to feel 'normal', but not intoxicated, reduced clearance of CYP2E1 substrates will be seen, although when alcoholics drink to become intoxicated, they consume staggering amounts. This means that ethanol will probably fully occupy CYP2E1, so any other substrates clearance will be reduced. As a result, toxicity could occur, that is, if the alcoholic notices. Certainly, warfarin metabolism is accelerated in very heavy drinkers and higher doses must be given for the drug to show efficacy.

7.7.6 Alcoholic liver disease

Predisposition to alcoholism: ADH/ALDH polymorphisms

Like any addiction, alcoholism is effectively a mental illness, although there are many predispositions that can help or hinder the desire to become dependent on the drug. It is generally true that those who pride themselves on their high tolerance to ethanol are more likely to develop alcoholic liver damage than those with little or no tolerance. This is partly because there is more opportunity to build high tolerance and dependence at the level of the brain. Although ethanol is cleared at a leisurely pace compared with nicotine, cocaine

and heroin, craving is nevertheless promoted by relatively rapid removal of the drug. On a practical level, the longer you can stay conscious and able to drink, the addiction can be serviced and the more ethanol-related toxic species will damage you over your drinking lifetime. If you can detoxify the acetaldehyde rapidly and efficiently, you will recover quickly from the previous night's excesses and you are ready to drink more. These factors facilitate dependence and eventually health destruction.

In contrast, those who become hilariously incoherent after one or two drinks are less likely to develop such heavy dependence on alcohol. Indeed, those who cannot detoxify acetaldehyde and thus suffer its systemic toxicity after exposure to ethanol are virtually immune to alcoholism, although incredibly, there are exceptions. These general observations are supported by the polymorphic clearance of ethanol by both ADH and ALDH.

In terms of capability, the vast variation in ADH catalytic activity across the human race is mainly due to just a few SNPs that profoundly change the efficiency of the isoforms. ADH1B/*1 is the most effective variant and is the ADH wild-type, with arginine residues at amino acid positions 47 and 369. This variant is by far the most common in Caucasians and American Indians. ADH1B/*2 is common in Japanese, Chinese and Koreans and has a histidine at position 47 and its efficiency is more than four-fold lower than the wild-type. ALD1B/*3 is found in some African races and has a cysteine at position 369 and exhibits low capability. ALD1C/*1 and ALD1C/*2 are also poorly functional and are found in the Chinese/Japanese/Koreans as well as Caucasian and black populations. Part of a 'successful' career as an alcoholic depends possessing the ADH1B/*1 isoform. The other defective isoforms are found in low frequencies in alcoholics and cirrhotics. The respective incidences of poorly functional ADH and its effects can be reflected in differing attitudes to alcohol and drunkenness between Eastern and Caucasian cultures.

As mentioned above, in the vast majority of individuals, whatever their variant of ADH, they are able to process acetaldehyde to acetate and water, as the consequences of failing to do this are severe. With ALDH, the wild-type and gold standard is ALDH2*1/*1, which has the highest activity of all these isoforms and is the second essential component for an alcoholic career. The majority of cirrhotics have the maximally efficient combination of ADH1B/*1 and ALDH2*1/*1. With regard to ALDH, the variant ALDH2*1/*2 has less than a quarter of the wild-type's capacity and is found predominantly in Eastern races. The variant ALDH2*2/*2 is completely useless and renders the individuals very sensitive to acetaldehyde poisoning, although the toxin is removed eventually by ALDH1A1 which does not seem to be affected by polymorphisms. In a survey of 1300 Japanese alcoholics, there was nobody at all with the ALDH2*2/*2 variant. Although it might be thought that impaired clearance of acetaldehyde would make being dependent on alcohol rather more unpleasant than for most Caucasians, nevertheless, in Eastern races up to 20% of alcoholics have impaired ALDH capacity. This demonstrates epic self-loathing if nothing else. Interestingly, in a somewhat perverse triumph over genotype, an individual in one study who was ALDH2*2/*2 became an alcoholic through imbibing small amounts of ethanol during his every waking hour.

Alcoholic liver disease

Social drinking is of little direct effect on hepatic metabolism, but in chronic heavy drinking, long-term disruption of the liver results. As you probably know, the liver is

the toughest organ in the body in terms of its formidable detoxification systems and very high (6–10 mM) levels of GSH are maintained intracellularly. Consequently, it takes enormous quantities of ethanol to damage the liver beyond repair. An estimate of how much ethanol is needed to kill the liver is interesting. Assuming the average alcoholic drinks heavily for 20 years prior to liver failure using about a bottle and a half of spirits per day, this amounts to about 2000 litres of pure ethanol in many thousands of drinks ranged against a 1.5 kg liver. That the majority of extremely heavy drinkers die of something other than liver failure (about a third die of cirrhosis) is further testament to hepatic toughness.

In chronic drinkers, ethanol impairs the complex control of hepatic lipids, partly by inhibiting the production of the major lipid regulator adiponectin and other pathways such as sirtuin 1, AMP-activated kinase and PPARα, as well as the ADH-mediated excess of NADH formation. This causes alcoholic 'fatty liver' or steatosis, which is a well-known staging post to more severe liver disease. Glucuronidation can be disrupted, as the sustained high NADH levels affect UDP-glucuronic acid formation. At this stage, there are few symptoms beyond low blood sugar, although hepatic weight can more than double. The condition will progress under pressure from ethanol consumption gradually to alcoholic hepatitis (severe liver inflammation) and more visible effects such as jaundice due to impaired bilirubin clearance set in. At this stage, if the patient stops drinking and reforms their lifestyle, eating properly and taking care of themselves, they can walk away intact with a large fund of appalling 'back when I was drinking' stories.

Regarding those who continue to drink, one of the major toxic pressures in alcoholic liver disease at the cellular level appears to be CYP2E1 induction. It is thought that CYP2E1 mostly converts ethanol to toxic acetaldehyde that must be detoxified by other systems such as ALDH, which are at full stretch in alcoholism. CYP2E1 can also form the toxic hydroxyethyl radical from ethanol and on the rare occasions the alcoholic is not drinking, CYP2E1 not occupied in ethanol clearance emits a constant stream of nasty reactive oxygen radicals that must be detoxified by the hepatocyte. CYP2E1 is also induced in mitochondria as well as the SER and of course quantities of acetaldehyde formed in the cellular 'engine room' can cause mitochondria to release apoptotic factors which can cause cell death. In addition, damage to mitochondrial ATP production can cause cell death through cellular necrosis. It is thought that ethanol abuse and CYP2E1 induction leads to long term oxidative stress. Interestingly, if CYP2E1 does play a decisive role in the oxidative destruction of the liver in alcoholism, then its level of expression could explain why some individuals can survive decades of very heavy drinking with minimal cirrhosis. Perhaps if long-living alcoholics are associated conclusively in clinical studies with a low expression/low induction version of CYP2E1, such as RsaI; or alternatively, those who succumbed to fatal cirrhosis relatively quickly possessed versions associated with high reactive toxic species turnover, such as Dra1 (which forms high levels of 2,5, hexanedione, section 7.2.3.) and is associated with alcoholism, then this might illuminate the role of CYP2E1 in cirrhosis progression. Sadly, such information is likely to be of limited practical relevance to alcohol abusers.

The oxidative stress caused by alcohol metabolism also gradually erodes GSH maintenance as thiols are required to detoxify reactive species formed by acetaldehyde. As alcoholic liver disease progresses, liver enzymes, such as serum ALT and AST, climb steadily from ten to fifty times the normal limits towards the thousands. Chronic acetaldehyde toxicity leads to hepatocyte death and the simultaneous stimulation of collagen formation that leads to a scarring effect in the liver.

During the inflammation phase the scarring process involves progressive replacement of hepatocytes with fibrotic connective tissue. This is cirrhosis, where nodules and lumps of this fibrous tissue appear all over the liver and disrupt its blood circulation and the removal of bile. The liver hardens to the touch and shrinks, developing varicose veins known as vascular 'spiders'. At this stage provided they stop drinking, there is still a chance of survival with some residual liver function. Although there are other causes of cirrhosis (hepatitis, drug therapy, genetic conditions), over 90 per cent of cases are sustained by ethanol consumption. Biochemically, the liver fights a losing battle as production of essential proteins gradually falls as the drinking progresses the disease and cholesterol, sugar and triglyceride metabolism is compromised. Elsewhere, the gut is damaged by ethanol intake to the point that it becomes excessively porous and even undigested food particles enter the blood, which are recognized by the immune system. This leads to virtually permanent gut inflammation and variable drug absorption.

As cirrhosis progresses, hepatic back pressure becomes so high that the blood from the portal vein has difficulty in entering the liver to the point that blood starts to leave the liver and run back down the portal vein. This causes swollen varices, or varicose veins in the oesophagus and stomach. Fluid is forced out into the tissues, causing abdominal ascites. By the time the alcoholic sustains this level of damage, there is no way back and death usually results. Alcoholics in end-stage liver failure have particularly poor outcomes in hospital intensive care units, especially in hepatorenal syndrome, where kidney failure occurs as a result of the liver disease. Approaching end-stage liver failure, serum ALT and AST levels are well in the thousands and bilirubin appears in the blood in quantity leading to severe jaundice as the liver effectively waves the proverbial white flag in desperation. Cirrhotic liver damage is permanent and five-year death rates even if the drinker stops can be more than 50 per cent from cardiac arrest, coma, malnutrition (alcoholics never eat properly) and renal failure. At this stage, the only therapy is a new liver, provided the alcoholic has been 'dry' for six months or more, or is exceedingly rich and famous. It could be argued that alcoholism is unlike many other addictions, as there are more than an average number of warnings, signs and chances to 'get off the bus' as the liver and the body in general both put up an exceedingly stout long-term defence against ethanol's cytotoxicity and solvent-like effects. Tragically, with many confirmed alcoholics it is probably easier to 'rescue' a fish from the sea than to stop them drinking.

Women and alcoholic liver disease

Women are much more vulnerable to ethanol damage and on average die in half the time it generally takes for a male alcoholic to drink himself to death. Women drink much less than men also-one study indicated that a group of women consumed about 14,000 drinks to induce cirrhosis, whilst men required more than 44,000 to achieve the same effect. Ethanol distributes in total body water only, so in women their greater fat content means that blood ethanol levels are higher than men of similar weight and age. In addition, their stomach ADH is less effective than men's, which also promotes the entry of more ethanol into the blood, although the role of stomach ADH in ethanol clearance is disputed. There is some evidence that hepatitis B progresses to liver cancer and fibrosis in women less rapidly than in men and postmenopausal women and it has been hypothesized that this is

related to the protective effect of oestrogens on cytokine levels, as well as its antioxidant effect. This does not appear to apply in alcoholic liver disease. Regarding the role of CYP2E1, background expression of this CYP is said to be lower in females than males. However, studies with NASH, (section 7.2.3), which has similar pathology to alcoholic liver disease, have indicated very high induction of CYP2E1 in *5B variant females which was linked to liver injury. Female susceptibility to alcoholic liver failure is probably multifactorial, a combination of excess reactive species formation, possibly linked to CYP2E1 induction, hormonal factors (insulin and oestrogens), cytokine effects and compromised detoxification in terms of GSTs and thiols. In general, women's vulnerability to alcoholic liver disease may be more than just hepato-physiological. Women are less likely to seek help than men to control their drinking and if they do complete a rehabilitation course and they are detoxified, they are more likely than men to return to drinking. Female five-year survival in cirrhosis is also worse than men.

7.7.7 Effects of cirrhosis on drug clearance

High extraction drugs

The main effects of cirrhosis on the liver include the reduction in blood flow (indeed, the total derangement of the portal system), the loss of functional hepatocytes and changes in protein binding. Mainly, CYPs 3A and 1A are much reduced in activity, although CYP2 series activity often survives. Sulphation is reduced, but glucuronidation is often preserved. Regarding the clearance of high extraction drugs, where blood flow is the major determinant of clearance, clearance of these agents will be severely compromised. The clearance of beta-blockers (propranolol, metroprolol, labetalol), as well as pentazocine, lignocaine and opiates such as pethidine and morphine can be reduced by between 30 and 50 per cent. The key issue here is that this results in major increases in bioavailability after an oral dose, which in turn means plasma concentrations increase by two- to seven-fold, or in the case of chlormethiazole, 17-fold. So for alcoholics, it is recommended to reduce oral dosing by five- to ten-fold and intravenous dosing by around two-fold.

Low extraction drugs

Clearances fall by more than 50 per cent in drugs such as naproxen and cefoperazone, but may fall by 70–90 per cent in theophylline and sulindac, whilst paracetamol clearance falls by around 20 per cent. Again, plasma levels may double or quadruple. Protein binding is much less in cirrhotics due to the failure of the liver to make enough albumin and the various fluid accumulations affect drug distribution. The net effect is to increase levels of unbound drug, which also contributes to the general two- to four-fold increase in drug plasma levels.

Overall, the extent of cirrhosis can be judged from general indices of hepatic health and usually the more severe the disease, the greater impact on drug clearance will occur. Mild cirrhosis generally does not significantly impact drug clearances. Interestingly, cirrhosis has less effect on some drugs that are cleared by conjugative processes only, such as lorazepam.

7.8 Artificial Livers

There are several systems currently being examined which act as extracorporeal liver support in patients who are in liver failure which may be due to a variety of reasons. The drive to develop such technologies is rooted partly in the scarcity of livers for transplant, as well as the benefits of temporarily taking over from a damaged liver while it recovers from disease or chemical-induced damage. These systems operate in a similar fashion to renal dialysis machines to remove endogenous toxins such as bilirubin from blood, as well clearing drugs in overdosage situations. SPAD (single pass albumin dialysis) operates by routing blood past an albumin-rich fluid that binds the toxins and/or drugs and then it is discarded, like in total-loss lubrication systems in very early cars. A more sophisticated version is MARS (molecular adsorbent recirculating system) that uses a combination of albumin and activated charcoal to bind and remove drugs, hepatotoxins and accumulating metabolic waste (bilirubin and ammonia). In a study in pigs, it was shown that the MARS system could remove midazolam and fentanyl. In humans, the system was shown to be beneficial in renal problems caused by cirrhosis. In MARS, the albumin system is regenerated by a system of adsorbents that detach the toxins from the blood to the albumin and out to the adsorbents.

The SPAD system can be a useful temporary support, although some studies have mentioned that it does not affect clinical outcome in liver failure patients. MARS has improved patient survival by up to six-fold in some cases, keeping bilirubin levels acceptable and avoiding removing too many clotting factors. The Prometheus system, or FPSA (fractional plasma separation adsorption) is a further innovation. This is a complete unit that separates the albumin-mediated removal of toxins from the clearance of water-soluble toxins. It uses the patients' own albumin to achieve this, whilst sparing fibrinogen for adequate clotting. The Prometheus unit uses two high affinity adsorbers to clear off the toxins and then the albumin is returned to the patient's circulation. The unit then removes water-soluble toxins using high-flux haemodialysis. The Prometheus system is used in several 5–6 hour sessions and preliminary data on patient survival looks very promising, as it is more efficient than other systems, does not require exogenous albumin and its ability to remove water soluble toxic agents is superior to MARS. Systems such as Prometheus and MARS will be useful in patients suffering from overdoses and is likely in the future to be valuable in modulating the concentrations of drugs such as anaesthetics, analgesics and antibiotics in patients awaiting liver transplants, as well as removing cytokines and antibodies, such as those suffering from the devastating paralyzing condition, Guillain-Barré syndrome. In the distant future, more life-like systems are under development which may involve packs of human hepatocytes which will be able to actively biotransform and process toxins, as well as eliminate them in bile and carry out hepatic 'housekeeping' tasks which no machine will ever be capable of doing.

7.9 Effects of disease on drug metabolism

Interferon (IFN-2b) treatments for melanomas can selectively impair CYP metabolism, with CYP2E1 unaffected and CYP1A2 significantly inhibited. Some alcoholics have been shown to express autoantibodies to CYP2E1 and to 3A4. Chronic hepatitis C and autoim-

mune hepatitis antibodies have been found against CYP2D6 and 2A6, as well as anti-UGT antibodies.

Over 2 per cent of the US population has the antibodies to the Hepatitis C virus. This can result in chronic liver disease, such as cirrhosis or even liver cancer. It is usually, but not always, associated with a history of youthful excess such as intravenous drug usage. Few studies have been carried out, but some evidence suggests that CYP expression (such as CYP3A4) declines as the disease progresses, although in liver cancer induced by hepatitis C, some CYP isoforms' expressions increase. This, coupled with the previously described effects of cirrhosis, indicate that hepatitis C patients may show reduced clearance of many of the major therapeutic drugs. Liver cancer linked with Hepatitis C and B leads to partial suppression of hepatic detoxification processes such as GST expression, as well as much reduced GSH formation. In one study the ratio of GSH to GSSG in these patients is around 6 : 1 when it should be more than 20 : 1 in healthy individuals. Similar findings in alcoholics, diabetics and those suffering from HIV suggest that these patients are much more at risk than healthy individuals of hepatic oxidative stress and vulnerability to reactive species formation by drugs. Effectively, the 'window' where reactive species generated by oxidative metabolism of drugs or environmental toxins have the opportunity to bind irreversibly within cells before they can be detoxified may well be considerably longer in these patients, thus predisposing them to greater hepatotoxic risk compared with healthy patients. This oxidative stress process is probably linked with the development of hepatic cancers in the first place.

Indeed, in diabetes, oxidative stress is complicated by CYP2E1 induction, but this only seems to occur in obese Type II diabetics, who can clear the CYP2E1 marker chlorzoxazone more than 2.5 times faster than healthy individuals. In animal studies, CYP2E1 induction in untreated diabetic individuals disappears once they receive insulin therapy and it has been suggested that insulin is part of the control of CYP2E1 expression. It is possible that Type II diabetics might be more at risk from paracetamol overdose and carcinogenicity linked with CYP2E1 metabolism. There does not seem to be a CYP2E1 inductive effect in well to moderately controlled Type I diabetics.

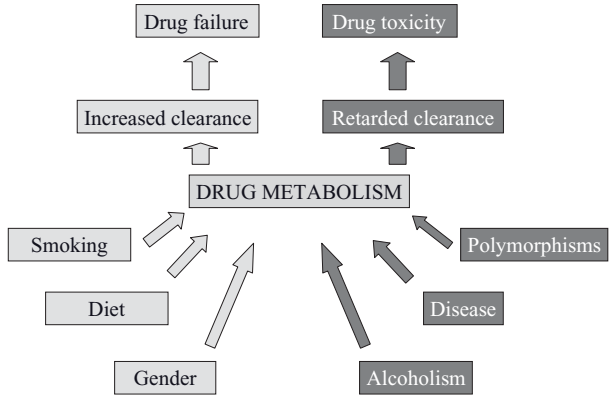

Figure 7.4 Generalized scheme of factors which influence drug metabolism, leading to changes in clearance, which are responsible for extremes of drug response

7.10 Summary

Overall, there are a large number of factors that can influence drug metabolism, either by increasing clearance to cause drug failure, or by preventing clearance to lead to toxicity. In the real world, it is often impossible to delineate the different conflicting factors which result in net changes in drug clearance which cause a drug to fall out of, or climb above, the therapeutic window. It may only be possible clinically in many cases to try to change what appears to be the major cause to bring about a resolution of the situation to restore curative and non-toxic drug levels. Figure 7.4 tries to form a summary picture of the major influences on drug clearance.

8 Role of Metabolism in Drug Toxicity

8.1 Adverse drug reactions: definitions

It is important to see the role of metabolism in drug toxicity as one component of the bigger picture of all adverse reactions suffered by patients during drug therapy. One view of this picture is on Figure 8.1. Different authors and textbooks classify these effects in various ways, but a convenient way to look at things can be to resolve all drug adverse effects as either reversible (Type A) or irreversible (Type B). At this point, it is worthwhile being more precise over the terminology of drug adverse reactions. The term 'toxicity' is a loose one and it has been used flexibly in this book so far. However it is useful when looking at drug metabolism-mediated effects to define toxicity more accurately for the cell in particular:

Irreversible change in structure leads to irreversible change in function.

The key here is 'irreversible'; it follows that a process that is not irreversible is not actually 'toxicity' in the strict sense of the word. Reversible reactions are often either an intensification of the usual pharmacological response, or, as has been mentioned previously, an 'off -target' pharmacological effect. There are, of course exceptions: this strict definition of toxicity is fine for the cell, but is not so clear for the relationship between the patient, their organs, tissues and individual cells. Obviously, the effects of an anticancer alkylating agent are irreversible and toxic to a cell, but could ultimately save the life of the patient. Conversely, a heroin overdose reversibly inhibits central control of respiration and this leads to death, which is unarguably irreversible. In general though, it is probably fair to say that cumulative irreversible damage at the cellular level usually leads to patient morbidity and mortality.

8.2 Reversible drug adverse effects: Type A

Type A describes the vast majority of adverse effects (Figure 8.1) and patients experience these in direct proportionality to the quantity of the drug and/or its metabolites in their tissues. These effects are sometimes described as 'toxic' but these are not usually irreversible effects. They can be resolved into two main causes, as follows.

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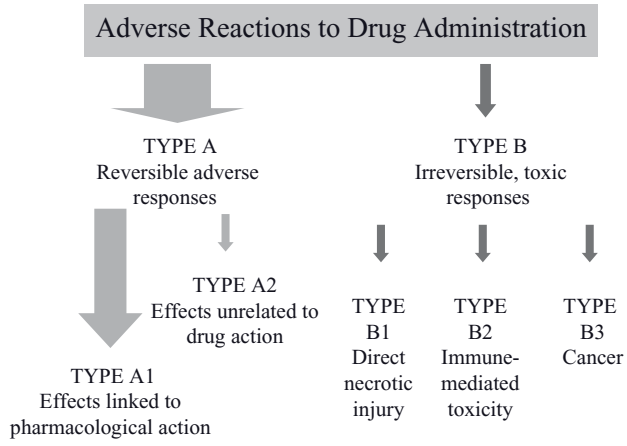

Figure 8.1 Summary of reversible (Type A) and irreversible (Type B) drug effects

8.2.1 Intensification of pharmacologic effect: Type A1

This is proportional to drug concentration and happens if drug levels climb well above the therapeutic window. Anticonvulsants are membrane stabilizers, so at high concentrations they cause sedation and confusion. Similarly, high concentrations of anticoagulants cause excessively long clotting times. Some drugs may display a series of known pharmacodynamic effects at therapeutic window levels, but exert other unwelcome effects at high concentrations. Some beta-blockers at higher doses cause central effects such as nightmares. When drug levels fall, the excessive pharmacodynamic effects subside also. These adverse effects, also known generally as 'side effects', are mostly an intensification of the 'main effects', and are thought to be the cause of more than 80 per cent of patient problems with drug therapy. Many patients will experience an unpleasant concentration-dependent drug effect at some point in their lives. As we have seen in earlier chapters, drug metabolism-related changes in clearance caused by induction or inhibition of biotransformation systems can have profound and occasionally lethal effects on the patient. Other reasons for the intensification of drug effects include renal problems, overdosage and problems with dosage calculations, or being too old, too young or too sick.

8.2.2 Off-target toxic effects: methaemoglobin formation – Type A2

As well as off-target pharmacodynamic effects caused by parent drugs, it is possible that at least one or other of the metabolites may disrupt cellular function in a way that is unrelated to the pharmacological effects of the drug, but the disruption is reversible and predictable. This situation is again dose related, in that the more drug that is absorbed, the more is cleared through the pathway which forms a 'problem' metabolite. This type of adverse effect, though reversible, has the potential to make a drug almost intolerable to take from the patient's viewpoint and can even be lethal. A good example of a reversible

adverse effect is methaemoglobin formation, where the appearance of the patient's blood becomes a passable impression of chocolate milk.

To see the link between a product of drug metabolism and an adverse drug effect, it is first necessary to understand the endogenous system involved. Haemoglobin is a molecule that normally becomes reversibly oxygenated, rather than irreversibly oxidized. Our continued existence depends on the difference between those two terms. Haemoglobin, as you know, is oxygenated to transport the gas from lung to tissues and release it where required. It is a tetramer, which means it has four protein subunits, each of which contains an iron molecule as Fe^{2+}. The iron molecules bind the four oxygen molecules. The oxygen binding is dependent, like many enzymes, on the ability of the metal to gain and lose electrons, rather like charging and recharging a battery as mentioned previously. It is clear that a molecule like haemoglobin is potentially reactive and is going to need protection from oxidation so it can continue to function, so the erythrocyte is designed to maintain haemoglobin so that it carries oxygen and is not damaged or changed in structure. It accomplishes this with the second highest level of GSH in the body after the liver and a series of protective enzymes, more of which later. Normally, the haemoglobin iron molecules (Fe^{2+}) bind the O_2 and form superoxoferrihaem complexes ($Fe^{3+}O_2^{-}$.). These dissociate at the tissues and 99 times out of 100, the Fe^{2+} is restored and O_2 is delivered to the tissue. The one time out of 100, the oxygen retains the electron and becomes superoxide ($O_2^{-}\cdot$) and the Fe^{2+} becomes Fe^{3+}. This is very bad, as Fe^{3+} will bind anything but oxygen, such as anions. So the Fe^{3+} means that this haemoglobin monomer is now methaemoglobin and useless for oxygen carriage. Since this is a natural consequence of transporting a reactive gas with a reactive protein, the erythrocyte carries two systems for reducing the oxidized Fe^{3+} to Fe^{2+}, so restoring the function of haemoglobin. The systems are NADH diaphorase and NADPH diaphorase. The NADH diaphorase is similar to the cytochrome b_5 REDOX partner discussed in Chapter 3, (section 3.4.7) and it operates for 95 per cent of the time, as the NADPH system is incomplete and requires an artificial electron acceptor for full operation. Why this is the case is unknown. If it were not for NADH diaphorase, methaemoglobin formation would be around 4–6 per cent per day, more if you smoke. If you measured your methaemoglobin levels now, they would be less than 1 per cent, so the system is adequate to maintain haemoglobin in normal circumstances.

It is possible for some xenobiotics to react with haemoglobin to form methaemoglobin. Nitrites are moderately efficient at this process, but the aromatic hydroxylamines are particularly effective in forming methaemoglobin. They oxidize haemoglobin to methaemoglobin in two stages. Initially, the hydroxylamine directly reacts with oxyhaemoglobin to form methaemoglobin. This is known as a co-oxidation, as the hydroxylamine will be oxidized to a nitrosoarene. This alone would not account for the speed at which methaemoglobin can form in patients with high levels of hydroxylamine metabolites in their blood (Figure 8.2). The nitrosoarene formed by the initial reaction is then 'helpfully' reduced to the hydroxylamine by GSH, so allowing the hydroxylamine to oxidize another oxyhaemoglobin molecule. The resultant nitrosoarene can then be re-reduced to the hydroxylamine and oxidize another oxyhaemoglobin and so on.

The initial oxidation is thus amplified by GSH and the presence of millimolar levels of GSH is like pouring fuel on a fire. Each hydroxylamine molecule can oxidize at least four oxyhaemoglobin molecules. The result is a very rapid process, where as soon as the metabolite is produced, methaemoglobin starts forming in significant quantity, in direct

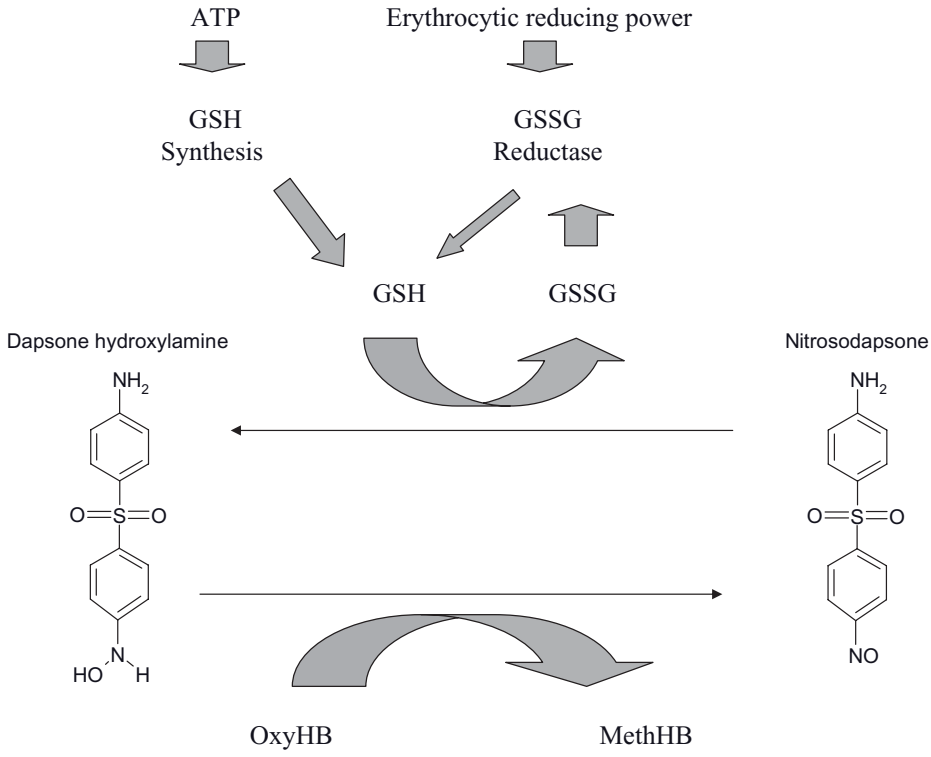

Figure 8.2 Basic process of methaemoglobin formation in the human erythrocyte. This is repeated many times per second in a futile cycle that is limited only by the levels of GSH in the erythrocyte

proportion to the level of the metabolite released by the liver, which is of course in proportion to the drug dose.

8.2.3 Clinical consequences of methaemoglobin

Methaemoglobin is measured as a percentage of haemoglobin. However, even modest levels exert a potent effect, due to the Darling–Roughton effect. You might recall that haemoglobin is a tetramer and binds four oxygen molecules as oxyhaemoglobin. If an Fe^{2+} of one of the monomers is oxidized to an Fe^{3+}, then the remaining oxygen molecules are much more tightly bound and it is harder for them to escape and oxygenate tissues. If two monomers are oxidized then it is even harder for the oxygens to be released. This means that a small percentage of methaemoglobin, say 5–10 per cent, has a greater effect than just removing 5–10 per cent of haemoglobin. The effects on the patient of methaemoglobin formation are a reflection of increasing inability of their tissues to receive enough oxygen. So at low levels, 4–6 per cent, people of fair complexions may look slightly bluish and may have a mild headache. As levels are increased to 10–15 per cent, this extends to headache, fatigue and sometimes nausea. At levels of 20–30 per cent,

hospitalization is usually required, with the intensification of the previous symptoms plus tachycardia and breathing problems. Higher levels (50 per cent plus) lead to stupor and loss of consciousness, with 70 per cent plus leading to death. Methaemoglobinaemia is a drug-dependent effect that may be either acute or chronic.

8.2.4 Acute methaemoglobinaemia

The most dangerous situation for methaemoglobin formation can be as a result of benzocaine usage in anaesthetized patients. The local anaesthetic is sprayed on the oropharynx prior to intubation, although not used much in the UK, it is still employed in some countries. Many formulations of the drug are around 14–20 per cent and three 1-second bursts of the spray could deliver perhaps 600 mg of the drug. A number of reports have shown that methaemoglobin levels of 30 per cent or more can result in 30–40 minutes (Figure 8.3). This can be a serious problem, as blood oxygenation is usually measured by pulse oximeters and these units overestimate blood oxygenation in the presence of increasing methaemoglobin.

This problem, coupled with the lack of recognition of the visual signs of methaemoglobin in a patient on the operating table, can lead to dangerously high methaemoglobin levels without awareness of the staff. If in doubt, a CO oximeter must be used to reliably measure methaemoglobin formation. Benzocaine is unable to cause methaemoglobin itself, so it is thought that it is oxidized to a hydroxylamine, almost certainly by CYP2C9 and possibly 2C8, although CYP2E1 is thought not to be involved.

Acute methaemoglobin formation has actually been a therapeutic goal: this would appear bizarre, but cyanide is an anion, so it binds very strongly to methaemoglobin, forming cyanomethaemoglobin. The methaemoglobin has the effect of ‘vacuuming’ the cyanide out of the tissues and holding it in the blood, before a thiosulphate is given to finally facilitate the urinary excretion of the cyanide. Sodium nitrite has been employed as an antidote to cyanide poisoning; although it does not form large amounts of methaemoglobin quickly enough in cyanide overdoses where, as you can imagine, time is of the essence. A series of phenones were designed in the 1950s and 1960s that were intended to be oxidized to potent methaemoglobin formers that would be used to protect military personnel prophylactically from cyanide toxicity. How these personnel might cope with 15–20 per cent methaemoglobinaemia and maintain their combat capabilities seems difficult to imagine. However, cyanides are one of the cheapest ‘weapons of mass destruction’ so they remain a long-term threat. The most extensively tested agent was 4-aminopropiophenone, which is also oxidized to a hydroxylamine and acts in a similar way to any aromatic hydroxylamine (Figure 8.3).

8.2.5 Chronic methaemoglobin formation

It is unlikely a new drug with an aromatic amine group would be approved for clinical usage today, on the grounds of toxicity. Sulphonamides retain their therapeutic place in HIV patient maintenance and their hydroxylamine metabolites are cytotoxic, and immunotoxic; fortunately, they form negligible levels of methaemoglobin. The sulphone, dapsone, is still the mainstay of the treatment of leprosy, dermatitis herpetiformis and

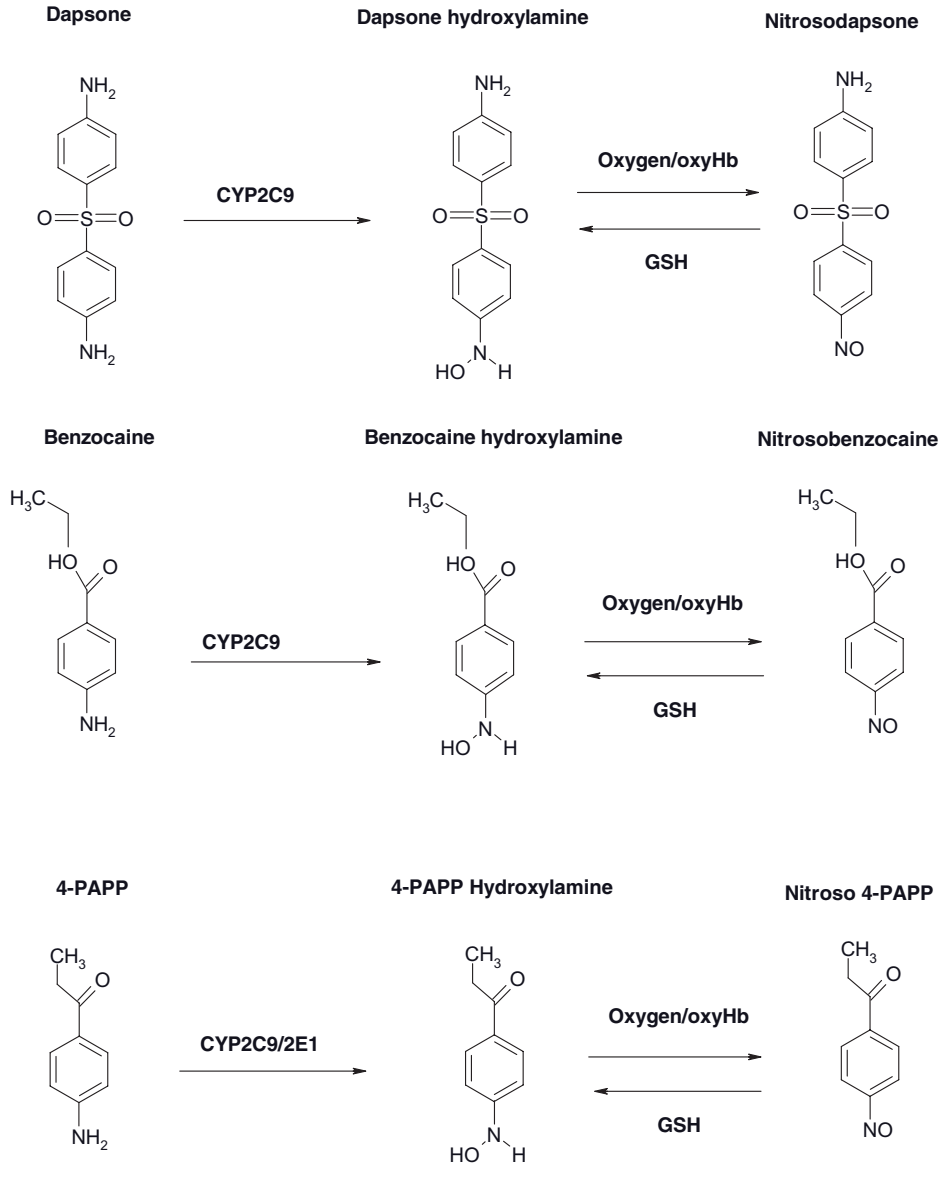

Figure 8.3 Production of various methaemoglobin-forming species from dapsone, benzocaine and 4-aminopropiophenone (4-PAPP). In each case, the hydroxylamine is formed from the parent compound by CYP-mediated oxidation and the presence of oxygen and/or oxyhaemoglobin (oxyHb) converts the agents to nitrosoarenes, whilst erythrocytic GSH re-reduces them to their hydroxylamines within the co-oxidation cycle

conditions where neutrophil migration into tissues leads to inflammatory damage. Despite successful efforts to develop non-methaemoglobin forming analogues of these drugs, the market is just not large enough to sustain their development. So patients are essentially stuck with dapsone and its dose-dependent methaemoglobin formation. Dapsone is usually

given at 100 mg/day for leprosy and anything from 25 mg/week to 400 plus mg/day for dermatitis herpetiformis. Methaemoglobin peaks at around three hours post-dosage and the patients complain of a sort of permanent 'hangover' effect, although tolerance varies widely. For an individual taking 100 mg/day, methaemoglobin levels may peak at 5–8 per cent, depending on their level of hydroxylamine production. The half-life of methaemoglobin reduction is just under 1 hour, so after the initial pulse, the deleterious effects of the methaemoglobin wear off within three to five hours. Many patients have to take dapsone for several years, leading to significant impact on quality of life, although co-administration of cimetidine can ameliorate this situation (Chapter 5, section 5.6.3). Primaquine, the 8-aminoquinoline antimalarial, also forms methaemoglobin, although this is used in much shorter courses for the elimination of *Plasmodium vivax, malariae* and *ovale* (recurrent) malaria in those who are not returning to the area where they were infected. Fortunately, the drug can be effective in eliminating recurring malaria from a single 45 mg dose, so methaemoglobin formation is only a temporary problem. If longer courses are used, then methaemoglobin formation can be considerable.

