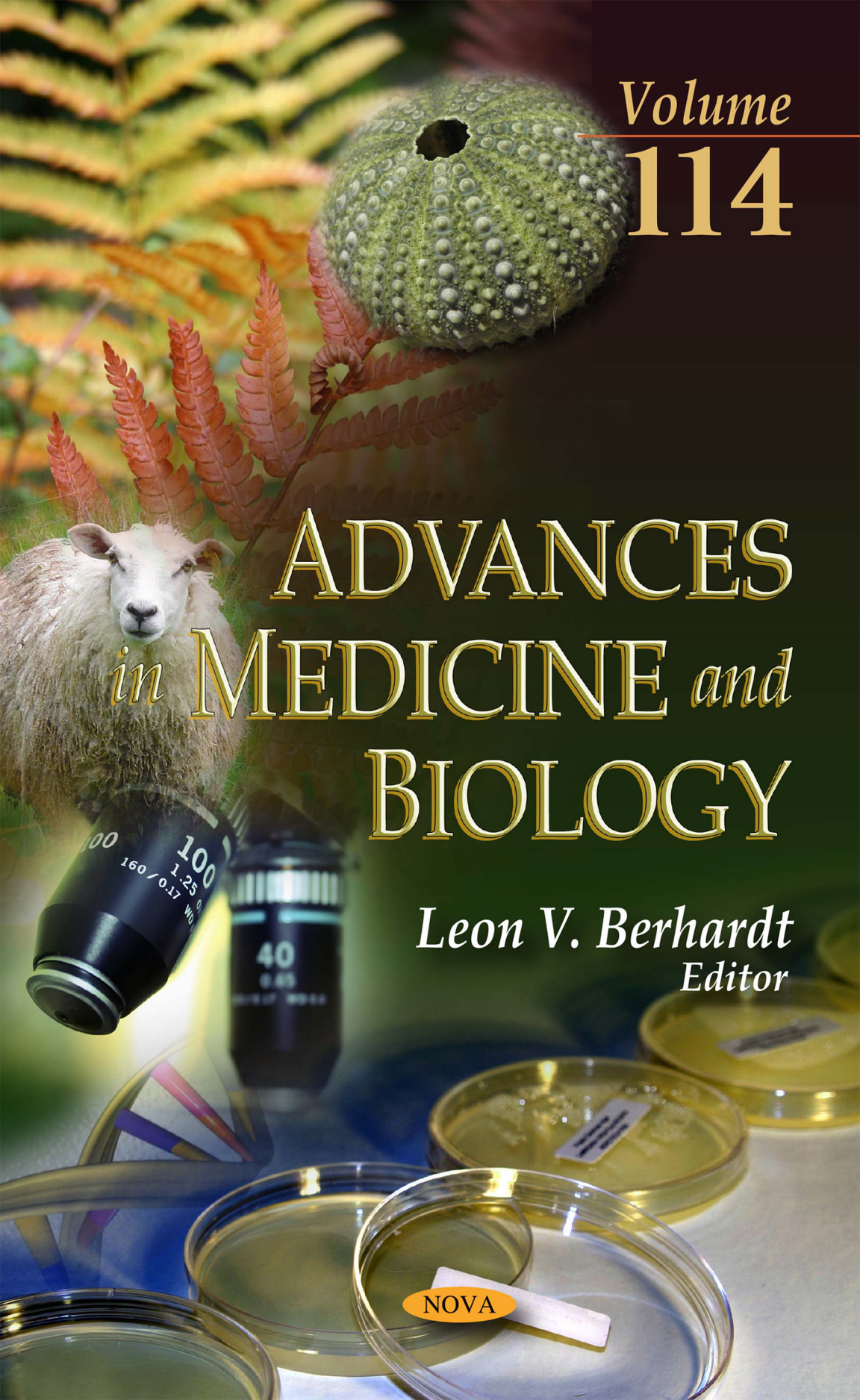
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ADVANCES
in MEDICINE and
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Leon V. Berhardt
Editor
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ADVANCES IN MEDICINE AND BIOLOGY

VOLUME 114

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ADVANCES IN MEDICINE AND BIOLOGY

ADVANCES IN MEDICINE AND BIOLOGY

VOLUME 114

LEON V. BERHARDT
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CONTENTS

PREFACE

The chapters in this volume present the latest developments in medicine and biology. Chapters One focuses on the synthesis of bisphosphonates and its biomedical applications. Chapter Two reviews polymeric nano-vectors based siRNA delivery. Chapter Three discusses the use of thrombophilia and pharmacological throboprophylaxis screening in women. Chapter Four provides insights into *Geobacillus* species through an overview of the model strain HTA426. Chapter Five examines how glycans provide molecular recognition motifs which regulate endoplasmic protein folding, transport, lysosomal targeting, and also how they are used by pattern recognition receptors in pathogen surveyance and innate immunity. Chapter Six discusses the effect of dehydroepiandrosterone (DHEA) on diabetes, obesity, and atherosclerosis. Chapter Seven discusses the various classifications, clinical features, diagnosis, and management options of juvenile idiopathic arthritis (JIA). Chapter Eight studies several surgical approaches for knee arthroplasty with their relevant advantages and disadvantages. Chapter Nine explores the antioxidant potential of lipoic acid in ameliorating the oxidative stress produced during lung carcinogenesis.

Chapter 1 - Bisphosphonate is a class of drugs used to slow down bone damage. They have been found to exhibit antimicrobial, anticancer, antiplasmodial activities etc. However, bisphosphonates suffer from limitations such as toxicity, poor bioavailability and low intestinal adsorption. Due to the aforementioned limitations, there have been several investigations by several researchers towards the development of delivery systems for delivery of bisphosphonates. This chapter will be focused on the recent classes of bisphosphonates synthesized and biomedical applications.

Chapter 2 - A plethora of encouraging studies have proposed siRNA based therapeutics as a potent and promising strategy towards treatment of various diseases, including, cancer. Several nanoparticle based vectors have been tailored in order to develop an effective delivery system. However, several barriers such as poor cellular uptake, instability and immunogenicity in physiological conditions needs to be resolved before successful clinical application. Hence, a clinically suitable and stable nanoparticle-siRNA formulation needs to be developed. In this context, polymeric nanoparticles have revolutionized drug-delivery systems owing to its ability to protect nucleic acids from degradation, intracellular uptake, low toxicity and controlled release. Amongst the available polymers, cationic polymers are one of the predominantly investigated molecules for the formation of stable complexes with the negatively charged nucleic acids. Further, these polymers also confer enhanced protection against nucleases. Manipulation of certain parameters such as molecular weight, charge density and hydrophobicity/hydrophilicity balance have shown to result in improved vectors.

Chapter 3 - Assisted reproductive technologies (ART) are associated to an increased risk to develop venous thromboembolism (VTE) compared to spontaneous pregnancy. Previous studies have found that VTE after a spontaneous conception is nearly 1 of 1000 pregnant women, while in women undergoing ART it may raise of three-four times. The pathophysiological mechanism by this increase is related to medical ovarian stimulation, a pharmacological ovarian stimulation that increases the number of oocytes available for ART associated to the presence of other risk factors as thrombophilia. Pharmacological ovarian hyperstimulation, in fact is able to increase procoagulant status of all women that may lead to thrombosis, as showed by several studies. However, available data about the magnitude and the duration of VTE risk in pregnancy after ART are conflicting, just as data about the risk to develop VTE for all women that perform ART and not only for those that show pregnancy after ART. Actually, the authors have only data by women that develop VTE during pregnancy after ART, as reported by clinical series or studies but the incidence of VTE for women that perform ART with or without a following successful pregnancy is unknown and only case reports or small clinical series are reported in the Literature. Several clinical aspects are lacking of full understanding as the VTE risk for women that will perform ART. The potential different role of such drugs and their dosages as the role of thrombophilia for the outcome of VTE and ART are

involved in this clinical setting; the potential of other thrombotic risk factors should be better understood.

Chapter 4 - The genus *Geobacillus* comprises Gram-positive, aerobic or facultative anaerobic, endospore-forming thermophiles that preferentially grow at temperatures ranging from 55°C to 65°C. The members of this genus were originally categorized into the genus *Bacillus*, a large family containing more than 305 species, but have been reclassified into *Geobacillus* as phylogenetically related thermophiles. *Geobacillus* species exist in a wide variety of niches; hence they have attracted interest in the fields of microbiology and biotechnology. For example, *Geobacillus* species often show remarkable properties in combination with thermophilic traits and are therefore used in microbial bioprocesses at high temperatures. In addition, they have historically served as sources of thermostable enzymes, which can be used as stable industrial catalysts and model proteins for biochemical and structural analyses. *Geobacillus* species are also used for thermoadaptation-directed enzyme evolution. Moreover, their diversified genomes and habitats may yield information on the mechanisms of organic evolution. In this chapter, the microbial properties, genomic features, bacteriophages, thermostable proteins, genetic tools, high-temperature bioprocesses, and thermoadaptation-directed enzyme evolution related to *Geobacillus kaustophilus* HTA426 are centrally reviewed. This strain was isolated from deep-sea sediment in the Mariana Trench and was the first among *Geobacillus* species to undergo whole-genome sequencing. Subsequently, the establishment of various genetic tools has led *G. kaustophilus* HTA426 to become one of the most intensively studied thermophiles. This paper aims to provide insights into *Geobacillus* species through an overview of the model strain HTA426.

Chapter 5 - Glycans are ancient highly conserved molecules which occur throughout 500 million years of vertebrate and invertebrate evolution. Glycans represent a significant repository of "recognition" information encoding functional data rivalling that of RNA, DNA and proteins. A diverse range of lectins have evolved to identify glycans and are used to regulate many essential physiological processes including protein folding, endoplasmic transport and secretion and the recognition of pathogenic organisms through molecular pattern signatures arising from pathogen cell wall components. Viruses and pathogenic bacteria have also developed their own glycan binding proteins which recognise cell surface glycans of the host cells and use these as docking modules for infection. A greater understanding of glycan interactive processes in health and disease is expected to deliver useful information

relevant not only to tissue homeostasis and functionality but also to the development of preventative measures to combat deleterious glycan-pathogen interactions in disease.

Chapter 6 - Dehydroepiandrosterone (DHEA) and its sulfated ester (DHEA-S) are the most abundant adrenal steroids in human blood. Peak levels of DHEA and DHEA-S occur around the age of twenty and decrease gradually thereafter. DHEA has been reported to have beneficial effects on diabetes mellitus, obesity, and atherosclerosis. Meta-analysis of DHEA supplementation to elderly men or women showed no effects on blood glucose and total cholesterol levels. However, DHEA supplementation to patients with type 2 diabetes has not been fully elucidated. Type 2 diabetes is characterized by an impaired capacity to secrete insulin, insulin resistance, or both. It has been reported in animal models that DHEA and DHEA-S increase not only insulin secretion of the pancreas but also insulin sensitivity of the liver, adipose tissue and muscle. The authors investigated the effect of DHEA on glucose metabolism in animal models and reported that DHEA decreased liver gluconeogenesis. Recently, the authors reported the effect of DHEA on liver and muscle by using insulin receptor substrate 1 and 2 (IRS1 and IRS2)-deficient mice. DHEA increased insulin-stimulated Akt phosphorylation in the liver of C57BL6 IRS1- and IRS2-deficient mice fed a high fat diet, suggesting that the increase in Akt signaling induced by DHEA is sufficient in the presence of IRS1 or IRS2. Here, the authors introduce the effect of DHEA on the liver, muscle, adipose tissue, and insulin secretion in diabetes mellitus and its effect on obesity and atherosclerosis in human and animal models.

Chapter 7 - Juvenile idiopathic arthritis (JIA), a term referring to a group of disorders characterized by chronic arthritis, is the most common chronic rheumatic illness in children and is a significant cause of short- and long-term disability. There has been a lot of development in the understanding of the disease pathogenesis and etiology in the last decade. Recently, the introduction of biological agents in the management of JIA has changed the approach and outcome in these children. This article discusses the various classifications, clinical features, diagnosis, and management options for JIA.

Chapter 8 - Arthroplasty remains the gold standard for end stage arthritis. Excellent long term results can be expected from the well-executed knee arthroplasty and adequate exposure is key to this success. Of the several approaches to the knee, there is no clear consensus as to the best approach. The medial parapatellar approach is by far the most frequently used, in particular in the setting of a primary knee arthroplasty. While most arthroplasty surgeons choose one approach as standard for most of their

patients, a working knowledge of other approaches is important as a standard approach may not be suitable for all patients.

Chapter 9 - Modulatory role of lipoic acid on oxidative stress produced during benzo(a)pyrene (B(a)P) induced lung cancer was studied in mice. Administration of B(a)P to mice caused a significant increase in oxidative stress indicated by abnormal changes in lipid peroxidation, enzymic antioxidants (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase) and non-enzymic antioxidants (reduced glutathione, vitamin C, vitamin E and vitamin A). Treatment with lipoic acid prevented all the above abnormal changes and restored cellular normalcy suggesting the protective role of lipoic acid in curbing the oxidative stress produced in B(a)P induced lung carcinogenesis in mice.

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Chapter 1

SYNTHESIS OF BISPHOSPHONATES AND THEIR BIOMEDICAL APPLICATIONS

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ABSTRACT

Bisphosphonate is a class of drugs used to slow down bone damage. They have been found to exhibit antimicrobial, anticancer, antiplasmodial activities etc. However, bisphosphonates suffer from limitations such as toxicity, poor bioavailability and low intestinal adsorption. Due to the aforementioned limitations, there have been several investigations by several researchers towards the development of delivery systems for delivery of bisphosphonates. This chapter will be focused on the recent classes of bisphosphonates synthesized and biomedical applications.

Keywords: bisphosphonates, polymer-drug conjugates, drug delivery systems, anti-cancer

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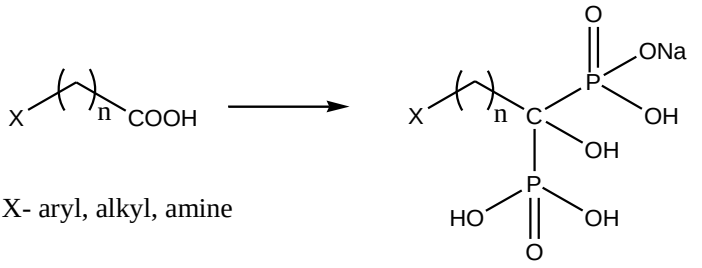
1. INTRODUCTION

Bisphosphonates are drugs used to treat bone diseases such as osteoporosis. However, they have been reported to be effective for the treatment of cancer and they also exhibit anti-inflammatory, antimicrobial and antiplasmodial activities *etc*. [1, 2]. Bisphosphonates have (P-C-P) bond with two phosphonate (PO_3) groups which are linked covalently to the same carbon atom. The carbon side chain is useful for modification that can result in compounds with various pharmacological properties. They are grouped into two classes namely: the nitrogen containing and non-nitrogen containing bisphosphonates. The nitrogen containing bisphosphonates are characterized by (N-PCP) bond. They enhance antiresorptive potency and do not destroy the mineralization of newly formed bone [3]. Examples of nitrogen containing bisphosphonates are zoledronate, alendronate, minodronate, ibandronate, neridronate, olpadronate, risedronate and pamidronate. The non-nitrogen containing bisphosphonates act by incorporation into ATP and examples are etidronate, medronate, clodronate, and tiludronate [4].