8.2.6 Methaemoglobin as a protective process

Although potentially lethal, methaemoglobin formation is not toxicity, as the erythrocyte has two processes capable of reversing it. Once methaemoglobin is reduced to haemoglobin, it can resume its oxygen carriage function, the erythrocyte replaces its thiols and is physically undamaged. The co-oxidation process effectively 'ties up' the hydroxylamines in the cycle and eventually releases them (via glutathione conjugates) as the parent drug. The erythrocyte thus performs a temporary detoxification of the hydroxylamine. Although methaemoglobin formation seems like an 'own goal', effectively the structure of the erythrocyte has been protected from the protein reactive nature of the nitroso derivative. The released parent drug will be either acetylated or reoxidized by the liver, but then it has some chance of conjugation as an N-glucuronide, so with each 'cycle' through the erythrocyte, the hydroxylamine fails to damage it, whilst cells such as mononuclear leucocytes are easily destroyed by nitrosoarenes.

8.2.7 Glucose-phosphate dehydrogenase deficiency (G-6-PD)

This condition occurs in all races, but is most common in those of Afro-Caribbean descent, with about 100 million individuals affected worldwide. This genetic polymorphism of G-6-PD is normally not an issue, except when the individuals are exposed to methaemoglobin forming drug metabolites. This enzyme supplies the majority of the reducing power of the erythrocyte and if it is poorly or non-functional, there is only sufficient reducing power available to supply GSH to protect erythrocytes from background levels of reactive species. As soon as the cells are exposed to high concentrations of hydroxylamines or primaquine metabolites, there is insufficient reducing power to tie up nitrosoarenes in the co-oxidation cycle and no detoxification occurs as little methaemoglobin formation happens. The nitrosoarene is then free to react with the structure of the erythrocyte. This causes the normal system of the erythrocytes' 'sell by date' to be prematurely activated (they usually last 120 days) and the spleen automatically removes them

from the circulation. This can happen so quickly that anaemia can result in days, with associated hepatic problems with the processing of large amounts of now waste haemoglobin. G-6-PD patients that must take either dapsone or primaquine must only receive half or less than the recommended dosage of the drugs. G-6-PD effectively converts what would be a reversible drug effect into true toxicity. If the individual is homozygous for Gilbert's syndrome (Chapter 6, 6.2.4), their inadequate UGT1A1 expression will be rapidly overwhelmed by the large amounts of bilirubin formed during the erythrocytic destruction.

8.3 Irreversible drug toxicity: Type B

8.3.1 How drugs can cause irreversible effects

All chemicals and of course drugs, interact with each other through three main routes. These range from relatively weak associations, such as van der Waals forces and hydrogen bonds, through to more potent ionic bonds and finally covalent bonds. Perhaps analogies for these interactions could be a child's toy magnet for van der Waals and hydrogen bonds, an electromagnet for ionic bonds and a spot-weld for a covalent bond. The first two interactions are reversible, varying in strength, but leading to no permanent changes in the participants. A covalent bond is the product of a chemical *reaction*, rather than just an interaction and is not desirable unless the drug is intended to 'weld' itself to its receptor to destroy its functional capacity. This is the case with anticancer drugs as they cross-link DNA or drugs which react with steroid receptors. However, the vast majority of drug action is propagated through reversible bonds, where a receptor is activated and the drug leaves to interact with other receptors, just as their endogenous counterparts do.

Unfortunately, drugs do cause unintended irreversible changes to organelles, cells and tissues, leading ultimately to organ damage. There are several ways that drugs might cause irreversible toxic effects involving three possibly interrelated pathways.

1. Drugs may alter the expression of key genes in cellular homeostasis that may cause irreversible damage.
2. Drugs may act to cause one group of cells to destroy another, such as by eliciting an immune response, which recruits cellular or antibody-mediated attacks on tissues.
3. Drugs might chemically react directly with a variety of cellular structures, changing their structure and thus their function.

These observations lead to several key questions: firstly, how would any given drug cause these undesirable effects, what chemical interactions are involved and is it the parent drug and/or metabolites that are responsible? Starting with pathway 3, reaction with cell structures requires covalent binding and this would only occur in a highly unstable drug. With some exceptions (penicillin and alkylating agents) highly reactive entities are always weeded out in the drug discovery process, so pathway 3 is most likely to be caused by prior biotransformation to a reactive species either in or near to the tissue involved. It is not really possible to be precise about the processes occurring in Pathways 1 and 2, which may be caused by parent drug, stable or reactive metabolites. So biotransformation is

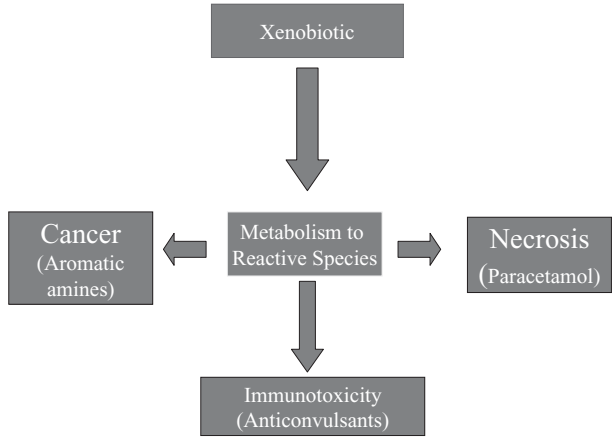

Figure 8.4 Main consequences of reactive species formation due to xenobiotic metabolism in different organs and tissues

probably the major determinate of irreversible drug toxicity, but it is important to recognize that it is not the only factor involved (Figure 8.4).

8.3.2 Role of biotransformation in causing drug reactivity

There are many routes whereby reactive species may be formed. As you will have gathered, CYP enzymes can radically rearrange the structure of a molecule to make it more water-soluble and during this process molecular stability is usually reduced. Oxidation can transform some molecules from innocuous agents to highly reactive species that are lethal to cells if formed in sufficient quantity. In general, the less stable a compound is, the more likely it is going to react with cellular structures.

The CYP enzymes function in a similar way that machine tools do, where, say, a robot welds a piece of bodywork onto a car. The metal is subject to an intense, concentrated assault in a specific area that is designed to form a product. You can imagine what would happen if a live grenade was subject to this treatment. That would be the end of the robot. Obviously the robot is pre-programmed to weld anything of the appropriate dimensions that it is presented with, even something that could destroy it. You might feel that this is an 'Achilles heel' in drug metabolism; however, 1 billion years of research and development has indicated that there is really no other way to metabolize otherwise stable chemicals and for the occasional reactive by-product, there are sufficient repair and protection systems (GSTs, GSH, etc.). Just as you are fully insulated inside your car from the engine's noise and emissions, the efficiency of the whole biotransforming system is reflected in the relative rarity of organ damage in most therapeutic drug use.

On a molecular scale, some chemical structures that are subject to the rigours of CYP-mediated oxidation form reactive products because of unique inherent features of their structure. Examples include strained three-membered rings and epoxides. These structures are the chemical equivalent of the old explosive, nitroglycerine, which could be detonated

by shock alone. Some reactive species, such as the mechanism-based inhibitors discussed in Chapter 5 (section 5.3.5), can bind covalently to the active site and the enzyme is no longer functional. For biological function to continue, more enzymes must be synthesized. Drugs or chemicals that cause this effect are often termed suicide inhibitors and grapefruit juice and norfluoxetine cause this effect. The long period of inhibition of the CYPs that formed the metabolites reflects the time taken for more enzymes to be synthesized. These metabolites are so reactive that they are paradoxically no problem, as they just destroy the enzyme that formed them and cannot reach the rest of the cell. Indeed, after all the available CYP has been inactivated by these metabolites, no more metabolite will be formed until more CYP is synthesized (Chapter 5, section 5.3.5). The rest of the parent drug may well leave the cell by other pathways. At the other extreme are metabolites such as hydroxylamines, which are no immediate threat if they are stabilized by cellular thiols or antioxidants such as ascorbate. These metabolites are so stable they can travel through cells and leave the organ in which they were formed and enter the circulation. Erythrocytes can thus detoxify them as described previously. In certain conditions, they can spontaneously oxidize forming nitroso derivatives, which are tissue reactive and cytotoxic. Further metabolism is necessary before they can be detoxified. In between these extremes are metabolites that can react with any cell structures that are short of electrons and seek electron-rich structures. Potent electrophiles such as nitrenium ions (N^+) will react with nucleophiles, which are electron rich. If a CYP or any other enzyme forms a species that is missing electrons and seeks them, or is too electronegative, the net effect is a reactive species which has the potential to attack cellular proteins, DNA and membrane structures, forming covalent bonds, which can do sufficient damage to necessitate the resynthesis of that structure. It is important to consider also that reductive metabolism can form equally reactive species which are capable of causing similar cellular damage to oxidatively produced species. Whatever process forms reactive species, the likely result will be some form of irreversible binding to cellular macromolecules.

8.3.3 Cellular consequences of CYP-mediated covalent binding

The obvious question is how covalent binding is linked to cell damage; indeed, what are the processes that lead to cell damage? When a reactive species is formed in a cell, it can react with cellular organelles, enzymes, nuclear membranes, DNA, and the structure of the cell membrane. However, the hypothesis that a high rate of protein binding leads directly to cell death is an oversimplification. When animals have been treated with antioxidants prior to exposure to a reactive species, the animals survive, despite their levels of covalent protein binding, which are as high as untreated animals that have developed fatal organ toxicity. So the fate of the cell is subject to a competition between various factors:

- the rate, quantity and reactivity of the toxin formed;
- the extent of reactive ‘secondary’ toxins (superoxide, various free radicals) formed from the initial reactive species;
- the extent the cell can defend itself from the reactive toxin by rendering it harmless;

- the period of time elapsed before cellular defensive resources are overcome;
- which specific molecules are damaged in the cell where irreversible damage occurs;
- the extent of possible repair and restoration;
- whether the intra- and extracellular damage attracts the attention of the immune system.

These competing processes might lead to three main cellular outcomes; necrosis, where destructive forces overtake the cell, or apoptosis may be triggered, leading to an orderly dismantling of the cell and its contents, or the damage may be attenuated and repaired leading to survival.

8.3.4 Sites of biotransformational-mediated injury

Barrier tissues

It is usually the case that although biotransformational capability exists in all tissues to varying degrees, a specific xenobiotic will show metabolism-mediated toxicity in a particular organ, tissue or group of tissues. These are often 'barrier tissues' that represent the front line against exposure to environmental toxins; these include the lung, gut, liver and the skin. These tissues are very rich in detoxification enzyme systems and have at their disposal the full apparatus for the control and expression of these enzymes. Although these organs are well defended, reactive species generation can still cause irreversible damage, either locally or distant from the site of formation. This is partly due to the nature of our acute and chronic exposure to environmental and dietary xenobiotics that overtaxes the capability of our defences and partly due to our genetically differing capabilities to resist these toxins over our lifespans. The skin does possess considerable local biotransformational activity, but lacks the in-depth protection of high hepatic GSH levels and it is subject to many local torsional movements, which may influence tissue stress. The following sections give some examples of agents that can cause irreversible damage to a number of different cells, tissues and organs, although the liver features most prominently.

Hepatotoxicity

Ironically, although the liver boasts extremely comprehensive detoxification defences and astonishing powers of recovery, hepatotoxicity is one of the commonest reasons for the failure of a candidate drug in clinical trial and even the withdrawal of a new drug from the market. This is mainly because the liver contains the largest concentration of biotransformational enzymes in the body and bears the greatest burden in clearing drugs and other xenobiotics. This means that it is more likely to form reactive metabolites in quantities sufficient to cause cellular injury or to trigger an immune response. In addition, the central position of the liver in homeostasis makes severe hepatic injury a life-threatening event that may only be remedied by a transplant. Unlike toxicity in many other tissues,

hepatotoxicity has been studied extensively in detail over the last few decades and there are accurate diagnosis criteria for drug induced liver injury. These validated criteria are applied to existing or newer drugs by regulatory organizations. They are termed 'Hy's Rule' after their originator, the renowned hepatologist Dr Hyman J. Zimmerman. The rule states that if ALT levels equal or exceed three times the upper limit of normal (ULN) and serum bilirubin equal or exceed twice the ULN, then mortality can range from 10–50 per cent, depending on the drug and patient pathology. Hepatotoxicity may have many causes and be the result of different drug or dosage levels, but the commonest clinical manifestation of liver failure remains necrosis due to drugs, either by overdose or idiosyncratic causes.

8.4 Type B1 necrotic reactions

8.4.1 General causes of necrosis

This process describes the effect where a cell sustains a great deal of damage to key cellular systems in a relatively short time (rather like the end of a James Bond film) that it cannot either protect itself from the toxic species or repair its systems fast enough to keep pace with the damage. In extreme cases, the cells may die before they can even initiate apoptotic processes. The main cause of this effect is if a drug promotes cellular oxidative stress. This is usually defined as marked imbalance between reactive species production and detoxification. It is more likely that reactive drug metabolites, rather than the parent drug, may cause oxidative stress by themselves as well as by promoting REDOX cycles which generate more reactive oxygen species as well as a host of destructive free radicals, which in turn accelerate GSH consumption, consuming reducing power and ATP. Reactive species may derange other cellular processes, such as fatty acid metabolism and may also react with membrane lipids; this effect triggers a cascade where the lipids will generate their own radicals as they oxidize each other in a chain reaction. This process is like a 'forest-fire' effect on the membrane lipids and the structure of the membrane will eventually break down, causing cell contents to escape. Although cells such as hepatocytes are extremely robust, if the reactive species are produced in very large quantities over a short timescale, such as after an overdose, then the drug is oxidized in sufficient quantity to form enough toxic species to overwhelm even hepatic cellular defences, such as with paracetamol. Other drugs may promote a more gradual but sustained oxidative stress that could be tolerated for some time, but may eventually defeat cell detoxification, but at lower dose levels over a longer period of time. In both cases, recent research has concentrated on the emerging and critical role of mitochondria in drug-induced organ toxicity.

8.4.2 Mitochondria and drug toxicity

As you should know from basic biochemistry, mitochondria provide virtually all of our energy and whilst a mononuclear leucocyte might only possess a few of these organelles, cells requiring high energy output, such as in the heart or the skeletal muscle have thousands of them and if more demands are made on the cell, more mitochondria duly appear. They have a smooth outer membrane that is permeable to nutrients and many other

substances and a complex folded inner membrane, which is impermeable and anything that enters must have a specific transporter. This means that they have a negative membrane potential with respect to the rest of the cell, which is essential for them to make large quantities of ATP from the oxidation of our food components. In some high-energy output cells, they arrange themselves like large banks of batteries, rather like those in submarines. They have their own DNA with the means to express and control it and they don't even replicate at the same time the cell does. In essence, they look suspiciously like they may have been separate cells at one time and it is now believed they were once indeed free-living bacteria. Along with our CYPs and other biotransformational enzymes, we have stolen them and their technology lock, stock and barrel to power ourselves. Mitochondrial function can be upset by any event that disrupts the inner membrane potential, which leads to a fall in ATP output and various apoptotic factors can be released which promote cell death. Reactive oxygen species, various radicals and the oxidative stresses they encourage are some of the main causes of the failure of inner membrane potential. These species may also derange electron transport and inhibit mitochondrial replication, as well as threaten the protein structure of the organelles. It is likely that mitochondrial damage is a key feature of drug-induced cell death through a number of processes besides necrotic damage. Interestingly, it was only the widespread toxicity of the first anti-HIV drugs, such as AZT that highlighted drug-induced toxicity of mitochondrial function, as these agents inhibited the organelles' DNA polymerases so preventing them from replicating.

8.4.3 Paracetamol (acetaminophen)

Although a number of compounds can cause liver necrosis and death in overdose, none of them are extremely cheap and available in virtually every retail outlet in the country. Every year in the UK, we get through around 1700 tonnes of paracetamol in various formulations and approximately 70,000 people overdose on this drug. The drug still leads to around 130 deaths per year from liver failure in the UK. Paracetamol is the first choice for those who wish to use an overdose to end their lives, although paradoxically, it's the safest way to commit suicide. Less than 0.15 per cent of paracetamol overdoses result in death. It seems that intentional overdoses are less lethal than unintentional ones (very rare) and children are less susceptible to fatal overdose than adults. The public is obviously aware that paracetamol can kill, hence the numbers taking it in overdose; however, despite more than 30 years of publicity, few are aware of the drawn-out and painful process that can lead to death with this drug. It is still used in suicide attempts because it works eventually (if the patient does not find medical help) and it is freely available. It is possible that those who take overdoses feel that paracetamol is an ideal 'cry for help' drug, where there is time to reconsider and this behaviour is associated with the aged 16–24 group, who are most likely to overdose. Ironically, you are much more likely to die of a paracetamol-related overdose if you are 40 and above. This is due to factors such as delays in seeking medical help and liver damage due to alcohol.

If the patient presents, or is carried to treatment, the rescue therapy usually works, but if it fails it is often believed that there is always the fallback position of a new liver. However, it is worth considering that less than one-third of patients with hepatic failure fulfill the clinical criteria for a transplant. Even in the light of restricted paracetamol pack sizes, which have made some impact in death rates, it remains incomprehensible

to many health professionals that paracetamol is still available at all. Suicide is usually a result of the 'balance of the mind being disturbed', so it does not make sense to provide a readily usable method that can be obtained by the simple expedient to a trip to three or four convenience stores. It is interesting that a number of much more therapeutically valuable drugs have been withdrawn from the market after accounting for a fraction of the deaths attributable to paracetamol.

Mechanism of toxicity

In the kidney, paracetamol can be deacetylated to 4-aminophenol, which is a nephrotoxin. In overdose, nephrotoxicity is much less pressing a problem than the effects in the liver. Around 95 per cent of paracetamol is cleared hepatically to an O-glucuronide and sulphate conjugates and about 2 per cent escapes unchanged (Figure 8.5). A small fraction, maybe between 1–3 per cent, is oxidized mainly by CYP2E1 (with a fraction cleared by 1A2 and 3A4) to the exhaustively investigated reactive species N-acetyl-*para*-benzoquinoneimine (NAPQI). Recent evidence suggests that CYP2D6 is also involved, which raises the question of the link between severity of liver toxicity and the polymorphic expression of this CYP (see Chapter 7, section 7.2.3). There are a number of other putative reactive species, such as dibenzoquinoneimines, but it is thought that NAPQI is the toxicologically important species. This compound is known to bind covalently to hepatocytes in various cellular areas, such as the cell membrane. Normally, however, there is negligible binding as the NAPQI is efficiently conjugated to GSH. It was believed that GST Pi was responsible for catalyzing this reaction, although it may be a combination of direct reaction with GSH and catalysis through other antioxidant enzymes. The net result is the excretion of NAPQI mercapturate in urine in small amounts. In its therapeutic dosage range, it must be stressed that paracetamol is harmless. The most a 70 kg adult can take a day without problem is about 4 g. For children, this is adjusted to 90–100 mg/kg in children, depending on which authority is cited. Most agree that no child should receive more than 4 g/day in total.

Overdose of paracetamol is considered to be around 7–8 g (100–115 mg/kg) in an adult and more than 150 mg/kg in children. In overdose, the same profile of paracetamol metabolism is maintained. The proportion of the dose cleared to NAPQI in overdose does not change much either, however the key here is that proportionally more NAPQI will be formed during overdose compared with a recommended dose. Obviously, the demand for GSH will be high to detoxify the reactive species, and you might recall from an earlier chapter that GSH is effectively 'thermostatically' controlled, in that if more is used, more will automatically be made to maintain organ concentrations at their normal preset level (Chapter 6, section 6.4). Accelerated GSH consumption leads to an increase in the recycling of GSSG to GSH and the synthesis of more GSH from cysteine, glycine and glutamate. Up until 10–16 hours after the overdose, the GSH system responds to the increased load and GSH levels are maintained through recycling and resynthesis. The one component of GSH manufacture that is in limited supply in a hepatocyte is unfortunately the key one, the thiol-containing cysteine. More can be transported in and another sulphur-containing amino acid, methionine, can be converted to cysteine to meet the demand. Between 8 and 16 hours or so, GSH consumption by NAPQI gradually exceeds demand and cellular supplies of cysteine are exhausted. Since GSH is acting as the cellular 'fire-extinguisher'

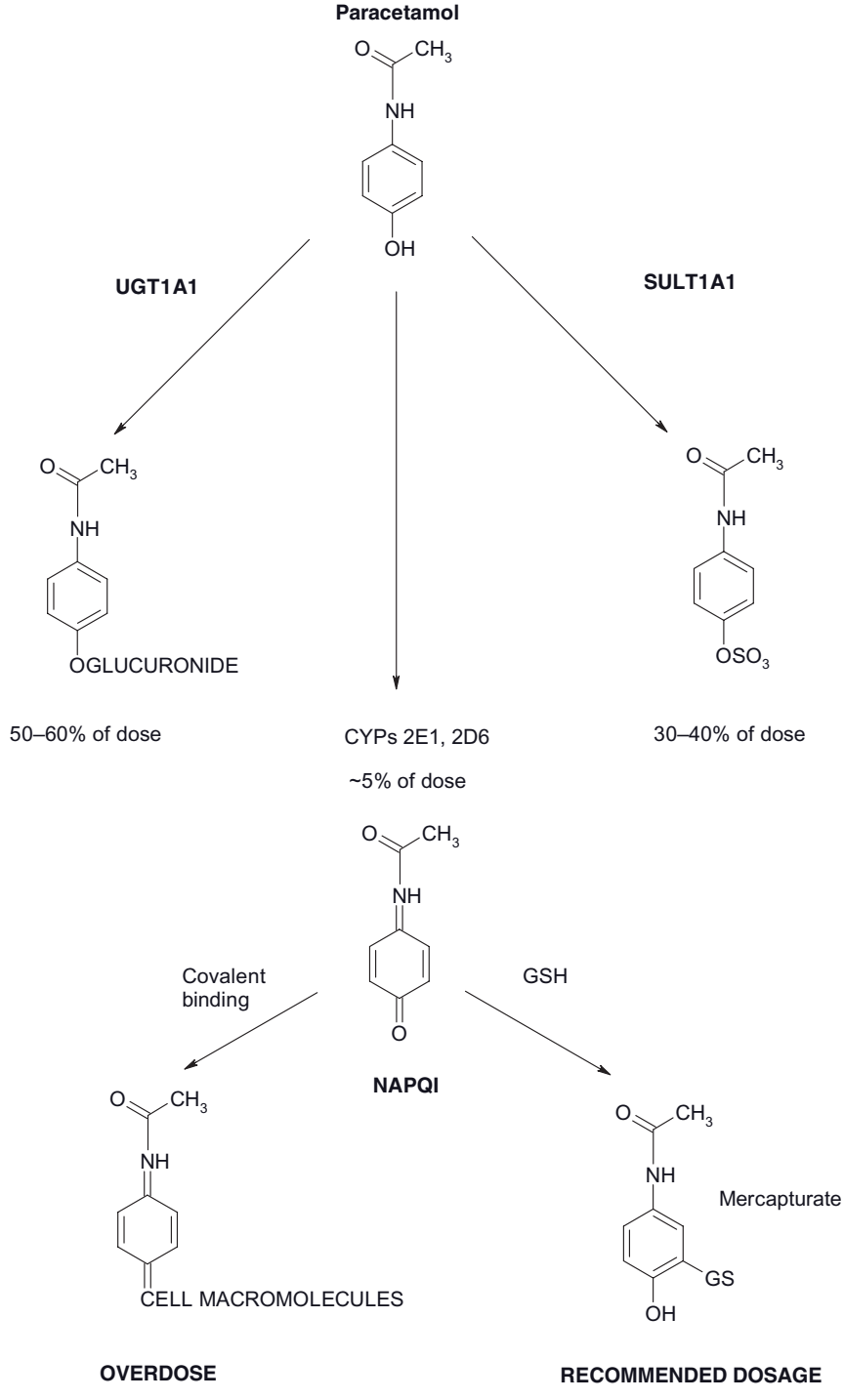

Figure 8.5 Main features of paracetamol metabolism. Aside from the toxic NAPQI (N-acetyl *p*-benzoquinoneimine), a 3-hydroxy derivative is cleared harmlessly by GSH to a catechol derivative. It is possible other reactive species are formed as well as NAPQI

Table 8.1 Time frame and clinical markers of paracetamol toxicity

Post-overdose Time (h)	Hepatic GSH (%)	Transaminases (units/litre)	Symptoms
6	100	Normal range	None
16–24	40–70	300–500	Anorexia, nausea/vomiting
24–48	10% or less	>1000–1500	Right upper quadrant pain, tenderness
48–72	Zero	>3000	Jaundice, bleeding, organ failure/death

in this acute context, this is the cellular equivalent of running out of foam in the face of steadily encroaching flames. After 24 hours or so, there is nothing to prevent the NAPQI from binding to the hepatocytes and gradually killing them through necrosis. The key to the toxicity of NAPQI is which cellular macromolecules it binds. It is still not really clear (after 30 years of research) as to precisely which structures must be heavily bound before the cell necroses. Eventually, such numbers of hepatocytes are killed that the central area of the liver starts to die also. It is termed *'Acute centrilobular hepatic necrosis'*. This translates clinically to a series of symptoms shown in Table 8.1.

Each of the three phases of paracetamol hepatotoxicity is reckoned to last around 24 hours. The first phase, up to 24 hours after the overdose, involves nausea and vomiting and general reluctance to eat. Interestingly, some individuals do not show any symptoms at all during this time. Many do complain of a general 'sick' feeling and start to look very pale and 'washed out'. At this stage, the individual might be lulled into a false sense of security and might not think that he or she is in any real danger. In addition, many overdoses are accompanied by copious quantities of ethanol in various forms and the devastating hangover that ensues can effectively mask the hepatic paracetamol-induced symptoms. It is also likely that in moderate drinkers, the ethanol may compete with the drug for CYP2E1, thus delaying NAPQI formation. The second phase, 24–48 hours, involves the appearance of some physical pain in the area of the liver. Heart rate can rise significantly and blood pressure fall. At this point, the liver is sustaining serious cellular damage and liver enzymes such as ALT and AST (Chapter 6, section 6.5.3) can be 30–50 times normal and this is an obvious sign of many disintegrating hepatocytes. In the third phase, the liver damage starts to become acute, although in less than 1 in 20 patients will this lead to organ failure. Symptoms of severe liver damage include jaundice, gut bleeding, general anticoagulation due to failure in clotting factor production and even encephalopathy. If organ failure does occur, then death follows within hours from cerebral oedema, renal failure and even blood poisoning.

Rescue therapy

Clearly the chances of survival depend on whether the patient undergoes treatment before too much liver damage occurs. If the individual does not seek medical help, then it is a matter of how much of the drug they consumed and their own liver's resistance to damage, as well as luck, as to whether they will survive. Habitual ethanol consumption simultaneously weakens the liver's resistance to paracetamol toxicity and the induced CYP2E1 forms much more NAPQI (Chapter 7, section 7.7.5) If the patient presents for treatment

in less than an hour or so after the overdose, then it is likely to be worthwhile flushing out the stomach, as the entire drug dose will not be fully absorbed. If the patient appears within 3–4 hours of the overdose, then activated charcoal is sometimes given, which is controversial as to how effective it is and it may interfere with subsequent antidotes. Upon admission to hospital, a blood sample will be taken and paracetamol levels measured. This sample is most informative as soon as possible 4 hours post overdose, as earlier samples are not relevant as drug absorption is still proceeding. Using a 'Rumack–Matthew nomogram', the amount of hepatotoxicity that might occur is read off from the drug level. Hepatotoxicity is usually seen when paracetamol levels reach and exceed 1–2 mM. The R–M nomogram is a product of data derived from hundreds of previous patients, so it is fairly accurate in its prediction of liver damage, but not necessarily of liver failure. If the levels are above the upper line, then there is a greater than 60 per cent chance of liver damage being sustained. Successive blood samples track the elimination of the drug and as treatment progresses, should show the patient gradually moving out of the hepatotoxicity 'danger zone'. The nomogram tracking starts 4 hours after the overdose and ends around 24 hours. The patient's INR, creatinine and ALT levels are checked before discharge to ensure hepatic recovery is proceeding.

In any case, a confirmed overdose will be treated with N-acetyl cysteine, which acts to supply cysteine to GSH synthesis, so that hepatic GSH levels are maintained until all the paracetamol has been eliminated. This is not as straightforward as it sounds, as orally, NAC tastes truly disgusting and the smell can make patients vomit, so it can only be given as a 5 per cent solution in a fruit juice. The effects of NAC and the aftermath of a serious hangover from alcohol used to wash down the overdose must be exceedingly miserable to the patient. If the patient vomits within one hour of NAC administration, then that one 'doesn't count' and must be repeated. The standard treatment involves oral NAC every 4 hours for three days after the overdose. As ever, it is imperative that the whole course is followed, even if paracetamol levels fall below toxic levels. Even though the stuff tastes so bad that antiemetics are sometimes given for people to keep it down, it is preferable to the intravenous route. Orally, the side effects include headache, diarrhoea, skin flushing and can lower blood pressure in a small proportion of patients. This is typical of the administration of any thiol and happens with radioprotectant drugs also. Intravenously, in less than 10 per cent of cases, anaphylactic shock can occur, with severe hypotension and bronchospasm, so do not try this at home. There is some confusion around the point that NAC is effective and when it is less effective, but current studies have drawn some main conclusions:

- If an individual presents for treatment at 8 hours or less after the overdose, then no matter how much paracetamol he or she has taken, no hepatotoxicity will be sustained.
- It is of particular importance to initiate the NAC therapy in pregnant women, as the NAPQI is very toxic to the foetus.
- If the NAC is started at 0–4 hours, there is no difference to the outcome; so early NAC treatment offers no advantage over later treatment up to the 8 hours 'cut off'.
- NAC is still effective up to 24 hours post-overdose, even though some hepatoxicity will have been sustained and can even be effective after this time, if paracetamol is still in the plasma and even if the liver is already damaged.

Methods for prevention of overdose

You might think that incorporation of a thiol with the paracetamol would solve the overdose problem instantly. It was first thought of in the early 1970s, soon after the mechanism of the drug's toxicity was discovered in the US. Unfortunately, nobody has ever determined the correct proportion of the thiol required to prevent liver damage in humans, nor are they likely to for ethical reasons. So to err on the side of caution, the only available product in the UK (Paradote) contains methionine in a 1 : 5 ratio with paracetamol. It also means that everyone who takes this preparation would be ingesting methionine and there are some long-term concerns about its possible toxicity, as well as some mild side effects, such as drowsiness, flatulence and headache. Unless every paracetamol preparation (and there are hundreds of them) contains methionine, then the idea is unlikely to prevent lethal overdoses, due to the vast number of preparations that contain paracetamol without an antidote. The antidote/drug preparations are obviously much more expensive than plain paracetamol, which also means that they are unlikely to be commercially viable in the long run in the face of hundreds of cheap competitors.

Paracetamol use in children

Opinion is divided as to the value of paracetamol for children in treating fevers and mild discomfort. Certainly in children under three years old, there can be considerable reliance by parents on expensive liquid paediatric paracetamol preparations to counteract the often painful effects of various inoculations in babies and toddlers. In hospitals, paracetamol is often used for what is termed minor discomfort in mild infections and for post-operative pain, so reducing the opiate consumption. It is not always easy to demonstrate its effectiveness and children can easily be given repeated doses that exceed the 4 g/day limit. Although in general children are more resistant than adults to paracetamol toxicity, inadvertent overdosage in febrile children can lead to elevated transaminase enzyme levels and liver toxicity. This can occur where the doses of paracetamol have not been properly recorded, leading to hepatotoxicity from apparently low doses of the drug. Chronic paracetamol toxicity in febrile children is more likely to happen if they are less than three years old and are not eating or drinking enough fluid. Paracetamol is useful in cases of febrile children in heart and respiratory failure, but has little value in those who do not have heart or lung problems, as the fever can be beneficial. This is because viruses and bacteria have specific temperatures where they multiply most efficiently and the febrile response ensures that the body temperature is higher than the infectious agents' optimum temperature. This is borne out by observations that children do less well in cases of mild and severe infection when dosed with paracetamol. If it is used, then it is strongly recommended by a number of sources that paracetamol dosage should not exceed 90–100 mg/day in children with the risk factors described above.

Paracetamol: conclusions

This drug will always be a best seller, despite its toxicity in overdose. This is partly due to the toxicity of alternative drugs, such as non-steroidal anti-inflammatories, which cause

severe gastric bleeding in overdose. There is no safe, mass marketed mild analgesic to replace paracetamol or aspirin, so it is important to be aware of the dangers of this drug and how it can lead to the destruction of the most resilient and best protected organ in the human body. Paracetamol's mode of toxicity is so obvious that if it had been submitted for testing in drug development systems over the last 30–40 years it would have been shown up as hepatotoxic and quietly dumped, alongside thousands of other failed agents. Interestingly, co-proxamol (325 mg paracetamol, 32.5 mg dextropropoxyphene) was withdrawn in the UK in early 2005. You might think that currently it would be almost impossible for a new drug to reach the mass market and cause necrotic hepatic damage, either from normal dosage of overdose.

8.4.4 Tacrine

You might think this, but you would be wrong. Although it is not unexpected that drug overdose might cause organ failure, it is generally much more difficult to explain how a drug might cause serious organ damage when it is taken at the recommended dosage by a comparatively high proportion of previously healthy individuals. The anticholinesterase and anti-Alzheimer's disease drug tacrine (1,2,3,4-tetrahydro-9-aminoacridine; THA) underwent the normal battery of animal test systems (rodents and dogs) in its development and was not found to be toxic. In clinical trials it was found to be a potent dose-dependent hepatotoxin in humans and has since been found to be mutagenic in the Ames test (see Appendix A). However, development went ahead and the drug is currently part of the treatment of Alzheimer's disease and it is still not clear how it causes hepatotoxicity. The reason this drug was not abandoned is similar to the experience with other toxic drugs, such as the early anti-HIV agents. These drugs at the time filled a niche that was not being occupied by any other effective drug and there was a pressing therapeutic need to have something to treat patients, until a really effective agent appeared. Indeed, in the developed world, there may be as many as 12–14 million Alzheimer's sufferers. So in the face of such a huge and increasing therapeutic problem, the considerable expense of monitoring the drug's hepatotoxicity must be borne. The anti-Parkinsonian drug tolcapone was treated similarly. When liver enzymes exceed a value of about twice the normal range, the patient must cease therapy until the levels subside. Although tacrine was designed only to treat the symptoms of a reduction in CNS cholinergic transmission (by prolonging acetylcholine action) it has since emerged that it does have a number of other beneficial effects in Alzheimer's disease.

Tacrine toxicity

Initially it was discovered that between 30 and 50 per cent of patients suffered from elevated ALT and AST levels after 6–8 weeks on tacrine, which was reversible and known as tacrine transaminitis. If the drug is continued, it is capable of inducing fatal midzonal and pericentral hepatic necrosis. *In vitro* studies have shown that tacrine depletes GSH and it was logical to suggest that a reactive CYP-mediated species may have been responsible. In man, tacrine is cleared to a number of CYP1A2-mediated hydroxylated metabolites (1–4 hydroxy derivatives) that then undergo glucuronidation, although its metabolic

fate has not been completely identified. The parent drug or its metabolites are capable of inhibiting the clearance of CYP1A2-cleared drugs such as theophylline. From a toxicological standpoint, the process of tacrine oxidative metabolism appears to be a 'red herring', as it was quickly established that liver injury is not related to metabolite levels of the drug, rather to blood parent drug levels. It seems that tacrine is toxic through several pathways, none of which are directly related to its biotransformation. Several studies in animals have tried to determine the route of tacrine-mediated necrosis, although this is somewhat ironic when you consider that studies in animals failed to predict the toxicity in the first place. However, work has also proceeded using human hepatic cell lines (HepG2s, etc.) as well as animal models, so some of the hypotheses on tacrine toxicity may well be as close to the human situation as we are likely to get for some time. It is apparent that there are several potential routes to hepatotoxicity.

Tacrine causes an increase in reactive oxygen species formation, which can be ameliorated by the use of antioxidants such as vitamin E. Tacrine also causes hypoxia: the autonomic sympathetic nervous system controls liver microcirculation, so an increase in sympathetic output should shut down liver blood flow and cause hypoxia leading to necrosis. If you remember your basic autonomic pharmacology (adopt blank, slightly shocked expression at this point) then you will know that the sympathetic nerves all output noradrenaline, but they are different from the somatic (voluntary) nerves that are all one unit. Autonomic nerves have junctions (ganglia) that are cholinergic. Tacrine promotes cholinergic effects by preventing the destruction of acetylcholine, thus stimulating the cholinergic ganglia that in turn increase noradrenaline release from the sympathetic nerves, so shutting off liver circulation. This also causes hypoxic reperfusion injury when circulation is resumed, as neutrophils and other immune systems are activated and cause tissue destruction, as seen in coronary thromboses. The tissue destruction is through reactive species released by the activated neutrophils, which will deplete GSH, as described above. In effect, this starts as a reversible adverse effect, which gradually turns into toxicity and is caused by the intended pharmacological mechanism of the drug.

Tacrine also increases hepatic membrane fluidity and damages various membrane-bound proteins and transport systems, leading to hepatic disruption. The drug can derange mitochondrial function as well as mitochondrial DNA replication. This is thought to be due to its ability to damage topoisomerase enzymes and disrupt DNA synthesis. This in turn triggers apoptosis in hepatocytes. An association with IL-6 expression haplotype and susceptibility to tacrine transaminitis has also been suggested.

All these mechanisms are plausible, if not fully explained, and it is likely that future research may be more illuminating. Certainly the liver's unique susceptibility to tacrine toxicity is currently not clearly understood. Overall, this drug demonstrates:

- The vulnerability of the drug development system to 'stealth hepatotoxins', which do not affect animals in the standard context of a toxicity test.

- The likelihood that a deleterious pharmacological effect of a drug may be organ-specific only in humans.

- That even a manifestly toxic drug can be therapeutically valuable if sufficient investment in clinical monitoring is made to prevent the transition from reversible to irreversible toxicity.

8.4.5 Troglitazone

This drug demonstrates one of the worst nightmares of both the drug regulatory system and pharmaceutical companies; a drug that is the 'flagship' of a very promising class of agents intended to treat a relatively common condition that also induces a relatively rare, unpredictable but aggressive hepatic failure. This effect was apparently unconnected to any of its pharmacological actions and did not seem to be linked to the immune system. Troglitazone was the first of three new thiazolidinedione insulin sensitizers that were originally developed by a Japanese drug company, for the treatment of the massive problem of Type 2 diabetes, a condition that is linked strongly to obesity. The drug was interesting because it had new mechanisms of action, one of which was the stimulation of glucose uptake by skeletal muscle. The drug achieved this effect through activating PPARγ and it also had many other beneficial anti-inflammatory actions, particularly in conditions related to Type 2 diabetes such as NASH. Soon after it was introduced, reports were received of several fatal incidents involving hepatotoxicity in Japan and the USA. There were at least 35 deaths after approximately two million patients received the drug worldwide for just three months in late 1997. The FDA recommended that the drug should be monitored for hepatoxicity, but further clinical studies showed that the drug was causing threefold higher AST and ALT levels than normal in nearly 2 per cent of those taking it, suggesting that there was a danger of significant hepatoxicity with the drug. Toxicity could occur within two days of starting therapy, although liver failure could occur up to three years after the drug was discontinued. In 2000, the drug was withdrawn worldwide. To give some idea of the scale of this problem, between 1970 and 2004, only paracetamol killed more people through drug-induced liver failure than troglitazone.

Unlike tacrine, troglitazone did not 'survive' its toxicity despite its usefulness. This was partly due to the availability of other effective anti-Type 2 diabetic agents, but it was also linked to the arrival of two sister compounds, rosiglitazone and pioglitazone, which were initially believed not to be subject to hepatotoxicity risks, due to structural differences. It is important to note that the tolerance of regulatory authorities to drug toxicity is strongly influenced by the intended disease target and the alternatives available. Hence the hideous toxicity profiles of anti-cancer agents can be accepted, whilst much less damaging and rarer effects in other drugs for non-life-threatening conditions where there are also many alternatives lead to withdrawal.

Troglitazone's toxicity has been investigated (as with tacrine) in the very models that did not alert the drug company to its problems, that is, animal systems. Studies with human hepatocytes, cell lines and clinical data have indicated that the drug is almost entirely cleared to a 6-O-sulphate. Rosiglitazone forms an *N*-desmethyl-*para*-O-sulfate as well as a *para*-O-sulfate. Over 70 per cent of troglitazone is sulphated by SULT1A3 (a phenol sulphotransferase) and to a lesser extent, SULT1E4, an oestrogen sulphation isoform. Around 10 per cent is cleared to a quinone and the rest is glucuronidated with only a few per cent excreted in urine unchanged. The main route 'out' for the sulphate is in faeces, which suggests that the bile is the major excretion route of troglitazone. Interestingly, rosiglitazone is cleared almost entirely in urine. In animal studies, those species that do not have the same high sulphation capacity as humans tend to eliminate the drug as glucuronides. This provides some clues as to why it is toxic in humans and not animals, although the mechanism is only gradually becoming apparent. The next section describes what is currently known from a variety of models, human and animal.

Troglitazone mechanisms of toxicity

Troglitazone is metabolized by several CYPs, as human hepatocyte studies have shown that CYP3A4 inhibitors do not abolish its cytotoxicity. Not surprisingly, these studies have also demonstrated that the drug is much more toxic in human than rat hepatocytes and it causes oxidative stress in the cells. How it does this is not entirely clear. There is evidence that the parent drug is responsible, but also reactive quinine species are formed by CYP3A4. In addition, it is possible for the thiazolidinedione ring to be cleaved, forming reactive alpha-ketoisocyanate or sulfenic acid derivatives. It is not known whether these GSH-reactive species would be formed *in vivo*, but the drug is a known inducer of several CYPs (3A4 and the 2C series), P-gp, MRP-2 (acting through PXR) and its reactive oxidative metabolites probably activate Nrf2, so it is certainly possible.

The drug orchestrates hepatocyte oxidative stress almost certainly through derangement of mitochondria, which seem to be the main sub-cellular target of troglitazone's toxicity. It has been suggested that troglitazone and its sister agents can disrupt mitochondrial energy formation and metabolism, causing reductions in ATP and increases in reactive species formation. This leads to damage to the organelles' ultrastructure that is accompanied by a fall in membrane potential. It was also apparent that any pre-existing hepatic insufficiency or fatty liver rendered patients much more susceptible to troglitazone hepatic failure. Unfortunately, Type II diabetics, the target population for this drug, have a high incidence of such liver pathology, as well as significant CYP2E1 induction that increases oxidative stress by itself. As if this were not enough, the cholestasis seen in patients was followed up with rat studies. These showed that troglitazone can cause a potent reduction in bile salt transport by inhibiting the various transporter efflux pumps. Other cellular and animal studies show that toxicity seems to correlate strongly with parent drug concentrations and troglitazone itself appears to be able to curtail not only liver protein synthesis, but also its own sulphation in human hepatocytes. Regulatory processes often record liver failure linked with a drug, but not necessarily permanent and debilitating liver injury. Troglitazone is toxic through several pathways and it is likely that many thousands more patients than actually reported suffered permanent hepatic dysfunction.

Predictably, it has been a priority in clinical and basic research over the last decade or so to determine whether this whole class of highly successful drugs is innately toxic, or whether it is just troglitazone alone. The clinical evidence shows that rosiglitazone and pioglitazone can cause hepatic dysfunction, probably through similar pathways to troglitazone, although this is rare. Several factors may make these drugs much safer than troglitazone. Pioglitazone and rosiglitazone are used at much lower doses and they don't cause cholestasis or appreciable oxidative stress at these doses. Experts in the field nevertheless suggest that healthcare professionals should be aware of the propensity of the thiazolidinediones to cause hepatic dysfunction in vulnerable patients, particularly obese and hepatically compromised individuals.

Although most of the focus on the investigation of troglitazone's toxicity was on reactive species generation and its relationship with oxidative stress, some preliminary DNA microarray studies in human hepatocytes revealed that the drug also had a strong impact on several hundred 'tox' genes. These genes, which coded for a wide range of genes associated with metabolic control and response to stress, were noticeably not affected by pioglitazone or rosiglitazone. The role of microarrays in revealing the impact of a hepatotoxin on gene expression has been explored in more depth in another idiosyncratic cause of liver failure, trovafloxacin.

8.4.6 Trovafloxacin

In contrast to troglitazone, trovafloxacin was developed as part of a long and established line of safe fluoroquinolone antibiotics, such as ciprofloxacin, ofloxacin and norfloxacin, which have become firmly established in infection control. These drugs are intended to be selective for bacterial DNA gyrase and topoisomerase IV. Trovafloxacin had a broader spectrum of activity and a better pharmacokinetic profile than the older drugs and was introduced in 1997 after trials involving more than 7000 patients. By the time it was withdrawn in 1999, more than two million people had used it and it had caused 150 cases of liver toxicity. Of these, 14 individuals went into liver failure and 4 received transplants, whilst 5 died. The drug is available now in the US only *in extremis*. Interestingly, several other new fluoroquinolones, such as grepafloxacin and temofloxacin were also toxic and also had to go. Again, preclinical studies revealed no problems in the rat and initial suspicions were based on the possibility that the drug was metabolized to reactive species that caused oxidative stress. The exact species has not been conclusively identified to date, but it has been suggested that the cyclopropylamine area of the molecule could be responsible for the drug's toxicity, as various chemical and enzyme systems have been shown experimentally to oxidize it to a reactive α,β-unsaturated aldehyde. It has been suggested that this can be achieved by CYPs or myeloperoxidase enzymes, but is yet to be proved. However, much more information has been discovered about the molecule from studies with HepG2 cell lines and human hepatocytes. Unlike its sister fluoroquinolones, trovafloxacin is a potent generator of oxidative stress at relatively low concentrations in human cell systems, causing production of reactive oxygen species and depleting cellular GSH-indeed, its depleting effect is more than ten-fold that of ciprofloxacin and nearly 15-fold greater than that of levofloxacin. DNA microarray studies in human hepatocytes were particularly damning, showing more than a thousand gene expression changes, including off-target pharmacodynamic effects which showed that trovafloxacin's DNA gyrase and topoisomerase inhibitions are not actually selective for bacteria at all. The arrays also revealed widespread disruption of mitochondrial and signal transduction genes, as well as fatty acid and glucose metabolism. The main problems appeared to centre on mitochondrial function (loss of membrane potential and damage to electron transport), whilst GSTs and other Nrf2-controlled oxidative stress response systems were also activated. Perhaps the most profound problem was that trovafloxacin severely impairs HNF4α expression; you might remember from Chapter 4 that this factor governs liver function at virtually every level. As the drug causes so much oxidative stress, it is probable that its major toxic effect is caused by reactive species, although the role of the parent drug cannot be ruled out in the gene suppression effects, on the grounds that the reactive species might be too unstable to reach the nucleus. The discovery of the human locus of trovafloxacin's toxicity with the combination of arrays and human hepatocytes should mean that no drug that causes this scale of interaction with human DNA should ever reach the market again (see Appendix A).

8.4.7 Summary of Type B1 necrotic reactions

- Fatal organ-directed toxicity can occur from a relatively modest overdose.
- Organ death can occur from the recommended dose.

- The organ/tissue affected is often not related to the pharmacological target of the drug.
- The toxicity may be linked with both off-target pharmacodynamics, oxidative stress and mitochondrial damage.
- Predisposition may be greater in the patients the drug is intended to treat.
- Onset may take days or weeks.

8.5 Type B2 reactions: immunotoxicity

8.5.1 The immune system: overview

Most people are aware that one of the basic functions of the immune system is the detection and destruction of bacteria, viruses and infected cells. The system in humans has evolved to protect us from relatively minor incursions of infectious agents as well as rapid and potentially overwhelming invasions, although often the immune system appears to operate on the 'better massively destructive than sorry' principle. Its responses are designed to eliminate all trace of the infectious agent, no matter how much 'collateral damage' may occur. This trait has been ruthlessly selected for by successive waves of pandemics, such as the 'Black Death' of the Middle Ages, which killed 40 per cent of the European population. In the early twentieth century, influenza caused several times more deaths than the entire Great War. If you also consider the range of potentially infectious agents that we can encounter, you can see why the immune system's evolution has encouraged flexibility, sensitivity and persistence. Foreign invaders appear in all shapes and sizes, combining incredible ingenuity and aggression with sheer weight of numbers. For example, intracellular pathogens are extremely adept at escaping and even manipulating the immune system and some of the most successful viruses, protozoa and mycobacteria can actually thrive within immune cells. At the other extreme, schistosomes blatantly sit unmolested in the portal circulation causing havoc for 30 years or more. These particularly loathsome trematodal worms can be an *inch* long. Similarly, the filarial worms of onchocerciasis (river blindness) can also exist untroubled by the immune system as they too have a 'cloaking device' that is torn away by a drug called diethylcarbamazine. The resultant immune activity as the worms die is so intense that it can be fatal and this drug has been replaced by ivermectin. So perhaps we should forgive the human immune system some measure of paranoia, as everything 'out there' really is out to get us.

Given that infections can and do kill in hours rather than days, it is essential to find and control them now and thus be around to repair the damage at a later date. This is similar to the expectation that cancer patients will withstand severe toxicity during therapy, on the basis that survival should be attained at any cost. With the immune system, this means in practice that it is capable of destroying cells, tissues and whole organs, which can inadvertently lead to death of the organism. The immune system can be awesomely destructive and it must be reined in by a strict gradation of response to avoid huge overreaction to minor amounts of infectious agent. If you look at just one component of the immune system, a single activated neutrophil can generate enough oxidizing agents to kill 160 bacteria in one second. Lesions seen in diet-induced autoimmune diseases like dermatitis herpetiformis show what several thousand activated neutrophils can do to non-infected tissue.

8.5.2 Context of drug hypersensitivity

Whilst most of us respond in a predictable way to a cold or 'flu, perhaps only one individual in several thousand will suffer severe hypersensitivity to a drug. These situations are feared because they are rare and often exceptionally severe, causing graphic tissue destruction accompanied by high fatality rates. Stevens–Johnson syndrome can lead to toxic epidermal necrolysis (TEN), where the patient's entire skin is attacked and virtually destroyed. Anticonvulsant hypersensitivity syndrome can cause severe, widespread tissue and organ damage. Other reactions, such as agranulocytosis, are more specific and lead to the complete shutdown of neutrophil production and death from sepsis. Doctors treating patients suffering from these reactions are often faced with extremely limited treatment options and a rapidly deteriorating patient. In addition, withdrawal of the causative drug does not always lead to amelioration of disease progression. Obviously the immune system should not respond in this way, but predicting whether a new drug might cause a catastrophic immune reaction in a small number of patients is still a long way off. A serious drug-induced hypersensitivity reaction might affect maybe 1 : 5000, or even 1 : 10000 patients, so it is not realistic to organize clinical trials with numbers high enough to hope that these reactions can be detected. Great progress has been made in the understanding of the immune system's normal operations, but we still do not fully understand how the system operates and how drugs become immunogenic is still controversial. There are conflicting theories and views as to how some immune reactions occur and it is possible to challenge many of these ideas, as they are still under development. This is mainly due to a lack of useful animal and *in vitro* models and difficulties in studying the reactions in patients, due to their understandable reluctance to be rechallenged by the drug that might have recently nearly killed them. However, modern cloning techniques have facilitated the laboratory study of many populations of immune cells and our understanding of how the system regulates itself is increasing rapidly.

8.5.3 The immune system: aims and normal operation

Before we can study how drugs can cause immune-mediated toxicity, it is important to review the main aims and functions of our immune system's activities:

- detection of non-self, or foreign material at a specific site, or sites;
- initiation of an appropriate response which will lead to the destruction of that material;
- retention of the 'memory' of that material.

Tolerance and autoimmunity

If the immune system is viewed mainly in terms of its ability to destroy pathogens, then you might think that it is engaged in a constant battle to detect 'non-self', such as invading organisms. The system does respond strongly to certain structural features of these organisms, such as bacterial lipopolysaccharide (LPS), which are termed 'adjuvants'. The

system detects these 'signature' molecules of infection that are 'non-self' and mounts a destructive response. This is sometimes termed the 'stranger' hypothesis and is well established. However, it is often ignored that the immune system is also programmed to attack and destroy our own cells and tissues in certain circumstances and this is actually an evolutionary advantage, as malignant and damaged cells can be eliminated to protect us from future cancers. Unfortunately, this same programming is responsible for the rejection of transplanted organs. The immune system also mounts sustained, damaging and apparently insane 'autoimmune' campaigns against our own tissues, ranging from diabetes to arthritis. All these processes occur without the presence of any bacterial or viral adjuvants and the obvious question is how the immune system actually becomes aware that cancer cells, foreign tissues or even our own apparently healthy tissues should be targeted. To account for this, it has been proposed that the immune system does far more than just hunt for the obvious clunking great LPS molecules and bacterial antigens. The 'danger' hypothesis suggests that the immune system controls its own state of readiness and sensitivity through a highly sophisticated 'listening' system, where perhaps hundreds of different 'danger signals' are processed every second to provide a picture of the health of a tissue. At present, we are not fully aware of what all these danger signal molecules are, but they are likely to be key proteins or peptides which are not normally found in the circulation. At first, heat shock proteins were thought to be responsible, but it now appears more likely that certain interleukins as well as cytokines such as HMGB1 (high mobility group box 1 protein) may be among one of hundreds of signals ranging from large molecules such as DNA/RNA fragments to smaller agents like uric acid. Other 'danger signals' may be the products of damaged cellular plasma or nuclear membranes. This is logical as pathogens generally thrash about in our tissues wrecking cellular membranes and releasing their contents that include the danger signals. So it makes sense to detect these signals quickly to buy time in order for an effective immune challenge to be initiated before the infective organism starts to appear in numbers. Of course, potentially cancerous cells also need to be discovered and immediately killed off.

These danger signals are released during a number of processes aside from infections, such as during normal and abnormal apoptotic and necrotic processes which occur every second of life. The immune system probably monitors complex proportions and patterns of different danger signals, which may allow exquisite discrimination between a normal and faulty cellular disposal processes. Essentially, every individual has a pre-set immune cell sensitivity that is likely to vary from tissue to tissue, which is governed by a combination of levels of danger signals and the balance of cellular damage and efficiency of repair. This last point may hold a clue as to why the vast majority of us are immune tolerant of ourselves and whilst others are not.

8.5.4 Antigen processing and presentation

In order for the immune system to detect infection as well as danger signals from abnormal cellular behaviour, it must search for them inside the cells themselves as well as outside in the circulation and interstitial fluid. B and T lymphocytes usually detect antigens after they have been *processed* into forms where they can be *presented* to the B and T cells in such a way that they will recognize it as foreign and not self. The process can be resolved into intracellular and extracellular antigen detection; presentation and the sensitivity

of the process is crucial in determining whether an individual develops an autoimmune disease or a severe drug hypersensitivity reaction.

Intracellular antigen detection and presentation

Normally within any given cell, there is a continuous process where various proteins are removed, shredded and then loaded onto a molecule known as MHC I, or major histocompatibility complex I (Figure 8.6). A protein called TAP handles this loading process and it happens in the rough endoplasmic reticulum of the cell. MHC I is related to immunoglobulins and can bind practically any peptide, so it can carry any given piece of protein to the cell wall, where it expresses the protein on the surface. This is a form of constant quality control, a monitoring or sampling of the presence of self inside the cell followed by a clear presentation of 'self' at the exterior of the cell. The MHC molecular system is a vital link in the process that alerts the immune system to possible internal foreign antigens. In the case of a viral infiltration, the viral proteins are picked up and shredded, loaded onto the MHC I which then exhibits them, rather like a 'hot dog in a bun', with the viral or foreign proteins acting as the 'hot dog'. Cytotoxic T cells (CD8+) recognize the MHC I as self (self MHC restriction), which means they will then automatically accept the foreign material bound to the MHC I as non-self and then destroy the cell. This recognition by the T cell of the self of the MHC I before antigen recognition prevents the T cell from indiscriminately attacking anything in sight and channels their cytotoxicity to where it is needed.

Extracellular antigen presentation and detection

Most bacteria and toxins are extracellular and viruses must be extracellular until they find a victim cell, so a series of antigen presenting cells (APCs) cruise the interstitial fluid

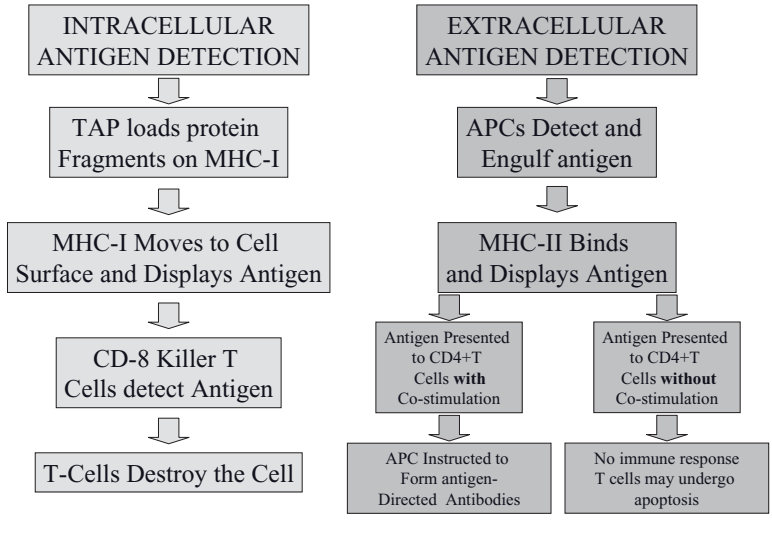

Figure 8.6 Basic events of intra- and extracellular antigen processing

around cells, as well as inside tissues hunting for antigens and 'listening' for danger signal molecules. These include B lymphocytes, macrophages, dendritic and Langerhans cells in the skin. These cells carry Immunoglobulin (Ig) molecules on their surfaces and they detect smaller entities than whole proteins, such as nucleic acid fragments, lipopolysaccharides and lipids. At this point the APC cells engulf the detected material, dismantle it and tie up the protein of the complex to MHC II, which is only found in APCs. This migrates to the APC surface as with the previous MHC I system, only this time the APC cell will present its processed MHC II protein fragments to helper T cells (CD4+), rather than the cytotoxic (CD8+) ones (Figure 8.6). There are usually twice as many CD4+ than CD8+ cells, although during disease states the proportions can equalize. The vast majority of the protein fragments presented will not elicit an immune response as there is a fail-safe built into this system called the 2-signal hypothesis. The presentation of MHC II and the fragments to the T cell surface receptors is considered 'signal 1' and although this results in a degree of T cell activation, without further stimulation, the cells do not proliferate and no further immune action is taken. This is essentially 'tolerance' to the MHC-presented material. However, if the T cell receives signal 2, which is known as co-stimulation, full activation should occur. Signal 2 comprises the binding of a B7 receptor on the APC with a CD28 on the CD4+ T cell. Once this happens, the CD4+T cell promotes the process by secreting lymphokines that instruct the APC to make antibodies. Signal 2 also instructs B cell proliferation (memory cells), ensuring that the next time the antigen is detected there will be a much more rapid and intense response. As the CD4+ cells enable this aspect of the immune response, they are called T 'helper' cells. The B cell amplification system is again designed to buy time. The immune system can respond to virtually any pathogen, provided it has the time to make enough cells and antibodies. This time period must not be slower than the proliferation rate of the infection, or death results by weight of numbers and secreted toxins. The memory pool of cells provides the ability for rapid response to the pathogen when it is next encountered. It seems that the key to tolerance or immune response is whether co-stimulation occurs. The APC and T cells are much more sensitive to successful co-stimulation if some of the various danger signals discussed earlier have been already detected.

8.5.5 How drugs might initiate immune responses

There are three current hypotheses that try to explain how drugs and other low molecular weight toxins manage to break through immune tolerance and elicit a major immune response. These hypotheses don't exclude each other and there is experimental and clinical evidence to support all of them, so it is probable that to some extent they all occur, depending on the nature of the drug, individual and their state of health (Figures 8.7 and 8.8).

Hapten hypothesis

This hypothesis assumes that small molecules of molecular weight of less than 1000–2000 daltons should not theoretically concern the immune system. It is true that bacterial and viral proteins or fragments, as well as LPS are all large entities, perhaps from 20,000 daltons upward. In the 1930s, Landsteiner showed that the immune system would recog-

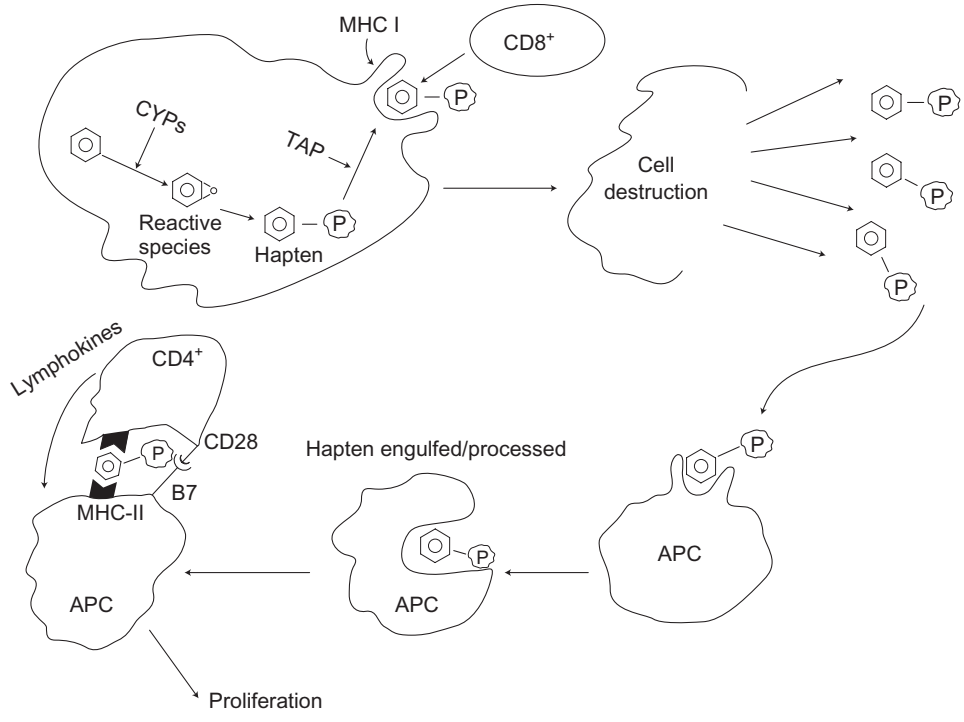

Figure 8.7 The hapten hypothesis as it might apply to CYP-mediated reactive species formation in drug hypersensitivity. The CD8+ MHC-I system may detect the hapten (reactive species bound to protein, P) and destroy the cell. The debris could then trigger the APC MHC-II system and both signal 1 & 2 are supplied, followed by cell proliferation and antibody formation. Spontaneously reactive drugs like penicillin probably trigger both MHC systems at once

nize very small molecules if they were to become covalently attached to much larger ones (40,000–50,000 plus), although there are examples of the immune system also responding to unbound small molecules. When a reactive small molecule does bind to a cell macromolecule, the process of 'haptenation' is said to occur. Injection of the hapten itself does not usually result in any reaction: the essential feature of this theory is that the hapten is bound to protein. The strongest support for this idea is penicillin, which will react covalently with proteins and thiols both intracellularly and in the circulation and tissues, so the penicillin-related haptens could be detected through MHC I and II pathways. Antibodies have indeed been found which are directed at penicillin-bound protein material and the drug is certainly powerfully immunogenic in a substantial proportion of patients. It is interesting that there must be a relatively high level of haptenated proteins after penicillin use and this can be easily detected by the intra- and inter-cellular systems, but still only a minority of individuals suffer severe reactions.

The hapten theory was extended to include reactive metabolites formed by CYPs (Figure 8.7). It is already known that suicide inhibition (Chapter 5) leads to CYP destruction and covalent binding and antibodies could be formed to the damaged CYP/hapten, or a slightly less reactive species may attack more distant macromolecules in the cell which could be equally antigenic. It seems more likely that this form of haptenation would be

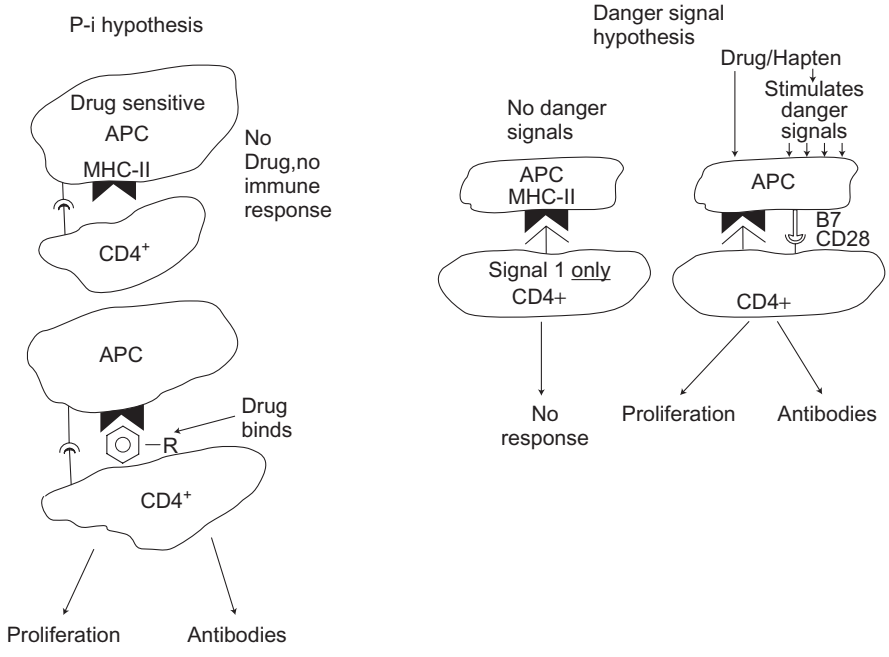

Figure 8.8 Aside from the hapten hypothesis, there are two other competing hypotheses on the cellular mechanisms of drug hypersensitivity: the pharmacological interaction (p-i) hypothesis concept and the danger signal hypothesis. It is probable that no single hypothesis is sufficient to explain all the available clinical and experimental data and elements of all three may be involved in many drug reactions.

detected initially by MHC I, rather than II, as the CYPs are intracellular. Many drugs that cause hypersensitivity syndromes are extensively metabolized, sometimes to reactive species, such as the AEDs carbamazepine and phenytoin. It could be speculated that once the CD8+s have destroyed the cell, debris-containing haptens could then escape the normal phagocytic 'clean up' and trigger the MHC-II system and APCs (Figure 8.7). Alternatively, the TAP system might detect the debris in the cells that 'cleaned up'.

There are many examples of chemicals and metabolites that spontaneously react with proteins and the degree of binding depends on the balance of 'activation' and detoxification in the individual, as well as the epitope density, that is, the number of haptens attached to the macromolecule. If MHC I is involved, then the involvement of killer T CD8+ cells might account for the hepatitis-like liver damage seen with the AEDs. However, there are many drugs that are activated to reactive species without any accompanied immune response, so there are limitations to the hapten hypothesis.

Pharmacological interaction hypothesis

Known as the p-i hypothesis or concept (Figure 8.8), this idea is based on experimental observations on the timeframe of activation of some populations of T cells that came from patients who had developed hypersensitivity reactions. In the presence of the drug that

caused the hypersensitive response in the patients, these cells produced a full 2-signal response so quickly that the cells would not have had time to process any antigen, haptenated or otherwise and present it. The cells reacted in seconds, which suggests that the drug bypassed the MHC II antigen presentation process and acted pharmacologically by either binding the MHC II complex or binding drug-specific T cell receptors and ultimately co-stimulating the T cells. At least one toxic agent has been shown to reversibly bind MHC, the direct thrombin inhibitor Exanta (ximelagatran). This agent caused the release of pro-inflammatory cytokines and chemokines that contributed to severe liver toxicity in some patients and was withdrawn in 2006.

There are perhaps two important features of the p-i concept. The first is that the drug acts reversibly on the receptors of the immune cells and covalent binding is not required. The second feature is that this process happens in cells *already* sensitized to the drug, so it is not intended to explain the events that led to the initial sensitization in the patients. Further studies suggested that the drug could also activate the T cell without the presence of the APC. This raises the possibility that drugs may in some circumstances switch on or even inhibit T cell activation, essentially as an off-target pharmacological response. The full implications of this hypothesis remain to be explored.

Danger signal hypothesis

As discussed above, the concept of the immune system listening for danger signals easily translates to drug-related material. It is reasonable to suppose that drugs, their metabolites or even haptens might act directly as danger signal molecules and may be 'read' by the APCs and T cells as advance warning of an immune problem (Figure 8.8). The drug-related material could also stimulate the production or perturbation of other danger signal molecules such as cytokines and this has been shown experimentally with sulphonamide and dapsone metabolites, as described below. Hence, the drug, its metabolites, or the haptens might cause the whole process of APC and T cell interaction to become more sensitive, so inducing a local immune response. However, not all reactive species or drugs may act as danger signals despite generating considerable oxidative stress (such as troglitazone and paracetamol) and other drugs may be detected as danger signals only in a minority of patients.

8.5.6 Nature of drug-mediated immune responses

The clinical presentation of drug hypersensitivity may depend on the time of onset, severity of symptoms and particularly the site of the reaction. The first two types of reaction, anaphylaxis and anticonvulsant syndrome, lead to widespread reactions throughout the body. The second probably involves a more localized cause, such as a systemic loss of neutrophils (agranulocytosis). Other reactions are confined to particular organs or sites in organs, leading to hepatotoxicity and skin toxicity.

Anaphylaxis

This can happen in response to a variety of foodstuffs (particularly nuts), food colourings, handling latex, contact with animals, insect bites and stings as well as drugs such as

penicillins, NSAIDS and some intravenous preparations. The symptoms include: fainting, swelling of the throat (laryngeal oedema), asthmatic symptoms as well as difficulty in breathing, rashes, swelling, vomiting and diarrhoea. If anaphylaxis progresses to anaphylactic shock, blood pressure plummets, tachycardia and arrhythmia occur alongside a racing pulse, coupled with extreme bronchospasm. Anaphylactic shock can be lethal in minutes.

This condition is caused by some exposure to an antigen that provokes the formation (by B lymphocytes) of a unique Immunoglobulin E (IgE) for that antigen. The IgE sits on the surface of mast cells and basophils, rather like a primed grenade. Another exposure to the antigen, or something similar in structure, and the IgE causes the cells to release massive amounts of vasoactive substances such as histamine. This process is known as degranulation. Since mast cells are found in all the tissues, particularly the vasculature and bronchi, blood vessels are relaxed causing the fall in blood pressure and the spaces between cells widen, leading to swelling in the tissues. The constricting effect on the bronchi is basically dependent on the amount of these agents released. Subsequent exposure to the antigen raises many more antibodies that cause more and more violent and widespread reactions through the body. This is the worst form of immunological 'own goal' as it is grotesquely out of proportion to the threat involved and can lead to death of the patient. The treatment is immediate injection of 300 micrograms (150 for a child) of adrenaline, which should control the metabolic mayhem, although recovery times can be considerable.

Since haptenation is one possible route to a drug-related immune response, it would appear to be a bad idea to market drugs that were directly protein reactive, but penicillin's spontaneously degrade into a number of reactive derivatives in all patients that take them. The result of these species binding covalently to proteins leads to the formation of various haptens such as the penicilloyl derivatives. The B lymphocytes form IgEs in response to these groups and the next administration of the drug to the patient could be their last. This is essentially a disseminated reaction, i.e. a reaction that occurs in many areas of the body, including crucial ones such as the blood vessels and the lungs. About 10 per cent of the population could be at risk from this type of reaction from penicillin or related antibiotics. Around 75 per cent of fatal anaphylactic shock cases are linked to penicillin and its related drugs. Concurrent usage of beta-blockers makes anaphylaxis much worse as they prevent the beneficial effects of catecholamines released in response to the condition as well as those administered to treat it. Animal studies suggest that only a minuscule fraction of the dose needs to bind to provoke a reaction, so the degree of covalent binding itself is not the issue, rather it is the shape of what is bound and where it is bound. Penicillin originated from a eukaryotic organism (mould) and many fungus-type organisms are pathogenic to humans. Therefore it is not really surprising that the structure of the penicillin-related material is so intensely antigenic. What is also interesting is that penicillin is protein reactive in everyone without exception, but only a minority of patients develop anaphylaxis.

Anticonvulsant hypersensitivity syndrome (AHS)

This multi-organ syndrome is similar to that seen with allopurinol and sulphonamides. It affects a small proportion of epileptics taking the older anticonvulsants, particularly carbamazepine and phenytoin, although the third most common trigger is the newer agent

lamotrigine. Valproate is not usually associated with the syndrome, although it can promote the effect with lamotrigine, as valproate retards its clearance. AHS is not as rapid in onset as anaphylaxis, but can begin within 2–12 weeks of starting therapy. Virtually all patients present with fever and this can persist for weeks after the drug has been withdrawn. A rash affects the trunk and limbs; the face and lips may swell and blister appreciably. Around half the cases also have hepatitis; this is the most dangerous area of the reaction and up to a third of patients die from liver failure. Kidney toxicity can also be apparent and high white cell counts are recorded, with general haematological disruption. Some patients go on to develop Stevens-Johnson syndrome and toxic epidermal necrolysis (TEN), which is the rather gruesome skin reaction equivalent of hepatic necrosis. Severe forms of TEN are often sent to hospital burn units. A serious clinical problem arises when the patient recovers, as the reaction may occur again with another anticonvulsant. The reaction will be much worse and appear more rapidly the second time, so patch testing is recommended before another anticonvulsant is started, although many authorities recommend that valproate be used as a safe substitute AED, although it too can cause hepatotoxicity in rare cases. As so many of the symptoms resemble infection, valuable time is often lost treating the patient with antibiotics whilst the offending drug is not always stopped immediately. Generally, the 'triad' of fever, rash and internal organ involvement should alert medical personnel to the possibility of AHS in a presenting patient. Treatment usually involves doses up to 80 mg/daily of steroids such as methylprednisolone for up to a month, which is then tapered along with resolution of the symptoms and eventual recovery. Antihistamines are often used to suppress itching and irritation.

All the drugs responsible for this syndrome are extensively cleared by CYPs, often to reactive arene intermediates that are cytotoxic and tissue reactive *in vitro*. With carbamazepine, polymorphisms of microsomal epoxide hydrolases (EPHX1: Chapter 7, section 7.2.4) are known to be responsible for altering the clearance of the 10, 11 epoxide, but it has been more difficult to link a slow version of the enzyme such as Y113H with predisposition to developing anticonvulsant syndrome. Interestingly, once the syndrome develops, its progression is more rapid in patients who have undergone recent radiotherapy, which generates free radicals and other reactive species in bystander tissues. This oxidative stress, together with their underlying conditions, would tend to compromise hepatic thiol levels and this could account for the patient's vulnerability to hepatic failure in the syndrome. It is believed that a cytotoxic T cell response is behind the reaction and this is likely to be linked with MHC I intracellular antigen presentation.