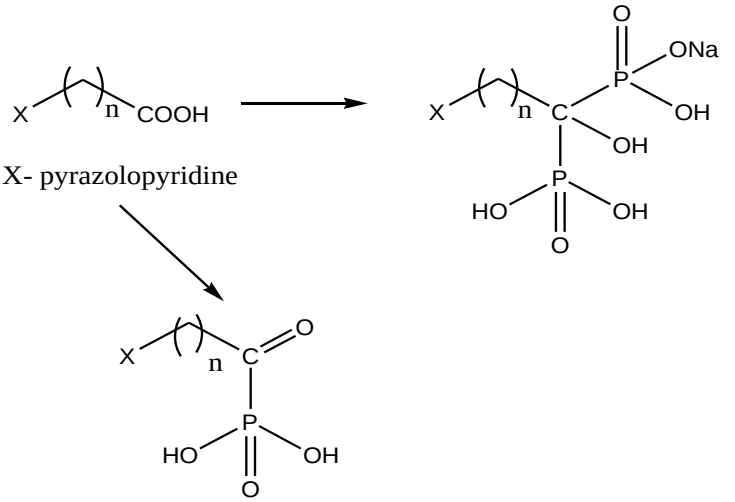
Bisphosphonates are used to treat bone diseases. However, they suffer from side effects when administered orally or intravenously, such as inflammation of the oesophagus, flu-like symptoms, low calcium level, toxicity, *etc*. [5, 6]. The side effects of bisphosphonates have prompted several researchers to develop delivery systems that can reduce these side effects with controlled release mechanism of the bisphosphonates to the target site. Some researchers have also prepared modified bisphosphonates. This chapter will be focused on the recent synthesis of bisphosphonates and their biomedical applications.

2. SYNTHESIS OF BISPHOSPHONATES

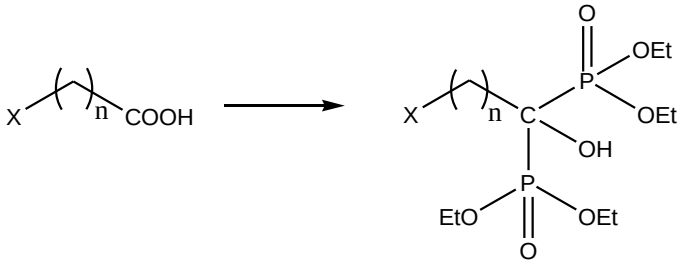
There are few recent reports on the synthesis of bisphosphonates in good yields. Lenin et al., employed microwave-assisted procedure for the synthesis of bisphosphonates containing sulphur and nitrogen in solvent-free medium [7]. The reaction was performed at 80-100^{o}C for 3 minutes. However, 80 ^{o}C was reported to be the appropriate temperature that resulted in good yields between 70-90% (Scheme 1).



Scheme 1. Microwave assisted synthesis of bisphosphonates.

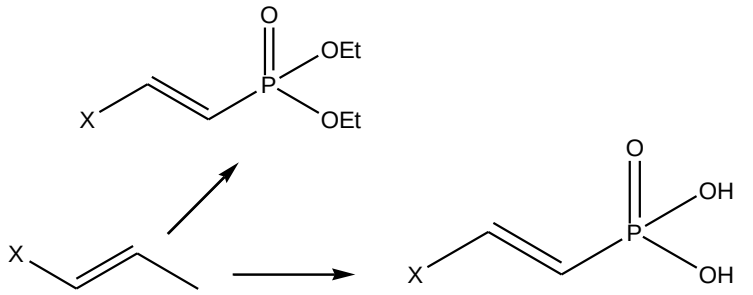


Scheme 2. Synthesis of pyrazolopyridine based bisphosphonates.



X- cyclohexanone, cyclopentanone, indanone

Scheme 3. Synthesis of cyclic ketones and piperidone based bisphosphonates.



X- 5(4H)-oxazolones

Scheme 4. Reaction of 5(4H)-oxazolones with tetraethyl methanediylbis(phosphonate).

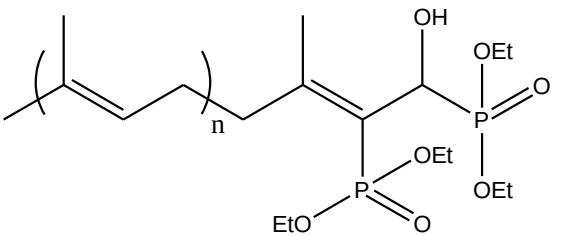


Figure 1. Synthesis of α-Hydroxy(polyprenyl) Bisphosphonates.

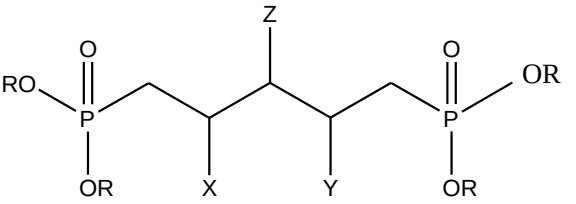
Teixeira et al., synthesized pyrazolopyridine based bisphosphonates and monophosphonates from carboxylic acids derived from 1*H*-pyrazolo[3,4-*b*] pyridine by conventional method in low yields [8] (Scheme 2).

Philip and Barros reported Michael addition reaction of cyclic ketones and piperidones to a vinyl phosphonate in good yields [9] (Scheme 3).

Boulous et al., reported the reaction of 5(4H)-oxazolones with tetraethyl methanediylbis(phosphonate) which afforded 1,1-bisphosphonate and 1,1-bisphosphonic acid analogues [10] (Scheme 4).

Kolodyazhnaya et al., reported bisphosphonates prepared from natural terpenes by phosphonylation of corresponding aldehydes [11] (Figure 1).

Samarat et al., developed a simple and efficient methodologies for the synthesis of novel hydroxy- and aminobisphosphonate derivatives by reductive amination of γ,γ′-diphosphonyl ketones (Figure 2) [12].



X and Y- aryl and alkyl groups
Z- amine and OH

Figure 2. Hydroxy- and aminobisphosphonate derivatives.

Matthiesen et al., reported stereo-controlled synthesis of isoprenoid-substituted bisphosphonates [13].

3. HYBRID OF BISPHOSPHONATES

Hybrid drugs are obtained from combining multiple existing drugs into one novel drug. The drugs are covalently linked to form molecule with synergistic properties that can exhibit a higher potency than the individual drugs. The hybrid compounds is based on combination therapies that can potentiate the effects of single drugs, overcome drug resistance, increase bioavailability with reduced toxicity resulting in enhanced therapeutic efficacy [14]. Few researchers have reported hybrid compounds in which one of the drug is a bisphosphonate.

Bekker et al., reported a one-step synthesis of a bisphosphonate derivative from folic acid and 1-(2-aminoethylamino)-1-(diethylphosphoryl) ethylphosphonic acid diethyl ester (Figure 3) [15]. Herczegh et al., conjugated bisphosphonates to fluoroquinolone analogues via an intermediate carbon. The hybrid compound exhibited and retained good antibacterial activity against most Gram-negative bacteria (Figure 4) [16]. Sturtz et al. prepared hybrid compound containing bisphosphonate and methotrexate [17]. Houghton et al., reported hybrid compounds containing bisphosphonate and fluoroquinolone [18-19].

Liu et al., synthesized hybrid compounds containing zoledronate and quinolone derivatives which were active against cancer cells [20] (Figure 5).

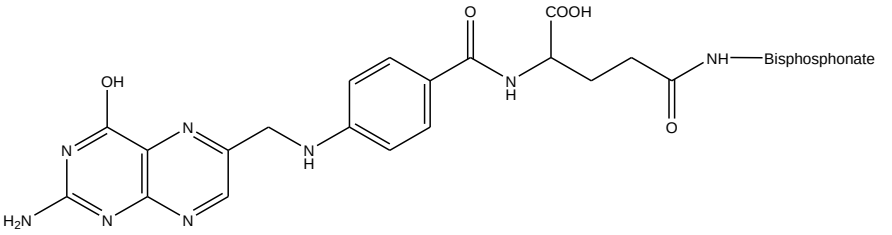


Figure 3. Hybrid compound of bisphosphonate containing folic acid.

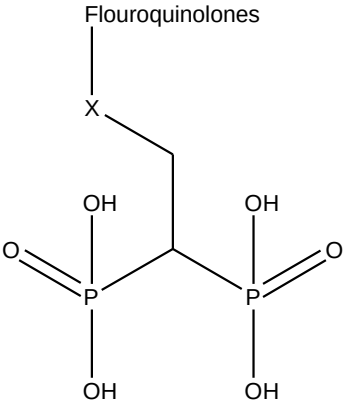


Figure 4. Hybrid compound of bisphosphonate containing fluoroquinolone.

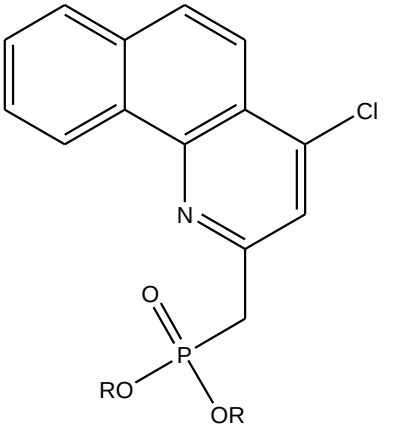


Figure 5. Hybrid compound containing bisphosphonate and zoledronate.

Abdou et al., prepared 2-chloroquinoline-based bisphosphonates in excellent yields. The compounds exhibited excellent antitumor activity against breast, prostate, ovarian cancer and melanoma. The compounds also exhibited anti-inflammatory properties at a dose of 50 mg/kg body weight [21]. Nakatake et al., conjugated platinum (II) complex to dialkyl bisphosphonic acid as a drug for metastatic bone tumor. The complex showed good hydroxyapatite affinity and tumor growth inhibitory effect than the free platinum drug [22].

4. APPLICATIONS OF BISPHOSPHONATES

4.1. Antimalarial

Presently, the number of drugs which target *Plasmodium* liver stages is very limited and example of such drug is primaquine. However, primaquine suffer from toxicity and there is a risk of hemolysis when administered to persons with glucose-6-phosphate dehydrogenase deficiency [23].

Bisphosphonates are potent inhibitors of the enzyme farnesyl diphosphate synthase (FPPS). It catalyzes the condensation of the isoprenoids dimethylallyl diphosphate and isopentenyl diphosphate which are produced in mevalonate pathway resulting in geranyl diphosphate. Geranyl diphosphate that is formed combines with another molecule of isopentenyl diphosphate molecule to form farnesyl diphosphate and geranylgeranyl diphosphate. FPPS inhibition then occurs and bisphosphonates block protein prenylation, as well as sterol, ubiquinone, dolichol, and heme biosynthesis [23]. Bisphosphonates stimulate human γδ T cells and interfere with antiparasitic activity. Bisphosphonate can also result in the inhibition of the mitochondrial adenine nucleotide translocase and induce apoptosis [23].

Singh et al., reported lipophilic bisphosphonates activity against *Plasmodium* liver stages. In vivo evaluation of the drugs on mice with *Plasmodium berghei* sporozoites revealed complete protection after 28 days. Activity against blood stage forms *was observed in vitro.* The lipophilic bisphosphonates exhibited activity against a *Plasmodium* geranylgeranyl diphosphate synthase and a low nM activity against human farnesyl and geranylgeranyl diphosphate synthases. The findings suggested that bisphosphonates are promising drugs for liver stage infection [23]. No et al., reported lipophilic analogs of zoledronate and risedronate. The analogs were active in cells and *in vivo* evaluation on mice with a major decrease in

parasitemia and 100% survival [24]. Zhang et al., synthesized lipophilic bisphosphonates which were tested in malaria parasite killing and human γδ T cell activation. Shorter chain-length species exhibited low activity whereas longer chain length species were poor enzyme inhibitors [25].

4.2. Application of Bisphosphonates for Treatment of Neglected Protozoan Infections

Protozoan infections are referred to as neglected diseases affecting millions of humans in the poor regions of the world populations. Some of such infections are leishmaniasis, trypanosoma, toxoplasma, schistosoma and cryptosporidium.

Leishmaniasis is a disease caused by the *Leishmania* protozoan parasite and is common in the tropical regions of Africa, Asia, Central and South America [26]. Bisphosphonates have been found to be effective as anti-leishmaniasial drugs. Christensen et al., reported alkyl bisphosphonate compounds which were active against *Leishmania tarentolae*. Complexes of vanadium/alendronate and vanadium/zoledronate were prepared and tested against *L. tarentolae* and they were found to be effective in inhibiting the growth of *L. tarentolae* [26]. Martin et al., investigated the effects of a class of bisphosphonates on the proliferation of *Trypanosoma cruzi, Trypanosoma brucei rhodesiense, Leishmania donovani, Toxoplasma gondii*, and *Plasmodium falciparum*. The nitrogen-containing bisphosphonates exhibited significant antiparasitic activity suggesting their potential application for treatment of parasitic protozoan diseases [27].

Szajnman et al., studied the antiprotozoal activity of a series of bisphosphonates derived from fatty acids as potent inhibitors against *Trypanosoma cruzi* proliferation [28]. Bouzahzah et al., reported the application of risedronate for the treatment of CD-1 mice infected with Brazil strain of *Trypanosoma cruzi* [29]. Demoro et al., prepared metal complexes with two bioactive bisphosphonates namely: alendronate and pamidronate. The complexes were active against *Trypanosoma cruzi* and exhibited no toxicity to the cells [30]. Huang et al., demonstrated the effects of nitrogen-containing bisphosphonates against *Trypanosoma cruzi* [31]. Yang et al., screened bisphosphonates against *Trypanosoma brucei* [32]. Recher et al., reported 2-sulfur containing bisphosphonate derivatives which were active against intracellular form of *Trypanosoma cruzi* and *Toxoplasma gondii* [33].

Liang et al., evaluated bisphosphonates against the replication of *Toxoplasma gondii in vitro* and *in vivo*. N-alkyl bisphosphonates containing long hydrocarbon chains were the most active. *In vivo* evaluation on Smith-Webster mouse model showed 80% protection from death with good effects against *Toxoplasma gondii* growth [34]. Rossi et al., investigated long-chain 2-alkylaminoethyl-1,1-bisphosphonates against proliferation relevant form of *Trypanosoma cruzi* and tachyzoites of *Toxoplasma gondii* [35]. Ziniel et al., investigated bisphosphonate activity against schistosomiasis [36]. Moreno et al., reported bisphosphonates ability to inhibit the growth of *C. parvum* in a mouse xenograft model [37]. Artz et al., reported nitrogen-containing bisphosphonates with anti-cryptosporidium activities in low micromolar concentrations [38].