Blood dyscrasias

These conditions are usually characterized by the loss of a group of cells within the blood system, such as erythrocytes, platelets or entire white cell populations. Although the various mechanisms of the reactions could fit all three of the theories of drug hypersensitivity, the net effect can be either the targeting of the cells themselves, the organ or tissue that makes them, or a combination of the two.

Haemolytic anaemia

The premature intravascular destruction of erythrocytes can be caused by many circumstances, from physical damage to the cells (passage through surgical life-support systems)

and accelerated wear and tear caused by drugs such as sulphones, through to immune-mediated causes related to drug therapy. A number of drugs are associated with immune-mediated erythrocytic loss, such as methyldopa, penicillin, cephalosporins, quinidine and some NSAIDS. As far back as the 1960s it was shown that haptenation of the cells' membranes by penicillin-related material led to recognition by B cell-mediated immunoglobulins, which led to complement activation, although the other mechanisms besides haptenation cannot be ruled out. As mentioned previously, red cells already have a 'sell-by date' system, where normal wear and tear (red cells can be reversibly deformed to an amazing degree) induces the appearance of various wear indicator molecules on their surface. The spleen 'reads' the number of these molecules and when the number corresponding to the 120-day lifespan appears, the spleen removes and destroys the cell. Clearly a process such as haptenation could accelerate the spleen's removal of the erythrocytes, as well as alerting other cytotoxic immune cells. Clinical presentation involves jaundice and dark urine, due to the liver struggling with the disposal of so much haem in such a short time, as well as the high levels of bilirubin produced. Symptoms appear that are related to tissue hypoxia, such as fatigue, shortness of breath and tachycardia. Usually once the drug is stopped, the liver will recover and transfusions can tide the patient over until normal red cell production eventually replaces the lost erythrocytes.

Aplastic anaemia

This condition is due to destruction of the bone marrow, which can be caused by radiation, infections or by an immune-mediated process. This results in the gradual reduction of vascular levels of all blood cells, including white cell populations, erythrocytes and platelets. The symptoms include those for haemolysis, alongside those of immune deficiency, such as oral thrush. Several drugs have been shown to cause aplastic anaemia, which include anticonvulsants, anti-cancer agents, chloramphenicol and phenothiazines. Interestingly, the chronic use of illegal drugs, such as opiates and cocaine, increases the risk of aplastic anaemia. Aromatic organic solvents such as benzene and toluene are also linked with this condition as well as a number of insecticides. The prognosis is poor, as even after a bone marrow transplant, survival over five years can be less than 60 per cent. The damage is probably linked to CD8+ killer T cells and the reactive nature of many of the metabolites of drugs that cause this suggests that haptenation could be linked with this condition.

Agranulocytosis

There are many causes of neutropenia, where neutrophil levels fall to low levels in cycles, but agranulocytosis is characterized by the 'one-off' decline to the point of almost disappearance of neutrophils in the circulation. The condition is stealthy, in that it is symptomless until cell levels fall below 0.5×10^9 per litre. Agranulocytosis underlines the importance of neutrophils, as in spite of the existence of so many other immune cell types, life is only possible for a few days without neutrophils. The first symptoms are of a serious infection, with fever, malaise and chills, leading to acute sepsis. The type of bacteria that infect the patients in this context includes *Staph. aureus* and various pseudomonads.

A considerable number of drugs cause this condition, such as clozapine, sulphonamides, dapsone, antithyroids and phenothiazines. It is an uncommon reaction and the overall rate

for most drugs is very small. Clozapine is about the most dangerous drug in this respect, with a frequency of more than 1 per cent.

The condition usually appears from 8 to 14 weeks after initiation of drug therapy. Agranulocytosis is unusual, as it is the loss of a separate population of cells. Once the causative agent has been withdrawn, neutrophil production resumes as if nothing had happened. A combination of intravenous antibiotics and stimulation of bone marrow with filgrastim should attenuate the infections. Normal neutrophil blood levels should be attained in less than 14 days. Examination of bone marrow shows no actual damage, although myeloid precursor cells may be absent. That cell production resumes so quickly after the withdrawal of the drug reinforces the idea that agranulocytosis in some cases is not really toxicity, more a reversible interruption in the cell assembly line. The first drug to show this effect was in the 1930s, but was atypical. Aminopyrine therapy caused antibodies to be formed to neutrophils and they were destroyed in the circulation, but not in the bone marrow. The condition occurred within a week and would occur even earlier on rechallenge. Drugs such as dapsone and clozapine, which cause agranulocytosis, show the 8–14 week latency, which in the case of clozapine does not change on rechallenge. If the immune system were involved, the pool of memory cells would cause an effect in less than half that time. This suggests that the effect is probably not connected to the immune system and is probably not related to haptenation. So it may be that agranulocytosis is less to do with reactive species and more to do with some form of a direct pharmacological modulation of neutrophil maturation. Since so many small molecules are so crucial in the modulation of cellular responses, it would not be surprising that a drug could act on certain individuals' receptors that were atypical in some way due to genetic variation. It is probable that a form of the p-i concept best describes the causes of agranulocytosis.

Site-directed drug-mediated immune responses

Most other drug-mediated immune reactions are targeted at an organ, where deposition of various immunoglobulins (IgA, IgG and IgM) may result in a hypersensitivity reaction. This could be part of a disseminated or local effect. These reactions could be linked to haptenation, the p-i concept or the danger hypothesis. Both the liver and the skin are vulnerable to drug-induced hypersensitivity reactions and the clinical impact of these organ directed hypersensitivities can range from mild disruption to terminal organ damage.

Hepatic immunotoxic reactions

For the immune system to be linked with hepatotoxicity, it is generally accepted that some symptoms of hepatitis should be present, which involves organ-wide inflammation, presence of inflammatory cells in the sinusoids as well as disruption of normal hepatic processes which lead to jaundice and high liver enzyme levels. Viral hepatitis will generally show these symptoms, but it can be difficult to distinguish an infected liver from a drug-hypersensitivity affected liver. A case of hepatitis without indication of viral or bacterial causes could well be due to drug hypersensitivity. This type of reaction is rare and rather drug specific: frequencies with the AEDs phenytoin and carbamazepine reported to be

1 : 10–50,000 and more than 70 per cent of phenytoin-induced liver toxicity is due to immune hypersensitivity, whilst only 30 per cent of carbamazepine hepatotoxicity is linked with immune response. Other agents associated with drug-induced hepatitis, include the anaesthetic halothane, isoniazid and some statins. Rechallenge with the drug brings about a rapid return of the symptoms and is not ethical. Onset of symptoms is usually within 4 weeks, but can occur up to 12 weeks after therapy initiation. The causes of immune-mediated hepatotoxicity are still controversial. As we have seen, hepatic injury can result through generation of reactive species through biotransformation and the species attack cell macromolecules and organelles, particularly the mitochondria, leading to oxidative stress-like conditions. Covalent binding used to be seen as the key event that might cause irreversible cell damage, either through physical attrition or by evoking an immune response. However, covalent binding is probably just a component of this picture, as even extensive binding of drug related material in some cases does not elicit an immune response such as with paracetamol. The main difficulty is discovering the sequence and nature of the critical events that lead to organ failure. It is not clear why some drugs cause progressive liver damage that does not involve the immune system, whilst others provoke an admittedly very rare aggressive immune response. Although haptenation, the p-i concept and the danger hypothesis all may play a role in how the immune system is recruited to damage the liver, the rarity of the reaction and its aggression in patients make it very difficult to treat, never mind study. Indeed, although steroids are often used to control AHS and phenytoin hepatotoxicity, they are not always effective as they can suppress the systemic symptoms and not necessarily arrest the progression of the liver damage.

There are some indications that there is a genetic component to predisposition to phenytoin-hypersensitivity, but it is likely that reactive species formation, followed by mitochondrial oxidative stress comprise the initial events in the process. A combination of overproduction of drug-related reactive species, failure to detoxify them, and compromised repair of the cellular damage caused due to enzyme polymorphisms are all stages which may or may not lead to immune involvement through MHC complex-related T-cell and APC activation.

Skin-directed immunotoxicity – sulphonamides

It is not generally appreciated that the skin possesses a significant battery of biotransformational systems, ranging from CYPs 1A1, 1B1, 2E1, 2B6, as well as CYP3A5. Normal human epidermal keratinocytes (NHEKs) also express FMO-3 and elsewhere in the skin, GST, NAT and UGT activity is easily detected. The MRP series is also present in skin and these efflux pumps can be upregulated by interleukins in NHEKs. Many of the OATPs also operate in various skin cell systems. Hence, the skin can biotransform, detoxify and influence influx and efflux of drugs, their metabolites and reactive species. These capabilities have been cited to support the contention that the skin can form reactive species locally that might cause oxidative stress and covalent binding, as well as hapten formation.

Aside from biotransformational capability, the immune system is represented in the skin by Langerhans cells, which are responsible for mounting immune responses, as they can become APCs that are thought to have a role in the development of cutaneous drug hypersensitivity. In addition, after an immune response has been triggered and antibodies bound to an area of skin, infiltration of neutrophils may occur and they also have the capability

of forming reactive species from certain drugs. Once they are activated, neutrophils use the iron-based enzyme myeloperoxidase to generate hydrogen peroxide, as well as hypochlorous acid to attack bacteria. It is established that these oxidants can form reactive species of sulphonamides and other drugs, which can covalently bind to tissues as well as form other reactive oxygen species.

Sulphonamides are among several drugs which are known for their tendency to cause a range of mostly skin hypersensitivity reactions which make patients, particularly those with a high risk of reaction and little clinical alternative, intolerant of the drugs. Around 5 per cent of healthy individuals show cutaneous reactions to sulphonamides, but skin reactions to the chemically related sulphone dapsone are much rarer. Notably, more than half those suffering from diseases such as HIV infection may show skin hypersensitivity to sulphonamides, particularly sulphamethoxazole. These drugs are aromatic amines, so they undergo the classic hepatic CYP (usually CYP2C9) oxidation to hydroxylamines that then spontaneously oxidize to nitrosoarenes that are very short-lived and reactive. Unlike dapsone hydroxylamines, sulphonamide hydroxylamines are poor methaemoglobin formers, and they are not concentrated by erythrocytes like some other aromatic amine hydroxyl derivatives. So they can be formed in the liver and apparently circulate in blood, so they could theoretically reach any organ, although it is more likely that the skin activates the parent drugs to the hydroxylamines locally, as this has been shown experimentally, where sulphonamide-related material has been found bound to proteins in NHEKs in cell culture. It is also apparent that FMO-3 has a role in the formation of sulphonamide bioactivation to reactive species that leads to haptenation of NHEKs. This was shown as CYP inhibitors did not stop the effect, whilst methimizole, an FMO-3 inhibitor did, probably by competition. At first sight, the pattern of sulphonamide activation appears to favour the hapten hypothesis as an explanation as to how the drugs trigger immune responses. However, it has been shown that experimentally the hydroxylamine metabolites of sulphonamides and dapsone are also capable of acting to induce the appearance of various danger signals, such as some heat shock proteins and cytokines. It is possible that the metabolites cause the haptenation and then trigger the co-stimulation of the APCs such as the Langerhans cells and T-cells that react to the haptens in certain individuals. Once the antibodies are formed this may amplify the process to involve the neutrophils, which may form more reactive species.

Clinically, a wide range of sulphonamide reactions are manifested, ranging from rashes caused by similar pathways to penicillin (IgE-mediated mast cell disruption) to the triggering of widespread cytotoxic T cell destruction. This is usually associated with the MHC I intracellular system for antigen detection and could be involved in the most severe hypersensitivity reactions, such as Stevens–Johnson/TEN syndrome. Catastrophic reactions such as these have contributed to the sulphonamides falling out of favour as antibacterials and antimalarials. Again, the obvious question is if everyone metabolizes sulphonamides into reactive species and their induction of danger signals is ubiquitous, why do most of us escape any immunological consequences of this?

8.5.7 Predispositions towards drug-mediated immunotoxicity

It will be many years before drug-induced hypersensitivity is fully understood and probably even longer before it can be predicted and averted in individual patients. It is probable

that the balance between the type of drug, the formation of reactive species, detoxification, production of danger signals and individual immune sensitivity governs the risk of a severe hypersensitivity reaction. It does seem apparent that elements of the hapten, p-i concept and danger signal theories are operating in concert, either sequentially or simultaneously, or both. The hapten hypothesis may well be the 'igniting' of the immune process, whilst the other two hypotheses are probably part of the propagation and amplification. However, this does not answer the key question as to why a certain individual, one in many thousands, suffers an unpredictable life-threatening reaction which is progressive, difficult to recognize, as it can masquerade as a common illness, as well as being hard to treat. From Chapter 7 the sheer variability in human biotransformational systems underlines the possibility of certain combinations of enzymes that predispose to overproduction of a dangerous chemical species, whilst the sensitivity of any given individual's immune system must be a combination of many genetic factors, as well as diet and general health. One possible clinical warning sign that a patient may be subject to a drug hypersensitivity reaction could be a current immune-related condition. In the case of dapsone-mediated agranulocytosis, the condition is very rare in immunosuppressed leprosy patients, around 1 : 8,000 in 'normal' individuals when used as an antimalarial and 1 : 250 in patients with dermatitis herpetiformis. Conversely, drug hypersensitivity can leave patients open to other immune events; individuals who have developed carbamazepine hypersensitivity reactions can suffer reactivation of viral infections such as human herpes 6 (HHV-6) at the same time.

Generally, the majority of individuals in a population will metabolize a drug into a reactive species and suffer a degree of oxidative stress, however only a minority will form antibodies to the drug-related material and an even smaller number will suffer a full-blown hypersensitivity reaction. Factors that govern the sensitivity of the immune system are probably crucial in these conditions. Interestingly, a development of the danger hypothesis may account for the increased sensitivity of certain individuals to drug hypersensitivity as well as autoimmunity. Whilst the level of danger signals and their patterns are likely to be constantly processed by the immune system, cellular repair mechanisms, particularly those engaged in sealing plasma membranes, should normally act rapidly to seal membranes and restrict the escape of the danger signal molecules into tissue fluids and the circulation where the APCs and T cells may detect them. There are two mouse models, *Syt VII*$^{-/}$and *dysferlin*$^{-}$ where single membrane repair genes have been deleted and these animals suffer from autoimmune diseases in tissues linked with mechanical stress that show some similarities with human conditions. It has been speculated that excessive immune sensitivity and predisposition to immune upregulation and activity by danger signals might be linked with inherited defective or partially defective membrane repair systems.

8.5.8 Summary of Type B2 immune reactions

- Reactions can be unexpected and unpredictable although they are most likely to occur within three months of initiating therapy.
- The organ/tissue affected is often not related to the pharmacological target of the drug.

- The effects can be severe, life-threatening and rapid in onset.
- The effects may lead to vulnerability to further injury on re-challenge.

8.6 Type B3 reactions: role of metabolism in cancer

8.6.1 Sources of risks of malignancy

It has become clear that oxidative and reductive metabolizing enzymes are responsible for the generation of some reactive species that interact differently with cellular structures than immunogenic or necrotic agents. Carcinogenic reactive species can have exquisitely precise structural characteristics that are necessary to react with individual nucleotides of DNA. This can lead to cross-linking the DNA, preventing transcription, or causing transcriptional errors.

One of the chief defences against the propagation of damaged DNA is apoptosis. Whether specific or non-specific binding is responsible for initiating the process, controlled and ordered cell death results, where reactive endogenous systems are shut down or made safe, DNA is destroyed and the cell dismantles itself. Apoptosis is a very complex process, but in terms of a response to reactive species, it is an attempt by the organism to contain damage and limit its consequences for other cells and most importantly, the organism itself. The destruction of the stricken cell's DNA prevents faulty genes from being propagated in cell division. The survival of the cell in this context could mean eventual death of the entire organism from a future neoplasm. If apoptosis is not triggered, the organism will rely on gene repair to prevent possible future malignancies. Gene repair in dividing cells is an endless process, like roadworks on motorways. The balance between enzymatic reactive species generation, degree of detoxification of the species, DNA damage, as well as the efficiency of DNA repair ultimately dictates an individual's risk of malignancy.

8.6.2 Risks of malignancy and drug development

Regarding drug development, there is a theoretical risk that a drug designed to treat a condition for perhaps many years could cause neoplasms. The drug industry is required to examine this possibility with carcinogenicity tests with new drugs. After *in vitro* studies, such as the Ames test (Appendix A) have shown a drug not to be mutagenic *in vitro*, the animals are exposed to the drug in carcinogenicity studies for their whole lifetimes with doses that greatly exceed the likely therapeutic level. These studies are usually still going on while the drug is in Phase I and II (human volunteers and patient) trials.

Although many drugs can interact with various nuclear receptor systems so causing profound changes in gene expression, the possibility that these changes could lead to malignancy can be detected to some extent through the use of DNA microarrays in human cells as well as *in vivo* animal studies. The other more likely route is through DNA damage caused by reactive species, formed by biotransformation either in the liver, lung or perhaps the kidney. Usually, but not always, reactive species may interact with human and animal DNA in a similar fashion. However, animal models may diverge from the human situation

strongly as to which CYP enzymes actually form the reactive species. In the case of aromatic amines like sulphonamides, dapsone, aniline, β-naphthylamine and 4-aminobiphenyl, different enzymes between the species form the same hydroxylamines. The recent examples of troglitazone, trovafloxacin and tacrine underline the problem that humans are more susceptible to certain toxic events than animals. So the drug industry is aware that there is the danger that a drug might be metabolized to a reactive and carcinogenic species in humans which does not happen in an animal model. This can be addressed by the use of human liver CYP enzymes to activate the compounds prior to inclusion in the Ames test, followed by the animal lifetime carcinogenicity studies. These studies can be backed up by the many DNA damage/repair assays (sister chromatid exchange, Comet etc; Appendix A) that can detect genotoxicity in human primary and cultured cell systems. Overall, it is likely that the chances of a new drug causing cancers in humans are virtually non-existent. The main causes of human cancers where drug-metabolizing enzymes are involved in the formation of DNA-reactive species include smoking, diet, occupation and atmospheric pollution.

8.6.3 Environmental carcinogenicity risks

Although there is a vast literature on carcinogens in animal species, there are surprisingly few proven carcinogens in man. It is perceived that our greatest risks of exposure to carcinogens lie in what we eat, what we breathe (or inhale, if it is tobacco) and where we work. The earliest hard information about human carcinogenic risk came from occupational sources. The first documented example (eighteenth century) was the detection of scrotal cancers in young chimney sweeps, followed by polycyclic aromatic-mediated disease in the early twentieth century. Aromatic amine-mediated bladder cancer risks in the dye and rubber industries were established well before the 1950s. This real-life 'experimental' system is from the human standpoint tragic and costly, whilst from the scientific standpoint, it is relatively uncontrolled and incomplete. Exposure to carcinogens is varied and confounded by factors such as genetic predisposition, age, smoking habits, changes in recommended exposure levels and working environments. Individuals are often exposed to carcinogens for up to 40 years and in some cases, their exposure is relatively brief and the latency period before the cancer appears can be several decades. It is also increasingly clear that protective mechanisms that maintain DNA are also extremely sophisticated and vary in their effectiveness between individuals. So what is perhaps remarkable, is that so many thousands of individuals smoked heavily, worked in the rubber or dye industries and died of old age.

8.6.4 Occupational carcinogens

Aromatic amines: introduction

To understand why aromatic amines are so toxic and carcinogenic, it is first useful to look at some basic amine chemistry. It is easiest to see these compounds in terms of existing mostly in the environment as two main forms, both of which are essentially non-toxic, that is, they are stable and non-tissue reactive. The first form is the amine itself and the

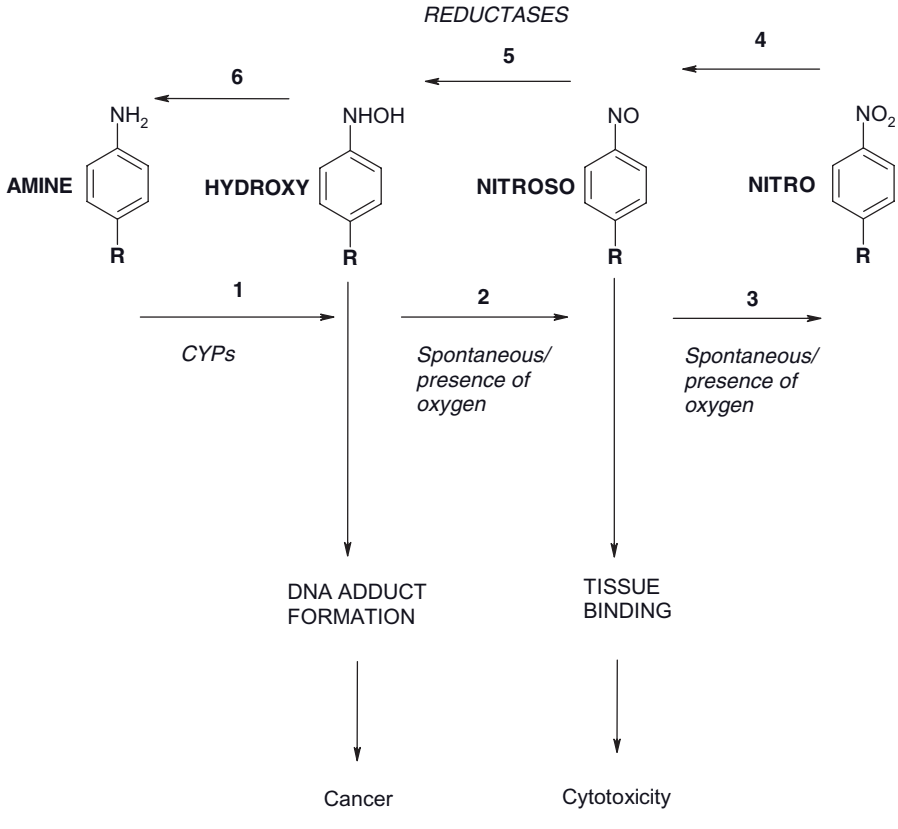

Figure 8.9 Stages of oxidation and reduction of aromatic amines

second is the nitroderivative (Figure 8.9). From this figure you can see that the amine can be oxidized at stage 1 to the N-hydroxy, or hydroxylamine. This usually requires some serious oxidative energy, which is usually supplied by CYP isoforms. The product hydroxylamine varies in stability according to the rest of the structure of the molecule. Stage 1 (Figure 8.9) essentially converts a stable entity into a relatively unstable one, in true CYP tradition. Two electrons are lost in the process. The hydroxylamine can now be conjugated, but this can lead to the formation of even more unstable products, which split, leaving a reactive nitrenium group (more later) that attacks DNA. The other alternative is the spontaneous oxidation (stage 2) and loss of two more electrons to form a nitrosoarene, which can often be highly tissue reactive and rapidly cytotoxic, although some are much more stable. Nitrosoarene stability again depends on the structure of the rest of the molecule. The further spontaneous oxidation and loss of two more electrons (stage 3) forms a stable nitro derivative, which is usually not a problem unless it meets a reductase enzyme, of which there are a large number, in the liver (NADPH reductases), erythrocytes (NADH reductases) and the cytosol of most cells. These return the electrons and form the (stage 4) nitroso and (stage 5) hydroxylamine and then the (stage 6) amine in turn. At each point, the same dangerous intermediates, the nitroso and the nitrenium ions, can be formed. So aromatic amines are a problem because they are relatively easy to oxidize or reduce, but

Table 8.2 The relationship between the stability of aromatic hydroxylamines and their mode of toxicity

Parent drug	Hydroxylamine half-life (pH 7)	Methaemoglobin formation	Immunogenicity	Carcinogenicity
S'onamides	<10 min	+	++++	No
Chlor'col	<20	++	+	No
Dapsone	<60 min	++++	++	No
4-ABP	>3 h	++++	No	++++
BNA	>10 h	No	No	++++

S'onamides (sulphonamides); BNA (β-Naphthylamine); 4-ABP (4-aminobiphenyl); Chlor'col (chloramphenicol).

essentially the same toxic intermediates are formed. So many different enzyme systems can activate them anywhere in the body, not just the liver. This explains their ability to cause toxicity and cancer in widely differing types of tissues.

As mentioned above the pattern of toxicity caused by aromatic amines depends very much on the stability of their hydroxylamines (Table 8.2). This is a generalization, but usually the more stable the hydroxylamine, it is less immunogenic, a poorer methaemoglobin former and the more carcinogenic it might become.

Hopefully you can see how aromatic amines can be so toxic, so the following examples should be easier to understand. It is highly unlikely that a new drug would appear on the market which contained an easily oxidized aromatic amine, or a free nitroaromatic group, as it would be toxic like chloramphenicol, where its nitro group is reduced to a hydroxylamine which caused the methaemoglobin in babies in the 1950s (grey baby syndrome). This drug only survives today as a topical preparation for minor eye infections, so its systemic absorption is virtually zero. Interestingly, a number of other drugs in the last 20 years have failed in clinical trials because they were metabolized to amines that were then toxic. There are many aromatic amines consumed as foodstuffs (overcooked burgers), environmental pollutants and occupational agents. I have cited some examples below, but there are many more in the scientific literature. As mentioned previously, there are two main ways aromatic amines can be metabolized to carcinogens: through oxidative or reductive means.

Aromatic amine carcinogens – oxidative metabolism

β-Naphthylamine (BNA) was used in the rubber industry for many decades as an antioxidant to maintain pliability in rubber products, such as tyres. It could be found in levels that exceeded 1 per cent. It was employed as an anti-rust additive in various oils used to coat metals between manufacturing stages. BNA was also used in the dye industry and it has been established as one of the few compounds that are accepted to be a human carcinogen even in a court of law, provided exposure can be documented. Benzidine, aniline and o-toluidine were all aromatic amines used along with BNA in the manufacture of various colourfast dyes. The pattern of aromatic amine carcinogenesis is interesting: it appears that in any given population of workers that were exposed to this agent, up to 10 per cent may develop bladder cancers. This may be from a relatively short exposure of as

little as 1–6 years. Up to 30 years after exposure ends, the bladder tumours appear and often the first realization that something is wrong for the patient is blood in the urine (haematuria), although many tumours are symptomless until quite late stages.

The following factors influence whether a worker might develop aromatic amine-related bladder cancer. If they:

- are slow acetylators;
- possess wild-type CYPs and Null detoxification genes (such as GSTs);
- are heavy smokers;
- have a diet low in vegetables and high in meat;
- work in a hot environment with poor fluid intake.

Mechanism

It seems that aromatic amine carcinogens all share a similar metabolic profile and it is possible to trace the metabolites formed and determine which are carcinogenic and which are not. It is also possible to explain each of the risk factors above. What is not easy to explain and is beyond the scope of this book, is why it takes 20–40 years before irreversible changes in the bladder lead to the appearance of a tumour.

From Figure 8.10, it is possible to see that there are several metabolic products of aromatic amine clearance. Usually low levels of parent amine are found in urine, but the main fate of these compounds is oxidative and conjugative metabolism. The hydroxylamine derivative is usually too unstable to be able to proceed through the bloodstream to be filtered by the kidneys to reach the bladder. It is much more likely to form an O- or an N-hydroxy glucuronide. The parent drug can also form an N-glucuronide. Free parent amine in the bladder is probably not a problem, as the bladder is unlikely to be able to activate it to its hydroxylamine in quantity. The acetylated derivative is not a problem, provided it is not deacetylated to the parent drug that can then be oxidized by the liver to the hydroxylamine. The fact that slow acetylators are more likely to develop bladder cancers suggests that the acetylation process does ‘hold up’ the amine and protects it from oxidative clearance. Some aromatic amines are excreted in urine as acetylated derivatives, but these may also be glucuronidated and oxidized to form the N-hydroxyacetylated derivative, although this is usually a relatively minor pathway.

The majority of the dose of aromatic amines undergoes oxidative metabolism and the various glucuronides are stable in blood, but not in acid urine. Most people in the developed world eat too much meat and not enough vegetables. This leads to acid urine, which accelerates the decay of these metabolites to only a few minutes in some cases. In addition, men working in hot environments often do not drink enough fluid. They might have a flask of tea or coffee, but the caffeine in these drinks causes a net dehydration throughout the day. This, coupled with possible restricted access to water due to the work environment, means that often the men’s urine is very concentrated throughout the working day, leading to high levels of the various aromatic amine metabolites in the bladder. The decomposition of the glucuronides leads to the formation of parent amine, hydroxylamine and possibly nitrenium ions through a number of different pathways (Figure 8.11).

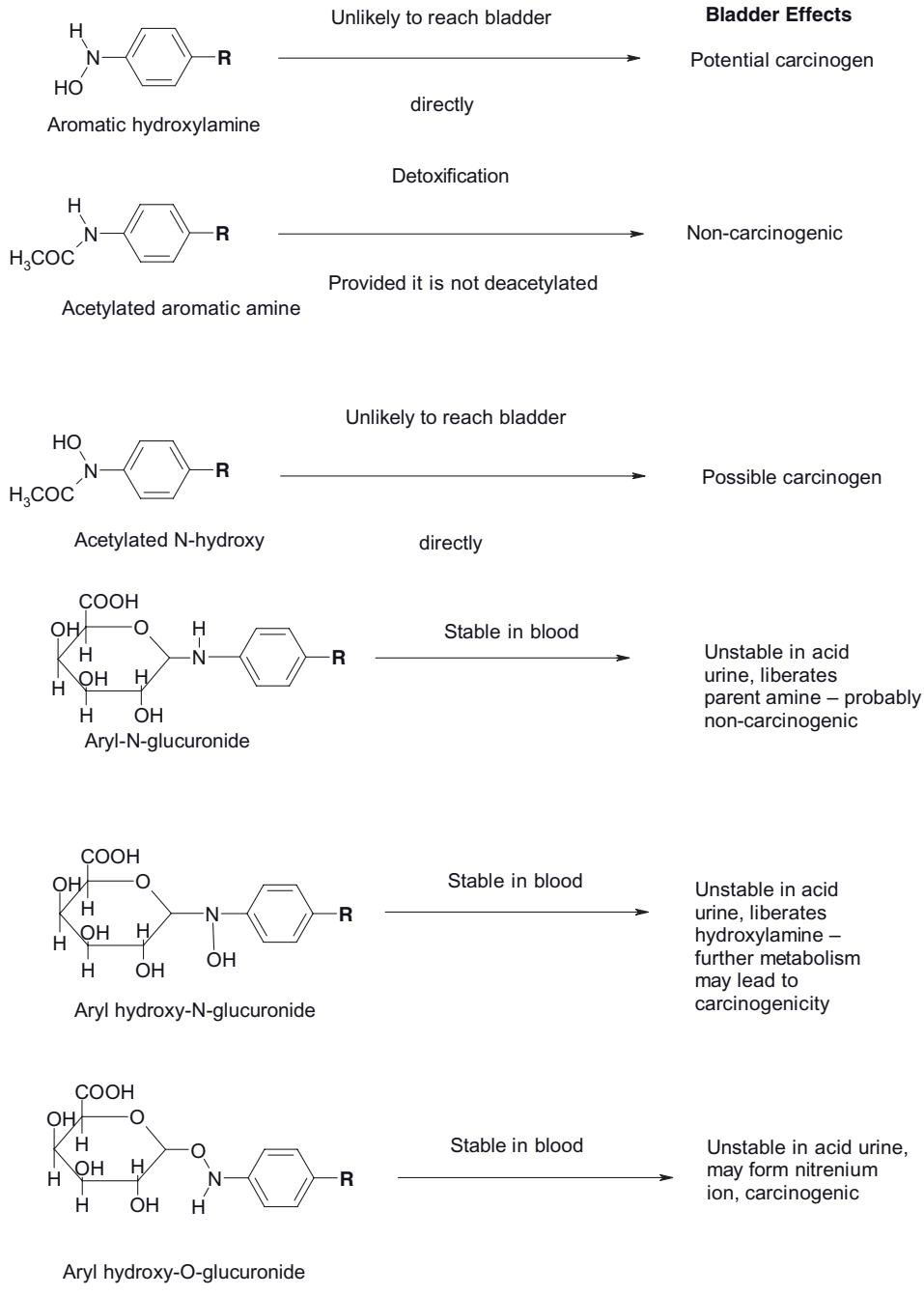

Figure 8.10 Some major metabolites of aromatic amines and their possible role in bladder carcinogenesis

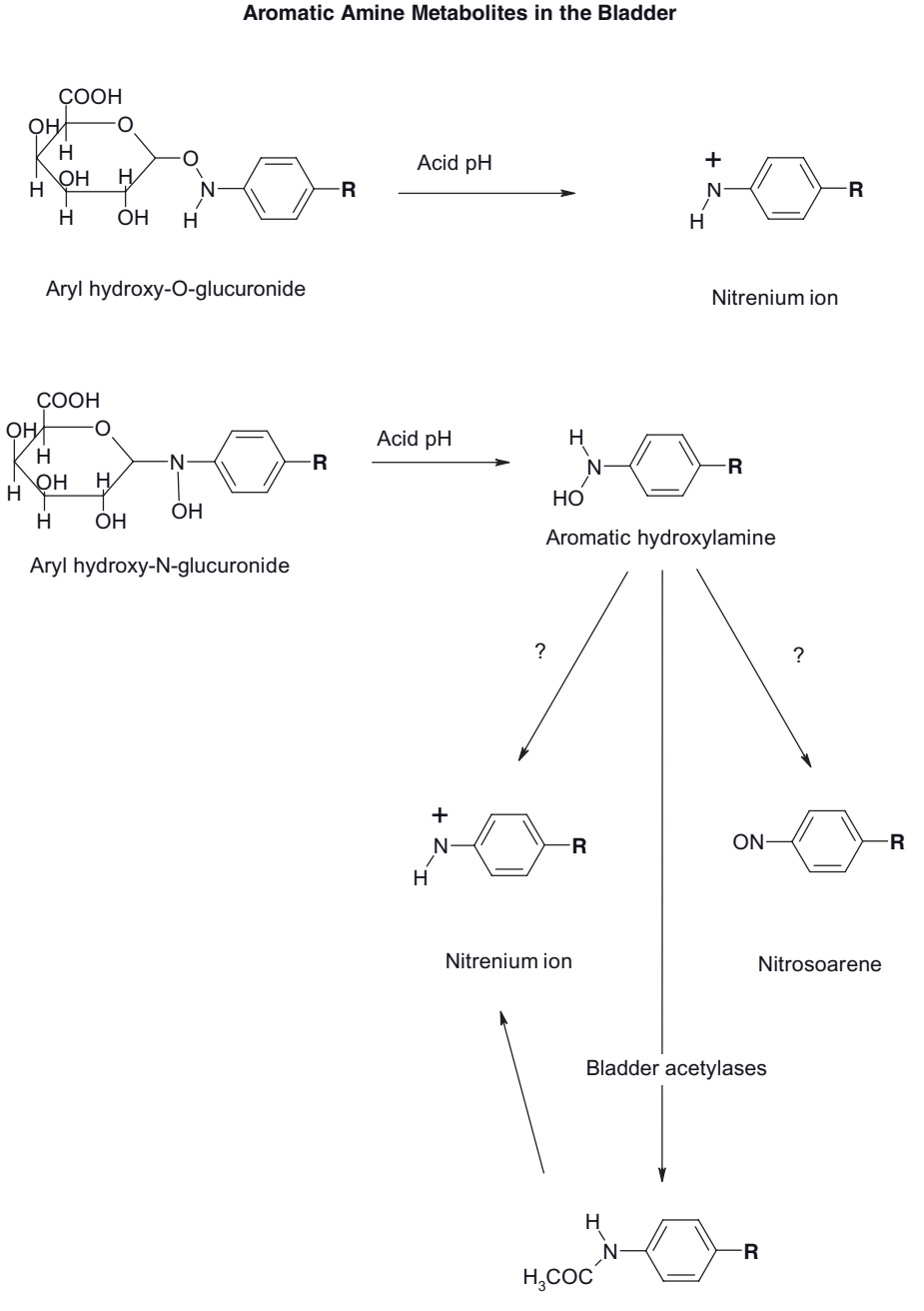

Figure 8.11 Final formation of aromatic amine-derived carcinogenic metabolites in the human bladder

Nitrenium ions (sometimes called aminylium ions) can be formed when a nitrogen/oxygen bond is broken, which leads to the formation of a positively charged nitrogen with two unshared electrons. This makes for a very reactive species that seeks to find its missing electrons from a rich source of them, perhaps DNA or other cellular macromolecules. The first pathway where these ions could be formed is by the decomposition of the aryl-hydroxy-O-glucuronide. There are other ways where nitrenium ions could also be formed (Figure 8.11), involving decomposition of the aryl-hydroxy N-glucuronide to yield the hydroxylamine. Nitrosoarenes would form in the presence of oxygen from the hydroxylamine and be protein reactive, although it is not clear whether they would be carcinogenic. It is suspected that nitrenium ions can be formed from reactions involving the hydroxylamine, but it is also possible that the bladder itself contributes to this by its acetylation of some of these metabolites, which may form more reactive species.

Effects of smoking

Smoking yields the same aromatic amines as the dye and rubber industries and as a result, the number one cause of bladder cancer is actually due to smoking, although it remains a relatively rare cancer; it is the eleventh most common cancer, affecting three males to every female. A smoker would be unlucky to contract it – they would be nine times more likely to die of lung rather than bladder cancer. Many men who worked in the dye, rubber, automotive and light engineering industries where they were in contact with aromatic amines in dyes, oils and rubber also smoked, but it is likely that the greatest contribution of amines came from the workplace, as considerable numbers of non-smokers who worked in these industries have developed bladder cancer. It is certainly true that smoking would add to the body burden of amines, but the chances of developing the disease are probably most strongly linked to the balance of carcinogenic metabolite formation, detoxification and DNA repair.

Human consequences of occupational amine exposure

Many men involved in these industries develop bladder cancer in their late fifties and early sixties, around the time when they would normally be looking forward to retirement. These individuals have often worked for a number of long-since defunct organizations, so they struggle to obtain compensation through the legal system. They also face a long battle with the Department of Health to establish that their condition is related to their occupations. The criteria laid down for this are strict and the onus is firmly on the individual to prove they were exposed to some or all of a list of specific aromatic amines (BNA, benzidine, etc.); they may well die before they even receive a disability pension. Since BNA was banned in the UK in 1949 and world manufacture was supposed to have been curtailed by 1971, it is increasingly hard for men to prove they were in contact with the described aromatic amines. However, recent studies with rubber residues in various tyre factories in Europe have shown high mutagenesis in the Ames test, suggesting that the chemicals used as antioxidants to replace BNA and its equally toxic derivatives (phenyl BNA) may be just as carcinogenic as their lethal forbears. The current treatment for

bladder cancer is removal of the bladder; five-year survival for bladder cancer is between 35 and 40 per cent and it is improving with earlier diagnosis and awareness. However, the pain and discomfort that these patients can suffer make their quality of remaining life relatively poor.

Aromatic amine carcinogens: reductive metabolism

As you know, most of atmospheric pollution is the result of burning fossil fuels. In general, the lighter the fraction of crude oil that is burned, the lower the polycyclic aromatic emissions become. Among the fractions used in transport, diesel is seen as a 'greener' fuel than petrol, in that it contains more hydrocarbons so you can travel further on a given volume of fuel, with lower carbon dioxide emissions. Early diesel engines put out seriously disgusting fumes, which contained large amounts of carcinogenic polycyclic aromatics alongside the particulates, and many studies over the last 25 years or so have shown the very high mutagenicity of diesel emissions. Although cleaner than they were, it seems that no matter which future emission standards they are said to be compliant with, diesels still smoke copiously on hard acceleration and emit a sulphurous stench at (a still very noisy) idle. They remain the main reason why urban air is barely breathable; in fact, diesel emissions are one of the primary sources of mutagenic polycyclic aromatics and nitrogen oxides in the atmosphere – indeed, the most mutagenic substances yet measured are found in diesel emissions. Although there are many mutagens in diesel, the nitroaromatics are the most potent and are discussed below.

Nitropolycyclic aromatics

These compounds are formed during the combustion of many fuels, but they appear to be produced in the greatest amounts in diesel exhausts, particularly when the engine is under high load and hard acceleration, such as in fully, or overloaded trucks. The previous record holder for the most mutagenic compound ever, 1,8 dinitropyrene, was supplanted in 1997 by 3-nitrobenzanthrone (3-NBA; Figure 8.12), which supplied 6 million mutations in the Ames test (Appendix A) per nanomole, compared with the 4.8 million caused by 1,8 dinitropyrene. The metabolic fate of nitroaromatics is a useful example of the conflicting roles that different enzymatic systems have in detoxifying, activating and excreting these toxins. They also illustrate the effects of reduction, rather than oxidation, which is a minor route in drug metabolism, but an important route in carcinogenesis.

Reductase enzymes are found all over cells in the cytosol and you might (or not) recall that they are one of the 'fuel pumps' for CYP isoforms, as they supply electrons that 'power' the enzymes. Reductases are found in tissues that are most likely to meet significant levels of 3-NBA and other nitropolycyclics, such as the lungs and the gut. Since these compounds are thought to be lung carcinogens, it is likely that the reductases form the hydroxylamine which either hits DNA itself through oxidation to a nitrosoarene, or more likely, undergoes sulphation and GST conjugation reactions which lead to the formation of esters and sulphates which decompose, yielding DNA reactive nitrenium ions (Figure 8.12). Whatever reactive species is formed, it certainly binds DNA but does not seem to be formed by CYP enzymes, so it appears that most of the activation is through reduction, followed by conjugation. All the enzymes necessary to activate these compounds

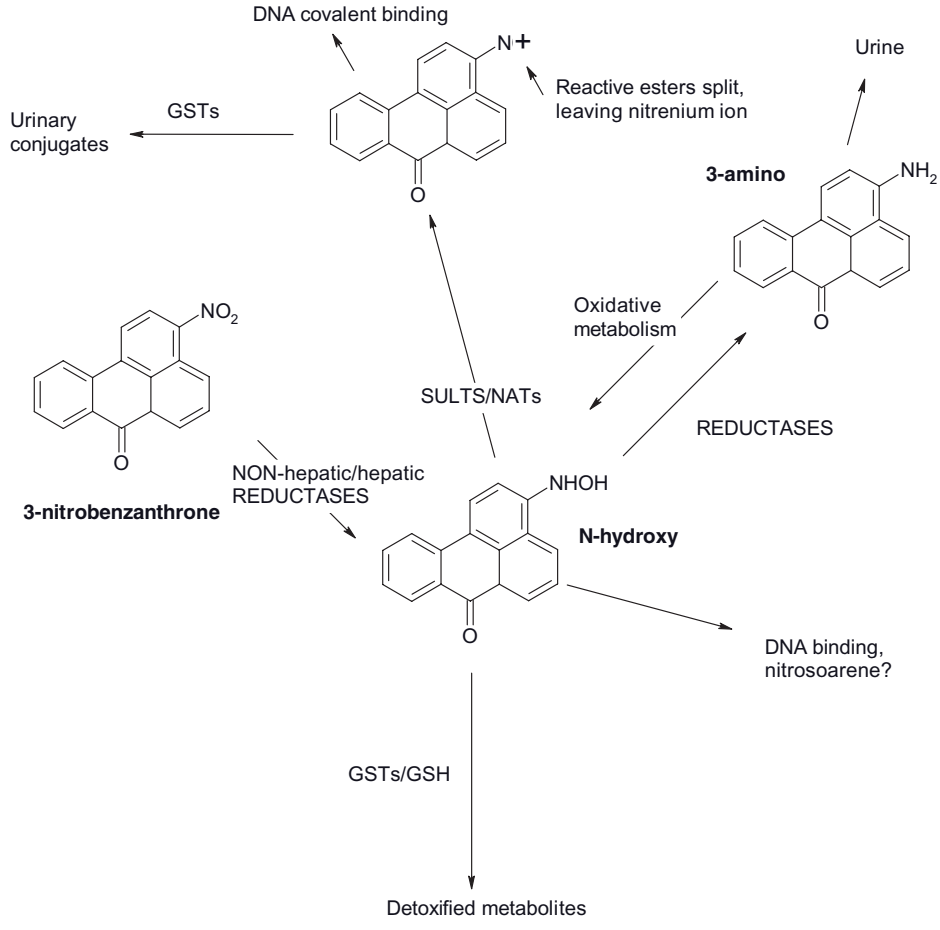

Figure 8.12 Processes of possible activation of 3-nitrobenzanthrone by reductive metabolism

(reductases, SULTs and GSTs) are found in the lung and the gut. Human reductases are particularly adept at this nitroreduction and many have suggested that nitropolycyclics are responsible for many cases of lung cancer in those exposed to diesel fumes, which, unfortunately, is all of us. The interesting feature of 3-NBA activation is that essentially the penultimate stage in carcinogenesis is the same as that for all aromatic amines, the formation of the hydroxylamine, which can then lead to nitrenium ion formation, via an unstable conjugated product. It is important to see the process in terms of varying stages of oxidation and reduction from aromatic amine to nitro derivative and back again. The nitrenium ions formed from 3-nitrobenzanthrone lead to transversion mutations, causing guanine/cytosine to change to Thymine/adenine. Recent calculations involving nitrenium ion stability in the benzanthrone series and Ames test results indicate that the more stable the ion, the more mutagenic it is. This is logical, as mentioned previously, extremely reactive species attack pretty much the first protein they encounter and are simply not stable enough to travel towards the nucleus and reach DNA. There is also some evidence that

3-aminobenzanthrone, one of 3-nitrobenzanthrone's human metabolites, is an inducer and substrate of the CYP1A series as well as NADPH reductases.

Other occupational carcinogens: 1,3 butadiene

Aside from its presence in exhaust emissions, this chemical is among the top 50 chemicals manufactured in quantity in the developed world. It is derived from the petrochemical industry and it is used in the manufacture of styrene-butadiene synthetic rubber in the automotive industry, as well as for making belts, hoses, seals and gaskets, among many products. It has dozens of other uses in the electrical and plastics industries. As this agent is used in such quantity, it is manufactured in many facilities and significant numbers of individuals are exposed to 1,3 butadiene. It is now considered a suspected human carcinogen, responsible for cancers of the lymphatic system, such as lymphosarcoma and reticulosarcoma. The compound has also been less strongly linked to stomach cancers. Like many other organic solvents, such as toluene and benzene, 1,3 butadiene is capable of causing intoxication and there is evidence it is also neurotoxic. It appears that its carcinogenicity is rooted in its oxidative metabolism. Indeed, 1.3 butadiene has been rated amongst the four most toxic agents to human health in the atmosphere alongside acrolein, formaldehyde and arsenic.

1,3 Butadiene is oxidized by CYPs 2E1 and 2A6 in human liver predominantly to a butanediol, which is not thought to be DNA reactive and is more polar than the parent and is conjugated by GSTs with GSH to form highly polar mercapturate-like derivatives, which are Phase III cleared from the hepatocyte. However, there are several minor pathways that involve attempts by CYPs and systems such as epoxide hydrolase to clear the compound to polar derivatives. What is interesting about 1,3 butadiene is the considerable number of highly DNA reactive products that are formed during its metabolism. These include epoxybutene, diepoxybutene, butenediol and epoxybutenediol (Figure 8.13). The diepoxy derivative, also known as butadiene diepoxide and 1,2,3,4 diepoxybutane, is believed to be the key genotoxic derivative. This same metabolite is also formed in patients when they are treated with treosulphan, which is used in ovarian cancers and is a mutagen and a carcinogen. There is also evidence that crotonaldehyde is also formed, which is also DNA-reactive. All of these potential carcinogens can be dealt with by GST-M1 to reduce the possibility of their reaction with tissue macromolecules and it is probably significant that liver cancer is not associated with this compound, despite the number of potentially reactive species it forms. The liver is known for its highly efficient protective systems, and it is possible that 1,3 butadiene is carcinogenic to the lymphatic system and the stomach because various extra-hepatic cell systems can oxidize drugs (either by CYPs or activated neutrophils) to toxic metabolites, but the local protective cellular systems are insufficient to detoxify these species, leading ultimately to neoplasms. The liver also maintains high thiol levels (see paracetamol) and it usually takes very high GSH consumption before critical components of the thiol (cysteine/methionine) are exhausted. Extrahepatic tissues, with the exceptions of the lung, gut and kidney, are likely to exhaust their thiol supplies much earlier than the liver, making them more vulnerable to DNA damage from reactive species such as those generated from 1,3 butadiene.

Much work has been carried out to determine biomarkers of 1,3 butadiene exposure and these include the main urinary metabolites known as M1 (1,2, dihydroxy-4-acetylbutane)

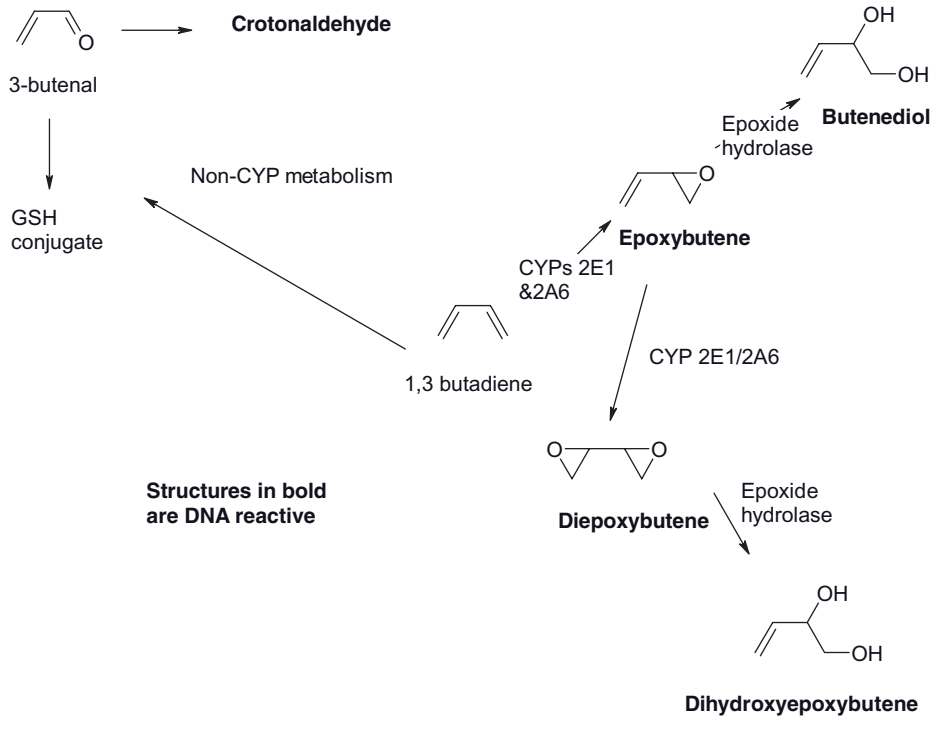

Figure 8.13 Formation of 1,3 butadiene into potential DNA-reactive carcinogens

and M2 (1,2, dihydroxy-4-acetylcysteinyl-3-butene). Interestingly, the key marker for the diepoxide is thought to be a haemoglobin adduct usually written as *pyr*-val. This marker was found in animal studies, but not yet in man, probably because the exposure in the workers was too low. There is a sex-difference in humans, with females forming less of the urinary metabolites than males, but there is no evidence yet that this translates to toxicity or malignancy risk. To date, from modest exposure to 1,3 butadiene, it has not been possible to prove beyond doubt that it is a human carcinogen, although it is unquestionably so in animals. It is likely that in the future the *pyr*-val adduct will probably be found in those more heavily exposed and preliminary findings on epoxide hydrolase and GST-M1 polymorphic status will be confirmed in determining the individual carcinogenic risk. It should be possible one day to screen prospective employees in 1.3, butadiene industries for those with detoxification enzyme profiles which are compatible with a low risk of carcinogenicity for this compound and unfavourable profiles will be rejected for their own good. Unfortunately, this does not help the rest of us who live in cities where the air is polluted with potential carcinogens such as 1,3, butadiene.

8.6.5 Dietary carcinogens

Many carcinogens are thought to be present in foods, although they should be less likely to affect the developed world, due to the degree of processing which occurs in the food industry. The exception is acrylamide, which is formed through the thermal processing of

various cereal and potato products and is ubiquitous in Western diets. Although CYP2E1 oxidizes it to the DNA-reactive epoxide glycidamide and despite its ability to form endocrine tumours in rats, the human carcinogenic risk of acrylamide has not yet been quantitated. Banned carcinogens such as the colouring Sudan I occasionally still appear in foods in the developed world due to oversights, stupidity and greed. Polycyclic aromatic amines are known to be a high risk in the cooking of meat products, however there are several classes of carcinogens that are found in foodstuffs that have been contaminated by moulds and fungi. The most potent are included in the general class of mycotoxins and these are known as aflatoxins.

Aflatoxins: introduction

This class of difuranocoumarins was unknown until 1960, when a bizarre disease wiped out over 100,000 turkeys in the UK. Eventually the disease was found to be caused by contamination of the birds' feed by *Aspergillus* fungus. These fungi grow on peanuts, corn, wheat, maize and many other oilseed crops. It is now believed that some form of mycotoxin contaminates perhaps a quarter of world grain production. *Aspergillus* grows best where conditions of storage are excessively damp and there is a lack of ventilation. The fungus forms dozens of different aflatoxins, which range from merely extremely, to ferociously toxic. They are unusual in that they are mutagenic, immunogenic and carcinogenic to anything living, from birds, animals and fish to humans. Their effects can be resolved into acute and chronic toxicity.

Acute toxicity

Since the 1960s threat to the UK's Christmas lunch, there have been much more serious outbreaks of human acute aflatoxin effects, which are termed aflatoxicosis, particularly in India. This has been due to poverty, where rural people had to eat mouldy cereals or nothing at all. The effects of aflatoxicosis include rapid and massive liver damage, shown by severe jaundice, portal hypertension, abdominal ascites and a condition similar to the effects of cirrhosis, which is quickly fatal in 60–80 per cent of cases. Only a few milligrams per day of the aflatoxins is necessary for these toxins to induce liver failure in days or weeks. In some of the areas in India affected, all the domestic dogs died of the disease just before the humans developed it. These toxins can also cause a disease similar to Reye's syndrome, which is rooted in damage to mitochondria. This complaint is usually fatal and has been described widely. This is rare in the developed world, but the risk of eating food contaminated with aflatoxins and other mycotoxins imported from the Third World is a recognized one for developed countries and monitoring of samples of potentially affected imported foodstuffs is carried out for mycotoxin contamination.

Chronic toxicity

Aflatoxins are potent inducers of hepatocellular cancers and this has been shown in a variety of environments, such as in workers in peanut processing plants in the developed world, as well as in diets in Third World areas. High liver cancer rates have been correlated with the presence of aflatoxin metabolites in urine of affected individuals. These compounds are found in human and cows' milk, as well as in various dairy products, so there

can be considerable exposure to these agents in human diets in many countries. Liver cancer rates in the developing world are far higher than developed areas and it is thought aflatoxins play a considerable role in these statistics. Aflatoxins are reasonably chemically stable, but roasting peanuts apparently decomposes them, so now you know. Aflatoxins affect growth in children and make them more susceptible to bacterial and other infections through immunosuppression.

Activation

The most toxic all these molecules is aflatoxin B1 (AFB1), followed by G1, B2 and G2 in terms of acute toxicity. The reason AFB1 is so dangerous is the presence of a double bond at the 8,9 position in ring 1 (dihydrofuran; Figure 8.14) as well as other substituents of the coumarin (rings 4 and 5). These compounds are thought to rely on CYP activation for their toxicity and the major isoform involved appears to be CYP3A4, but CYP1A2 and CYP3A5 may also be involved. Many metabolites are formed, but the key carcinogenic and general macromolecule-reactive agent is the 8,9 exo-epoxide, formed only by CYP3A4. If you look at Figure 8.14, the best way to visualize the shape of the molecule is to imagine that rings 2–5 are basically planar (flat) while ring 1 sticks up at an approxi-

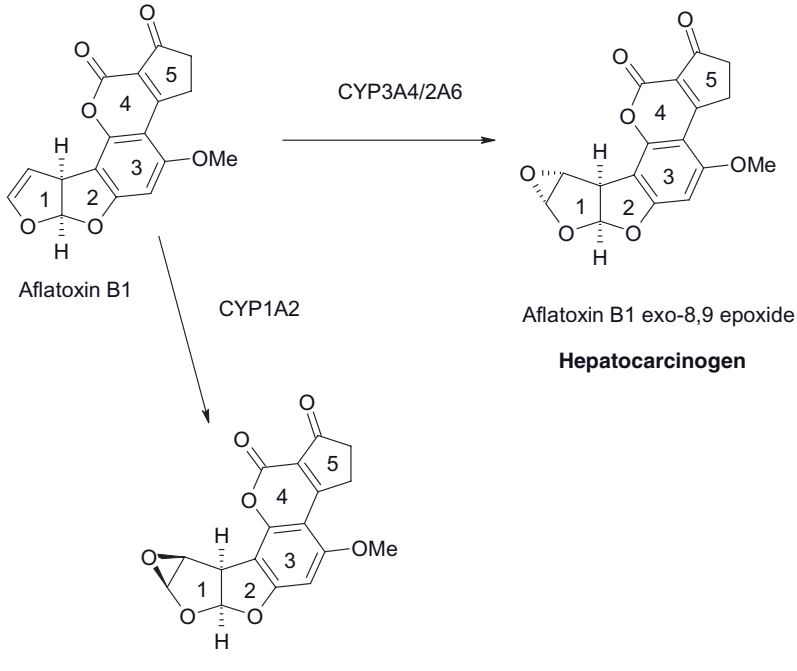

Figure 8.14 Structure of aflatoxin B1 and its carcinogenic and non-genotoxic metabolites

mately 45° angle to the horizontal. The term 'exo' for the epoxide means that the oxygen dips away from you as you look at the molecule on the page. There is an 'endo' epoxide, where the oxygen would be oriented towards you as you looked at the molecule.

The reason the 8,9 exo-epoxide is so dangerous is the precision where it interacts with DNA. It binds at the N7 position of guanine residues. This is obviously a direct consequence of the three-dimensional shape of the molecule, as the 8,9 'endo' epoxide and other aflatoxin metabolites do not intercalate with DNA in this position and are not as genotoxic. This intercalating effect of the epoxide causes a transversion of guanine and thymidine at codon 249 of the p53 gene of the liver. This may be linked with the route of induction of hepatocarcinoma. It is likely that dietary inducers of CYP3A4 and probably CYP1B1 will increase aflatoxin activation, in both humans and animals.

Detoxification

It would be expected that processes would detoxify the 8,9 exo epoxide and the most obvious candidates would be epoxide hydrolase, GSH and GST enzymes. The detoxification of this metabolite is not completely understood; although its half-life is so short (around 10 seconds), epoxide hydrolase may well not have a significant role in the formation of the diol, which apparently has an even shorter half-life. GSH does not react directly with the epoxide at pH 7, so an important route of clearance from the cell is likely to be via GST catalysed thiol conjugation. GST Alpha, Mu and Theta are thought to be involved with aflatoxin 8,9 epoxide clearance. Individuals with no expression of GST M1, (null individuals) have a much increased risk of malignancy due to aflatoxin B1.

Prevention of toxicity

Since these toxins make a massive impact in developing world health, the major concern is to educate ordinary food producers about the dangers of poor feed storage. In addition, the alleviation of poverty, although highly unlikely, would be invaluable in preventing the situation where there is no choice but to eat contaminated food. On a more realistic preventative level, it is known that it is possible to decontaminate feedstuffs with ammonia treatment, which has been shown to reduce the capability of aflatoxins to cause tumours in animals, most likely by chemical decomposition of the toxins by the ammonia under reduced pressure. Other systems are being developed which use UV light to destroy the aflatoxins on the surface of nuts, although this is not effective against all the toxin's derivatives as yet and takes several hours. For the food consumers themselves, inhibitors of CYP3A4 should be protective, although it is difficult to see how this would practically be applied to prevent aflatoxin carcinogenicity in everyday diets.

Studies with the chemopreventative dithiolthione oltipraz demonstrated that these agents have some promise in preventing the toxicity of dietary agents such as aflatoxins. The dithiolthiones operate through Nrf2 (Chapter 6, section 6.5.4) to increase GSH synthesis and they induce GST, thus stimulating detoxification of reactive species. Although clinical trials in China had mixed results, there was some reduction in urinary aflatoxin adducts, although there were issues with side-effects as quite high doses were necessary to demonstrate efficacy. Other newer members of the class of dithiolthiones, such as TBD, may

be more effective in future in clinical trials. Vitamin C has been shown to be protective in cellular systems, with Vitamins E and A less so. The process of aflatoxin carcinogenicity is now reasonably well understood and the main thrust of current research is to find a practical, yet inexpensive means to diminish the formation and effect of aflatoxin-related reactive species. In the real world, it is highly unlikely that aflatoxins can be removed from human diets. Therefore there is strong interest in dietary activators of the Keap1-Nrf2-ARE system such as sulforaphane (Chapter 7, section 7.4.3) as well as the new dithiolthiones, which will attenuate the toxic species, even if their formation probably cannot be prevented.

8.6.6 Type B3: cancer

- Linked to antineoplastic drugs, pollutants and dietary toxins.
- Irreversible changes in tissues often where cell growth is continuous.
- Future neoplasms may occur in tissues unrelated to the original tumour.

8.7 Summary of biotransformational toxicity

Although drugs and toxins may occasionally act as parent agents to cause irreversible changes in cell structure and function, the major part of the process of necrosis,

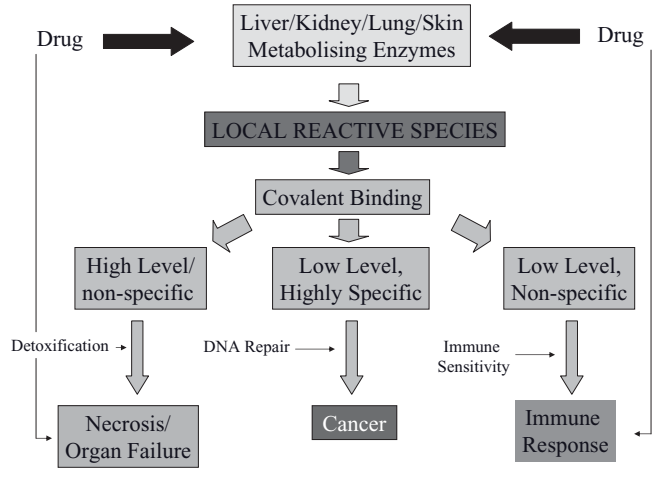

Figure 8.15 The links between drug metabolism, formation of active species, covalent binding and irreversible damage to the organism. Conjugative detoxifying (UGTs, SULTs and GST/GSH) defences are crucial to prevent organ failure from high levels of covalent binding, whilst detoxification mechanisms as well as DNA repair are crucial to avoid carcinogenicity as a result of specific DNA binding of reactive species. For an immune response, the sensitivity of the individuals' immune system may be the major determinate of whether a response occurs. Although covalent binding is linked with most routes of toxicity, the effects of the parent drug cannot always be ruled out

immunological damage and malignancy, is linked with the formation of unstable and damaging species from a xenobiotic compound (Figure 8.15) which leads to oxidative stress. Ultimately, virtually all of us form these species, but the combination of our net exposure to these toxins and our individual detoxification and repair mechanisms decide whether we will suffer Type B toxicity or tolerate and clear the agent without ill effects.

Appendix A
Methods in Drug Metabolism

A.1 Introduction

There are two main reasons why candidate drugs fail to reach the market: either they don't work or they are toxic. However, if a marketed drug is abruptly withdrawn, then toxicity alone is likely to be the reason. You might be aware that the costs of developing a new drug now exceed the billion mark, at least in dollars. What is not generally appreciated is that this huge sum is dwarfed by the costs of withdrawing a drug from the market. So human toxicity is a key issue with the development and successful marketing of drugs and it is now clear that biotransformation is a highly significant component of this toxicity. This may be through inhibition or induction impacting on parent drug levels leading to off-target effects, or by direct bioactivation to a damaging reactive species. Although drug design and initial candidate selection will always begin with pharmacodynamic performance, the drug industry has learned the hard way that the link between biotransformation and toxicity must be evaluated thoroughly as early as possible in a lead candidate drug's lifetime as part of ADMET, (absorption, distribution, metabolism, excretion and toxicity) *in vitro* and *in vivo* studies.

In most countries it remains a legal requirement for basic toxicological whole animal studies to be carried out with a new drug and the hepatic metabolism of the drug will be determined *in vitro* using the same animal model. Although these initial 'pre-clinical' studies are intended to predict the potential human toxicity of the drug, in some instances they are poor value scientifically and financially. In reproductive toxicity, the predictive power of animal studies is so poor there is no real scientific justification for even carrying them out. In addition, current animal models only have a 1 in 2 chance of predicting human hepatotoxicity, which is probably the commonest reason for drug withdrawal, although animal studies are admittedly more predictive for gastrointestinal and cardiotoxicity. Unfortunately, aside from the examples quoted in this book in Chapter 8, there are many other instances of drugs that were safe in animals but toxic in man. Alternatively, we will never know how many other agents that were toxic in animals, such as tamoxifen, might have turned out to be safe and valuable in man.

Leaving aside ethical issues, current animal pre-clinical toxicity testing is not sufficiently predictive of human toxicity to protect patients from drug toxicity and drug company shareholders from huge losses. Fortunately, enormous strides have taken place

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in the past ten years in improving the efficiency and widening the scope and applicability of existing human-based models and developing new ones that can provide evidence of possible drug toxicity which will be more relevant to man than basic animal studies. Now it is possible to make reasonably accurate determinations of the essential features of a drug's biotransformation which will minimize the current risks involved during Phase I initial human volunteer trials, as well as reducing unnecessary animal suffering. Humanized mouse models are under development to attempt to explore the 'whole system' effect on a candidate drug. With the accumulation of data from a variety of sources, *in silico* systems are actively assisting in the design of drugs by considering pharmacokinetic and toxicokinetic standpoints as well as pharmacodynamic performance. Other techniques on the distant horizon include the application of stem cell systems, which may one day supplant key techniques such as hepatocytes, although this work is very much in its infancy.

Overall, as part of the drug development process, there are various attributes of a new drug that pertain directly to biotransformation and toxicity which need to be determined, or at least estimated, prior to the instigation of clinical trials in man.

- *Rate of its metabolism:* drugs which are very rapidly, or very slowly, cleared can present problems in accurate control of plasma levels and with very long half-life agents, the risk of toxicity can be considerable.

- *Multiple CYP metabolism:* this is a very positive attribute, as if many CYPs can clear the drug and one or two are inhibited by co-administered drugs, there will usually be a route of metabolism to prevent serious accumulation.

- *Likelihood of DDIs, inhibition:* if a drug is likely to inhibit a major CYP isoform, or its metabolism is blocked by a known series of inhibitors, then the likelihood of DDIs or drug–drug interactions will be high.

- *Likelihood of DDIs, induction:* a powerful inducer will be problematic in complex regimens and will accelerate the clearance of other drugs, as inducers tend to be very broad in their effects on the main human CYPs. DDIs will be promoted through induction and treatment failures may result.

- *Single CYP clearance:* this can be seen as vulnerability, as inhibition of the CYP will easily lead to accumulation and potential toxicity. Conversely, induction of this CYP could lead to treatment failure.

- *Polymorphisms:* if the metabolism of the drug is dependent on a CYP isoform that is polymorphic, this erodes the mass-marketability and safety of the drug. Clinically, a drug that was subject to a single polymorphic CYP clearance may well be restricted in its usage or would require close medical supervision, as the risks of toxicity and drug failure would be considerable.

- *Linearity of metabolism:* most drugs are cleared at a rate proportional to their intake (linear metabolism). Some (ethanol, phenytoin) are cleared at a constant rate, irrespective of intake (non-linear metabolism). If the metabolism of the drug is non-

linear, then it will be difficult to predict plasma levels with ascending dose, which will also make the drug difficult to use and subject to toxicity and potential failure.

- *Metabolism to toxins:* drug metabolism may lead to the formation of toxic products in some or the entire target patient group.

- *Off-target gene expression effects:* the drug and/or its metabolites may cause marked changes in gene expression in certain organs or specific patient groups with unique pathologies.

The first step before the investigation of these features of a candidate drug can begin is the selection of some practical means of detecting the drug and its various metabolites in biological fluids.

A.2 Analytical techniques

When a candidate drug progresses to the point where it will be toxicologically and metabolically tested, it is necessary to choose a method or assay which will quantify the drug and its metabolites reliably in various *in vitro* and *in vivo* experimental models, which are usually aqueous and contain biological material such as enzymes and other proteins. The assay requirements are sensitivity, selectivity and reproducibility and some form of chromatography is usually employed. High-pressure liquid chromatography (HPLC) has always been one the most practical methods used to detect most low molecular weight (<400) drugs and related material, usually 'reversed phase'. This means that the column contains a non-polar stationary 'phase' (the packing) and the mobile 'phase' is water-based and polar, involving solvents such as acetonitrile, methanol and different additives. As most drugs are usually fairly non-polar, they are repelled by the mobile phase and tend to associate with the non-polar stationary phase for much longer than the more polar metabolites and the other cellular material from an experimental incubation. This leads to good chromatographic separation from unwanted material and facilitates accurate drug quantification.

HPLC assay development usually starts with authentic standards of the drug; this is to establish the individual solvent proportions and other conditions to ensure that the drug is retained on the column long enough to be well clear of the more polar metabolites. At this point, another chemical entity that is very close in structure and physicochemical properties to the drug is selected and chromatographically tested to be an internal standard (IS). The IS becomes a yardstick with which to measure the drug and compensate for variation in the assay and it should be retained on the column in a similar fashion to the drug. To 'see' the drug, some form of detection is necessary, which most often exploits UV or fluorescence absorption, as these are very common properties amongst therapeutic drugs. Other agents that do not absorb UV or fluoresce can be measured using systems such as electrochemical detection. Once the chromatography is established, some form of extraction must be used to remove the drug and the IS (added just before the assay) from the aqueous matrix. This can be through the use of organic solvents or disposable miniature solid phase columns. The solvents dissolve the drug and metabolites, but not too much of the biological material and they can be quickly evaporated and the sample

resuspended in an alcohol. The solid phase columns are prepared and the sample introduced. The column holds the drug and metabolite and the residual biological material is flushed through. The drug and metabolites are then 'eluted' with methanol or a similar solvent. The main aim of this stage is to leave behind the proteins and cell contents and produce a relatively 'clean' uncontaminated sample containing drug and hopefully some metabolites. Once the extraction procedure is optimized, the suspected metabolites can be separated and routed to more intensive analytical systems, such as mass spectrometry or more often NMR (nuclear magnetic resonance) machines which can provide enough information to assign a structure to the metabolites, so authentic standards can then be made to study their stability and toxicity. Although there are other chromatographic methods, such as gas liquid chromatography, its advantages of high sensitivity can be outweighed by its operation mode, where the sample must be vaporized at high temperature to be analyzed. Many unstable metabolites may not survive this process. The isolation and assignment of structure to reactive species is much more difficult and often the best hope is to use a 'trap' molecule such as glutathione which reacts with the species and forms a GS-adduct, which can be analyzed with mass spectroscopy or NMR to determine which area of the molecule has attacked the thiol. Other systems may use proteins to trap a species, such as the haemoglobin adduct used as a butadiene diepoxide marker (Chapter 8, section 8.6.4).

There are several other systems that can quantify drugs of a variety of different structures and sizes, particularly peptides and high molecular weight antibiotics, such as immunoassays. These use the high specificity of fluorescently labeled antibodies to bind drugs and these systems are often used for therapeutic monitoring of steroids and other agents and are capable of detecting sub-picomolar concentrations. Overall, most drug analysis systems can now be fully automated and are highly sensitive and selective. They are essential to determine the fate of the drug and its related metabolites in the different ADMET experimental systems. The following sections describe some of the main techniques employed in the evaluation of biotransformation and toxicity in drug development.

A.3 Human liver microsomes

The most basic tool for the determination of the metabolic fate of a candidate drug is the fractionation of human liver through differential centrifugation. The liver is homogenized on ice and centrifuged at around 10000*g* to remove cellular and nuclear membranes, as well as larger cellular organelles. The supernatant is then centrifuged at 100000*g* to provide a pellet that contains the smooth endplasmic reticulum (Chapter 3) where the CYP isoforms and UGTs reside. This is termed a 'microsomal' pellet and it is then stored at least −70°C until the content of the total CYP content is measured using a standard method that exploits the ability of carbon monoxide to bind the CYPs. Every human liver also has a unique profile of individual CYP isoforms and the livers are usually characterized in terms of their ability to metabolize certain substrates that are cleared by particular CYP isoforms. As there is so much variability in human liver microsomal systems, in trying to determine the metabolic profile of a candidate drug, it will be studied using many human livers to build up a picture as to which isoforms predominantly oxidize the drug. The products of the oxidation are usually extracted and purified, then analysed as described

above. It is possible to confirm which CYP isoforms are oxidizing the drug by the use of specific CYP inhibitors as well as antibodies which have been raised to CYP isoforms, which also block their activity. It is also possible by using specific substrates of various isoforms to determine if the new agent has any inhibitory effects on the main CYP isoforms.

Once the CYP or CYPs have been identified which are responsible for the oxidation of the drug, other microsomal studies can be carried out using human lung, kidney and gut to determine what contribution these organs will make to the overall oxidative metabolism of the drug. The addition of UDGPA as a co-factor will activate the UGTs in the liver to provide a preliminary evaluation as to the degree of glucuronidation that may occur. Human liver cytosolic fractions (S9 fractions) can also be used to determine levels of sulphation and GSTs. In addition, cytosolic and microsomal epoxide hydrolase activity can also be measured.

Supplies of human liver have dwindled somewhat, happily because liver transplants are now more common and more successful than in previous years. The disadvantages of human liver homogenate preparations include the wide variation and the detailed observation of only some parts of a complex system of metabolism. If a very reactive oxidative metabolite is detected which is cytotoxic, this suggests that the drug could be a hepatotoxin or may be reactive elsewhere. However, what cannot be determined with human microsomes is that detoxification and transport systems might just as well avidly clear the toxic metabolite and no systemic or organ-directed toxicity occurs. In microsomal studies, a drug may be a potent CYP inhibitor but *in vivo* it would not penetrate cells due to its particular physicochemical properties or lack of a suitable transporter. That microsomal incubations are not a complete system also prevents realistic estimation of hepatic intrinsic clearance (Chapter 1, section 1.4.1). Clearly microsomal studies are a useful but far from definitive stage in determining a candidate drug's metabolic profile.

A.4 Human hepatocytes

Although obtaining human liver suitable for the preparation of hepatocytes is always likely to be expensive and problematic, modern effective cryopreservation techniques mean that their use in drug discovery and academic study is now much more practical and prevalent. Hepatocyte preparations remain the key experimental technique for modelling drug biotransformation from a number of standpoints, indeed the phrase often used in publications is 'gold standard'. They can demonstrate that a drug is physicochemically able to enter the liver, with or without influx transporter assistance. The complete oxidative and conjugative fate of a drug can be explored in a single model and their use in a suspension allows rapid diffusion of drug into the cells as would occur *in vivo*, which notably does not occur in other techniques, such as liver slices.

Hepatocytes are often sourced from livers unsuitable for transplants, as they are too old, fatty, young, diseased, damaged or unsterile. The best quality hepatocytes are obtained from livers that have been cold preserved for 24 hours or less. The standard two-step method of cell preparation involves a body temperature perfusion with calcium-free isotonic buffer containing a chelator to remove all divalent metals and to flush out any blood. This is followed by another warm flush, this time with collagenase to break up the tissue matrix. Generally, if a whole liver is treated, only around 10 per cent of the hepatocytes

(there are 300 billion in an adult healthy liver) will be harvested, although the yield is better from smaller liver fragments. Many studies have shown that cryopreserved hepatocytes (stored with DMSO in liquid nitrogen) show similar CYP, conjugative and OATP transporter viabilities to freshly isolated cells.