4.3. Antimicrobial

Bacterial arthritis is a severe, progressive and erosive disease with high morbidity and mortality [38]. Staphylococcus aureus is the most common cause of non-gonococcal arthritis and it has high degree of selectivity for the joints [38].

Verdrengh et al., studied the effect of combination of zoledronic acid with antibiotics and corticosteroids for the treatment of staphlococcus aureus-induced arthritis. *In vivo* study of *S. aureus* mice treated with bisphosphonates in combination with antimicrobial agents and corticosteroids decreased the activity of osteoclasts in septic arthritis significantly resulting in the risk of skeletal destruction [39]. Steinmetz et al., reported the antibacterial activity of bisphosphonates. A thin coating composed of poly(styryl bisphosphonate) grafted onto oxidized polypropylene films were prepared for anti-biofilm applications. The anti-biofilm formation was effective against Gram-negative and Gram-positive bacteria [40].

4.4. Antiviral

Bisphosphonates have also been reported to exhibit antiviral activity. Lacbay et al., developed pyrido[2,3-d]pyrimidine bisphosphonates which were screened and were effective at inhibiting HIV-1 reverse transcriptase [41]. Tu et al., reported aminobisphosphonate effects against influenza virus infected cells. It inhibited viral replication *in vitro* [42]. Richter et al., invented

formulations of bisphosphonate that specifically inhibited the activity the number of the monocytes and/or macrophages resulting in reduction or elimination of HIV reservoirs [43]. Hostetler et al., invented phosphonate compounds containing phosphonate monoester formed by covalent linking of monophosphonates to selected antivirals such as: cidofovir or tenofovir [44].

4.5. Anticancer

Bisphosphonates inhibit tumor cell adhesion, migration, invasion, proliferation and induce tumor cell apoptosis when used alone or in combination with chemotherapeutic agents [45]. The antitumor effects of nitrogen containing bisphosphonates from *in vitro studies* suggests that the inhibitory mechanisms of action of bisphosphonates is via the blockade of the mevalonate pathway [45]. Fehn et al., compared the antitumor effects of zoledronate with docetaxel, adriamycin, cyclophosphamide, 5-fluorouracil, epirubicin and cyclophosphamide in primary breast cancer cells. The combinations resulted in IC_{50} which was significantly lower than the IC_{50} values for the selected anticancer drugs. The median IC_{90} value for zoledronate was significantly higher than that of some of the anticancer drugs [46]. Rashner reported that zoledronic acid inhibited MDA-MB-231 cell proliferation after 72 h, and induced apoptosis via activation of caspase-3 and -7. Minor effects was observed in MCF-7 cells [47]. Fakai et al., evaluated the potentials in the use of zoledronate as an adjuvant to temozolomide in human malignant glioma cells. In vitro study of the combination of temozolomide with zoledronate indicated a significant decrease in cell growth. *In vivo* evaluation on subcutaneous xenograft models revealed significant decrease of tumor growth [48]. Guillaume et al., reported the therapeutic efficacy of zoledronate when used alone or in combination with chemotherapy in Ewing's sarcoma. Enhanced inhibitory effects on primary bone tumor development was significant. Inhibition of tumor progression in the bone in a model induced by intra-tibia injection of Ewing's sarcoma cells in vivo was also significant in vivo [49-50]. Aviles et al., studied the application of zoledronate in combination with a high dose of dexamethasone followed by autologous stem-cell transplantation on previously untreated multiple myeloma patients. The combination did not result in any significant toxicity [51]. In a study by Ueno et al., hormone-naive patients were randomized to a combined androgen blockade group or combined androgen blockade with zoledronate. The combination of androgen blockade with zoledronate improved progressive free

survival rate for patients with prostate cancer [52]. Okamoto et al., reported that zoledronate ability to inhibit growth of mesothelioma cells bearing the wild-type *p53* gene through apoptosis induction with activation of caspases, or S-phase arrest by up-regulated cyclin A and B1 [53]. Morgan et al., reported the efficacy of zoledronic acid in patients with newly diagnosed multiple myeloma for the prevention of skeletal-related events [54]. Whalen and Bush, reported the inhibition of osteotropic cells in a 3D spheroid culture by zoledronic acid [55]. Ashimoto et al., evaluated the anticancer effects of alendronate on ovarian cancer. *In vivo* evaluation was performed and alendronate was administered10 days after tumor inoculation. Alendronate administration resulted in a decreased in the serum CA-125 levels of mice bearing disseminated ovarian cancer than in the untreated mice [56]. Hodgins et al., proposed zoledronic acid and alendronate as sensitisers in γδ T cell immunotherapy in preclinical and clinical studies. Liposomal alendronate was used in combination with γδ T cell immunotherapy. Inhibition of tumour growth was significant after treatment with both liposomal alendronate and γδ T cells in pseudo-metastatic lung melanoma tumour-bearing mice [57].

Administration of bisphosphonates in combination with selected anticancer drugs to patients with advanced-stage solid tumors, was reported to result in delayed skeletal morbidity associated with bone metastasis [58]. Yin et al., developed polylactide nanoparticles loaded with doxorubicin and coated with pamidronate for targeted treatment of malignant skeletal tumors. The nanoparticles exhibited enhanced bone tumor accumulation with prolonged retention ability [59].

4.6. Osteoporosis and Bone Related Diseases

Bisphosphonates inhibit bone resorption by inducing apoptosis of osteoclasts resulting in the prevention of bone loss and deterioration of bone microarchitecture [60]. Nitrogen-containing bisphosphonates have enhanced potent antiresorptive properties and are commonly used in the treatment of osteoporosis [60]. There are different diseases that affects the bones namely: osteoporosis, paget disease, osteogenesis imperfecta, tumor of the bone *etc*.

4.6.1. Osteoporosis

Osteoporosis is a disease characterized by deterioration of bone structure resulting in bone fragility with increased risk of fracture [61]. This disease has devastating consequences such as high risk fractures, injuries that can often be

physically debilitating. It can occur in localized parts of the skeleton as a result of conditions that reduce muscle forces on the bone e.g., limb paralysis. Osteoporosis is classified as primary or secondary osteoporosis [61]. Bisphosphonates were first approved for the treatment and prevention of osteoporosis in 1995. These drugs bind to hydroxyapatite in bone and are incorporated into the mineral matrix. The release of the drug from the bone mineral matrix involves bone resorption and reduction in bone resorption is the primary mode of action of bisphosphonates [62]. The terminal half-life of nitrogen containing drug such as alendronate in humans has been reported to exceed 10 years. This report suggest the slow release of alendronate from the skeleton and that the bone is a reservoir for bisphosphonates [62]. Many physicians have suggested that patients should limit the use of bisphosphonates to not more than 5 years [62].

Wells et al., evaluated the efficacy of administration of risedronate for primary and secondary prevention of osteoporotic fractures in postmenopausal women. Women receiving risedronate over a period of one year for postmenopausal osteoporosis were compared with those receiving placebo or concurrent calcium/vitamin D or both. Fracture incidence was studied and for the fracture of the spine, 14 out of 100 women had a fracture when taking a placebo and 9 out of 100 women had a fracture when taking risedronate [63]. McClung et al., evaluated the efficacy of amino-bisphosphonates to reduce fracture risk at the spine, hip, and other non-vertebral skeletal sites. Patients receiving bisphosphonates and having bone mineral density in the osteoporosis range were reported to benefit in a continuing therapy with low risk of harm [64]. Khosla et al., evaluated the risks and the benefits of bisphosphonates. The prevention of fractures in patients with established osteoporosis and in those who are at high risk of fracture was proven when administered bisphosphonates. Cases of major complications associated with bisphosphonate use was not significant [65]. Ward et al., reviewed the impact of bisphosphonates in primary and secondary childhood osteoporosis and it was reported to be effective with some side effects [66]. Some of the side effects reported were ocular toxicity, nephrotoxicity, anterior uveitis, atrial fibrillation and esophageal cancer *etc*. Bisphosphonates were reported to interfere with the process of continually changes in bone size and shape of paediatric skeleton [66]. The use of bisphosphonates have been reported to result in side effects such as: severe irritation of upper gastrointestinal, hypocalcemia, severe bone, joint, muscle pain, osteonecrosis of the jaw and fractures [62]. Although the use of bisphosphonates is associated with risks,

report shows that the benefits of bisphosphonate treatment still outweigh the risks.

4.6.2. Paget Disease

Paget's disease of bone is a chronic and metabolic bone disorder resulting in rapid bone resorption and excessive disorganized bone formation [67]. Some of the common features associated with paget disease are coarsening of bone trabeculae with focal osteosclerosis, cortical thickening, and bone enlargement [67]. The typical sites affected are pelvis, femur, lumbar spine, skull, and tibia [67]. Nitrogen-containing bisphosphonates are first line medications used to treat Paget's disease of bone. They act by suppressing the disease activity by reducing osteoclast-mediated bone resorption and bone turnover. Their action is usually evident by the reduction or normalization of the raised serum ALP level [67].

Silverman reported the percentage of change in serum ALK-P between oral alendronate administration of 40 mg daily and oral etidronate 400 mg daily over a period of 6 months. There was a significant difference in serum ALK-P reduction between alendronate and etidronate [68, 69]. Miller et al., compared oral administration of risedronate (30 mg daily for 2 months) with oral administration of etidronate (400 mg daily). The serum ALK-P concentrations normalized in 73% of risedronate-treated patients and in 15% of etidronate-treated patients after a period of 12 months [69, 70]. Reid et al., performed a re-treatment study on patients from the phase 3 trials of zoledronic acid in Paget's disease, who had received zoledronic acid initially with subsequent relapsed. The patients reached absolute nadirs of serum alkaline phosphatase similar to those after the first dose. There was no occurrence of significant adverse events [71]. Baykan et al., reviewed the remission and relapse status of 12 patients with Paget disease of bone after treatment with zoledronate over a period of two years. The levels of alkaline phosphates, osteocalcin, deoxypyridinoline were measured before and at 6th, 12th, 18th months of treatment. No statistically significant difference between the levels measured at 6th, 12th and 18th months of treatment was detected [72]. A study by Reid et al., investigated why enhanced therapeutic effect of zoledronic acid in the treatment of Paget disease of bone was more than risedronate. Administration of zoledronic acid resulted in lower bone turnover markers and the mean alkaline phosphatase remained within the reference range in the patients. Patients administered risedronate exhibited mean alkaline phosphatase above normal from the first year. Relapse rates were greater in the risedronate group than in those treated with zoledronic acid [73]. Merlotti et

al., did a comparison study of the long-term effects of the same neridronate dose given as an intravenous or intramuscular regimen in patients with paget bone disease. The patients received calcium plus vitamin D supplementation throughout the study period. The therapeutic efficacy of both routes of administration was the same [74]. There are several reports on the application of bisphosphonates for treatment of paget bone disease [75-78].

4.6.3. Arthritis

Arthritis is the inflammation of the joints. It has been reported to be treated with bisphosphonates. Nagashima investigated the effects of selected bisphosphonates such as: etidronate, alendronate, and risedronate when administered alone and in combination with statin, on the bone mineral density and bone metabolism of rheumatoid arthritis patients. Long term administration of bisphosphonates alone resulted in bone resorption and formation. The combination of bisphosphonate and statin resulted in less marked inhibition of bone metabolism [79]. Moreau and colleagues reported that subcutaneous administration of tiludronate to dogs after surgical induction of osteoarthritis reduced pain responses [80]. In a report by Bingham et al., the efficacy of risedronate in providing symptom relief and slowing disease progression in patients with knee osteoarthritis was studied. Risedronate compared with placebo did not improve symptoms of osteoarthritis or alter its progression [81]. Arai et al., reported that minodronate inhibit osteoclastic bone resorption more strongly than alendronate. Mice intraperitoneally injected with minodronate once a week showed higher clinical arthritic scores with a significant increase in serum concentration of tumor necrosis factor (TNF)-α [82]. Wolfe et al., evaluated the efficacy of bisphosphonate in reducing the risk of myocardial infarction in a rheumatoid arthritis population with high prevalence of bisphosphonate use and vascular disease. Among the patients who were treated with bisphosphonates at some time during the study period, the risk of myocardial infarction in patient on bisphosphonate when compared to those not on bisphosphonate was 0.56 [83]. Laslett et al., studied the beneficial effects of treating osteoarthritis with bisphosphonates. In bisphosphonates users in the first three years, the numeric rating scale pain scores were significantly reduced [84]. Xing et. al., evaluated the therapeutic efficacy of bisphosphonates in osteoarthritis treatment from literature databases from inception to February 28, 2016. Based on several studies, bisphosphonates therapy are useful at relieving pain, reducing stiffness and enhancing functional recovery in osteoarthritis. However, the outstanding limitation with reported studies are: differences in duration of bisphosphonates

used, the doses and types of bisphosphonates and the lack of long-term data on joint structure modification after bisphosphonates therapy. More studies are required to evaluate the effectiveness of bisphosphonates for osteoarthritis treatment [85].

Kawabata et al., evaluated analgesic property of etidronate, a non-aminobisphosphonate. In vivo studies on rat with adjuvant-induced arthritis suggested that etidronate exhibited antiallodynic effect similar with alendronate, an aminobisphosphonate.

CONCLUSION

Bisphosphonates are drugs used to treat bone diseases. However, they have been found to be effective for the treatment several diseases such as cancer, and they also exhibit antimicrobial, antiviral, antibacterial and antiplasmodial activities. Despite their unique biomedical applications, they suffer from severe side effects such as toxicity, poor bioavailability, *etc*. In their application for treatment of cancer, they are used in combination with chemotherapeutic agents. They also delay skeletal morbidity associated with bone metastasis in advanced-stage solid tumors when used in combination with anticancer drugs. The use of bisphosphonates have also been reported to result in side effects such as: severe irritation of upper gastrointestinal, hypocalcemia, severe bone, joint and muscle pain, osteonecrosis of the jaw and fractures. Despite the risks associated with the use of bisphosphonates, several reports have also confirmed that the benefits of bisphosphonate treatment outweigh their limitations.

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Chapter 2

POLYMERIC NANO-VECTORS BASED siRNA DELIVERY: A BLOOMING FIELD TOWARDS BETTER THERAPEUTICS

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ABSTRACT

A plethora of encouraging studies have proposed siRNA based therapeutics as a potent and promising strategy towards treatment of various diseases, including, cancer. Several nanoparticle based vectors have been tailored in order to develop an effective delivery system. However, several barriers such as poor cellular uptake, instability and immunogenicity in physiological conditions needs to be resolved before successful clinical application. Hence, a clinically suitable and stable

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nanoparticle-siRNA formulation needs to be developed. In this context, polymeric nanoparticles have revolutionized drug-delivery systems owing to its ability to protect nucleic acids from degradation, intracellular uptake, low toxicity and controlled release. Amongst the available polymers, cationic polymers are one of the predominantly investigated molecules for the formation of stable complexes with the negatively charged nucleic acids. Further, these polymers also confer enhanced protection against nucleases. Manipulation of certain parameters such as molecular weight, charge density and hydrophobicity/hydrophilicity balance have shown to result in improved vectors.