Limitations of hepatocyte usage include variability and the short duration of experiments. The cells lose around half their CYP expression every 24 hours and many studies are only carried out over 5 hours or so. Transporter expression does not always survive freezing and GSH levels can be low when the cells are revived. However, the donor variability problem is usually circumvented by using cells from at least five livers to conduct studies and some cryopreserved cells can be grown on collagen coated plastic plates as monolayer cell cultures and these can last for up to three days. Of course, with any human tissue there remain risks linked to human infections, such as human variant CJD, hepatitis and HIV.

The initial use of hepatocytes in drug development is to calculate the intrinsic clearance of new agent. To predict whether a drug might be a low or high extraction agent, the intrinsic clearance must be related to whether a drug is strongly protein bound or not *in vivo* as well as to blood flow. To compensate for the issue of free bound drug some studies have included serum or various proteins in hepatocyte incubations, although this is controversial. Other authors have shown that hepatocyte data combined with mathematical models for protein binding and liver blood flow can yield convincing estimations of *in vivo* clearance. These values are very important for the estimation of the dosage ranges that will be used for drug development in human trials. Hepatocytes are also used to determine metabolic pathways, such as those involving conjugative products. Indeed, if the major metabolites are conjugates, then it is not really necessary to look at drug clearance from microsomal fractions.

Drug metabolism inhibition studies are well established in hepatocytes and they also demonstrate that both inhibitor and 'victim' drug can attain cellular concentrations that are relevant *in vivo*. Both reversible and time-dependent inhibition can be studied in hepatocyte cultures, often by the use of cocktails of CYP-specific substrates and various inhibitors in six-well plates, which maximize the data extracted from the valuable cultures. Some studies in hepatocytes demonstrate lower inhibitory drug effects compared with expressed systems (see next section) and some are the reverse. This may be linked to cellular binding concentrating effects and other metabolizing systems, which are again more representative of the 'real world'.

The advent of the monolayer-plated cultures described above has been a major advance in the modeling of enzyme induction, which is tracked by measuring increases in the appropriate mRNA as well the CYP itself. Hepatocytes have the full functioning complement of nuclear and cytoplasmic receptors necessary to respond to any inducer and all *in vivo* inducers show their effects in hepatocytes and it is thought unlikely that a new agent shown to be a hepatocyte inducer would not act similarly *in vivo*. The ability to model induction and inhibition realistically is a powerful advantage of hepatocytes in the prediction of drug-drug interactions (DDIs) *in vivo*. The US FDA defines a new agent as an inducer in hepatocyte studies if it doubles control CYP expression or produces more than 40 per cent of a positive control's inductive effect.

Hepatocytes also have immense value in the study of influx transporter effects on drug metabolism (OATPs) as well as in DDIs. Indeed, confirmation that the interaction between cerivastatin and cyclosporine was caused by inhibition of transporters rather than by

metabolism was through hepatocyte studies. In contrast, it is important to note that efflux transporters, such as the MRPs can only really be studied in so-called sandwich cultures, that is, they are grown between two gel layers (sandwich cultures), which can also maintain CYP expression. The use of human hepatocytes as a model is under continuous development and their role in drug toxicity has been expanded significantly with the use of DNA arrays that will be described in a later section.

A.5 Human cell lines

Although primary cultures isolated from humans or animals are probably most scientists' preferred models for studying different processes in complete cells, they are logistically difficult to handle, as they require access to donors and specialized isolation procedures and infection risks are ever present. Experimental studies are likely to be fairly short, as once separate from the organism, primary cells gradually cease to express the features that make them specialized. Immortalized cell lines were introduced as an alternative to overcome these disadvantages, as they are easy to prepare, propagate and maintain. They originate from tumours and if you have either witnessed or even endured the remorseless effects of malignancy you will appreciate that these cells display rather too many 'Terminator'-like highly abnormal characteristics of their parent tumour, such as resistance to toxicity, overexpression of detoxification and transport systems, manic growth and abnormal energy metabolism. One of the main reasons why hard tumours are resistant to radiotherapy is that this treatment acts by creating reactive oxygen species and oxygen is almost absent in anaerobic tumours, as they only build themselves a minimal blood supply while they grow. The cells derived from these tumours retain this anaerobic profile, using glycolysis instead of oxidative phosphorylation and they are usually grown in a high carbon dioxide, reduced oxygen atmosphere. Although their mitochondria are functional, they are greatly underused, so these cells lines are often highly resistant to reactive species or metabolic toxins that target mitochondria. Hence, many immortal cell models underestimate the effects of candidate drugs that cause oxidative stress through this route. Nonetheless, the convenience and robustness of these lines make them a well-used screen for both toxicity and pharmacodynamic drug effects and most lines retain enough characteristics of the original tissue to have some relevance to the *in vivo* situation. Amongst the many lines employed, HepG2 cells were isolated in the early 1980s from a hepatoblastoma and they are used to model some aspects of hepatocyte function, such as CYP induction. Unfortunately, their basal CYP expression is on the whole very low and variable. Indeed, some CYPs are switched off completely. Several studies have shown that mRNA levels are low in these cells and translation to actual CYP protein, with CYPs such as 1A1 and 1A2 is very much lower than intact animals, or human hepatocytes. Often it is necessary to stably transfect human CYP genes to model a cellular response to a metabolite. HepG2s do lend themselves well to automated high-throughput toxicity assays, known as high content screening (HCS), which pre-loads cells for three days with potential toxins at up to 30-fold higher concentrations than intended clinically. Fluorescent dyes are used to measure multiple markers of response to toxicity and these models have been reasonably predictive of some human toxicity. The most sensitive markers have been shown to be cell proliferation and nuclear membrane area. Other studies have shown that if culture conditions are modified, such as by using galactose instead of glucose, many cell lines

become more aerobic in their metabolism and their response to mitochondrial toxins and oxidative stress inducers becomes more realistic. This is particularly relevant in the light of the critical role of mitochondrial toxicity in several human hepatotoxins, such as troglitazone and trovafloxacin. Although HepG2s will never replace human hepatocytes, they nevertheless occupy a useful niche in drug development.

A.6 Heterologous recombinant systems

Once the basic parameters of the metabolism of a new drug have been established, such as which CYPs and conjugative systems are involved and it is established in whole cell studies such as hepatocytes that the drug enters cells and reaches relevant intracellular concentrations, specific components of the metabolic profile can be focused on through the use of heterologous systems. Although many of these data can be obtained from microsomes, the obvious advantage of heterologous systems is that they are easy to obtain and don't depend on a problematic supply of human livers. In the late 1980s and early 1990s, the technology for the excision of complete human CYP and conjugative genes as well as their reductases and ancilliary support systems was developed and insertion into bacterial, eukaryotic or viral vectors was perfected. This allowed the almost unlimited expression of specific human CYP isoforms, as well as many UGTs, SULTs and GSTs. This facilitates extremely detailed studies of how a particular isoform metabolizes a candidate drug, along with inhibition studies and detailed kinetic measurements, such as K_is and the rate of formation of the metabolite, which is free from the influence of other metabolic pathways. These systems are useful for examining the clearance of drugs with individual rCYPs (recombinant CYPs) in a variety of concentration ranges, so teasing out the detailed profile of clearance systems in all dosage ranges. This might provide data on how dependent on cellular concentration particular DDIs might be. Recombinant CYPs can also be adapted to high capacity nanolitre assays where the CYP is embedded in alginates and fluorescent visualization allows very high throughputs of test agents to screen for metabolite formation. The burgeoning study of polymorphisms also allows detailed evaluation of the catalytic activities of various polymorphic isoforms, which might again inform on how a particular population may clear a drug. This is extremely relevant in the global marketing of drugs that occurs in the face of such radical human diversity, which was described in Chapter 7. Heterologous systems are also used to provide plentiful supplies of expression transporters such as the OATPs, P-gp and the MRPs, to determine whether they are inhibitors, substrates or both. There is an assumption that the gene cloned and expressed is identical to the CYPs in all patients, which may not necessarily be true. Of course, rCYPs are not applicable for enzyme induction studies.

A.7 Animal model developments in drug metabolism

A.7.1 Introduction

Although hepatocytes and other models may provide a great deal of relevant information on the clearance of a drug, they cannot model the complete living system of an intact

human. Extensive research as shown that current animal models cannot achieve this either, due to large numbers of species differences at virtually every level of hepatic function, ranging from nuclear receptor-mediated induction, PXR, CAR and the various detoxification and transport systems. The failure of conventional rodent systems to highlight the toxicity of the mitochondrial hepatotoxins underlines the depth of these species differences. Despite these problems, the aim of drug development is to explore ADMET in a whole system, so there will always be a drive to create such a system capable of transcending species differences, but be applicable to humans.

The mouse has emerged as the most commonly used animal model for human drug metabolism study, although the range of rodent CYP expression is nearly double ours and the catalytic activity of roughly equivalent rodent isoforms (usually written in lower case) does not resemble that of humans closely enough, with the exception of cyp1a2 and CYP1A2, as well as cyp2e1 and CYP2E1. In addition, the processes that impact most strongly on human drug clearance, such as induction and inhibition, are too different in mice to be meaningful for man. However, the mouse genome is relatively easy to manipulate and in the sections below, you can gain some idea of the current state of the development of the mouse as a model for drug metabolism and toxicity in man.

A.7.2 'Knockout' mice

Since the mid 1990s, 'knockout' animals have been produced where a complete cyp or enzyme system has been deleted. Although the deletion of some enzyme systems is fatal (such as POR, Chapter 3, section 3.4.6) much can be learned of the homeostatic roles of various isoforms, and the technology has progressed to the point that several knockout mouse cyp models now exist such as cyp1a2 and cyp2e1 null strains. These cyps are relatively applicable to the human versions, so if a knockout mouse does not suffer the toxicity or carcinogenicity of a given toxin, then it is likely that the absent cyp would have been responsible for clearance of the agent and production of reactive species in the wild-type. Cyp1a2 knockout mice have shed light on the activation of the aromatic amine carcinogen 4-aminobiphenyl (Chapter 8, section 8.6.4), with the result that this amine is probably cleared by a number of CYPs in man rather than just CYP1A2. The complex role of cyp2e1 has also been explored in null animals and has been informative in the study of benzene activation and the role of lung cyps, which are revealed in the absence of the hepatic cyp. Overall, knockout technology has been useful toxicologically, but not necessarily from the drug development perspective. However, the application of knockout methods has been instrumental in the 'humanization' of mouse models, as described in the next section.

A.7.3 'Humanized' mice

'Humanization' of mouse models has progressed from the insertion and deletion of genes all the way to the creation of 'transgenic' mice. This work has been a high priority to try to improve the extrapolation of data derived from animals to the human situation. Several approaches have been tried to achieve this aim. The idea of 'knock-in' animals is now established, where complete human genes, have been inserted into mouse genomes,

although this can be problematic as they have to operate surrounded by mouse genes. This has been progressed where the mouse genes that express similar cyps to human CYPs are knocked out and the human genes inserted. Humanized CYP2D6 mice have been used to reproduce the gene expression of PMs, IMs and EMs. The role of HNF4α was understood by deleting this gene to determine its effects on CYP2D6 expression in the humanized mice. It is thought that this model will be of some value in polymorphic research as well as drug development. The most successful humanized CYP2E1 model is also cyp2e1 null, so eliminating the endogenous cyp expression. This model may facilitate the study of alcoholic liver disease and CYP2E1 control of expression. Although a CYP3A4 mouse model would be highly desirable, the first one in 2003 only expressed the CYP in the gut and not the liver. CYP3A4 is highly gender specific in mice and this complicated the production of then humanized animals. There are also doubly humanized CYP3A4/CYP2D6 models available, although models that employ the complete CYP3A4 gene are under development. One interesting CYP3A4 humanized mouse model had a luciferase gene inserted close to the CYP gene and this acts as a 'reporter' of CYP upregulation, as the luciferase expression can be measured by an imaging system *in vivo*. This system allows the study of CYP3A4 expression under various stresses and stimuli. However, even when CYP3A4 humanized animals are cyp3a null, there other mouse cyps are still present and may affect the human CYP's expression and *in vivo* performance.

To address the problem of the mouse CYP environment, the obvious answer was to replace the mouse's liver with a human one. In 2004, this was achieved, where immunodeficient mice that had liver failure had over 95 per cent of their livers replaced with human hepatocytes and these grafted cells are not rejected. These hepatocytes function and retain the characteristics of the donor, in terms of polymorphisms and responses to induction, inhibition and clearance. These 'chimeric' mice may have a role in examining the whole system aspect of ADMET, although the gut and the rest of the animal are obviously not human, so they are not a faithful human model in all aspects and they have to be 'constructed' and cannot be produced by breeding in quantity. However, during studies with warfarin and several human inducers and inhibitors, human, rather than mouse profiles of metabolism have been achieved and it is likely that these models will have value in predicting hepatic drug interactions in the future.

A.8 Toxicological metabolism-based assays

For an experimental drug, much work will obviously focus on the particular metabolizing system that might form reactive metabolites. However, it is also important to visualize what these species will actually do to a cell. So, in the last 30 years, considerable efforts have been made to determine what effect toxic metabolites might have on human cells or tissues. This requires the design of *in vitro* systems that can model the balance between the formation of toxic species and their effects on a human target cell. The simplest test of all is probably trypan blue exclusion, where recently dead cells cannot exclude the dye and swell as well as looking bloated and grotesque. However, it is essential to investigate much more subtle and early indications of drug and reactive species toxicity and there are several specialized assays that focus on reactive species, mitochondria and DNA-linked effects of drug toxicity.

A.8.1 Ames Test

Although originally developed in the 1970s to determine if a chemical was mutagenic, it was quickly realized that the effects of reactive species could easily be incorporated into the test to make it relevant to drug development. Briefly, the test uses *Salmonella typhimurium* bacteria, which have been altered to make them dependent on supplied histidine in their media. If they are plated out onto media without histidine they will not grow. If a mutagenic agent is added, it causes reverse mutations and the bacteria grow on the deficient media as they can now make their own histidine. Essentially there is a linear relationship between the colonies formed and the mutagenicity of the chemical. The addition of human microsomes and S9 fractions made the test useful for new drugs. Indeed, all the major human CYPs can currently be expressed in separate strains of Salmonella to determine if the test agent has the potential to form a mutagen. It is important to carry out this test before a large investment is made in a compound, as if it fails, then it's 'dead'. Interestingly, several older drugs do fail it, such as isoniazid. Aflatoxins obviously fail, as, of course, do loathsome diesel emissions.

A.8.2 Microarrays

These assays depend on amplifying the mRNA from cells or tissues exposed to toxic agents and comparing it with control mRNA expression. Single high-density microarrays are now available which contain virtually every known human gene. This creates a vast amount of data that can require complex software systems to process it all. In addition, it can be difficult to determine the practical relevance of specific gene changes in the expression of so many genes that may or may not have been relevant to a drug's effects on an organism. However, knowledge of genes specifically involved in responses to toxicity, as well as detoxification are now much better documented and as you will have read in Chapter 8, giant strides have been made in the focusing of arrays and using gene expression as an endpoint after toxic pressure is now termed 'toxicogenomics', which has become a new sub-discipline in toxicology. The combination of array data and toxicogenomic databases of known toxicants and their gene effects has facilitated the focusing of microarrays which are tailored to certain predictive 'biomarker' genes which are critical in the development of organ toxicity. Biomarker genes or 'tox' genes include inflammatory process genes, such as those expressing interleukins and other cytokines, as well as genes involved in the defence against oxidative stress, such as the GSH maintenance enzymes, superoxide dismutase and catalase. DNA repair, cholestasis, immunotoxicity and mitochondrial functional genes are amongst many that are radically changed in expression under toxic pressure. In addition, patterns of differential up and down regulations can occur in response to certain toxins; the hepatotoxin carbon tetrachloride will down regulate CYP3A4 and upregulate MDR-1, whilst other toxins might downregulate DNA repair, yet upregulate a CYP that tends to form reactive species, such as CYP2E1. In addition to the effects a toxin on groups of genes, there is evidence that arrays are compatible with other endpoints as they are dose-dependent. Microarray studies in human hepatocytes in the presence of known hepatotoxins troglitazone and trovafloxacin illustrated the power of the combination of two highly relevant techniques to man. These drugs induced oxidative stress in human livers and this was reflected in

their effects on gene expression. Troglitazone upregulated a large number of tox genes that its safer sister drugs, pioglitazone and rosiglitazone did not. Among the many genes affected by trovafloxacin, Bax and Mitofusin-1 were downregulated, which are important genes in the maintenance of mitochondrial health. Presently, drug development programmes have access to combinations of focused microarrays and toxicogenomic databases of predictive gene biomarkers so it should be unlikely that hepatotoxins displaying similar characteristics to troglitazone and trovafloxacin will reach the market again. In the future, databases will be expanded and biomarkers for immune-mediated hypersensitivity will be characterized and may yield clues on possible susceptible patient populations.

A.8.3 Comet Assay

This assay detects DNA damage in cells by subjecting the DNA to an electric current in a gel (electrophoresis) in solutions of different pH. The DNA moves through the gel forming a characteristic 'comet' like shape and DNA strand breaks are visualized using fluorescent dyes.

A.8.4 Micronucleus Test

This test can be carried out *in vivo* or *in vitro* and this detects micronuclei, which are formed during mitosis when a chromosome is lagging behind and may be lost (aneugenic event) or when a chromosome is broken (clastogenic event). These faults prevent the chromosomes integrating into the daughter nuclei and can be counted. The measured frequency of these events is correlated with increasing toxicity.

A.8.5 Cell viability assays

There are several basic assays of cellular health that can be routinely applied to any cell-related system in toxicity screening. Flow cytometry is an extremely flexible technique that can be used to study the effects of a toxin on the cell cycle, both from the apoptotic and necrotic viewpoints. Different fluorescent antibodies can be used to count and separate populations of cells. In addition, various dyes are used, which either bind DNA or become activated within metabolically active cell areas, such as mitochondria. Other assays measure a cell's functional capability, by reduction of a dye such as MTT. Although originally developed in the early 1980s, this assay is still used, although it is rather cumbersome and involves destruction of the cells to recover the blue dye for measurement on a fluorescent plate reader The Alamar Blue assay is easier to use and cells do not need to be killed to visualize the dye, so they can be monitored in real time for considerable periods, which is a real advantage over MTT. ATP is a useful measure of the cell's mitochondrial health and GSH depletion assays reveal if cells are under severe oxidative stress.

A.8.6 Bioassays

The production of toxic species can be studied using bioassays, where biotransforming enzymes can be brought into varying proximity with victim cells and the impact assessed on cell health.

A.8.7 'One Compartment' Models

The simplest bioassay system is the mixture in one sample tube of victim cells such as human mononuclear leucocytes, or adherent cell lines grown on a matrix with human liver microsomes, hepatocytes or even expressed human CYPs. After a period of time, the human cells would be separated from the mix and assayed for toxicity as detailed above. A more sophisticated version of this method is also used where human CYP systems are inserted heterologously into human cell lines, such as HepG2 cells. A reactive species could be made by the particular CYP and the degree of toxicity measured. These systems are very efficient at modelling short-lived species that cannot escape a cell or tissue before reacting with a macromolecule. This type of assay can be applied to a University or College practical course to illustrate biotransformation-mediated cytotoxicity.

A.8.8 'Two Compartment Models'

Toxins which are relatively stable like hydroxylamines, are better modelled in multi-compartment systems which separate the source of the reactive species from the victim cells by a porous barrier, which keeps cellular material apart, but allows small molecules to pass through. Dialysis systems comprising Teflon disks are useful for adapting to this purpose. The victim cells can be aseptically assayed and the ability of a toxin to travel some distance and still inflict toxicity can be evaluated. Modular systems like those described above can be used with virtually any cell type and many different sources of reactive species.

A.9 *In silico* studies

The availability of specific software packages designed to design new drugs combined with easy access to extremely cheap and fast PCs have left behind older laborious methods of synthesizing thousands of probably useless drugs. These packages rely on algorithms, which are stepwise instructions designed to accomplish the task of producing a viable drug structure with high efficacy and low toxicity. To achieve this, the software exploits huge databases accrued by either commercial or academic institutions to search and incorporate pharmacophores, i.e. specific areas of molecules which have high pharmacodynamic activity, and toxophores, i.e. areas which are likely to lead to either direct toxicity or the propensity to be metabolized to something unpleasant. The effectiveness of any package is reliant on the logic of the algorithm, but it is mainly dependent on the quality of the data in the database. This software design process is evolving extremely quickly and has been combined with combinatorial chemical synthesis. This involves using a form of

chemical 'mass production' to exploit many basic chemical structures, like modular platforms seen in the car industry. This means that hundreds of analogues can be designed and then synthesized in days using robots that make small quantities of relatively impure compound. These can be rapidly screened and if there is a 'hit', i.e. pharmacodynamic activity, the effect can be fine-tuned in days rather than years. Software-directed combinatorial synthesis did at first create 'bottlenecks' where thousands of potential drugs were designed and made in days, which would then take months to test. Much investment has accelerated the development of high-throughput pharmacodynamic test systems to 'catch up' with the potential flow of useful compounds.

In silico drug metabolism software has matched this targeted drug design by incorporating large databases on human CYP metabolism of hundreds of thousands of structures and systems such as the highly sophisticated SimCYP® software package which originated from the University of Sheffield in the UK. This system is intended to simulate all aspects of ADMET and contains a very large database on human genetic, physiological and metabolic systems, which is combined with the *in vitro* clearance data from many relevant human models. This provides a platform where the likelihood of a new entity to cause problems in the clinic can be modelled with a greater degree of certainty than has been the case to the present. A strength of the SimCYP® software is that population variabilities can be estimated concerning predicted DDIs as well as the impact of disease states, anatomical, physiological and factors related to polymorphic biotransformation. A number of leading pharmaceutical companies have developed their own in-house software predictive systems for major CYPs and their substrates and along with commercially available products like SimCYP® they can predict clinical interactions with CYP3A4 to a high degree of accuracy (>88–90 per cent). SimCYP® can also predict the specific consequences of inhibition as it impinges on alternative pathways of clearance, such as a shift to UGT1A4-mediated clearance of midazolam during CYP3A4 inhibition. These predictions are invaluable in estimating drug dosage and disposition that is necessay for the successful design of human trials that minimize toxicity and maximize efficacy.

Although predictions of drug disposition from *in vitro* data are highly advanced, toxicity as a whole is, of course, much less easily predicted. Due to the volume of data available on the liver, hepatotoxicity is still the most successfully predicted drug adverse reaction using these systems and recent advances in microarray technology has strengthened the ability to predict *in vivo* oxidative stress inducing effects based on preliminary data in human *in vitro* systems. Within hepatotoxicity predictions, risk factors from previous *in vitro* and *in vivo* data from immune-based hypersensitivity reactions as been built into the programmes, which improve the predictive power of the software in terms of human hypersensitivity. Overall, the drug industry is still investing strongly in *in silico* systems from a variety of sources to minimize costs and accelerate the time frame for candidate agents to reach the clinic successfully and safely.

A.10 Summary

Drug development is unusual amongst commercial concerns; it faces an enormous 'leap of faith' for every new agent. Despite testing on perhaps 10,000 individuals in 4–5 years, within a few months of marketing the power of the promotional machine propels it to millions of patients to fulfill the clinical need and recoup the investment. Cars that depart

from the road on corners, spontaneously combust, or fail 'elk tests' can be modified or adapted and no matter how uncharismatic or unreliable they will be bought in quantity. Even in the entertainment industry, certain individuals can anticipate public tastes and unreliable artistes can be retrieved from their downward slides, poured into rehab and returned to the studio more or less lucid. However, once a drug is withdrawn, it will never recoup its costs and is commercially 'gone' and sometimes it can take the entire pharmaceutical company with it. Hence, there are colossal pressures on the drug industry to market their products and although it is in their commercial interest for the products to be safe, this does not always occur and famously, many companies have not always exactly behaved appropriately in their handling of these situations from moral, ethical, or commercial standpoints. However, as you have read, far less is known of living systems than is known about popular tastes or mechanical engineering and in spite of their imperfections, only the pharmaceutical companies have the resources and expertise to fulfill the clinical need for new and old drugs.

Fortunately, there have been some spectacular advances in the past decade and it is as well to remember that the hepatotoxins described in Chapter 8, were initially tested with the technology of the 1980s. As we have seen, some agents will still be developed and reach the clinic because there is little or no alternative in the face of overwhelming clinical need and a considerably greater level of toxicity or DDI problems will be tolerated with these agents than with the latest in a long line of beta-blockers. However, regarding hepatotoxicity, there is now sufficient information and techniques available which can prevent another troglitazone or tacrine from reaching the clinic, but the spectre of drug immune hypersensitivity remains and may not be eliminated as a risk for some time. Overall, over the next decade, human diversity, which is often the main reason drugs are toxic, will be modelled more accurately and predictively using both *in vitro*, *in vivo* and *in silico* systems and it is reasonable to hope that drugs introduced in the future will be less likely than in previous years to be withdrawn due to biotransformational toxicity.

Appendix B
Metabolism of Major Illicit Drugs

B.1 Introduction

It is certain that the use of various plant and animal sourced chemicals by humans for their pleasurable effects predates recorded time. These agents have been and continue to be directly ingested, smoked, extracted, inhaled and manufactured using a variety of processes. Often it is the case that drugs that are abused have vital clinical roles, such as the opiates; however, others, such as cocaine, amphetamines and PCP, are of little or no current clinical use. In general, those seeking some form of illicit chemical stimulation will pay any price and often go to any length to obtain it, whilst tacitly condoning the human cost of the drug traffickers' firm hand on the tiller and the damage to the users' lives and those of their neighbours. This is nothing new and virtually every culture in history has used a particular favoured drug or group of drugs, based on availability and tradition. It is only relatively recently that the use of chemicals for pleasure has been subject to legal restraint, to the end that nicotine and alcohol are the only freely available drugs to the public in most Western countries. Most other 'hard' drugs are illegal and although judicial tolerance to milder agents such as cannabis is gradually building, developed world societies expend vast resources to stem their own unquenchable thirst for mind-altering substances.

The ethical and moral implications of drug and alcohol abuse are clearly outside the scope of this book; although it is worth mentioning that suffusing one's life with chemicals as if recreating the feasting scene in 'The Vikings' (1958), is clearly incompatible with generally accepted standards of behaviour. Scientifically, it is informative to discuss the metabolic fate of some of the more commonly used and interesting abused drugs and how this might impinge on their pharmacological effects and those of prescription drugs. Of course, whether or not illicit users may emerge from their pharmaceutical adventure unscathed is also of compelling interest to their parents and loved ones.

B.2 Opiates

These agents include those either extracted from opium (morphine) or are close structural analogues of morphine, such as diacetylamorphine (heroin), codeine and dihydrocodeine, oxycodone, hydrocodone and buprenorphine. Amongst the many synthetic opiates, these

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include methadone, fentanyl and pethidine (meperidine). Most of the morphine derivatives are converted to morphine or morphine-like agents. Others, such as fentanyl, methadone and buprenorphine act directly on opiate receptors.

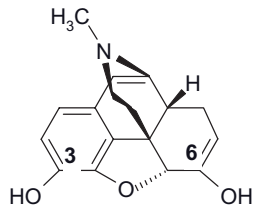
Morphine

Morphine and its relatives act on μ-opioid receptors and are the most effective agents of relief available for most types of pain, although interestingly, around a third of patients receiving these drugs do not receive adequate analgesia. It remains controversial whether this is due to inadequate dosage and all pain should respond eventually, or whether some types of pain (e.g. neuropathic pain) are insensitive. The euphoric aspects of morphine and its derivatives effects have been the basis for their abuse and they induce tolerance and dependence. Addiction to morphine-like compounds is a worldwide problem, which is as acute in the countries that grow opium poppies as it is in the developed world. During opiate addiction, aside from dangers associated with the lifestyle and the particular attendant dangers of the intravenous route of administration, fatal respiratory depression is a consequence of overdose.

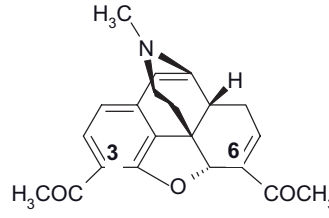
The pharmacological potency of morphine resides in several aspects of its structure (Figure B.1). The tertiary nitrogen and its methyl group are required for analgesic activity and changes to either moiety will reduce activity – indeed substitution of the methyl group with larger groups yields antagonists. Substitutions to the 3-hydroxyl group on the phenolic group usually result in loss of activity, whilst changes to the 6-hydroxy group can increase activity. The drug is not well absorbed orally, with bioavailability levels of around 20 per cent, but interestingly, its plasma levels can be doubled if oral diacetylmorphine (heroin) is used.

Morphine metabolism

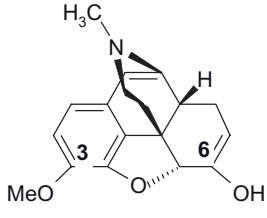
Just over half of a morphine dose will be cleared to the 3-glucuronide, whilst 10 per cent appears as the 6-glucuronide. Of the remainder, about 5 per cent forms a 3-sulphate and small amounts of a diglucuronide and a 3,6-glucuronide are also formed. Less than 1 per cent of morphine is methylated to codeine (3-methoxymorphine), whilst around 10 per cent of the dose appears in urine unchanged. In addition, N-dealkylation occurs at the tertiary nitrogen, leading to an N-oxide as well as the formation of normorphine. It appears that virtually no gut wall metabolism occurs in man, although the main metabolites are formed in a number of other organs as well as the kidney and liver. The liver is probably the major site of morphine metabolism, as cirrhotic patients show a substantially increased half-life of the parent drug. Renal failure also increases morphine half-life in man and when renal function declines, the drug and its 6-glucuronide accumulate unless the dose is reduced. Although the 3-glucuronide has no analgesic activity it is thought to be rather toxic, having a stimulant effect as well as causing irritability and even hallucinations. By contrast, the 6-glucuronide is believed to be up to 50-fold more potent as an analgesic compared with the parent drug. The relationships between the clearance of morphine and its analgesic activities in different groups of patients remain to be fully explored, although UGT2B7 is thought to be responsible for the glucuronidation of the drug. This UGT is



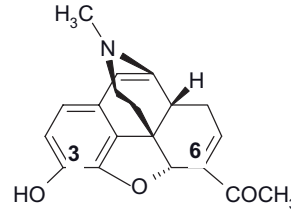
Morphine



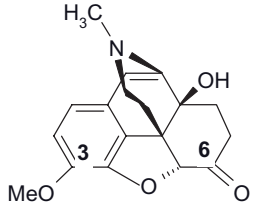
Heroin



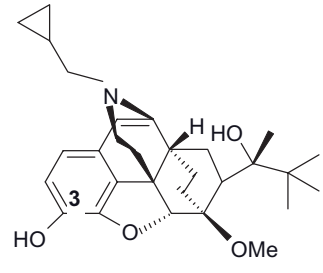
Codeine



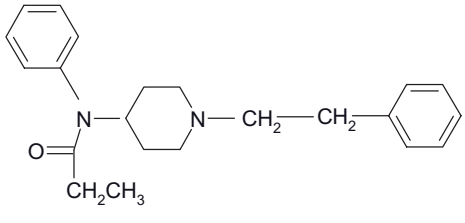
6-Acetyl-morphine



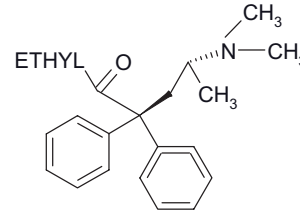
Oxycodone



Buprenorphine



Fentanyl



Methadone

Figure B.1 Structures of morphine-based opiates and the synthetic opiates, methadone and fentanyl

polymorphic and subject to inhibition by NSAIDs such as mefenamic acid. It is thought that differences between patients' morphine responses may be linked with UGT2B7 expression, although the opposing effects of the glucuronides makes this difficult to study. In addition, there is considerable variation in the *OPRM1* gene that codes for the opiate receptors which has a major impact on sensitivity to the opiate effect. Some studies have suggested that induction of UGT2B7 might reduce the effectiveness of morphine, as it increases the toxic 3-glucuronide at the expense of the parent drug, although more of the 6-glucuronide should also be formed. A study with rifampicin, an inducer of UGTs, did erode the analgesic effect of morphine, although this was thought not to be linked with an induction effect. Inhibition of UGT metabolism can increase morphine plasma levels, but this area is surprisingly under researched given that morphine has been used in clinical practice for so long and it is remarkable that more is not known about optimizing morphine's therapeutic performance, particularly in terminal patients with intractable pain.

Heroin

Diacetylmorphine, or heroin, was first synthesized in 1874 with the aim of increasing the potency of morphine; it was even marketed in the early twentieth century as a non-addictive cough suppressant. Whilst it is undoubtedly an effective cough suppressant, heroin is the preferred drug of abuse of the opiates, as its onset or 'hit' is as rapid as it is potent. It can be injected, smoked or snorted according to the consistency and purity of the available drug. It can range in appearance from pharmaceutical grade white powder all the way to the 'black tar' version manufactured in Mexico and popular in the US. Obviously intravenous dosing is the most efficient method of administration, as the dragon chasing inhalation method shows only about a 50 per cent bioavailability, although a very impure drug can be used with the latter method. The half-life of heroin in man is around the 5–7 minute mark, indicating rapid clearance. In addition, the two acetyl groups make the drug much more lipophilic than morphine so it reaches the CNS more rapidly. In addition, during its progress to the CNS and when it penetrates the blood–brain barrier, heroin can be hydrolysed rapidly by various hepatic, plasma and CNS esterases. These include butyrylcholinesterase (BChE), human carboxylesterase-1 (hCE1) and acetylcholinesterases. Heroin is a pro-drug, as it shows little binding to opiate receptors, but if the 3-acetyl group is hydrolysed away, the 6-acetylmorphine does bind, and it is probably more potent than morphine. The 6-acetyl derivative is then hydrolysed to morphine. The 6-acetyl derivative of morphine has a half-life of 6–25 minutes in blood and is stable in urine and is thus used as conclusive proof of relapsed heroin abuse in those enrolled on methadone programmes. This is because no other opiate is metabolized to the 6-acetyl derivative. The metabolic profile of heroin use is the same as morphine, with the exception of the 6-acetyl derivative. Although heroin addicts are exposed to many different substances and are subject to various systemic abnormalities (e.g. effects on glucose tolerance), there is evidence that heroin addicts form much more of the pharmacologically active morphine 6-glucuronide than the non-active 3-glucuronide, in comparison with non-addicts receiving heroin. Interestingly, aside from the usual risks of hepatitis and HIV, intravenous users of black tar heroin rapidly incapacitate their exposed veins and are reduced to sub-cutaneous administration, which can then lead to the development of botulism, from spores introduced when the heroin is 'cut' with whatever is to hand, such as boot polish or soil.

Buprenorphine

Introduced in the 1980s (Figure B.1), this semi-synthetic opiate is of interest pharmacologically, as it can act as a partial as well as full agonist on μ-opioid receptors, but as antagonist on K-receptors This means that it shows the usual complement of opiate effects and is 30-fold more potent than morphine, but at higher doses it is less likely to depress respiration and opiate effects are then diminished. However, this is negated by the concurrent administration of benzodiazepines and other CNS depressants that potentiate its effects leading to fatalities. The drug still causes euphoric effects and is an efficient, long-lasting (1–3 days) analgesic, although in around a third of patients it has a sedating effect. It has found increasing use in managing opiate withdrawal instead of methadone. Interestingly, its partial agonist status means that it is not as physiologically dependence-inducing as heroin or morphine and that the withdrawal is not quite as severe as from the major narcotics. Unfortunately, naloxone is not very effective at reversing severe respiratory depression if it does occur. This is believed to be due to more potent binding and reduced dissociation from the opiate receptors, which is an effect that also contributes to its long-lasting action. One formulation of the drug, Suboxone, contains naloxone and is intended to abolish the euphoric effect when injected, to prevent abuse of the preparation. The drug is still abused in its pure form, often alongside benzodiazepines, of course. The drug is marketed under several other brand names such as temgesic and buphenex. The best the drug abuse fraternity can do for a street name appears to be ‘bupe’, although there are others. Buprenorphine is cleared predominantly by N-dealkylation to norbuprenorphine and the hydroxylation of the hydroxytrimethylpropyl sidechain also occurs prior to glucuronidation. There is a buprenorphine and norbuprenorphine 3-glucuronide formed and buprenorphine-3-glucuronide is the main urinary metabolite. There are at least five hydroxylated derivatives and it is thought to date that CYP3A4 is responsible with some element of CYP2C8. Concurrent efavirenz induced buprenorphine’s clearance, although much is still to be discovered about the involvement of other isoforms such as CYP2B6 and CYP2D6 in the clearance of buprenorphine.

Oxycodone and hydrocodone

Oxycodone (Figure B.1) has been available for nearly a century and is a semi-synthetic opiate originally designed to be a less dangerous alternative to heroin, although it has nearly double the opiate effect of morphine. The less potent hydrocodone was synthesized a few years later and is structurally related to codeine, but is a much stronger analgesic. Both drugs are part of several popular analgesic combinations; oxycodone is found in Percocet, Percodan and OxyContin, a potent sustained release version known as ‘Hillbilly heroin’ as it was a popular choice in deprived areas of the US. Hydrocodone is found in Vicodin, which is often linked with more ‘white collar’ addiction, as well as media personalities, both real and fictional. Rather bizarrely, Vicodin abuse and unintentional overuse has been linked with sensorineural irreversible hearing loss. It is not understood why this occurs with this agent and not with other opiates, but the only remedy is the somewhat drastic one of a cochlear implant.