1. INTRODUCTION

Recently, gene therapy has gained tremendous amount of attention for the treatment of various incurable and inherited diseases. Gene therapy is a process of treatment of disease either by replacing the defective gene or by modifying the expression of target gene, using therapeutic gene or any other genetic material into the cells. Owing to its immense potential, gene therapy has been widely investigated. The first clinical trial for the treatment of severe combined immunodeficiency (SCID) was conducted in the year 1990 employing gene therapy. Since, the inception of gene therapy, only small number of gene therapies has been successful (Blaese, Culver et al. 1995). A prominent limitation to these strategies is the adapted gene delivery methods. As the traditional carrier system faces various obstacles such as degradation by nucleases, stability of genetic material in the cellular environment, release from endosome and nuclear localization, there arises a need of a delivery system that is stable, biocompatible, non-toxic which exhibits high transfection efficiency. The gene delivery vectors can be categorized into two groups, namely, viral and non-viral vectors. Viral vectors exhibit high gene delivery efficiency but carry certain limitations that include safety concerns, antigenicity, inflammation and possible insertional mutagenesis. On the other hand, non-viral vectors possess potential benefits such as low immunogenicity and toxicity. However, non-viral vectors are not as efficient as viral vectors in terms of gene transfer efficiency (Merdan, Kopeček et al. 2002, Schagen, Ossevoort et al. 2004).

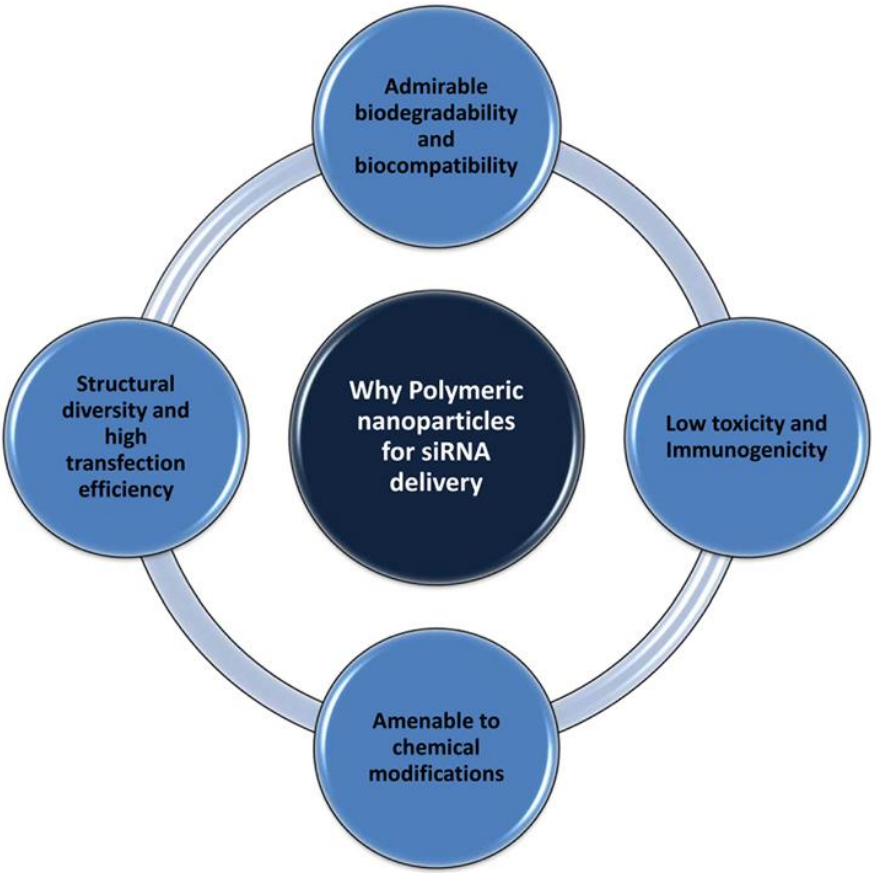

Figure 1. Properties of Polymeric nanoparticles.

Amongst the available non-viral gene delivery systems, nanoparticles are considered to be the most potent carriers. Nanomedicine involves combination of both nanotechnology and healthcare that holds great potential for the development of better therapeutic products with faster imaging, diagnosis and efficient drug delivery (Zhang, Gu et al. 2008, Han, Ma et al. 2013). In this context, remarkable advancement has been made in the field of nanotechnology towards designing a suitable vector that can efficiently deliver genes *in-vitro/in-vivo*. Nanoparticles can be divided into two categories; organic nanoparticles and inorganic nanoparticles. Organic nanoparticles usually comprise of lipid or polymer based complexes and inorganic consists of quantum dots, magnetic and metallic nanoparticles (Zhang, Gu et al. 2008). These nanoparticles can be considered as potential candidates for drug/gene

delivery as they possess same size as that of several proteins and effective binding ability due to presence of functional groups. Also, their size, drug/gene release properties and surface characteristics can be controlled. Nanoparticles also carry certain limitations such as low transfection efficiency that demands frequent administration that could result in cytotoxicity. In order to increase the efficiency, the nanoparticles are usually subjected to modifications such as PEGylation and coating with polymers such as chitosan, poloxamer, and poloxamines. This modification reduces the toxicity and other biological issues by reducing opsonisation in the physiological milieu as well as increases the internalization of nanoparticles by the cells. Since the inception by Felgner (1987) cationic lipids are currently widely explored as non-viral vectors for *in vivo* and *in vitro* gene delivery due to their high efficiency. These liposomes also carry certain limitations such as reproducibility, colloidal stability and cytotoxicity. Amongst the discussed aforementioned nanoparticles polymeric formulations are the most preferred non-viral vectors owing to their inherent advantageous properties such as biodegradability, biocompatibility and low cytotoxicity. Also, polymers can be easily tailored for the purpose of efficient gene delivery to cells (Zhou, Liu et al. 2012, An, Kuang et al. 2013, Han, Ma et al. 2013, Yang, Hendricks et al. 2013). Cationic polymers are more often used for this purpose as the positively charged polymers can easily interact with the anionic genetic material forming nanometer sized polyplexes. They also confer high stability against nuclease degradation with narrow size distribution. Biodegradable polymers such as poly lactide-co-glycolide (PLGA) and chitosan have been extensively investigated as carrier for therapeutic agents, as they are less toxic and immunogenic to the cells in physiological milieu. Further, the polymeric formulation aids in the slow release of payload leading to sustained gene expression (Panyam and Labhasetwar 2003).

The biological activity of nanoparticles is mainly governed by some important properties such as size, zeta potential, chemical and structural features. Overall, to design a stable nanoparticles based vectors a complete and thorough optimization of synthesis strategies is required. In this chapter we have focussed on various synthesis strategies for preparation of nanoparticles. In addition, we have accounted for major barriers encountered during nucleic acid delivery as well as role of commonly used polymers towards siRNA delivery. On the final note, we have discussed about the clinical relevance of polymeric nanoparticles in siRNA delivery.

2. METHODS FOR SYNTHESIS OF POLYMERIC NANOPARTICLES

Polymeric nanoparticles of size 10-1000 nm are generally prepared from biocompatible and biodegradable polymers. These could be prepared by encapsulation, entrapment or attachment of the therapeutic compound over the nanoparticles. Broadly nanoparticles can be divided into two types: Nanocapsules and Nanospheres.

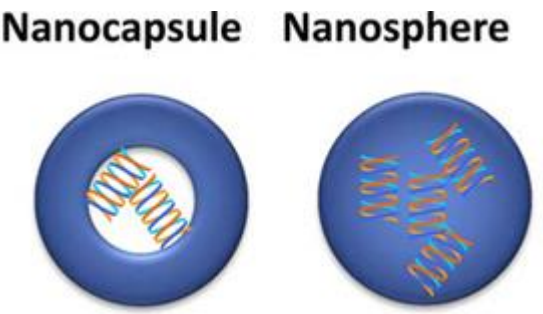

Figure 2. Types of Nanoparticles: A. Nanocapsule B. Nanosphere.

Nanocapsules consist of a core surrounded by polymeric membrane that can carry either lipid soluble or aqueous soluble compounds depending on the dispersible content. On the other hand, nanospheres are solid nanostructures having therapeutic molecules loaded or adsorbed on the matrix. These polymeric nanoparticles are effective in carrying drugs, proteins as well as nucleic acids to the cells and organs. Moreover, these are more stable in physiological system. Thus, these are synthesized with varying properties to make it suitable for medical applications. Different methods for the preparation of nanoparticles have been developed depending on the chemical and physical properties of polymer (Soppimath, Aminabhavi et al. 2001, Mohanraj and Chen 2006). Along with this, the synthesis of nanoparticles is further classified according to its involvement in polymerization followed by nanoparticles formation or synthesis directly from preformed polymer. Amongst, most of the polymers available, only few of them have been approved by the health authorities for parenteral administration, while others are accepted for topical or oral administration. Over the years, different copolymers possessing polyethylene glycol or polysaccharides have also been

synthesized with tuneable surface properties for synthesis of stable nanoparticles.

Polymeric nanoparticles are prepared either by dispersing the preformed polymers or from polymerization of monomers. The polymers employed in the preparation can be natural (e.g., chitosan, gelatin, sodium alginate, albumin) or synthetic (e.g., polylactides, polyglycolides, poly(lactide-co-glycolide), polyglutamic acid, polymalic acid, polyethyleneglycol etc.) (Ghosh 2000, Mohanraj and Chen 2006). Thus, the methods employed for preparation of polymeric nanoparticles from preformed polymers are usually solvent evaporation, solvent diffusion, nanoprecipitation, salting out, dialysis and supercritical fluid technology. In case of polymerization of monomers, emulsion, mini-emulsion, interfacial polymerization, micro-emulsion and controlled/ living radical polymerization are employed.

2.1. Synthesis from Polymers

2.1.1. Polymeric Nanoparticles Preparation by Solvent Evaporation

Solvent evaporation method was first pioneered for the preparation of polymeric nanoparticles. This involves dissolution of polymer in volatile solvents followed by diffusion through continuous phase. Thereafter, nanoparticles are prepared by evaporation of solvent of the emulsion. Single and double emulsions i.e., oil-in-water (o/w) and (water-in-oil)-in-water (w/o)/w is the commonly used strategy for the preparation of polymeric nanoparticles. These are accompanied with high speed homogenization or ultrasonication followed by evaporation. The resultant polymeric nanoparticles are further subjected to ultracentrifugation, washing and lyophilisation. Initially, the most commonly used solvents were dichloromethane and chloroform that are replaced with ethyl acetate due to its better toxicological profile (Allemann, Gurny et al. 1993, Anton, Benoit et al. 2008). Several polymers have been employed for the preparation of nanoparticles with different solvents and particles of size 60-300 nm can be synthesized (Song, Labhasetwar et al. 1997). Besides this, studies have also been done to determine the role of other parameters such as temperature, phase volume, surfactant concentration and molecular mass of polymer on particle size, zeta potential and polydispersity index of polylactide nanoparticles. It was observed that higher (3% w/v or more) concentration of surfactant lead to the formation of smaller size nanoparticles, while no significant differences was seen with varying molecular mass of the polymer. Similarly, duration and

intensity of sonication also influenced the particle characteristics in double emulsion method, where the particle size decreased with increase in time period during second emulsification process. In addition copolymer nanoparticles were also synthesized. Besides, being a facile method for nanoparticle synthesis, it is time consuming and has the possibility of coalescence of the polymeric nanoparticles during evaporation procedure (Zambaux, Bonneaux et al. 1998, Bilati, Allémann et al. 2003).

2.1.2. Salting Out

This method does not require the use of surfactants or chlorinated solvents as used in solvent evaporation method. Instead, polymeric nanoparticles are prepared employing water miscible solvents such as acetone. In this process, high concentration of sucrose, salt or electrolytes is used for salting out. Reverse salting-out is also attained by dilution with excess water causing precipitation of the polymer from the emulsion. This is induced by reduction of sucrose or salt concentration in the continuous phase. Allemann et al. performed similar set of experiment using electrolyte or non-electrolyte stabilized aqueous solution and polymer suspended in acetone. After preparation of oil-in-water emulsion excess of water was added to the emulsion for complete diffusion of acetone into aqueous phase leading to the formation of nanospheres. Similarly, Zhang et al. prepared poly(trimethylene carbonate) (PTMC) nanoparticles with a size range between 183 and 251 nm (Bindschaedler, Gurny et al. 1990, Allémann, Gurny et al. 1992, Ganachaud and Katz 2005, Zhang, Grijpma et al. 2006).

2.1.3. Nanoprecipitation

The nanoprecipitation method also known as solvent displacement method works on the basic principle of interfacial deposition of polymer after displacement of solvent. For the synthesis of polymeric nanoparticles, polymer, solvent (miscible in water) and non-solvent are required, where slow addition of organic phase to aqueous phase under moderate stirring leads to formation of nanoparticles. The physicochemical properties of synthesized polymeric nanoparticles mainly depend on the organic phase injection rate, aqueous phase agitation rate and the concentration of the components. Also, the nature and concentration of the surfactant has also been known to influence polymeric nanoparticles characteristics. Basically, the particle formation involves three stages: nucleation, growth and aggregation. The key determining factor for the formation of uniform sized particles is the separation between nucleation and growth stages. This method is widely used

for the preparation of nanospheres and nanocapsules (Fessi, Puisieux et al. 1989, Ferranti, Marchais et al. 1999, Blouza, Charcosset et al. 2006, Cheng, Wang et al. 2008, Dalpiaz, Vighi et al. 2009, Mishra, Patel et al. 2010).

2.1.4. Dialysis

Dialysis is one of the simple and effective methods for synthesis of polymeric nanoparticles with narrow size distribution. In this approach, dialysis is performed with a dialysis tube containing polymer in organic solution against non-solvent that is miscible with the organic solvent. The diffusion of solvent through the membrane towards non-solvent allows aggregation of polymers inside the tube that ultimately leads to production of homogenous nanoparticle suspension. The exact mechanism for the formation of polymeric nanoparticles is yet to be understood. Several polymers such as poly(benzyl-lglutamate)-β-poly(ethylene oxide) and poly(lactide)-β-poly(ethylene oxide) have already been used in the preparation of polymeric nanoparticles using dimethylformamide(DMF) as solvent. Similarly, Akagi et al. prepared polyglutamic acid nanoparticles using different solvents such as dimethylsulphoxide (DMSO), DMF, dimethylacetate (DMAc), n-methylpyridine (NMPy). From this experiment, it was evident that morphology of the particles varied with the type of solvents used. Polymeric nanoparticles prepared using DMSO showed spherical morphology with diameter ranging from 100 to 200 nm whereas in case of NMPy various sized nanoparticles were formed with broad particle size distribution (Fessi, Puisieux et al. 1989, Oh, Lee et al. 1999, Lee, Cho et al. 2004, Akagi, Kaneko et al. 2005).

2.1.5. Supercritical Fluid Technology

Continuous use of organic solvents for preparation of nanoparticles that are toxic to the environment, demands for a more eco-friendly approach. Introduction of supercritical fluids for the preparation of polymeric nanoparticles have paved way towards safer approach. Use of supercritical fluids facilitated synthesis of nanoparticles with high purity without any remanants of organic solvents. Nanoparticles synthesis using supercritical fluids is done by two methods: Rapid expansion of supercritical solution (RESS) and Rapid expansion of supercritical solution into liquid solvent (RESOLV). In the RESS method, solute dissolved in a supercritical fluid is made to expand across an orifice or capillary nozzle into the air. Supersaturation and decrease in the pressure results in the formation of well-dispersed particles of both nanometer and micrometer size. In case of

RESOLV the expansion of supercritical fluid is done in a liquid solvent instead of air. Several supercritical fluids are available for nanoparticles synthesis e.g., carbon monoxide, n-pentane, water etc. However, the major drawback in utilising supercritical fluids is poor solubility or non-solubility of the polymer in supercritical fluid (York 1999, Kawashima 2001, Weber and Thies 2002, Nagavarma, Yadav et al. 2012).

2.2. Methods for Synthesis of Polymeric Nanoparticles by Polymerization of Monomers

Synthesis of polymeric nanoparticles by polymerization is mainly achieved by three techniques: emulsion polymerization, mini- and micro-emulsion polymerization. Other techniques involve interfacial and living controlled radical polymerization.

2.2.1. Emulsion Polymerization

Emulsion polymerization is one of the widely employed methods for the preparation of polymeric nanoparticles. It involves the use of water as dispersion medium and the technique is further classified as conventional and surfactant free emulsion polymerization.

2.2.1.1. Conventional Emulsion Polymerization

In conventional method, the nanoparticles are prepared from polymerization of monomers soluble in water and an initiator as well as surfactant. The initiation of polymerization occurs when the monomer molecule in the continuous phase collides with an initiator. The reaction progresses using hydroxyl groups of water or by other nucleophilic groups present in the reaction mixture. Moreover, the diameter of nanoparticles depends on the type of surfactant added to the system. The small size nanoparticles of 50 nm were synthesized using polyethyleneoxide lauryl ester, whereas, addition of anionic surfactant led to the formation of larger sized nanoparticles (Couvreur, Kante et al. 1979, Vranckx, Demoustier et al. 1996, Bertholon, Lesieur et al. 2006, Nicolas and Couvreur 2009).

As noted, the conventional technique utilizes surfactants which are difficult to remove during synthesis process as these are time consuming and causes increase in the production cost. Surfactant free emulsion polymerization uses distilled water, water soluble initiator and monomers, such as, vinyl and acryl monomers. This method is emerging to be a simple

and green approach for the synthesis of nanoparticles without addition or removal of surfactants.

2.2.2. Mini and Micro-Emulsion Polymerization

Microemulsion is similar to that of emulsion polymerization, the difference lies in the number reaction intervals. Emulsion polymerization and mini-emulsion process are similar to each other. The major key difference between them is the use of low molecular mass compound as a co-stabilizer with the use of high shear device. Mini-emulsions require high shear to reach steady state condition and has interfacial tension greater than zero. Miniemulsion possesses three reaction intervals whereas there are only two reaction intervals in case of microemulsion polymerization. The polymerization starts in the swollen micelles in micro-emulsion containing an initiator. This mixture possesses high amount of surfactant that later coats the nanoparticles after polymerization. Further, due to some elastic and osmotic differences, nanoparticles destabilize and produce large number of smaller micelles and leads to secondary nucleation (Puig 1996, El-Aasser and Miller 1997, Qiu, Charleux et al. 2001).

2.2.3. Interfacial Polymerization

This method works by polymerization of two reactive monomers dispersed in two phases i.e., continuous and dispersive phase, respectively. The process of polymerization occurs between the interfaces of two liquids. Although, the technique is gaining attention, issues dictating size needs to be addressed. Use of membrane reactors allows separation of products from reactants as well as controlled addition of one phase to another phase.

The controlled/living radical polymerization (C/LRP) technique was introduced in order to control over the molar mass, molar mass distribution, architecture and function of nanoparticles. This technique uses environmentally benign solvents such as water, supercritical carbon-di-oxide etc.(Drioli, Criscuoli et al. 2003, Zetterlund, Nakamura et al. 2007, Zetterlund, Kagawa et al. 2008).

3. BARRIERS TO SUCCESSFUL GENE DELIVERY MEDIATED BY POLYMERIC NANOPARTICLES

Therapeutic nucleic acids hold great promise in gene therapy. Owing to their negative charge, therapeutic nucleic acids cannot cross the lipid membrane of a cell easily. Besides polymeric carriers, there are several other delivery strategies such as: nucleic acid backbone modification, covalent modifications of the vector and supramolecular assembly of molecules into therapeutic nanosized molecules. Locked nucleic acids have chemical bridging that locks the nucleic acids into A-form that allows efficient hybridization with RNA. In order to decrease the size of nucleic acids, double stranded DNA is replaced by single stranded DNA. In the process, 5′-phosphorylated region of RNA is replaced by 5′-vinylphosphonate for the formation of RNA induced silencing complex (RISC). Simultaneously, for providing optimal stability 2′-fluoro and 2′-methoxy nucleosides were introduced in the central strand. Effective gene silencing has been observed employing modified nucleic acids (Behlke 2008, Souleimanian, Deleavey et al. 2012, Wagner 2013). Although, different techniques have been implemented for better delivery, non-covalent complexation and encapsulation can prove to be better alternatives. In this context, polymer based system such as polymerosomes, polymer micelles, hydrogels and complexes formed from cationic polymers appear promising. However, there are number of barriers that need to be overcome for successful delivery of polyplexes.

3.1. Extracellular Barriers

Biodistribution and pharmacokinetics of the synthesized nanoparticles mainly depends on the size of the particles. Polyplexes are generated as small as 6 nm, 25 nm and as large as 100 nm (Blessing, Remy et al. 1998, Dohmen, Edinger et al. 2012). Nanoparticles of size 6nm are easily cleared off from kidneys (Choi, Liu et al. 2010) rather nanoparticles with size 400 nm get easily accumulated in solid tumors owing to enhanced permeability and retention effect of tumor tissues having leaky blood vasculature (Maeda 2001). However, the extent of accumulation depends on the type of cancer (Smrekar, Wightman et al. 2003). Nanoparticles of size 30-100 nm are poorly taken up the pancreatic tumors, whereas the penetration is good enough in other tumor models. Other problems associated with polyplexes are their stability in blood

and biological fluids. Loss of delivery efficacy are mainly due to interaction with the proteins, cellular surfaces leading to dissociation as well as binding of positively charged particles with serum proteins causing activation of complement system, aggregation of particles and erythrocytes resulting in life threatening conditions (Cabral, Matsumoto et al. 2011). Thus, a more stable nanosystem needs to be generated to overcome undesired interactions with the bio-environment. For this, nanoparticles could be shielded to prevent non-specific interactions. However, target cell binding and uptake is still not efficient in much type of cells and tissues such as blood-brain-barrier of brain tissues. The nanoparticles are further decorated with ligands for convenient approach towards target cell and receptor mediated cellular internalization.

3.2. Intracellular Barriers

The first challenge posed is the delivery of molecules across the cellular membrane. Direct transfer of molecules is mainly possible with small molecules that can pass through carriers or channel proteins. Non-enveloped viruses and protein toxins follow two step entry processes: first step involves engulfment of the organism followed by escape out of vesicles in cytoplasm. But in case of polyplexes the uptake and release of the payload is comparatively difficult to achieve. The main hurdle is the delivery and release of payload at the final site of action. Thus, to overcome this issue, polyplex needs to be dynamic in function to deliver the payload to its site of action. Further, the site of action also depends on the type of therapeutic nucleic acids: pDNA has its destination in the nucleus whereas siRNA, miRNA has its destination located in the cytosol. For efficient delivery of nucleic acids, the DNA is required to be imported via microtubules to nucleus or through other alternative pathway. Although several studies have already been established regarding nucleic acid delivery, to date there have been no efficient results for successful intra-nuclear delivery of polyplexes (Lukacs, Haggie et al. 2000, Itaka, Harada et al. 2004).

3.3. Nucleic Acid Packaging

Polyplexes formation is mediated by electrostatic interactions of positively charged polymer with the negatively charged nucleic acid. It has been observed that in comparison to the unprotected plasmid DNA, polyplexes

exhibit greater efficiency in providing protection from the DNase degradation by sterically blocking the nucleolytic enzymes. However, the structure and morphological characteristics of polyplexes mainly depends on the method of synthesis. Additionally, it is important to note that considerable binding efficiency of DNA to the polymer does not necessarily correlate to its gene delivery efficiency. Thus, a subtle balance is required between binding and delivery of nucleic acids as well as protection of enzymatic degradation (Zelphati and Szoka 1996, Schaffer, Fidelman et al. 2000, Abdelhady, Allen et al. 2003).

3.4. Cell Specific Delivery

Cell specific delivery of nucleic acids varies according to the type of applications, such as in case of haemophilia cell specificity is not required as long as sufficient levels of protein is secreted. Whereas, cell specific vectors are required in cancer therapy to kill the cancer cells. Polymer's flexible chemical properties allows surface modifications with targeting moieties that ultimately lead to increased uptake of the polyplexes in cells.

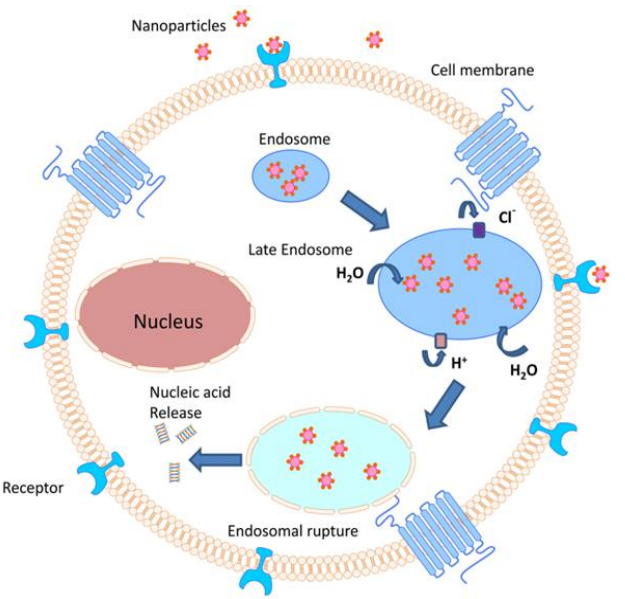

Figure 3. Mechanism of intracellular siRNA/nucleic acid release from nanocomplexes (e.g., Polyethylenimine) taken up by the cell.

Receptor ligands or proteins are deployed for facilitating uptake of polyplexes by receptor mediated endocytosis. Polymer derivatives with glycosidic moieties and other small molecules such as folate provide specific targeting of the nanoparticles to the cells. In addition, it may be noted that the efficient targeting of the cells can be achieved by adjusting the length of the spacer between ligand and polyplex, number of targeting ligands. For this purpose, many cross linking chemistries were applied including covalent bonds and biotin-streptavidin. However, there needs to maintain balance between specific interactions and non-specific interactions. Also, optimal ligand valency remains the matter of concern. Therefore, to achieve cell specific targeting careful optimization of all the parameters is required (Zanta, Boussif et al. 1997, Bettinger, Remy et al. 1999).

4. Design and Development of Recent Derivatives to Improve Systemic Delivery

Polymeric nanoparticles are most preferred in nucleic acid delivery as it is less immunogenic relative to other nanoparticles. However, polymeric nanoparticles are imposed with several challenges such as endosomal release before its degradation. A method developed to promote endosome lysis was the use of chloroquine with the formulation. Use of chloroquine was found to have a drawback of disrupting all endosomes and lysosomes that could probably result in damage of cells. Introduction of different cationic polymers such as poly(L-lysine) (PLL) and PEI, polyamidoamine (PAMAM) dendrimers, poly(lactide co-glycolide)(PLGA) and chitosan have revolutionised the field of gene delivery owing to their beneficial characteristics. Despite of having several advantageous properties, these polymers are not capable of overcoming some barriers on their path. Several polymers have been designed in order to address issues of stability, biocompatibility and endosomal escape. Herein, we have discussed some of the commonly used polymers designed to deliver siRNA with improved efficiency.

4.1. Chitosan

Amongst the available polymers, chitosan nanoparticles have been a promising non-viral vector both in *in vitro* and *in vivo* delivery of nucleic acids. Chitosan is a polymer composed of glucosamine and N-acetyl glucosamine that have been already approved for several biomedical applications such as in wound dressings. Being a natural polysaccharide chitosan is biodegradable, biocompatible, mucoadhesive and a non-toxic polymer. It has been widely explored in therapeutics for drug and gene delivery depending on the current advancements.

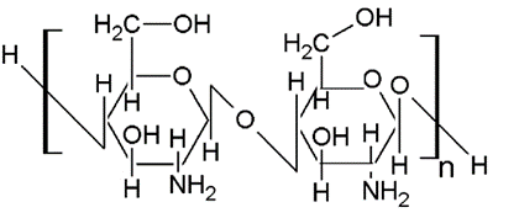

Figure 4. Basic structure of chitosan.

4.1.1. Chitosan in siRNA Delivery

Chitosan has gained tremendous interest for the delivery of siRNA due to its cationic nature, biocompatibility and biodegradability. Studies have suggested successful delivery of siRNA with fully deacetylated chitosan with intermediate chain lengths (degree of polymerization (DP)(n) 100-300). Efficient delivery of siRNA was seen under lower concentrations with long term silencing of both exogenous and endogenous target genes with minimal cytotoxicity (Malmo, Sorgard et al. 2012, Ragelle, Vandermeulen et al. 2013). Chitosan/siRNA complex is formed after several optimization steps done either by simple complexation or using different cross linkers such as tripolyphosphate (TPP), dextran sulphate (DS) and poly-D-glutamic acid (PGA). Amongst all the formulations prepared by these cross linkers CS-TPP-siRNA were found to be more stable with small size along with better protection of siRNA. Additionally, cellular uptake studies showed efficient uptake by colorectal cancer cells (DLD-1) (Raja, Katas et al. 2015). In addition, chitosan/siRNA were also synthesized using ultrasonication method at specific amplitudes and time of sonication. The optimum conditions obtained for the synthesis of nanoparticles of size 456 nm were 60.6, 30.0 (seconds), 28.0, and 12.5 (minutes) for amplitude, time of sonication, N/P, and

stirring time, respectively. Further, amongst varied types of chitosan low molecular weight have been found to be of much use due to its ability to form small sized nanoparticles. To further decrease the toxicity and enhance the transfection efficiency, polymers are generally conjugated with ligands. Upon folate conjugation, chitosan of 25 kDa and 50 kDa was found to form complex with siRNA of size 220 nm with chitosan:siRNA ratio 50:1 (Fernandes, Qiu et al. 2012). siRNA were also employed for treatment of viral diseases such as hepatitis C virus. Scavenger receptor class B type 1 (SR-B1) are responsible for attachment and internalization of pathogen that are markedly present on liver and steroidogenic tissues. Thus, siRNA against (SR-B1) successfully silenced the gene with the use of low molecular weight chitosan nanoparticles prepared by ionic gelation method (Farid, Hathout et al. 2014).