Both drugs are mainly N-demethylated by CYP3A4 to weak opiates; oxycodone to noroxycodone and hydrocodone to norhydrocodone. CYP2D6 O-demethylates about 10

per cent of the dose of these drugs, leading to oxymorphone and hydromorphone. Both metabolites are 10 and 30 times respectively more potent than the parent drugs and are cleared by UGT2B7 and UGT1A3 to 3-glucuronides. Unlike oxymorphone, the 3-glucuronide of hydromorphone can cause seizures in animals, although it is not known if this occurs in man. The effect of CYP2D6 polymorphisms with oxycodone and hydrocodone appear similar to tramadol, codeine and dihydrocodeine, as PMs show reduced efficacy but pain relief is not entirely diminished, suggesting that the parent drugs and other routes of metabolism make significant contributions to the analgesic effects of the drugs. Inducers should diminish their potency through CYP3A4 formation of weaker metabolites and UGT-mediated clearance of the active metabolites. CYP inhibitors will cause the parent drugs to accumulate and it is interesting that in one study of fatalities associated with oxycodone, a high proportion of the victims were CYP2D6 PMs.

Fentanyl and designer opiates

With a basic knowledge of synthetic chemistry and structure–activity relationships, extremely potent 'home-made' opiates have been made over the last 30 years or so. Some of the most notorious agents were derivatives of the synthetic opiate fentanyl (Figure B.1) introduced in the 1960s. This agent is already more than a 100-fold more potent than morphine and is used in clinical practice in a variety of forms, including lozenges, various tablets and transdermal patches as well as many parenteral forms. Its rapid onset and potency makes it highly effective in chronic pain management as well as in surgical pain control. It is abused due to the intensity of its euphoric effects. It is cleared by CYP3A4 by N-dealkylation mainly to norfentanyl as well as hydroxynorfentanyl. Azoles markedly inhibit the drug's clearance, as will any CYP3A4 inhibitor. If fentanyl accumulates, respiratory depression is a real danger. In the 1970s methylated fentanyl derivatives were made illicitly and these are thousands of times more potent than heroin and their metabolism/clearance is virtually irrelevant as they may stop the addict breathing in a few minutes. Carfentanyl is a derivative of fentanyl manufactured legitimately that is 10,000 times the potency of morphine, which is useful if you need to tranquillize an inmate of Jurassic Park. It is believed that fentanyl derivatives in a halothane aerosol were used in the Moscow Theatre Hostage Crisis of 2002. The strongest opiate obtainable currently may be a fluoro analogue of ohmefentanyl, which is nearly twice the potency of carfentanyl.

Other home-synthesized products contain extraordinarily toxic impurities. The best example is 'synthetic heroin', a pethidine (meperidine) analogue known as MPPP (1-methyl-4-phenyl-4-propionpiperidine). When the synthesis is poorly managed, MPTP (1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine) appears, which causes a permanent Parkinsonian condition, as it is metabolized by MAO-B to MPP+, a potent and selective neurotoxin.

Methadone

This synthetic opiate was first synthesized in Germany during the Second World War in response to opiate shortages. Although it does not resemble other opiates chemically, it does act on opiate receptors and is cleared relatively slowly. Methadone has been unfairly stigmatized as its best-known use clinically is as a substitute for heroin, so aiding gradual

withdrawal from addiction; this is because its half life is much longer than other opiates. The drug is used in a variety of formulations worldwide, but in the UK, it is dispensed to addicts as the ubiquitous sweet green methadone 'mixture' (1mg/mL), which is distinct from the less potent (2mg/5mL) methadone linctus. The latter preparation is intended for severe coughing in terminally ill patients. The green methadone mixture was so coloured to prevent confusion with other medicines, but tragically it has been irresistible to children on several occasions and now opiate addicts are required to consume their methadone mixture whilst supervised in a pharmacy. However, methadone is really wasted on heroin addicts, as it is extremely effective in cancer patients who are unable to control their pain with morphine or who have experienced severe morphine side effects. However, clinically, it is recommended that if there is no medical practitioner available who is well acquainted with its use, a different opiate should be used. There are several reasons for this; the drug's clearance is astonishingly variable, with reported half-lives ranging from a few hours to more than six days. Consequently steady state may take more than a week to achieve and the analgesic effect can be dissociated from its half-life, which means that the patient may take the drug more frequently to maintain analgesia, but the drug quietly accumulates. As detailed in Chapter 7 (section 7.2.3) methadone is pharmacologically active through its R-isomer but toxic through its S-isomer. Although it can cause torsades des pointes rarely (Chapter 5, section 5.5.2) the real danger, as with all opiates, is respiratory depression. However, it is believed that low initial doses, conversion of the dose to reflect the previous opiate tolerance and gradual dosage escalation to achieve adequate analgesia can exploit the capabilities of this drug without the attendant toxicity. This approach has been summarized by some authors as 'start low, go slow'.

The biotransformation of methadone has been investigated in considerable depth but has been until recently rather controversial. What is not in dispute is that the drug undergoes two sequential N-demethylations (Figure B.2); the first methyl group is removed from the tertiary nitrogen and the molecule essentially reacts with itself and immediately cyclizes forming EDDP (2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine) which is then demethylated again to form EMDP (2-ethyl-5-methyl-3,3-diphenylpyraline). Both metabolites are inactive. Methadone clearance is clearly CYP-mediated, although it has been difficult to determine which isoforms were chiefly responsible. The drug is highly vulnerable to change caused by various CYP inhibitors and inducers. Clinically, inducers such as phenytoin and rifampicin can accelerate the clearance to the point that not only is analgesia lost, but also opiate withdrawal is precipitated. The azoles (fluconazole, ketoconazole and cimetidine) and fluvoxamine all retard clearance-causing accumulation of methadone. This pattern of induction and inhibition was assumed to be due to CYP3A4, however, as you will recall from Chapter 4, if a drug induces CYP3A4, it will induce most others with the exception of CYP2D6 and the Ah-receptor controlled CYP1A series. However, nelfinavir is a potent inhibitor of presystemic CYP3A4 and clinically will simultaneously inhibit concurrently administered CYP3A4 substrates whilst virtually doubling methadone clearance. This suggests that CYP3A4 has little or no role in methadone's metabolism, although at least one study suggests that CYP3A5 could be involved. Nelfinavir and rifampicin induce CYP2B6 in human hepatocytes and it is now established that CYP2B6 is most likely to be the major influence in methadone's residence time in the body. Indeed, CYP2B6 clears the S-isomer preferentially and thus has a direct influence in the ratio between the isomers. There is also evidence that methadone acts through PXR to induce CYP2B6 and CYP3A4, although it has not been shown to be a P-gp inducer

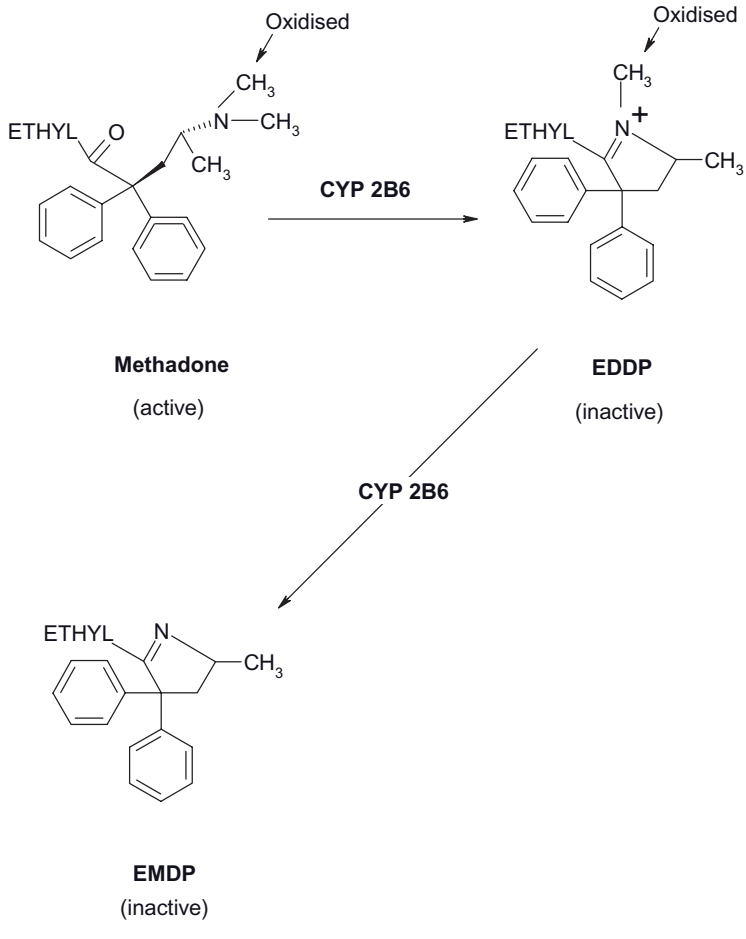


Figure B.2 Dual, sequential N-demethylation of methadone to inactive products by variants of CYP2B6 in the gut and liver

in human cellular systems. Interestingly, although the CYP2B6 methadone clearance is stereoselective, the PXR binding does not appear to be. Methadone is a substrate for P-gp that governs its entry into the CNS as well as many other tissues. From Chapters 4 and 5 you will recall that P-gp can be inhibited and induced markedly. Considering that CYP2B6 is the most polymorphic human CYP, overall, there are many reasons why methadone's clearance and pharmacological effects can be so variable. The full process of methadone clearance remains to be fully explored.

B.3 Cocaine

Cocaine originated in Latin America and most of the world's supply still comes from this continent. The drug is a potent inhibitor of presynaptic dopamine and noradrenaline reuptake by transporter systems and the pharmacological effect is through the persistence

of the neurotransmitters. Judging from its popularity and the cravings that it causes in addiction, to classify the drug merely as a stimulant is a spectacularly inadequate description. Cocaine also acts to block sodium channels and inhibit action potentials in peripheral nerves. During its use for its local anaesthetic effects, its vasoconstrictive actions are useful to restrict bleeding. However, many other safer analogues can be used which do not have the attendant cardiotoxic effects. Although its stimulant effects when smoked as the base (crack) or when injected intravenously occur within 10–15 seconds and are intense, this does not last more than 5–10 minutes. This is due to the extremely rapid clearance of the drug.

As with heroin, butyrylcholinesterase (BChE; Chapter 7, section 7.2.4) and human carboxyesterases (mainly hCE-1 and hCE-2) metabolize most of the drug (Figure B.3) as they are found in the plasma as well as most other organs, particularly the liver. The hCE enzymes are high capacity, low affinity and are found in the endoplasmic reticulum in the liver, although their presence in plasma appears variable. Drugs like cocaine are subject to widespread and rapid attack by several esterases, hence their short half-lives. About half the dose is de-esterified to ecgonine methyl ester, which is low in toxicity, as it has little vasoconstrictor effects or cardiotoxicity. The hCE group of enzymes is capable of

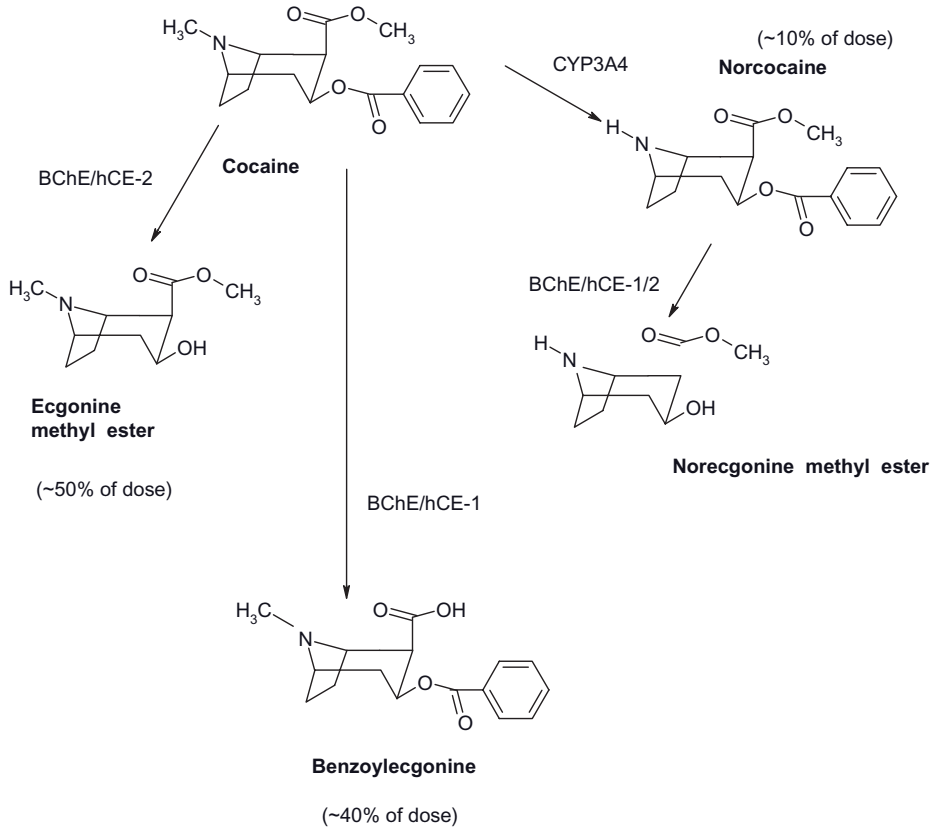


Figure B.3 Cocaine metabolism, showing the role of CYP3A4 in demethylation (toxic pathway) and human carboxylesterases (hCE-1 and 2) and butyrylcholinesterase (BChE). Esterases are found in virtually every tissue, clearing cocaine extremely rapidly.

metabolizing a very wide variety of substrates and probably has a defensive role against xenobiotics, as well as myriad vital endogenous functions. Cocaine is optically active and the (–) isomer is the natural version, whilst the (+) isomer is found when it is synthesized illegally. The (+) is poorly cleared by esterases but 200 times less potent than the natural version.

Demethylation to norcocaine is seen as a toxic pathway, as norcocaine is a more potent vasoconstrictor than cocaine and it retains the rest of cocaine's pharmacological effects. CYP3A4 catalyses this reaction and if the individual is regularly using inducers of this CYP (rifamycins, St John's Wort and barbiturates) then the possibility for toxicity due to excessive norcocaine effects depends on how quickly the hCEs and serum cholinesterases can clear it (Figure B.3). If the individual has poor plasma cholinesterase performance (BChE polymorphisms, Chapter 7, section 7.2.4), then the chances of them exhibiting cocaine toxicity (tremors, agitation, paranoia, high blood pressure, weak pulse) are much higher. It has been known that cocaine users will self-administer insecticides, such as organophosphates or carbamates, which will block the esterases to prolong cocaine's survival in the plasma. Clearly, this could increase the high but equally go horribly wrong and induce severe toxicity, such as convulsions and arrhythmia, leading to fatal cardiotoxicity. Regular cocaine use is believed to induce CYP2B1, whilst acutely, it is thought to exert some inhibitory effects on several CYPs, such as CYP1A2.

As recreational drug use often involves several drugs, it was noticed that cocaine's effects were 'improved' when the user had consumed ethanol. This is because, rather unusually, the use of cocaine with ethanol leads to the formation of a separate and unique 'hybrid' metabolite of both agents, known as cocaethylene. It is thought that human carboxylesterase 1 (hCE-1) transesterifies cocaine with ethanol to form cocaethylene. This is followed by clearance by either hCE-1 or 2 to the more usual cocaine ester metabolites (Figure B.4). Cocaethylene is thought to induce a more potent state of euphoria than cocaine and the dysphoria (down) is much less unpleasant. Cocaethylene seems to be able to retard the clearance of cocaine and prolong its effects. The downside is that it is more cardiotoxic and can induce convulsions at lower concentrations. Although any drug of addiction can be destructive, cocaine in all its forms is particularly efficient at destroying its victims remorselessly, both physically and mentally.

B.4 Hallucinogens

LSD

In 1938, Albert Hofmann synthesized lysergic acid diethylamide whilst trying to design a respiratory stimulant. Unimpressed with the results, he let the work lapse until 1943 when he thought it would be a good idea to make some more and consume 250 μg (as you would). He then rode home on a bicycle and spent the next six hours or so inventing the first acid 'trip'. He suffered no obvious ill effects, living on to the exceptionally impressive age of 102. The drug company Dr. Hofmann worked for marketed LSD to try to get it established in psychotherapy. The US military flirted briefly with the drug as a non-lethal weapon and entertainment figures such as Cary Grant used it under supervision as part of various experimental psychiatric treatments in the 1950s and 1960s. Increasing abuse and accidents ('Look, I can fly!!') led to its proscription in the late 1960s. It is more than 100 times more potent than psilocybin in magic mushrooms (first synthesized in the laboratory

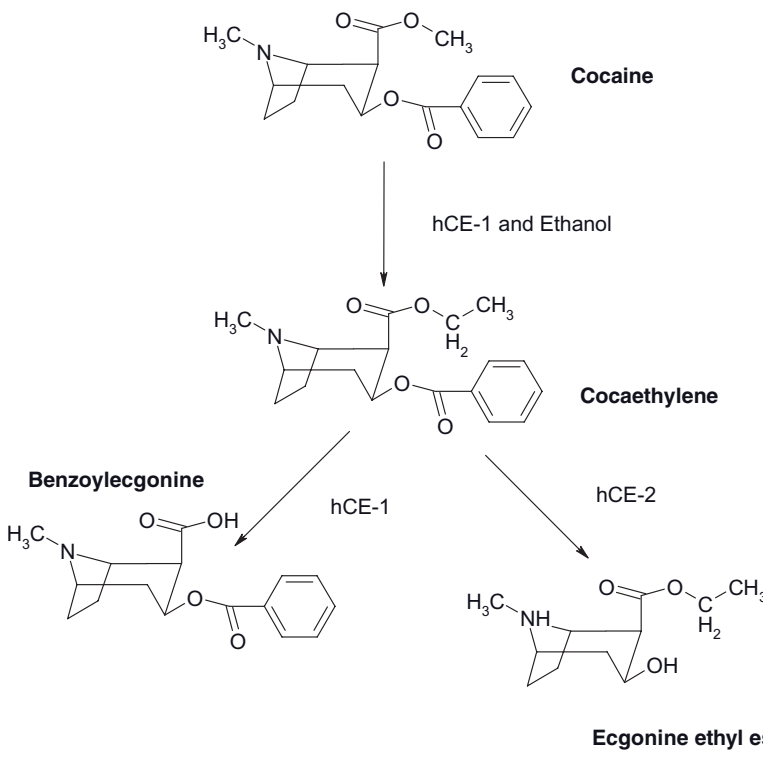


Figure B.4 Formation of cocaethylene by esterification with ethanol and the metabolism of the product to other cocaine esters and metabolites by esterases (hCE-1/2)

by Dr Hofmann) and 5000 times stronger than mescaline. The d-isomer is active and the rest of the usually racemic mixture that is synthesized illicitly (iso-LSD) is inactive. The drug is usually taken in very low doses (50–100 μg) and is extensively metabolized, so is difficult to detect and is not usually included on standard laboratory drug screening systems. Excessive use quickly leads tolerance within only a few doses, although the chief danger from the drug is not really from direct toxicity, but from accidents during intoxication. LSD does have the tendency in some users to unmask psychotic reactions from which recovery is very slow if it occurs at all ('acid casualties'). Of course, it is not really possible to know one's own susceptibility to such an unpleasant event in advance of using the drug and many hardened chemical abusers refuse to take it. The seeds of the 'Morning Glory' plant contain structural relatives of LSD (lysergic acid amide and isolysergic acid amine), which have milder hallucinogenic effects, although these agents cause nausea and vomiting in most people, as well as occasional panic attacks and even a hangover. Of course the good Doctor Hofmann was also the first to officially discover this effect also.

The effects of LSD begin within 1 or 2 hours and may last for up to 12 hours. It has been likened to a 'mental rollercoaster' that you cannot get off. Despite its long period of effect, it is rapidly metabolized, primarily by dealkylation, or de-ethylation, to be precise (Figure B.5). The main metabolite in the urine of individuals who have used the drug is 2-oxo-3-hydroxy LSD, which is not found in the plasma, presumably as it is rapidly filtered by the kidneys. This metabolite is found in much greater quantity than the parent drug in

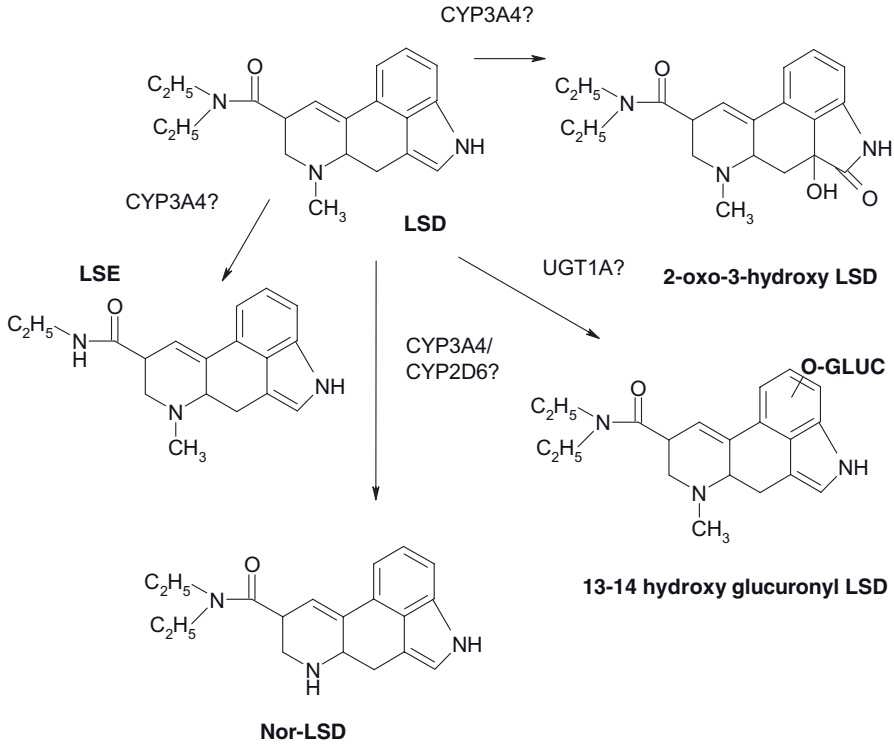


Figure B.5 Metabolism of LSD: formation of lysergic acid ethylamide (LSE), nor (N-demethylated) LSD and its hydroxylated derivatives

urine. Aside from the de-ethylated derivative (lysergic acid ethylamide) a demethylated metabolite (nor-LSD), the side chain and the top phenyl ring (13/14 position on the molecule) can be hydroxylated and at least one glucuronide is formed from the 13/14 hydroxylated derivative. Presently, no CYP has been assigned to these products, although the molecule is quite lipophilic (log *P* of 1.3) and vaguely resembles a steroid shape, so it would not be unreasonable to suggest that CYP3A4 and possibly CYP2C9 and CYP2D6 might be involved in the hydroxylation and dealkylation of LSD. The drug may also be a substrate of P-gp. Interestingly, Dr Hofmann always believed that LSD did have potential medical benefit and he did not approve of the stance of 1960s counterculture luminaries such as Dr Timothy Leary, who championed the drug's use as a psychedelic recreational agent.

Serotonin-based hallucinogens

The Sonoran desert toad, or Colorado River toad (*Bufo alvarius*) found in Arizona, as well as many other species such as the Australian cane toad, are much sought after in some circles for the hallucinogenic properties of their skin secretions. These secretions are exuded when frightened or alarmed to deter predators. It is not generally realized that toad

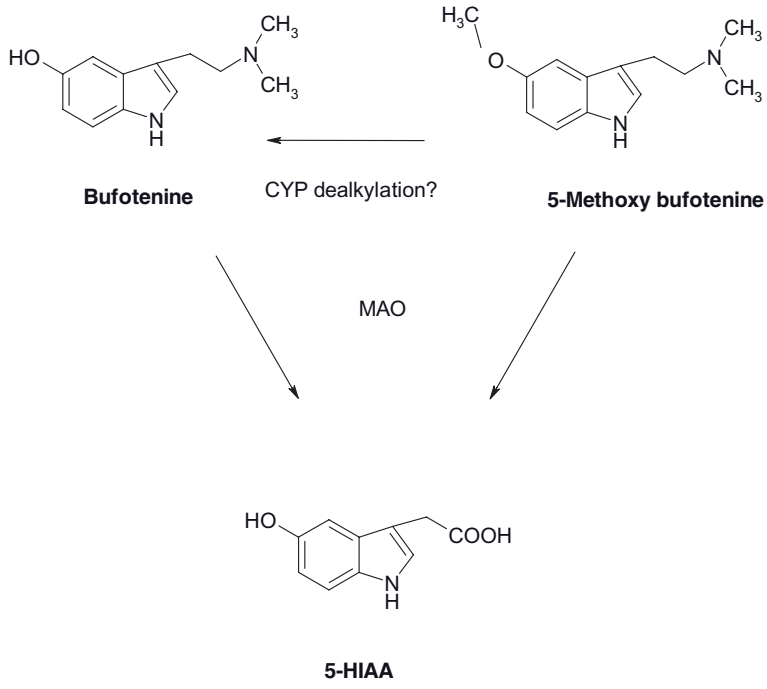


Figure B.6 Some routes of dimethyltryptamine derivative clearance leading to 5-hydroxyindole acetic acid (5-HIAA)

venoms contain many other exceedingly toxic agents, such as bufagins (bufendienolides) that have cardiac glycoside-like effects and catecholamines. The net result is heart failure, seizures and vasoconstriction leading to death in animals and at least some recorded cases of seizures in children. The collective effect from an entire toad skin can be lethal to the average weight adult. The hallucinogenic fraction of the venom contains a series of serotonin-like compounds, the indolealkylamines, which are commonly named bufotenines. Bufotenine itself (5-hydroxy-N,N-dimethyltryptamine) is a weak hallucinogen, which has potent cardiodepressive effects, sufficiently severe to induce circulatory crises. 5-Methoxy-N′N′ dimethyltryptamine (known also as 5-MeO-DMT, O-methylbufotenine and 5-methoxy bufotenine; Figure B.6) is a very potent hallucinogen. Orally, they have no effects, as MAO clears dimethyltryptamines to 5-OH indole acetic acid in the gut, although the 4-hydroxy substitution of the close structural analogue psilocybin (O-phosphoryl-4-hydroxydimethyltryptamine) makes it orally active. 5-Methoxybufotenine was first identified in the 1930s but it was not until the late 1960s that it was found in the toad exudates. 5-Methoxybufotenine is highly active when inhaled as an aerosol through smoking the dried toad exudate as well as intravenously. Due to the variability of the active 5-methoxybufotenine content of the venom exudates, the effects have been described as ‘from bliss to horror’, which sounds somewhat perturbing. Chemically synthesized bufotenines are now available illicitly, although they are Class ‘A’ drugs in the UK. In the US and Australia, opinion is predictably divided as to whether licking the toads is

harmful to them, although this could upset the animals and cause them to produce more exudate, which may be unpleasant for the toad 'user'. Interestingly, the dimethyltryptamine derivatives are found in the urine of schizophrenic patients. The metabolism of these agents is complex, involving MAO and methylation as well as demethylation. It is unclear which CYPs may be involved.

B.5 Amphetamines

Introduction

Amphetamines have been popular drugs of abuse for more than 50 years. They retain some slender clinical uses, such as in narcolepsy and some weight control effects. MDMA may well be their most popular manifestation in current usage (see below). Amphetamines are known by the usual litany of tedious street-names and the most popular variant of the more serious forms at the time of writing is methamphetamine, known mainly as 'ice' or 'crystal meth'. Amphetamines and their derivatives can be dosed intravenously, orally or smoked, depending on the physical form of the drug and the speed of effects onset desired. They act to cause CNS and peripheral biogenic amine effects to be strongly potentiated by preventing their re-uptake and destruction. These sustained elevated amine levels, (particularly dopamine) lead to the characteristic stimulatory effects, which include feelings of well-being, euphoria, and boundless energy; they may also cause hallucinations. There is also evidence that methamphetamine addicts show impairment in seratonergic systems as well as dopaminergic areas. The general effects of amphetamines are similar to that of cocaine, but with an important difference. Cocaine is a very transient 'high', perhaps only a few minutes, whilst amphetamines can maintain their potent effects over more than half a day. The dysphoric effects of the drugs once they have been cleared are notoriously bad, due to severe synaptic biogenic amine depletion. Of course, the tight neuronal regulation of biogenic amine adapts rapidly through mechanisms such as receptor down-regulation. These erode the pharmacological effects over time, leading to tolerance and dependence with repeated usage. Addicts escalate their doses and try to beat the tolerance and the dysphoria by taking the drug for days alongside depressants such as ethanol or heroin. 'Tweaking', as it is known, leads to continuously wakeful states that may exceed two weeks and can make these individuals exceedingly dangerous to themselves and presumably they should definitely avoid operating machinery. Amphetamines can cause a long list of toxic effects, from hypertensive crises to strokes and seizures. They can even induce paranoid schizophrenia in some individuals, as well as repetitive stereotypic effects. The impact on the physical appearance of serious addicts over time is genuinely shocking.

The variety in amphetamine clearance routes reflects their closeness in structure to endogenous biogenic amines. Their main route of metabolism seems to be ring – hydroxylation by CYPs 3A4, 2D6 and 2B6. Potent inhibitors of 3A4 such as some of the HIV protease inhibitors are thought to be capable of causing fatal amphetamine accumulation from normally safe dosages as a consequence of inhibition. Amphetamines can also be N-oxidised by flavin monooxygenases (FMO-3) and deaminated (Chapter 3) by various enzyme systems such as the MAOs. MDMA is a good example of how the metabolic fate of an amphetamine has been gradually unravelled and how this might relate to the long-term consequences of its usage.

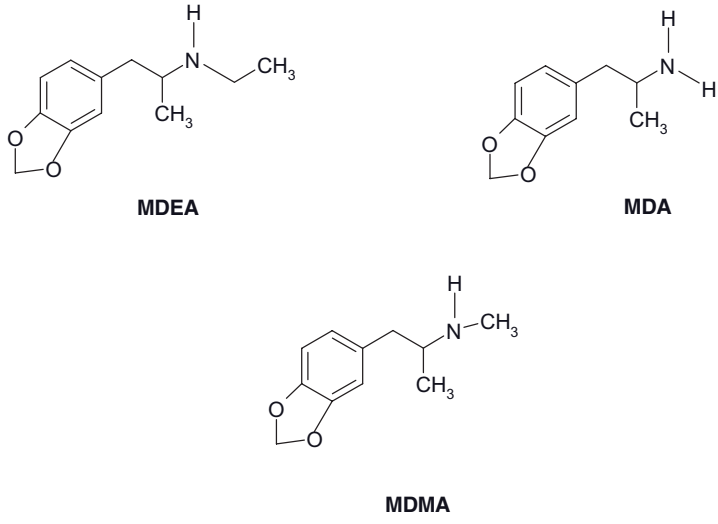


Figure B.7 Methylenedioxy amphetamine derivatives: MDMA (methylenedioxymethyl amphetamine: ecstasy), methylenedioxy amphetamine (MDA) and methylenedioxyethyl amphetamine (MDEA: Eve)

Ecstasy (MDMA) mode of action and acute toxicity

MDMA is well established as the illicit drug of choice for around 7 per cent of the male and 4 per cent of the female population in the 16–24 age group, although its use is widening in older people. The drug (Figure B.7) is the best-known representative of a group of N-substituted methylenedioxyamphetamine derivatives, which also include the N-ethyl derivative ('Eve') and MDA, the primary unsubstituted amine derivative. These agents are stimulants, although they are also reputed to induce feelings of empathy and warmth towards oneself and others; the word that has been coined to describe them is 'entactogen'. MDMA analogue toxicity can be resolved into acute and chronic toxicity – acute toxicity is well understood and described. Overdose of these agents can lead to hyperthermia, high blood pressure, rhabdomyolysis and kidney failure. Deaths attributed to these drugs are sometimes exacerbated by repetitive violent physical activity ('dancing') and its attendant dehydration. Overall, deaths due to MDMA are rare, although the chronic toxicity is still far from completely understood.

Unfortunately, there is a strong perception among users that MDMA derivatives are generally much safer than other drugs. However, those who take MDMA, alongside most illicit drug users, are curiously trusting, believing that someone they just met in a nightclub will sell them high quality MDMA free from unpleasant impurities. The designer MDMA analogue 4-methylthioamphetamine (4-MTA) is often sold as 'ecstasy' and this agent is much more toxic than MDMA. 4-MTA is known on the street as 'flatliners' and it is indeed far more likely to end it all for you than MDMA, according to the statistics. There are experimental data that suggest that CYP2D6 EMs could be at greatly increased risk of toxicity from 4-MTA. In response to these problems of purity and

adulteration, in some European countries MDMA testing facilities are available where the drug is sold to guarantee a reasonable standard of purity.

Chronic toxicity and metabolism

There is some evidence that low and infrequent use of MDMA may not lead to permanent neural impairment, as some authors report it is not associated with any changes in cognitive brain function (memory, attention, associative memory). Other studies have shown worrying low dose-mediated changes in brain microvasculature and some neural damage. As with other amphetamines, a much more serious effect seems to occur at lower doses in younger users below the age of 18. However, it is difficult to evaluate the true risk of neurotoxicity caused by these drugs due to problems estimating accurately how much is taken and how often. It is generally agreed that around 10 per cent of MDMA users could experience serious long-term CNS problems as a result of their high regular intake of the drug.

The metabolism of MDMA has been hotly pursued (Figure B.8) as it was discovered in the mid 1990s that these agents were specifically demethylenated by CYP2D6, forming a catechol product. The ethyl side-chain in 'Eve' and the methyl group in MDMA can also be dealkylated by CYP2B6, although this route is a minor one in humans, as MDA levels are around only 5 per cent of parent drug in plasma. In man, the major route is CYP2D6-mediated demethylenation, and initially it was thought that catechol formation might occur within the brain and lead to reactive species formation. However, the parent drug itself is not directly neurotoxic and neither are any of its catechol metabolites (HHMA/HHA) when injected into rat brains. It appears that the drug must be given systemically and the metabolites make their way across the blood-brain barrier and undergo further metabolism to damage 5-HT neurones. It is believed that in rats the route of toxicity is due firstly to the formation of catechol metabolites (HHMA), followed by the formation of quinones, which then react with glutathione-derived thioethers, which can be transported into the brain to form reactive species. Some *in vitro* studies have also suggested that MDMA may be oxidized to nephrotoxic thioether metabolites.

In humans, the situation is more complex. There is now evidence in MDMA users that HHMA and HHA are further metabolized and they are found in urine as thioether products, which is indicative of a reactive intermediate being formed, as occurs in rats. Interestingly, the thioethers were found at only a very low level in urine at 4 hours post dosage (0.002 per cent of the dose). Whilst this adds weight to the contention that the neurotoxicity of the drug could be linked with CYP2D6-mediated reactive species formation, the low level excretion of the thioether could also be analogous to the situation with paracetamol, where mercapturates can be found in urine at therapeutic doses and the reactive species, though formed, are fully detoxified. It is still not exactly certain if catechol-related MDMA reactive species are indeed formed in the human brain. What makes the situation in man even more labyrinthine is that CYP2D6 undergoes a rapid mechanism-based irreversible inhibition with MDMA, which occurs after one or two consecutive doses. Clinical studies with the CYP2D6 substrate dextromethorphan have shown that MDMA turns nearly 70 per cent of users into CYP2D6 PMs. Its inhibitory effects can cause a 10-fold increase in dextromethorphan plasma levels and the effect lasts for around 10 days. This suggests that if CYP2D6-mediated demethylenation was the main route of toxicity through catechol

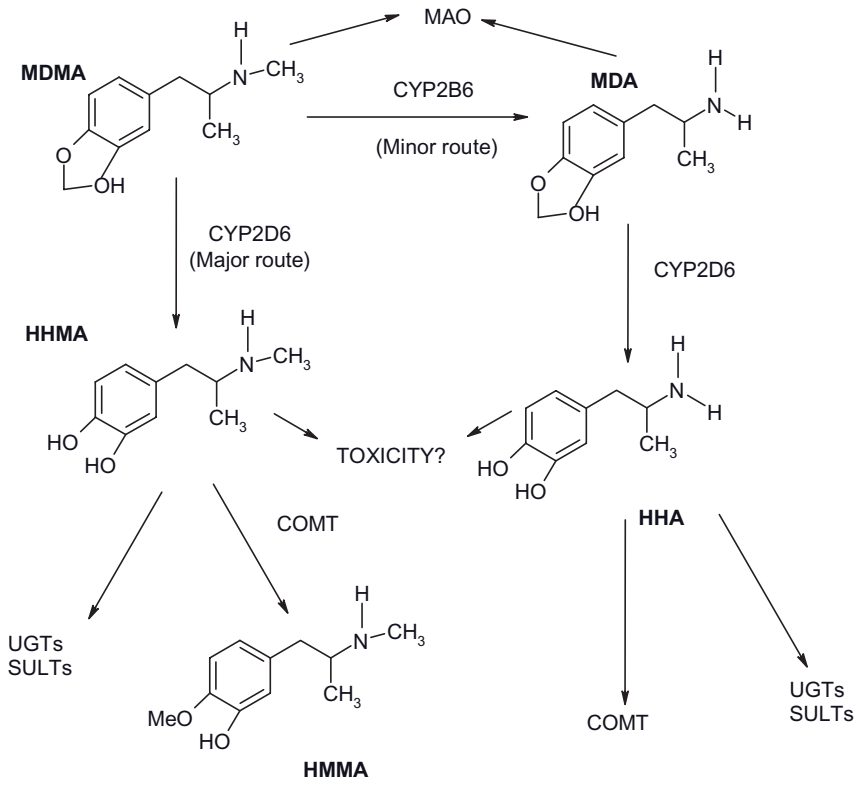


Figure B.8 Metabolism of MDMA: the main route of clearance is initially via CYP2D6 to HHMA (3,4,dihydroxymethamphetamine), which is cleared either by conjugation or may be methylated by catechol-O-methyl transferase (COMT) to HMMA (4-hydroxy-3-methoxymethyl amphetamine). The demethylated MDA may then be demethylenated to HHA (3,4 dihydroxyamphetamine) which can also be methylated and undergo conjugation. MAO may also oxidize MDA, or MDMA

formation, then bizarrely, repeated dosage of the drug could actually reduce the potential neurotoxicity. This does not appear to be the case and heavy use of MDMA is associated with CNS deficits, so CYP2D6 is not the only route of bioactivation. Indeed, although the CYP2D6 blockade causes MDMA accumulation on repeated dosage, several other CYPs such as 2B6, 3A4 and even 1A2 that can metabolize the drug, as well as MAO and conjugation systems that also clear it. It is difficult to estimate just how MDMA is toxic is likely to be in man, given the polymorphisms of CYP2D6 and other CYPs, as well as the differing activities of the other enzyme systems, such as COMT. There is also the variability of the dosage in each ecstasy pill to consider as well as whatever else the users are taking at the same time. What is certain, is that the inhibitory effects of MDMA on CYP2D6 are so potent that it is likely that the clearances of any prescription substrates of this isoform will be significantly extended: this might occur with antipsychotics, antidepressants (SSRIs and TCAs) and opiates.