Similarly, human papillomavirus (HPV) oncoproteins E7 responsible for progression of malignancy was silenced using HPV16 E7 siRNA in Ca Ski cells through chitosan nanoparticles (Yang, Li et al. 2013). siRNA mediated silencing could also be applied for the treatment of kidney disorders. This was ascertained by silencing the aquaporin gene in kidney cells that was uptaken by megalin mediated endocytic pathway (Gao, Hein et al. 2014). Moreover, chitosan nanoparticles were also exploited for co-transfection of complexed payload in cystic fibrosis cell line (CFBE41o-). The study showed increase in fluorescence intensity with CS-pEGFP and decrease in fluorescence with CS-pEGFP siRNA. Thus, this offers potential alternative for the treatment of cystic fibrosis (Fernandez Fernandez, Santos-Carballal et al. 2016). In addition, chitosan nanoparticles are mostly employed in cancer therapeutics as it is a multifactorial disease with complex gene mutation and dysregulation. A group of researchers used glycol-chitosan for co-delivery of dual siRNA in order to silence VEGF and BCL-2 for more effective cancer therapy (Lee, Yook et al. 2015). Similarly, in another set of work Mad-2 siRNA complexed with chitosan was used for silencing Mad-2 gene in A549 cell lines. The results showed complete reduction in Mad-2 siRNA levels with selective killing of cancer cells (Nascimento, Singh et al. 2014). Thereafter, Mad-2 siRNA-CS was also co-delivered with cisplatin for better treatment towards drug resistant tumours (Nascimento, Singh et al. 2016).

Delivery of drugs to brain has also imposed a major barrier in the event of cancer therapy. The major complication is due to the presence of efflux pumps (P-glycoprotein (P-gp)) overexpressed that transports drugs back into the blood. To overcome this problem, CS-siRNA complex against P-gp was delivered to the rat brain endothelial cells that ultimately mediated efficient knockdown of P-gp protein. Thus, this resulted in reduced level of drug

(doxorubicin) efflux in cancer cells (Malmo, Sandvig et al. 2013). Likewise, plethora of work have been done on cancer therapy. Recently, chitosan was used for co-delivery of siRNA and doxorubicin against high-mobility group AT-hook2 (HMGA2) gene in lung cancer cell line A549. HMGA2 is responsible for epithelial mesenchymal transition (EMT). Thus, co-delivery of the mentioned complexes showed alteration in EMT markers, apoptosis induction and migration inhibition (Seifi-Najmi, Hajivalili et al. 2016).

Despite the development of different methods for delivery of siRNA, its clinical application is hindered by poor cellular uptake and enzymatic degradation. To address this issue, siRNA was delivered against telomerase reverse transcriptase via N-((2-hydroxy-3-trimethylammonium) propyl) chitosan chloride (HTCC) nanoparticles (HNP). *In vivo* and *in vitro* results demonstrated HNP efficacy in delivering siRNA. On this basis, HNP was utilized for the delivery of anticancer drug (paclitaxel) and siRNA simultaneously to the tumour cells. A significant decrease in the tumour was observed (Wei, Lv et al. 2013). Interestingly, RNAi strategy has also been implemented to control pest insects. In relation to this, a study was conducted, where CS/dsRNA was targeted against the vestigial gene (vg) of mosquito *Aedes aegypti*. This resulted in malformation of adult wing and delayed growth of the pest (Ramesh Kumar, Saravana Kumar et al. 2016).

4.1.2. Polyethylene Glycol Modifications in Chitosan Nanoparticles

PEG modified chitosan have been widely developed in order to increase the stability and circulation of nanoparticles. Diffusion through extracellular matrix (ECM) is a critical step in delivery of genes to the target region. Collagen and collagen-hyaluronic acid (HA) gels were used to study the diffusion and stability of CS/DNA polyplexes. PEGylation increased the diffusion and DNA release property of the nanoparticles. In contrary, HA decreased its penetration depth into the gel (Lelu, Strand et al. 2011). Peptide tagged PEGylated chitosan were formulated for the treatment of neurodegenerative diseases. The formulation was successful in delivering siRNA against Ataxin-1 gene in *in vitro* model of spinocerebellara ataxia (SCA-1) overexpressing ataxin protein. The results depicted successful suppression of SCA-1 protein after 48h of transfection (Malhotra, Tomaro-Duchesneau et al. 2013). A pH responsive PEG grafted carboxymethyl chitosan (PEG-CMCS) nanoparticles were prepared using CaP for delivering siRNA in cancer. The formulation proved to be safe and effective in anticancer therapy in HepG2 cells and Balb/c mice possessing HepG2 tumour xenograft (Xie, Qiao et al. 2014). Besides using PEG for the purpose of combination,

different polymers could be used in combination to PEG to increase the beneficial properties of nanoparticles. In a recent study, magnetic nanoparticles containing polyethylene glycol-lactate polymer (PEG-LAC), chitosan and PEI nanoparticles containing surviving siRNA were synthesized. The cytotoxic results were even more effective with the nanoplex combination (Arami, Rashidi et al. 2016).

4.1.3. Hyaluronic-Acid Modification of Chitosan

Chitosan nanoparticles are surface modified with hyaluronic acid to increase endocytosis for cellular imaging and gene delivery. The study demonstrated the effect of substrate effects on the process of endocytosis by mesenchymal stem cells (MSCs). Caveolae mediated endocytosis was more predominant for CS/HA nanoparticles. This facilitates stem cell labelling or can be possibly used for gene delivery (Hsu, Ho et al. 2012). Also, it has been observed that CS/HA nanoparticles aid in release of genes by loosening CS-siRNA binding (Al-Qadi, Alatorre-Meda et al. 2013). In addition, CS/HA nanoparticles are successfully implemented in co-delivery of drugs and therapeutic nucleic acids. DOX-miR-34a co-loaded HA-CS NPs prepared by ionotropic gelation method showed synergistic effects on tumour suppression (Deng, Cao et al. 2014).

4.1.4. Polyethylenimine Modified Chitosan Nanoparticles

Chitosan-graft-(polyethylenimine-beta-cyclodextrin) (CPC) was prepared for siRNA delivery in HEK 293, L 929, and COS 7 cell lines. Further, PEGylation exhibited enhanced stability and transfection efficiency upto 84% (Ping, Liu et al. 2011). Similarly, cationic nanoparticle-polyethyleneimine-introduced chitosan shell/poly (methyl methacrylate) core nanoparticles (CS-PEI) were employed for the delivery of siRNA against the HPV oncogene E6 and E7. The silencing of the specific oncogenes were specific that suppressed the HPV oncogene in a much efficient manner (Saengkrit, Sanitrum et al. 2012). *In vitro* and *in vivo* studies were conducted with chitosan-PEI (CP) conjugates prepared by conjugating low molecular weight branched PEI (LMWP) with depolymerized chitosans (7 and 10 kDa) via their terminal aldehyde/keto groups. The formulation was further modified by tuftsin molecule that aids in targeting macrophage cells. Among the two formulations with 7 kDa and 10 kDa, nanoparticles prepared from 7 kDa chitosan showed higher transfection efficiency (Tripathi, Goyal et al. 2012). Recently, carboxymethyl chitosan (CMCS) prepared by chitosan alkalisation and carboxymethylation reactions was grafted with PEI by amidation reaction for

the purpose of stability, lower cytotoxicity and high transfection efficiency in 293T and 3T3 cell lines (Liu, Mo et al. 2016).

4.2. Dendrimers

Dendrimers are multivalent branched globular nano-structures having three different domains: (i) A central core possessing at least two functionalities for facilitating linking of branches, (ii) branches coming out of the core having repeating units in geometric progression arranged in concentric layers, (iii) terminal functional groups that determines its drug entrapment ability. Dendrimers are known to possess high solubility, reactivity and binding properties that allows most of the polycationic dendrimers such as PAMAM and polypropylamine (PPI) to be widely investigated as a vector for various applications such as drug and gene delivery (Buhleier, Wehner et al. 1978, Tomalia, Baker et al. 1985).

4.2.1. Poly(Amidoamine) Dendrimers

PAMAM dendrimers are generally rich in amine groups. Primary amines present on the surface of the molecule plays a main role in nucleic acid binding, whereas the secondary amines aids in proton sponge effect inside endosomes. A study using siRNA alone and delivery of antisense siRNA using PAMAM dendrimer conjugated to a peptide showed higher and efficient delivery using PAMAM generation five (G5). The siRNA delivered using the vector showed higher effects against P-glycoproteins compared to the results obtained from lipofectamine 2000 (Kang, DeLong et al. 2005). In a study, polycationic PAMAM dendrimers were developed for the delivery of siRNA where triethanolamine was used as core that gave rise of branching. It was observed that higher generation dendrimers formed stronger interactions with siRNA compared to low generation dendrimers. Further, complex was formed at neutral pH itself and showed greater gene silencing efficacy at higher N/P ratio that could be attributed to G7 buffering capacity. Further, it was noted that toxicity increased with increase in siRNA concentration. In addition to this, other studies using flexible triethanolamine core were capable of delivering Hsp27 siRNA in prostate cancer (PC-3) cells that resulted in silencing of this gene (Zhou, Wu et al. 2006),(Liu, Rocchi et al. 2009). Further, the dendrimers could be modified using other polymers for better results. In such an attempt, siRNA delivery was conducted using dendrimers in combination with α-cyclodextrin (α-CDE) to silence the luciferase gene. It was

seen that ternary complexes of pGL3/siGL3/α-CDE displayed RNAi effects efficiently and that α-CDE conferred interaction with the nucleic acid as well as protection from serum in NIH 3T3 cells. Dendrimer-RNA complex properties were analyzed using atomic force microscope (AFM). Finally, the study revealed that fabrication of nanoparticles mainly depends on the size of RNA molecules, dendrimers with higher generations and higher N/P ratios (Shen, Zhou et al. 2007, Tsutsumi, Hirayama et al. 2007).

In another study, Patil et al. converted PAMAM-NH_2 dendrimers into PAMAM-NHAc dendrimers in order to overcome the cytotoxicity issue (Patil, Zhang et al. 2008). During this process, the surface amine groups were modified by acetyl groups and the tertiary amine groups were quaternized. The AFM studies revealed that QPAMAM-OH and QPAMAM-NHAc formed stable nanoparticles. Also, the cytotoxicity reduced significantly. Studies done on A 2780 human ovarian cancer cells revealed higher uptake of QPAMAM-NHAc-siRNA compared to PAMAM-NH_2-siRNA and QPAMAM-OH-siRNA complexes (Patil, Zhang et al. 2008). Further, amine groups were acetylated using acetic anhydride. It was found that with 60% of modification with acetyl groups resulted in formation of nanocomplexes of size 200 nm using siRNA. The cytotoxicity decreased with increase in acetylation whereas, a significant decrease in buffering capacity was observed that resulted in reduction in delivery of siRNA (Waite, Sparks et al. 2009).

Dendrimers with ethylendiamine (EDA) core are generally not flexible. Complex formation from this polymer with siRNA was done on various conditions to determine the effect of ionic strength of media on different properties such as size, binding affinity, zeta potential and its uptake by the cells. It was revealed that the size increased with increase in ionic strength. Further, the uptake was dependent on size of the particle. The silencing efficiency was greater in the absence of NaCl. The siRNA-G7 complexes showed maximum (45%) EGFP suppression in phagocytic cells (J-774-EGFP) and 35% in non-phagocytic cells (T98G-EGFP) (Perez, Romero et al. 2009). Recently, dendrimers with several modifications are implicated in RNAi therapeutics. Efficacy of siRNA delivery was evaluated by modifying free amine groups with alkyl chains of increasing length for its preferential delivery to the liver and endothelial cells (Khan, Zaia et al. 2014). Similarly, the versatility of the dendrimer (PAMAM:G4) was further subjected to certain modifications using positively charged amino acids such as arginine in the terminal regions generating a arginine rich motif for efficient formation of dendriplexes with siRNA. The G4Arg generated dendrimers showed enhanced transfection efficiency in comparison to the non-arginine bearing PAMAM

dendrimers. The G4Arg dendriplexes showed potent silencing as well with good anticancer results in prostate cancer (Liu, Liu et al. 2014). Consequently, PEGylation of dendrimers were also done to reduce its toxicity. The amine groups in dendrimers are known to cause toxicity in physiological conditions. The inherent inert, non-immunogenic and non-antigenic properties of PEG make them overcome drug leakage, immunogenicity, systemic cytotoxicity as well as aids in solubilization of hydrophobic drugs (Luong, Kesharwani et al. 2016). Recently, in a study, RNAi technique was used for knockdown of vascular endothelial growth factor (VEGF) using PEGylated PAMAM dendrimers in cobalt-chloride induced neovascularization model in retinal vascular endothelial cells (RF/6A). The resulting formulation showed higher transfection compared to commercial transfection reagent as measured by flow cytometry (Xu, Shen et al. 2016).

4.3. Polyethylenimine

Polyethylenimine, a cationic polymer is widely used for gene delivery due its high transfection efficiency. Behr et al**.** used the polymer for the first time to deliver oligonucleotides. The polymer is available in two forms: linear and branched, depending on the arrangement of repeating units of ethylenimine. Branched PEI (BPEI) is prepared by acid-catalyzed polymerization reaction of aziridine whereas Linear PEI (LPEI) is prepared through ring opening polymerization method using 2-ethyl-2-oxazoline followed by hydrolysis. Further, these vectors are modified to enhance its biological function and other properties (Jones, Langsjoen et al. 1944, Boussif, Lezoualc'h et al. 1995, Brissault, Kichler et al. 2003, Neu, Fischer et al. 2005).

In context of delivering nucleic acids, the vector needs to maintain a balance between lipophilicity and hydrophilicity for its proper transfer across the plasma membrane. Although, positive charges on the surface of PEI aids in transfection by forming a complex with the nucleic acids. Excess positive charge can cause cytotoxicity in cells and a more stable complex can prevent endosomal release. Thus, by modifying certain structural and chemical properties, PEI can be manipulated for enhanced delivery and silencing. In one of the study, Oskuee et al. studied the effect of alkylated PEI (25 kDa) on biophysical properties and bioactivity. The formulation enhanced the efficiency of siRNA delivery to the cells (Oskuee, Philipp et al. 2010). Besides, other modifications such as low degree of carboxylation (<20%) have known to enhance endosomal escape. In addition, it was noted that with

increase in hydrophobic acyl chain length, the stability of the complex also increased and carboxyalkylation of PEI increased the luciferase knockdown (Philipp, Zhao et al. 2009).

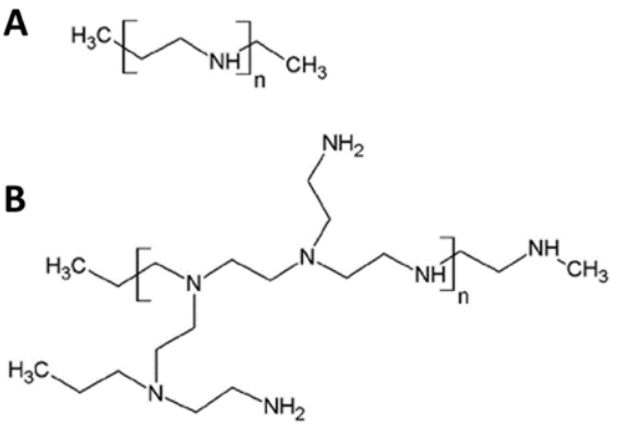

Figure 5. Basic structure of PEI.

Among many factors affecting efficiency of siRNA delivery and silencing the most important factor affecting the complex formation and release is the amine to phosphate ratio (N/P) of PEI and siRNA. Physicochemical studies showed that PEG-PEI/siRNA were not suitable for transfection until N/P of 15 was attained. Moreover, further increase in N/P ratio resulted in increased transfection efficiency upto N/P 30, as higher N/P produces more condensed formulations resulting in poor release of nucleic acids into the cells. To overcome the problem of instability and low transfection efficiency, the polymer are modified with certain hydrophilic molecules such as PEG. PEG (2kDa)-PEI (25 kDa) were prepared for targeting siRNA to CD44v6 in gastric carcinoma cells (Wu, Wang et al. 2010). For enhanced delivery, iron oxide were bound by chitosan-grafted-PEG and PEI (Veiseh, Kievit et al. 2010). Similarly, PEI was also modified using different concentrations of alginic acid, where it was observed that with increase in the concentration of alginic acid, the zeta potential decreased and the transfection efficiency increased when compared to native PEI. The maximum transfection efficiency increased with the PEI-alginate nanoparticles generated at w/w ratio 20:1. Further, these nanocomposites showed 80% green fluorescent protein (GFP) silencing in mammalian cells (Patnaik, Aggarwal et al. 2006). Additionally, acylation of PEI was done by propionic anhydride and nanoparticles were formed by conjugating PEG-bis(phosphate). The size of nanoparticles were obtained in

considerable range with a size of 100 nm. High efficiency of gene silencing was observed with inhibition of GFP expression upto 85% (Nimesh and Chandra 2009).

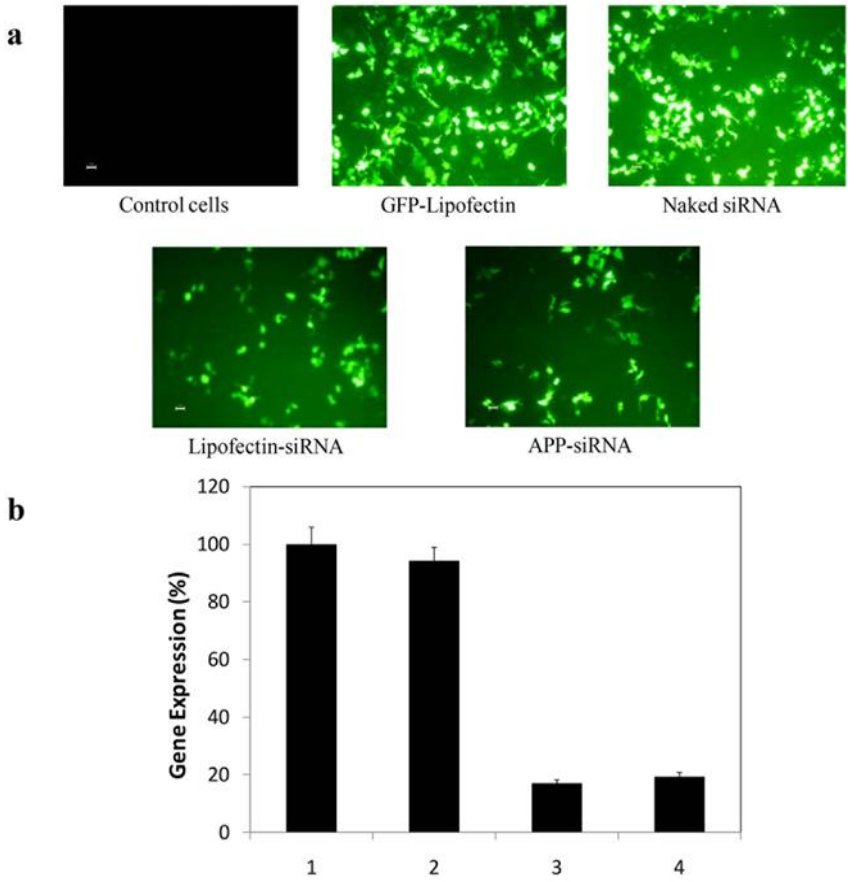

Figure 6. Comparison of gene silencing efficiency of various siRNA formulations after 48 h. (a) The GFP expression was observed under fluorescent microscope at 10X magnification. (b) The level of GFP expression was estimated by quantitation of green fluorescence after 48h. The data was recorded at optimal inhibition efficiency for APP nanoparticles i.e., APP nanoparticles: siRNA at 30:1 ratio. (1) Lipofectin–pEGFP, (2) Naked siRNA, (3) APP–siRNA complex, (4) Lipofectin–siRNA. (Results reproduced from Nimesh et al. 2009).

In addition to all the aforementioned approaches, PEI vector can also be exploited for targeted delivery. Folate conjugated nanoparticles were used for delivery of dihydrofolate reductase (DHFR) siRNA to inhibit the expression of

DHFR gene in folate receptor (FR) positive KB cells for targeted delivery. Folate-PEG-PEI (25kDa) (FOL-PEG-PEI) complex conjugated to siRNA showed higher transfection efficiency in A549 cell lines (Biswal, Debata et al. 2010). Transferrin-conjugated PEI was used for delivery of siRNA against inflammatory genes in activated T-cells (ATC) with an aim for the treatment of inflammatory lung diseases such as Asthma. It was observed that the formulation showed higher cellular uptake and knockdown in human primary ATCs. Further, the formulations were also tested in murine asthmatic model that displayed good biodistribution profiles (Xie, Kim et al. 2016). Similarly, in another study, folate was conjugated to triblock copolymer polyethylenimine-graft-polycaprolactone-block-poly(ethylene glycol) (PEI-g-PCL-b-PEG-Fol) for the delivery of siRNA to overcome chemotherapy resistance in ovarian cancer. The formulations of size 260 nm were obtained that showed efficient knockdown of toll like receptors-4 (TLR-4) in SKOV-3 cells. Consequently, the gene knockdown sensitized the tumour towards paclitaxel treatment (Jones, Lizzio et al. 2016). NGR-peptide conjugated PEI/siRNA nanoparticles were employed for silencing Bmi-1 and hTERT in MCF-7 breast cancer cell line. The size and zeta potential of nanoparticles prepared were analysed using AFM and particle size analyser that showed a size range between 100 and 200 nm. To determine the efficiency of transfection qRT-PCR and western blot was done. The formulation showed greater silencing of the targeted gene (Liu, Li et al. 2015).

4.4. Poly(Lactic-Co-Glycolic) Acid

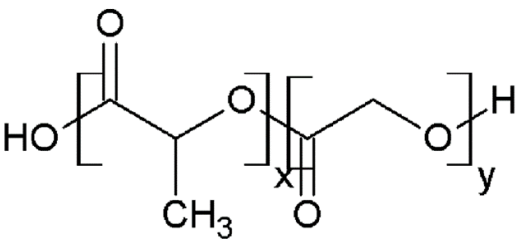

Figure 7. Basic structure of PLGA.

PLGA is one of the most effective polymer amongst the available non condensing nucleic acid delivery systems. PLGA nanoparticles are easily taken up by the cells through endocytic processes in a targeted/ non targeted manner (Panyam and Labhasetwar 2003). It was revealed that in acidic

environment, the PLGA nanoparticles undergo endosomal escape from the lysosomal compartment because of the surface charge reversal of PLGA nanoparticles (Panyam, Zhou et al. 2002, Panyam and Labhasetwar 2003). Subsequently, this enables the sustained release of the payload from the nanoparticles in the cytoplasm for longer duration more than 2 weeks (Panyam and Labhasetwar, 2004). Additionally, PLGA is biocompatible and biodegradable and gets hydrolysed in lactic and glycolic acid. The degraded monomers can then be metabolised through Krebs cycle and easily removed from the body thereby reducing the toxicity. So, PLGA nanoparticle holds a significant potential to deliver nucleic acid for gene silencing owing to its high biocompatibility, lysosomal escape, efficient cellular uptake, controlled drug release and reduced toxicity. Commercially, PLGA are available in different variants of molecular weight and lactic/glycolic acid ratio and are prepared in presence of metal catalysts through ring opening polymerisation of cyclic dimers.

4.4.1. PLGA as an Efficient Gene Carrier

The flexible nature of PLGA enables its modification and derivatisation for the efficient delivery of nucleic acids. In a study, PLGA surface has been modified with chitosan to impart positive charge on the nanoparticles thereby improving the adsorption of siRNA. Further, it was found that large size (400-1000 nm) and high PDI of PLGA-chitosan nanoparticles are ascribed to the molecular weight and concentration of both chitosan and PLGA. Hence, lower the molecular weight of chitosan, smaller will be the PLGA-chitosan nanoparticles. Moreover, the binding capacity of siRNA relies on various physicochemical properties. For instance, it was observed that an increase in the degree of uncapped end groups resulted in high siRNA loading capacity. Similarly, it was reported that the probability of particle aggregation during freeze dying were reduced with the use of trehalose. Also, to attain slow and prolong release of nucleic acids, formulated PLGA nanoparticles are prepared to regulate the degradation rate of PLGA (Katas, Cevher et al. 2009). Besides, it was evident from MTT assay that formulated PLGA-chitosan nanoparticles were non-toxic to the cells and resulted in 95% cell viability for the particles to siRNA weight ratio (100:1 to 500:1).

The chitosan modified PLGA nanoparticles facilitates the increase in binding efficiency and delivery of siRNA along with the enhanced bioavailability of PLGA nanospheres (Tahara, Yamamoto et al. 2010). The siRNA loaded nanospheres were synthesised from solvent emulsion diffusion method of size ~300 nm with positive zeta potential, the unloaded PLGA

nanospheres exhibited negative charge. It was reported that the cells showed high uptake efficiency with siRNA loaded PLGA-chitosan nanospheres in comparison to the native nanospheres. Moreover, higher luciferase knockdown efficiency was observed when modified PLGA was introduced in A549 cells compared to PLGA nanoparticles and naked siRNA (Tahara, Yamamoto et al. 2010). It was found that because of the presence of electrostatic interactions both the gene knockdown and cellular uptake studies were comparable. In a similar study, the addition of an emulsifier such as 1% PVA during the preparation, resulted in the formation of small sized uniform chitosan modified PLGA nanoparticles (Yuan, Shah et al. 2010). It was found that with the increase in the concentration of the chitosan in the formulated chitosan-PLGA nanoparticles, the observed size ranged from 204 to 543 nm. In another similar type of study, chitosan-modified PLGA showed efficient HBV gene silencing compared to delivery with PLGA alone (Tahara K. 2011). Also, these nanoparticles were investigated for siRNA transfection where they exhibited 50% EGFP silencing after 48 h post-transfection on H1299 cells (Abdelhady, Allen et al. 2003).

However, the efficiency of PLGA can also be enhanced by the fabrication of hydrophilic amino groups to the polyvinyl alcohol (PVA) that can further be added to the side chains of PLGA (Nguyen, Steele et al. 2008). It was observed that the size of the amine modified PVA-PLGA siRNA loaded nanoparticle were around 150-200 nm and zeta potential ranged between +15 to +20 mV in the presence of PBS. It was reported that the knockdown efficiency was 80-90% of gene expression when treated in H1299 luc, a human lung epithelial cell line.

To enhance the encapsulation efficiency of siRNA, cationic polymers such as PEI can be added with the PLGA nanoparticles (Patil and Panyam 2009). In a study, the formulated PLGA-PEI nanoparticles were prepared from double emulsion solvent evaporation procedure. It was observed that the size effectively increased from 280 nm to 568 nm with the increase in PEI concentration. From this it was evident that the PLGA-PEI (40KDa) nanoparticles size depends on both the concentration of PLGA and PEI. Also, it was found that the encapsulation efficiency was increased to 2 fold when PEI modified PLGA nanoparticles were used and consequently the release profile of siRNA was also improved when compared with the native PLGA nanoparticles. Moreover, the silencing efficiency was also effective in transfected cell lines EMT-6 G/L and MDA-Kb2 (Patil and Panyam 2009). The effective uptake of PLGA-PEI nanoparticles by the cells results in an

increased gene silencing efficiency because of the proper encapsulation of siRNA onto the PEI-PLGA nanoparticles.

5. POLYMERIC/SIRNA NANOPARTICLES IN CLINICAL TRIALS

Gene delivery using polymeric nanoparticles have gained tremendous attention in the field of nanotechnology. A number of formulations have been prepared that have successfully made into clinical trials. PEGylation and multifunctional modifications are some of the strategies to overcome barriers faced during gene delivery.

PEI based nanoparticle SNS01-T was designed in order to induce apoptosis in B-cell cancers via siRNA to suppress hypusinated elF5A with simultaneous over-expression of non-hypusinable elFA mutant by plasmid based vectors. Phase I and phase II studies were conducted to test safety and tolerability at low dose in group of patients with relapsed or refractory multiple myeloma or B-cell lymphoma (Francis, Taylor et al. 2014). Similarly, a PLGA-based therapeutic drug, siG12D LODER was prepared to knockdown the expression of KRASG12D and was enrolled in phase 0/I clinical trial by Silenseed to determine its safety and tolerability for the treatment of pancreatic ductal adenocarcinoma. It is known that mutations in KRASG12D is known for causing pancreatic ductal carcinoma. In this study, endoscopic ultrasound (EUS) biopsy needle was used for injecting the formulation into the tumour. A biodegradable polymeric matrix LODER was used for designing a desired formulation to release drugs in tumour. The study was conducted for over 8-weeks. Safety and tolerability of the drug was tested in phase 0, while dose escalation study was performed in phase I clinical trial (Khvalevsky, Gabai et al. 2013).