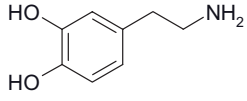
Animal studies have not been that helpful in the study of the relationship between MDMA, its metabolism and toxicity. Although 5-HT-system neurotoxicity caused by

MDMA can be shown in animal models, in mice, this is thought to be due to dopamine-mediated events, as the drug is not metabolized. In rats and monkeys, the profile of MDMA metabolism is markedly different again from man (mostly demethylation), so animal models appear to have limited value in the main objective of this type of research, which is to predict what exposure of MDMA will cause long-term neurological impairment. If anything, these models have been 'too positive' in underlining the apparent risks of these drugs, which patently flies in the face of the experience of the users. This leads to mistrust of official advice, no matter how well it is intentioned.

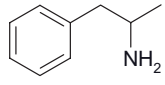
The multi-enzymatic complexity of MDMA metabolism and where it takes place in relation to potential sites of neural vulnerability means that accurate predictions of human toxicity may be some way away. However, it may be that factors such as the frequency of use, CYP2D6 status and age of the user might be greater determinants of possible long-term problems than dosage and co-administered drugs. It is likely that future epidemiological studies may determine the ultimate risks of MDMA and its relatives.

Piperazine derivatives (BZP, TFMPP)

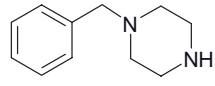
Although amphetamines remain popular drugs of abuse, their illegality led to the promotion of alternatives with similar effects that could be sold commercially as they were not prohibited in many countries, although since they were banned in the US in 2004 this situation is changing. At the time of writing they remain legal in New Zealand, where they are made in quantity, although they were made illegal in the UK in 2009. There are several of these drugs (Figure B.9), although two are encountered most often, N-benzylpiperazine



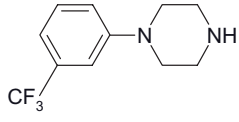
Dopamine



d-amphetamine



N-Benzylpiperazine (BZP)



TFMPP

Figure B.9 Structures of dopamine, d-amphetamine, N-benzylpiperazine (BZP) and trifluoromethylphenylpiperazine (TFMPP)

(BZP) and trifluormethylphenylpiperazine (TFMPP). These agents are either used singly or in combination, when they are intended to recreate the effects of ecstasy. Other popular derivatives include methylenedioxybenzyl-piperazine (MDBP) methoxyphenylpiperazine (MeOPP) as well as methylbenzylpiperazine (MPZP). Piperazines are often sold in pill form under a variety of jolly names, most usually, 'party pills'. Curiously, BZP was originally synthesized commercially during the Second World War as a possible drug to be used against tapeworms, although this property is not an added bonus to regular users who enjoy raw meat, as it was not very effective. BZP and its relatives do have many amphetamine-like actions, including the usual stimulant effects (increased blood pressure and heart rate). BZP itself is a sympathomimetic like amphetamine, as it releases dopamine in the CNS through its effects on the dopamine transporter. The drug has shown some addictive potential in non-human primate studies, whilst TFMPP, which is a seratinonergic agonist, did not. The combination of BZP and TFMPP does appear to promote an MDMA-like 'entactogen' effect, where a feeling of general well-being is accompanied by a bonus of hallucinations at no extra charge. Whilst the combination causes seizures in rats, there are enough 17-year-old volunteers to determine in due course whether this occurs in man and at what dosage range. However, if you are 17 and overdose just on BZP, it is already established that there is an approximately 20 per cent chance of a seizure and a likely spell in intensive care. A small number of deaths have been recorded as linked with BZP, although as is the wont of habitual drug users, several other agents were also taken. Animal studies seem to reinforce the message that BZP and its related agents taken during adolescence may cause behavioural problems in maturity, although this remains to be discovered and experienced by lots of lucky 'party animals'.

Piperazine metabolism

The possibility that BZP and its congeners may be problematic when taken with prescription drugs is borne out by preliminary studies on their human biotransformation and metabolism. *In vivo*, relatively little is known of BZP's clearance, although one study suggested that its bioavailability is fairly low. The half-life was estimated at 5.5 hours and it is cleared much more slowly than methamphetamine. Hydroxylation of the aromatic ring was noted at the 3 and 4 positions, which was followed by sulphation rather than glucuronidation. There is also sulphation at one of the nitrogens in the piperazine group. Using human liver microsomes, it was found that BZP and TFMPP were metabolized mainly by CYP2D6, CYP1A2 and CYP3A4, but not by CYP2C19 and CYP2C9, although BZP does not appear to block CYP2C19, it does inhibit the rest of the major CYPs. Indeed, these drugs as a class appear to be major CYP inhibitors. Interestingly, BZP and TFMPP also inhibit each others' metabolism, which may partly explain their mutually enhanced pharmacodynamic effects on the CNS. Although more studies on the biotransformation of these drugs needs to be carried out, it seems certain that the piperazines will cause drug-drug interactions with CYP2D6 substrates such as antipsychotics and SSRIs as well as CYP3A4 substrates. The toxic interaction between MDMA and BZP is probably at least partly caused by irreversible CYP2D6 inhibition leading to BZP accumulation. It seems that the general propensity of drug users to take combinations of various agents may be particularly risky when this involves the benzylpiperazines.

B.6 Cannabis

Introduction

The hemp plant *Cannabis sativa* is the main source of cannabinoids, a group of around 60 terpene psychoactive agents which are probably the most commonly used illicit substances for recreational purposes. In the plant itself, the richest source of the cannabinoids is the resin removed from the leaves of the female plant. The most potent of the cannabinoids is Δ-9 tetrahydrocannabinol (Δ-9 THC; Figure B.10), although Δ-8 THC is as psychoactive and is chemically more stable. Cannabinoids are usually prepared from the dried flowering tops and leaves (marijuana, up to 5 per cent Δ-9 THC), the resin itself combined with the flowers (hashish, up to 25 per cent Δ-9 THC) and the industrial strength version, hash oil. This is resin that has been extracted and purified with solvents and concentrated and it can exceed 70 per cent in Δ-9 THC content. As cannabis is rather bulky to smuggle and law-enforcement interception rates have improved, it was found that crossing *Cannabis*

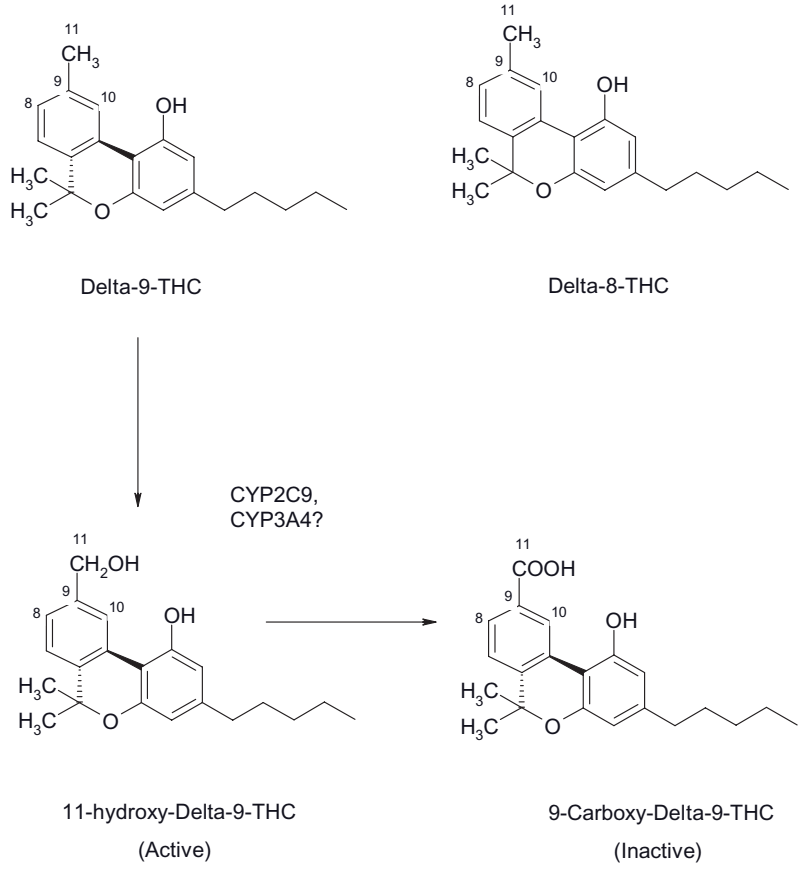


Figure B.10 The main active constituents of *Cannabis sativa*, Δ-8 and Δ-9 THC and the clearance of Δ-9 THC to its hydroxyl carboxy products by CYPs 2C9 and possibly CYP3A4

sativa with its less palatable cousin *Cannabis indica* happily yielded a hardier, smaller, more potent and profitable version known as 'skunk' which can be grown unobtrusively in quantity in a suburban house next door to you. Skunk can have three times the Δ-9 THC content compared with imported cannabis. A great deal of illicit research and development has led to the use of high intensity lighting which induces the plants to form more resin which is part of the effort to produce greater Δ-9 THC content. Indeed, the growing process is a race against time before the power company notifies local law-enforcement over the stadium-sized electricity bill.

The cannabinoids are quite potent and a dose of less than 10 mg is required for the standard 'high' effects, although they also have stimulant, sedative and even hallucinogenic properties. It is now clear that these agents they have more in common with opiates than other drugs of abuse such as cocaine, in that there are specific cannabinoid receptors known as CB1 and CB2. Endogenous agonists are anandamide and 2-AG, which are made on demand and then hydrolyzed by an enzyme known as FAAH, which is of interest as a possible drug target to potentiate the effects of the endogenous molecules. It seems that cannabinoid receptors act by inhibiting the release of neurotransmitters responsible for anxiety, like glutamate and GABA. THCs are agonists, whilst other agents in cannabis, such as cannabidiol (CBD) act as antagonists. Essentially, the mixture of psychotropic (mainly THC mediated) and anxiolytic (CBD) effects will vary according to the contents of the respective agents, route of administration and dose. Thus cannabis consumption leads to a complex mixture of effects targeted on areas of the brain rich in the CB1 receptors, such as those associated with cognition, memory and movement coordination. CB1 receptor activation by higher doses is noticeably different from lower doses and this may be linked the intense paranoia and psychosis, linked with heavy use, which can lead to users being 'sectioned' under the Mental Health Act. This process is exacerbated by the accumulation of active THC derivatives and skunk's low CBD levels. The CB1 receptors are also found in the periphery alongside CB2 receptors and their endogenous functions are wide-ranging and not entirely understood. Several synthetic agonists and antagonists have been synthesized. The first CB1 antagonist, rimonabant, was briefly introduced for treatment of obesity, but was withdrawn in 2008 due to CNS problems, which suggests that we have some way to go to understand the full profile of cannabinoid receptor function. In contrast, Sativex, a mouth spray that consists of equal proportions of CBD and Δ-9 THC, has shown promise in relief of pain and spasticity associated with multiple sclerosis and was approved in Canada in 2005. It is available at the time of writing in the UK on a 'named patient basis'.

It seems the main reason that cannabinoids are not very toxic is that unlike, say, opiate receptors, they are not found in vital neural areas that control respiration or heart rate. It is possible that more subtypes of cannabinoid receptors will be found and the full medicinal potential of these receptors will emerge in years to come. For instance, it is believed that the anti-angiogenic and antimetastatic properties of cannabinoids may be exploited in future anti-neoplastic agents.

Metabolism

THC-derivative clearance is extensive, with dozens of metabolites formed; indeed, only small amounts of parent Δ-9 THC appear in urine. The half-life of Δ-9 THC in blood is

around 20 hours, although the effects from smoking appear within 5–10 minutes. When cannabis products are smoked, Δ-9 THC is cleared within a few minutes to 9-carboxy Δ-9 THC (Figure B.10), which is pharmacologically inactive and is the major urinary metabolite. This metabolite is used to monitor cannabis usage in drug-testing protocols. It is unclear as to which CYP performs this function, although it is believed that CYP3A4 may form oxo-metabolites from 7- and 8-hydroxy derivatives of Δ-8 THC, which retain activity, although 2C9, 1A1 and 1A2 were also involved. It is interesting that school friends of mine who used the drug in the late 1970s insisted that it was far better consumed orally than smoked and that the effects were more pleasant and longer in duration. Indeed, it is now known that this is the case, as oral dosage promotes the formation of 11-hydroxy Δ-9 THC which is not only more potent than Δ-9 THC, but enters the brain more quickly. There is some evidence that CYP2C9 catalyses this reaction. It is possible that chronic use leads to induction of THC metabolism, but the metabolites are still very lipophilic and accumulate in fatty areas. After around a week, more than one-third of the dose is still in the user. The metabolites' oil solubility means that they are consequently difficult to clear into urine and complete excretion of the various metabolites usually occurs in faeces and can take months after high doses. Hence, the consumption of small quantities can lead to the failure of a random drug test several weeks later.

Carcinogenic activity of cannabis products

It is hotly debated by various groups as to whether cannabis smoking does lead to increased risks of lung cancer. Logically, it would appear to be very hard to separate the effects of tobacco, a known carcinogen, from the cannabis itself, as they are more often than not co-administered. There is evidence both for and against the possible carcinogenic properties of cannabis; in animal cell-line studies, cannabis was shown to be capable of inducing CYP1A1 activity, thought to be a key factor in lung carcinogenesis. Indeed, Δ-9 THC was shown to act through the Ah receptor system in classical fashion. However, Δ-9 THC was also shown to competitively inhibit the induced CYP1A1; what this might mean for possible carcinogenesis over many years of use is difficult to extrapolate. If Δ-9 THC exposure was fairly constant, then CYP1A1 levels would be maintained in accordance with exposure. It has been suggested that when CYP1A1 is 'idle', i.e. not oxidizing substrates, it 'leaks' reactive species that lead to DNA damage. Since THC derivatives are so persistent, it is possible that there may not be much time when the isoform is 'idle', so the THC might restrict reactive species formation. Clearly, arguments could be made in the opposite direction and on balance it is unlikely that occasional use would be carcinogenic, just as occasional use of tobacco is much less risky than heavy use. That the issue is not resolved after more than 25 years of study indicates that confounding factors such as the lack of filters on cannabis cigarettes, the variability of dose, the difficulty in estimating concurrent tobacco exposure and the effects of THCs on the immune system may well make it impossible to prove conclusively whether marijuana use is carcinogenic. It is, however, not unreasonable to suggest that heavy use, which is effectively abuse, will increase the risk of lung neoplasms. Some have suggested (hopefully ironically) that the more potent forms such as skunk would be less carcinogenic, on the grounds that users will limit their consumption and thus potentially their carcinogenic exposure.

B.7 Dissociative anaesthetics

PCP

Phencyclidine (PCP; Figure B.11), an arylcyclohexylamine, was developed in the 1950s as a dissociative anaesthetic, where the patient feels detached from themselves and their surroundings. PCP acts at the glutamate-binding NMDA receptor as a non-competitive antagonist. It had a potent analgesic effect, but at clinically effective doses it did not depress respiration or the cardiovascular system, which is highly desirable clinically. Although initially promising, the drug was withdrawn in the early 1960s after patients experienced extremely unpleasant hallucinations and agitation when they emerged from anaesthesia. Later in that decade, the drug began to be abused, although its effects were so unpredictable and often horrible, it never gained mass popularity. Once it was discovered it could be sprayed onto tobacco or cannabis and smoked to exert some rudimentary control of its effects, it gained more adherents, although it remains confined to some large cities in the US, notably Los Angeles and Philadelphia.

The drug is effective orally as well as through smoking PCP oil-soaked tobacco. The 'oil' is an ether extract of the drug when illicitly manufactured, which in the right circumstances is explosive and has been known to demolish buildings. In the pure form PCP is a white crystalline powder, but the street drug can be anything from a powder or pill to a

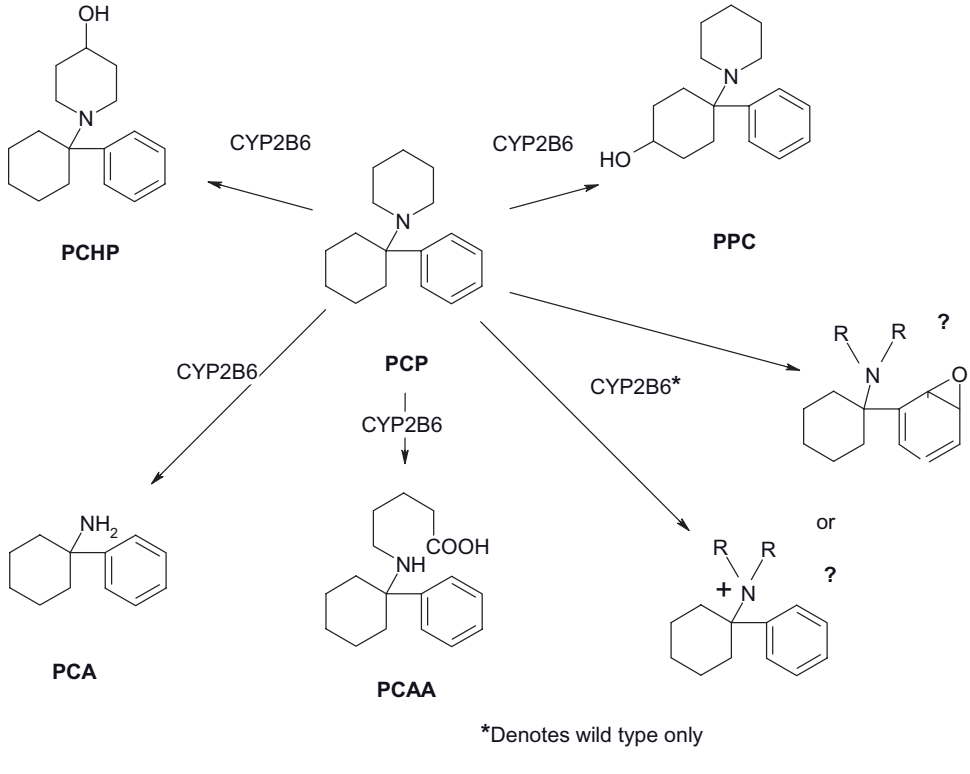


Figure B.11 Major metabolites of PCP in man; CYP2B6 K262R cannot form the reactive species which inactivates the enzyme.

brownish syrupy gum. The powdered form of the drug ('Angel Dust') is virtually pure PCP and is so lipophilic it can be absorbed through the skin in pharmacologically effective quantities. It enters the brain easily and interacts with so many neurotransmitter systems that it exhibits a very wide range of central stimulant and depressant effects, including paranoid delusions, depression and hallucinations. Those suffering from acute intoxication can be diagnosed by their generally bizarre and violent behaviour, nystagmus (eye oscillations and visual impairment) and a positive urine test for the parent drug. It causes seizures and can be lethal at only 1mg/kg, often due to a variety of severe reactions, from strokes, respiratory arrest, status elepticus and hyperthermia. The drug is spectacularly addictive on repeated use and it is said that users often die violently or commit suicide under its influence. Fortunately, for those who consider PCP as wholly inadequate for their recreational requirements, there are several designer analogues available, such as PCEEA and PCMEA, so good luck with that.

PCP Metabolism

PCP is extensively metabolized to several main hydroxylated derivatives, including PCHP (1-(1-phenylcyclohexyl)-4-hydroxypiperidine), PPC (4-phenyl-4-(1-piperidinyl)-cyclohexanol) and PCAA (5-[N-(1-phenylcyclohexyl)]-aminopentanoic acid; Figure B.11). Another metabolite has also been found, known as 1-phenylcyclohexylamine (PCA). The drug has a long half-life in man and the effects of one dose can last several days in chronic users. The metabolites are difficult to clear, but are eventually eliminated in the urine and faeces, where they can be detected up to 28 days after drug use. PCP appears to be able to affect CYP expression in animals, although it is unknown whether this might occur in man. It seems that early human liver microsome study showed that PPC and PCHP were cleared by CYP3A4, although there was high inter-individual variation in the pattern of its metabolite formation. Interestingly, a reactive species was detected and it was suggested that PCP could inhibit CYP3A4. More recent studies have found the CYP2B6 is probably the main isoform that clears PCP and its designer equivalents. The drug is also capable of forming a mechanism-dependent reactive species that inactivates wild-type CYP2B6 but not the K262R variant (Chapter 7, section 7.2.1), which is probably why PCP is so persistent in heavy users. Interestingly, the K262R variant can form all the dealkylated metabolites the wild-type can. The wild-type lysine is not near the active site, but it promotes substrate binding which facilitates reactive species formation. The substitution of the polymorphic arginine prevents this process. The reactive species can be trapped with GSH and is thought to be either a dihydroxylated iminium, or a monohydroxylated epoxide derivative. Hence, the effects of CYP2B6 inhibitors and inducers may depend on the drug user's CYP2B6 expression status. Overall, PCP is something of a 'Nantucket Sleigh ride' and is not recommended.

Ketamine

Ketamine, 'the drug PCP could have been' was synthesized in 1962 as an alternative to PCP and shares elements of its mode of action (Figure B.12). The drug entered the clinic in 1970 and has a number of desirable properties aside from its dissociative anaesthetic

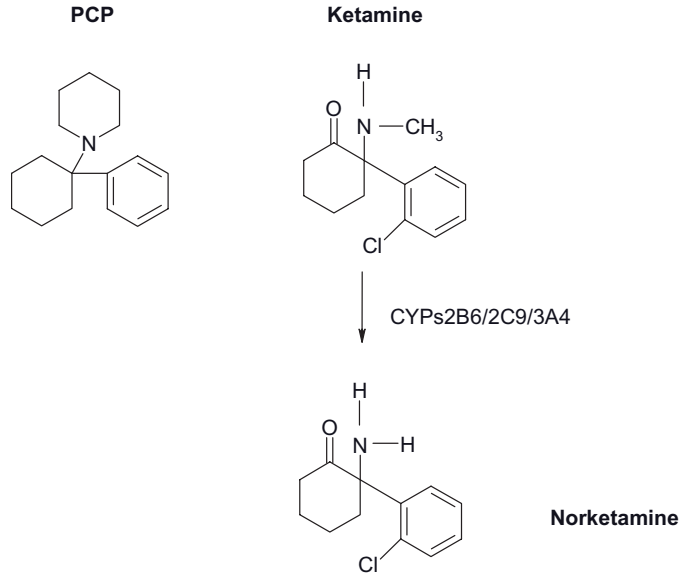


Figure B.12 Structure of ketamine and its major demethylated derivative norketamine, alongside the structure of PCP

effects, as it is a good analgesic and hypnotic and does not depress respiration. Although induction of anaesthesia is smooth, emergence can be problematic, with confusion and agitation. It is usually used intravenously or intramuscularly, as orally its effects are less predictable, with more of a sedative rather than an anaesthetic effect, unless the doses are high. It is still used clinically for some minor procedures and in Casualty/Emergency rooms. The drug is usually given as a racemate, although the S-isomer is four-fold more potent as an analgesic than the R-isomer. Interestingly, it has shown promise in patients with intractable depression, with one dose yielding a beneficial effect for a week or so. It is more commonly used now on animals, particularly horses, although it is less effective in cows. Ketamine is abused for its hallucinogenic, dissociative and narcotic properties and has been scheduled as a controlled drug in most countries. It appears to detach users successfully from who they are, why they are here and what they are doing, so it is not surprising that it has been said that its properties make it an 'ideal' date-rape drug. It is known by a number of street names, such as 'Special K', 'Kit-kat', 'wonk' and 'boing'. OK, so the last name I made up. Although not conventionally physiologically addictive, it can cause compulsive use and it does not inspire confidence that one of its leading advocates died in his bath while under its influence. Even when used medically, rapid intravenous injection can stop a patient breathing, so it is more than unwise to try this at home, unless your friends are particularly loyal, sober and adept at CPR.

Ketamine metabolism

The drug is cleared in man mostly through N-demethylation to norketamine (N-desmethylketamine), which is thought to have some analgesic action and more than

90 per cent of the dose is cleared in urine in 5 days. CYP2B6 is believed to be the main high affinity low capacity route of clearance, as well as CYP2C9, with some CYP3A4 involvement as the low affinity, high capacity isoform. This is supported by in vitro data where methadone and diclofenac (CYP2B6 and CYP2C9 substrates) are known to inhibit ketamine conversion to norketamine in human microsomes, probably by competition. The clearance of ketamine is likely to be affected by many factors, not least the high variability of CYP2B6 and to a lesser extent, CYP2C9, as well as the effects of inducers and inhibitors of these CYPs.

Were such things here that we do speak about, Or have we eaten on the insane root that takes the reason prisoner?

Act 1, Scene 3, *Macbeth*, by William Shakespeare

Appendix C
Examination Techniques

C.1 Introduction

This section does not just apply to drug metabolism, but to almost any life-science subject. Obviously, the type of examination you might be sitting for this subject could be anything from continuous assessment, through to multiple choice or essay-style questions. It is true that the vast majority of students extract from the university system more or less exactly what they put in. Every student, if they are honest, knows that they will get the degree they deserve and most put in enough effort to achieve this. If all you want is a modest degree, then you will not exert yourself, no matter what your lecturers say. However, there will be a fair number of students who would like to obtain the very best degree they can, but are unsure as to how to achieve this. Often, it is possible to emerge from 13 years of school examinations and still be none the wiser as to how to produce a first-class performance, *even though you are capable of it*. So how is it done?

C.2 A first-class answer

This does not consist of simply reproducing, like an elaborate living photocopier, the notes your lecturer gave you – you need much more than this. The detail of the lectures is a starting point, a platform, if you like, for building a first-class answer. If you have learned the course then you can understand and exploit the opportunity for extra reading and graft the extra knowledge onto your existing knowledge. This means you can show that you know and understand more than you were given. The initial source of this extra reading could be a textbook, but at the highest level it is better to use primary knowledge from journals. You may not be able to understand all of a scientific paper, but the introduction and discussion will provide an overview of the subject and if you read a few papers on the subject, you will see that they basically say the same thing. Another essential component in the construction of a first-class answer is the integration of your knowledge, perhaps with different courses and particularly in how you answer the question. Up to now, your answer will be clearly different from the run-of-the-mill student effort, but it still lacks a vital component for top marks. This final component can only appear once

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you have mastered the previous ones. You need to be 'creative'. Your answer must show depth of thought about a subject that means you have evaluated the available knowledge and come up with your own view and even your own interpretation on it. At the stage of a final degree, the subject is still evolving and 5, 10 or 20 years later, you might look back and see how wrong the prevailing wisdom of the time was, in terms of understanding of a particular phenomenon. So it is right to question the ideas and theories of current scientific literature, providing it is done through logical argument. So the five components are:

- learn and understand the lecture notes;
- extra reading of primary literature;
- demonstrate integration of knowledge;
- answer the specific question;
- show originality of thought and analysis.

If you can manage all this, your answer will practically leap off the page and stun your examiner, as the majority of students are either not prepared or not intellectually able to go to these lengths to succeed and this shows in their answers. It is unlikely that most of you can write first-class answers on every subject in your course. However, you should be able to, on courses that you find particularly interesting, as your interest makes the work easier to absorb. On courses that you really do not enjoy or have always had trouble with, work on them to the point where you can get a good second-class answer no matter what the question.

C.3 Preparation

From the above you can see what is *required* to achieve a first-class answer. You may be wondering exactly *how* you might achieve this. Up to university, most students have evolved a method of learning their work which has served them reasonably well, well enough indeed, to actually get to university. This may not be enough to compete at the highest level and the usual problems encountered are:

- lack of sufficient time devoted to revision;
- poor 'productivity' from time spent;
- inability to recall what was learned;
- exam terror/horror/panic (or worse).

Lack of sufficient time

This is obviously related to commitment and determination. Starting revision two or three weeks before a major examination is startlingly inadequate, even with a photographic

memory. To some extent, efforts must be made throughout the academic year to understand and process information accrued through lectures/practicals, etc.; certainly, several months before major examinations, revision should have been started. Many courses tend to communicate the bulk of the work required early in the year, so the majority of the course has usually been covered several months in advance of the finals. It is a case of 'how committed are you?' You may be capable of first-class work, but do not have the inclination to fulfil your potential. Regarding the future outside university, perhaps it is worth considering how much a good degree distinguishes you from 'the pack'. This gives you the widest possible choice in future directions, and in the context of near 50 per cent participation in higher education in the UK, you will need every edge you can get to find a specific job or position.

Poor productivity

It is very easy to sit in front of notes or a book for several hours and then adjourn to the nearest drinking establishment while transported in a haze of self-congratulation at your academic prowess. It is quite another thing to actively test how much you actually learned, by using past exam papers and setting yourself written tests. This can be a little demoralizing at first, but the truth must be found in terms of how much you have retained. Once you have been through your courses, you must start again and again and as many times as you can to commit them to long-term memory. After the second run-through, this is the time to incorporate extra reading and original thought, as you have worked out most of the basics and have something to build on.

Inability to recall

You may have been annoyed by a friend while watching an old film on the TV, who then announces who did it and why, having seen it once before in a drunken haze at party seven years previously. We appear to have almost unlimited space in our brains for storing information; the problem arises when we try to recall it. Obviously, you will remember where you went on holiday last year and have problems remembering what you did at 4.25 pm last Tuesday. The key appears to be some form of indexing system that provides a focus for recall. One way around this is to condense each lecture or parcel of a particular subject to a few lines, memorize them (word perfect), then memorize a list of these short condensates for each course. It is time consuming, but it means that you will be able to recall the entire course in serious detail. One of the keys to good performance in examinations is mastery of detail. This impresses examiners and can be attained with enough commitment. You may find your own way to 'reach' the knowledge you have learned. It is important that you use some method, as otherwise there is little point in learning your work if it cannot be recalled.

Exams – 'The horror ...'

This accounts for a considerable proportion of lack of fulfilment of potential and often has dogged a student's academic career. There is no easy answer, other than building confidence by using the techniques described above. Often poor examination performance in otherwise good candidates is due to 'rabbit in the headlights' – inexplicably strange choices made in the exam. Consider a crude and not entirely appropriate analogy: you will be aware that members of the armed forces, firefighters and the like, develop fears about what they have to face like anyone else, but they can overcome these fears and function during otherwise terrifying situations by sheer repetition in their training. How often do you read about some heroic individual who usually says something like 'I was staring certain death in the face, then my training kicked in and I saved the day. … I didn't have time to scared, we had trained for this for months, etc.'.

If they can do it, you can, by training yourself to face the exam horror by focusing on preparation, building confidence and looking forward to the exam. Before the exam you will have:

- learned the basic notes from the lectures;
- supplemented with primary literature;
- worked in your integration and understanding of the courses;
- where you can, applied original thought to the work;
- ensured that even courses which are less than thrilling, you can answer any question to high second-class standard.

This means you have trained yourself to turn a situation you dread into an occasion you actually want to arrive so you can shine.

C.4 The day of reckoning

It is possible to avoid the 'rabbit in the headlights' by using some important tips that should be burned into the consciousness so no amount of panic will erase them:

- Read the question: every word will have been scrutinized by maybe a dozen external and internal academics, so clearly every word, punctuation mark, etc. is essential and cannot be ignored.

- If there is a choice, *do the easiest question first*: you gain confidence with 'money in the bank'. You will save time also, which you can use in tackling harder questions. The questions should be done in order of difficulty.

- Do not write things that you know are not relevant to question: this is double jeopardy, you are getting no marks and losing time that you could have used to answer a question where you might have excelled.

Finally, you have done all you can do. A university/college is the last opportunity in your life where you will receive an absolutely fair deal, whether or not you might have extenuating circumstances or whatever might apply. The staff and the external examiners are guaranteed to give you every consideration so that the degree you received is a fair and accurate reflection of your commitment and aptitude. You will never again encounter a more ‘level playing field’, so make the most of it.

Appendix D
Summary of Major CYP Isoforms and their Substrates, Inhibitors and Inducers

This is not an exhaustive list and many drugs are metabolized by several CYP isoforms to varying degrees.

CYP	Substrates	Inhibitors	Inducers
1A1	Polycyclic aromatic hydrocarbons (PAHs), organochlorine insecticides	α-naphthoflavone	PAHs, organochlorines
1A2	Amitriptyline, imipramine, caffeine, fluvoxamine, clozapine, haloperidol, mexiletine, ondansetron, propranolol, tacrine, theophylline, verapamil, R-warfarin, zolmitriptan Polycyclic aromatic amines	Amiodarone, cimetidine, furafylline, fluvoximine, ticlopidine ciprofloxacin	PAH amines in barbecued/ flame-broiled meat, Brussels sprouts, broccoli, insulin, tobacco, omeprazole, phenytoin
1B1	Tamoxifen, polycyclic aromatics Aromatic amines, aflatoxins, oestrogens.	PCBs (e.g. pyrene) some flavonoids & coumarins, resveratrol, mitoxantrone, flutamide, taxols	PAHs, β-naphthoflavone tobacco PAHs
2A6	Coumarins, aflatoxins 1,3, butadiene, letrozole, valproate	Tranylcypramine, methoxsalen, grapefruit juice	Phenobarbitone, rifampicin
2B6	bupropion, coumarins, cyclophosphamide, mephenytoin, methadone, methoxychor (pesticide), PCP, ketamine, efavirenz	Tranylcypramine, thiotepa, ticlopidine	Phenobarbitone, rifampicin, efavirenz
2C8	Amodiaquine, cerivastatin, paclitaxel, tolbutamide	Quercetin, glitazone drugs, gemfibrozil efavirenz, montelukast	Phenobarbitone Rifampicin,

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CYP	Substrates	Inhibitors	Inducers
2C9	Amitriptyline, dapsone, fluoxetine, fluvastatin, NSAIDS, phenytoin, sulphonyl ureas, tamoxifen, S-warfarin	Isoniazid, fluvastatin, fluvoxamine, lovastatin, sulphafenazole diclofenac	Secobarbitone, Rifampicin
2C19	Barbiturates, citalopram, mephenytoin, proton pump inhibitors e.g. omeprazole, phenytoin, proguanil, R-warfarin voriconazole.	Tranylcypramine, cimetidine, fluoxetine, ketoconazole, ticlopidine	Carbamazepine, norethindrone, prednisone, rifampicin
2D6	TCAs, antipsychotics (haloperidol, etc.), anti-arrhythmics: flecainide, mexiletine, beta-blockers (e.g. timolol, S-metoprolol, bufuralol), MDMA, SSRIs, opiates (e.g. tramadol, codeine), venlafaxine	Amiodarone, cimetidine, ranitidine, histamine H-1 receptor antagonists (e.g. chlorpheniramine), quinidine, SSRIs (e.g. fluoxetine), St John's Wort	Not conventionally induced
2E1	Benzene, chlorzoxazone, ethanol, flurane anaesthetics (e.g. halothane), paracetamol	Sulphides (e.g. DDC, diallyl sulphide, disulfiram)	Ethanol, acetone isoniazid
3A4/5	Aflatoxin B1, antihistamines (terfenadine, astemizole), calcium channel blockers (e.g. felodipine), cannabinoids, cyclosporine, macrolides (e.g. erythromycin), opiates (buprenorphine), protease inhibitors (e.g. ritonavir), statins (except pravastatin), tacrolimus, paclitaxel THCs midazolam, nefazodone, dasatinib.	Amiodarone, azoles (ketoconazole fluconazole, voriconazole) cimetidine, grapefruit juice, macrolides, steroids, protease inhibitors	Barbiturates, carbamazepine, glucocorticoids, glitazones, nevirapine, phenytoin, rifampicin, St John's Wort

Suggested Further Reading

This section is intended for those interested in looking at some of the source literature that I consulted when I wrote the text. By the time you read this, many more up-to-date reports will be available which can be found easily using electronic systems. Indeed, these fresh reports will probably contradict some of the conclusions in the text as knowledge develops. As part of your study, it is important to realize that real understanding of many life-science processes is often difficult to acquire. In many cases, it will be necessary to read several authors' versions of an explanation before it is possible to form an understanding that makes sense to you. Someone once asked Isaac Newton towards the end of his life how he had made so many brilliant discoveries. He replied to the effect that he thought about the problems constantly, mulling them over in his mind ceaselessly until he solved them. So if this was not an instant process even for Newton, you must also give yourself time to absorb the arguments and explanations made in scientific literature. Sometimes, there are conflicting data available and leading authors come to different conclusions. In this case, have confidence in your own ability to follow the logic of the arguments and even if you feel that you lack experience in this field, you still bring the ability to follow a well-written argument. If you cannot follow or understand a theory or argument, then it is possible that it is not as logical as it appears and your interpretation of the process could be more valid. However, everyone has their limitations and even the greatest scientists can be spectacularly wrong-headed; even Newton spent many years in a doomed attempt to turn base metal into gold. Nonetheless, the next generation of discoveries must be made by somebody training now; remember that senior figures in science certainly have vastly superior knowledge of the field, but in terms of raw intelligence they may not necessarily be any cleverer than you!

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