Cyclodextrin based therapeutics have also managed to reach clinical level. Cyclodextrin (CD), a cyclic oligosaccharide is widely investigated in order to develop a better carrier molecule for delivery of therapeutic nucleic acids. Cyclodextrin is preferentially used as complexing agent attributing to its beneficial effects such as solubility, stability and bioavailability. Hence, a nanoparticle based formulation (CALAA-01) was generated by Davis et al. using cyclodextrin, siRNA (against M2 subunit of ribonucleotide reductase (RRM2)) and transferrin ligand to target the cancer cells. From the results it

was discerned that the formulations could be used for various cancers including melanoma cancer (Davis 2009, Davis, Zuckerman et al. 2010).

CONCLUSION

Cationic polymers have gained much attention in the field of gene delivery and silencing. However, these vectors are less efficient as compared to non-viral vectors due to extracellular and intracellular barriers. To overcome these issues polymeric nanoparticles are modified with different hydrophilic and hydrophobic molecules. In addition, with growing understanding about nanoparticles and gene delivery mechanisms, formulations with targeted approach can be obtained while maintaining low off-target effects. Thus, it could be inferred that polymeric nanoparticles can become an important tool in human gene therapy for treatment of different types of diseases.

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Chapter 3

THE USE OF THROMBOPHILIA AND PHARMACOLOGICAL THROMBOPROPHYLAXIS SCREENING IN WOMEN: THE BENEFITS OF THE RIETE REGISTRY

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BACKGROUND

Assisted reproductive technologies (ART) are associated to an increased risk to develop venous thromboembolism (VTE) compared to spontaneous pregnancy. Previous studies have found that VTE after a spontaneous conception is nearly 1 of 1000 pregnant women [1, 2], while in women undergoing ART it may raise of three-four times [3]. The pathophysiological mechanism by this increase is related to medical ovarian stimulation, a pharmacological ovarian stimulation that increases the number of oocytes available for ART associated to the presence of other risk factors as thrombophilia. Pharmacological ovarian hyperstimulation, in fact is able to increase procoagulant status [4] of all women that may lead to thrombosis, as showed by several studies. However, available data about the magnitude and the duration of VTE risk in pregnancy after ART are conflicting, just as data about the risk to develop VTE for all women that perform ART and not only for those that show pregnancy after ART. Actually, we have only data by women that develop VTE during pregnancy after ART [3], as reported by clinical series or studies but the incidence of VTE for women that perform ART with or without a following successful pregnancy is unknown and only case reports or small clinical series are reported in the Literature.

Several clinical aspects are lacking of full understanding as the VTE risk for women that will perform ART. The potential different role of such drugs and their dosages as the role of thrombophilia for the outcome of VTE and ART are involved in this clinical setting; the potential of other thrombotic risk factors should be better understood.

CLINICAL EVALUATION OF THROMBOPHILIA IN WOMEN ONGOING ART

Although the increasing of knowledge, the successful rate of ART remains low [5]. One of suggested causes of this low successful rate is the suboptimal vascular uterine perfusion during ART [6], so in these years an increasing attention has been given to thrombophilic state and to the possible sub-optimal perfusion induced by thrombophilia in these patients [6, 7]. This critical point has been also suggested by the increased number of patients carrying thrombophilia with repeated ART failure [8] and a recent case control study confirms that such thrombophilia is associated to recurrent ART failure [9,

10]. In other studies the increased rate of thrombophilia and its statistical significance, has not appeared but probably the different inclusion and exclusion criteria of these studies may have influenced results [11-13]. However, the suggested suboptimal uterine perfusion and the potential role of thrombophilia influences the current choice of physicians to add antithrombotic drugs to the regular treatment of women ongoing ART; and in particular for women with several ART failures in their anamnesis. Aspirin and/or low molecular weight heparin in fact, are constantly present as additional drugs in women ongoing ART after repeated ART failure [14-18]. This clinical and pharmacological approach may be frequently found in the daily clinical management of these patients and the clear data on the improvement of ART procedures with antithrombotic drugs is still a matter of discussion. A review from Dentali et al. underlined that the use of LMWH is associated with increased pregnancy rate in several studies, as previously already reported; but following studies did not confirm this protective role of LMWH [19]. On the other hand, several studies and reviews did not show a protective role of aspirin for women ongoing ART and their main outcomes in particular regarding the increased pregnancy rate [20].

Probably, a registry or double blind randomized trial with definite outcomes as the pregnancy rate and the evaluation of live-birth rate and the incidence of VTE and the type of used drugs to prevent VTE (i.e., LMWH, aspirin, LMWH plus aspirin or placebo), may be helpful to clarify this intriguing issue.

CLINICAL EVALUATION OF VTE RISK FOR WOMEN ONGOING ART

Venous thromboembolism is a rare complication of management of ART [21] and its real incidence is really difficult to be understood for several reasons; the main of them is related to the fact that frequently ART procedures are associated to antithrombotic treatment with aspirin and\or low molecular weight heparin per se, in order to have an increase of successful pregnancy [7]. Another frequent extra-trouble to evaluate the real incidence of thrombotic complications in women prompt to ART is related to the presence of several pharmacological protocols used to increase and to improve the hormonal *habit* of treated women [22].

However it is well testified that pharmacological ovarian hyperstimulation stimulates hypercoagulable state [4] and this may lead to thrombosis. Venous thrombosis seems to be more frequent than arterial thrombosis and the number of cases of atypical venous thrombosis is more frequent, in particular for upper limb deep vein thrombosis [23]. Several case reports or short case series are available in the Medline starting from last 20 years but case-control studies and clinical trials are not present in the Literature. Recently a cohort study showed a slight increase of VTE in pregnant women by ART compared to the VTE's rate present after a spontaneous pregnancy [24].

Thrombotic complications, in fact, may appear in thrombophilic so as in non-thrombophilic women underling so that the thrombotic event may be associated to a complex gene-environmental cause [23]. A previous retrospective analysis by Grandone et al. showed most frequent risk factors associated to the occurrence of VTE in women treated for ART [23].

Furthermore, a pharmacological thromboprophylaxis is not suggested by international guidelines in women that perform ART to prevent VTE, but the debate if it is indicated is still open and in particular a point of discussion is when to start the pharmacological thromboprophylaxis. Besides the hypercoagulable state induced by hormonal drugs for ART and the presence of thrombohilia, that could easily induce thrombotic complications in this clinical setting, women that perform ART seem to have further several aspects that should be considered; the surgical approach to perform ART, the suggested hypomobility after embryo transfer, the suggested prolonged hormonal treatment, are thrombotic risk factors too. All these items are considered in the PADUA score [25], a clinical score used for the daily clinical management of inpatients that may benefit of pharmacological thromboprophylaxis.

THE ROLE OF RIETE REGISTRY

The RIETE registry is an ongoing, international, multicentre, prospective cohort of consecutive patients presenting with confirmed symptomatic VTE (DVT and/or PE) [19,20]. It started in March 2001. Consecutive patients with acute symptomatic VTE (DVT and/or PE) confirmed by objective tests (contrast venography or ultrasonography for suspected DVT; pulmonary angiography, lung scintigraphy, or helical computed tomography scan for suspected PE) are eligible for inclusion in the registry. There are only two exclusion criteria: a likely follow-up of less than three months, and

participation in another therapeutic clinical trial. Patients are managed according to the clinical practice of each participating hospital member/centre.

All patients are followed up for at least three months. Any suggestive sign or symptom of DVT and PE recurrences or bleeding complications is noted. Each episode of clinically suspected recurrent DVT or PE is documented by further ultrasonography, venography, lung scan, helical CT scan, or pulmonary angiography.

All patients are recruited after having given their oral consent to participation in the registry, according to the requirements of the ethics committee within each hospital.

Usually patients ongoing ART are excluded from general clinical trials and registry so it is very difficult to collect data about this kind of patients in particular if they show complications during ART procedure.

Preliminary data available in the RIETE registry informed us about some information on 20 patients with VTE after ART procedures. There are few data available about thrombotic risk factors in particular concerning hormonal treatments (i.e., timing and dosages of hormonal drugs). The most common risk factors in evaluated patients seem to be smoking and hypomobility. Thromboprophylaxis has been used only in 30% of patients and low molecular weight heparin is the most common drug used for thromboprophylaxis. Few information are available concerning timing and duration of pharmacological prophylaxis.

Nearly 30% of women showed upper limb DVT. After diagnosis, treatment is usually based on therapeutic doses of low molecular weight heparin, while warfarin and other anticoagulants are less used. Life threatening complications as fatal pulmonary embolism and major or fatal bleeding are infrequent.

However, data are really preliminary because based on a small population and to increase the number of patients by the registry may improve informations about this topic. On the other hand, to increase the number of patients into the registry may give to the scientific community further data about risk factors, prophylaxis, clinical presentation, treatment and outcome of this dangerous complication that may occur in women ongoing ART. Moreover data on thrombophilia and how thrombophilia may influence the clinical features of VTE in these women may be collected.

TAKE HOME MESSAGES

The clinical approach to a woman that experienced repeated ART failure is really complex and a multidisciplinary approach may be helpful in the daily clinical management. If also thrombohpilia is considered as a possible cause of repeated ART failure, a thorough anamnesis focused on additional transient or permanent thrombotic risk factors (e.g., type of hormonal drugs, timing of exposition to hormonal treatment and dosages of hormonal treatment, smoking habits, obesity, suggested or prolonged bed rest after ART),should be performed in order to understand if antithrombotic treatment may be helpful. Moreover, in such cases, the use of pharmacological thrmboprophylaxis may help preventing VTE in such patients. In this case LMWH seems to have more efficacy versus aspirin if relevant outcomes as VTE prevention or the increase of pregnancy rate are considered. These aspects are emerging from a variety of studies performed with different inclusion and exclusion criteria. In order to have a better understanding, further clinical studies should be better addressed or alternatively, extended data from registries, as the RIETE registry or other type of registry, should be planned.

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Chapter 4

GEOBACILLUS KAUSTOPHILUS HTA426: A MODEL ORGANISM FOR MODERATE THERMOPHILES

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ABSTRACT

The genus *Geobacillus* comprises Gram-positive, aerobic or facultative anaerobic, endospore-forming thermophiles that preferentially grow at temperatures ranging from 55°C to 65°C. The members of this genus were originally categorized into the genus *Bacillus*, a large family containing more than 305 species, but have been reclassified into *Geobacillus* as phylogenetically related thermophiles. *Geobacillus* species exist in a wide variety of niches; hence they have attracted interest in the fields of microbiology and biotechnology. For example, *Geobacillus* species often show remarkable properties in combination with thermophilic traits and are therefore used in microbial bioprocesses at high temperatures. In addition, they have historically served as sources of thermostable enzymes, which can be used as stable industrial catalysts and model proteins for biochemical and structural analyses. *Geobacillus*

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species are also used for thermoadaptation-directed enzyme evolution. Moreover, their diversified genomes and habitats may yield information on the mechanisms of organic evolution. In this chapter, the microbial properties, genomic features, bacteriophages, thermostable proteins, genetic tools, high-temperature bioprocesses, and thermoadaptation-directed enzyme evolution related to *Geobacillus kaustophilus* HTA426 are centrally reviewed. This strain was isolated from deep-sea sediment in the Mariana Trench and was the first among *Geobacillus* species to undergo whole-genome sequencing. Subsequently, the establishment of various genetic tools has led *G. kaustophilus* HTA426 to become one of the most intensively studied thermophiles. This paper aims to provide insights into *Geobacillus* species through an overview of the model strain HTA426.

Keywords: *Geobacillus kaustophilus* HTA426, *Geobacillus*, *Bacillus*, thermophile, genetic engineering, high-temperature bioprocesses, thermostable enzymes, thermoadaptation-directed enzyme evolution

INTRODUCTION

The genus *Bacillus* comprises Gram-positive, aerobic or facultative anaerobic, endospore-forming bacilli. The members of this genus have been identified in a wide variety of niches, including extreme environments, and thus contain extremophiles that exhibit thermophilic, alkaliphilic, psychrophilic, halophilic, and/or balophilic properties. Although still phylogenetically related to *Bacillus*, several members have been now reclassified from *Bacillus* into over 15 new genera: *Alicyclobacillus* [1], *Amphibacillus* [2], *Aneurinibacillus* [3], *Anoxybacillus* [4], *Brevibacillus* [3], *Filobacillus* [5], *Geobacillus* [6], *Gracilibacillus* [7], *Halobacillus* [8], *Oceanobacillus* [9], *Paenibacillus* [10, 11], *Salibacillus* [7], *Thermobacillus* [12], *Ureibacillus* [13], and *Virgibacillus* [14]. The members of these new genera exhibit remarkable properties, such as the halotolerant traits of *Salibacillus* spp. and nitrogen-fixation abilities of *Paenibacillus* spp., and several genera comprise polyextremophiles. Examples for polyextremophiles include the thermophilic and acidophilic *Alicyclobacillus* spp., thermophilic and alkaliphilic *Anoxybacillus* spp., halophilic and alkalitolerant *Filobacillus* spp., and halotolerant and alkaliphilic *Oceanobacillus* spp. Thus, *Bacillus*-related bacteria are extremely diverse and exist in a wide range of environments.

A thermophile is an organism capable of growing at temperatures above 55°C. Numerous thermophiles have been identified and classified into three groups: moderate thermophiles, extreme thermophiles, and hyperthermophiles. Most hyperthermophiles are archaea, but moderate and extreme thermophiles include bacteria. *Geobacillus* spp. are *Bacillus*-related moderate thermophiles that preferentially grow at 55–65°C and are present in a range of natural and artificial hot environments (Table 1). Intriguingly, their niches include not only hot environments but also cool soils [15–19] and *Geobacillus* spp. account for > 80% of thermophiles isolated from soils in Northern Ireland [15]. One possible reason for this is the facultative nature of their thermophilic properties, which enables slow but substantial growth in cool soils. It is also possible that cells or endospores are distributed in rainfall and air [17, 19, 20]. Indeed, air samples collected above cool soils in Northern Ireland contained thermophiles (mostly *Geobacillus* spp.) at a concentration of 1.55 colony forming units per 1 kl of air, and rainwater at this environment contained 1.1 cells per 100 ml of water. This frequency implies that approximately 1.4×10^5 cells or endospores are deposited on 1 m^2 of soil surface each year [17, 19]. The wide distribution of *Geobacillus* spp. may also be attributable to their excellent capacity for environmental adaptation through genetic alteration. This idea is supported by the fact that *Geobacillus* spp. undergo frequent spontaneous mutations [21] and that *Geobacillus* genomes contain many genes presumably acquired from heterologous organisms via horizontal gene transfer [22].

Geobacillus spp. are attractive for biotechnological applications. For example, *Geobacillus* spp. have historically served as sources of a wide variety of thermostable enzymes, which can be used as stable industrial catalysts even at high temperatures. Moreover, *Geobacillus* spp. can be used for microbial bioprocesses, which have advantages compared with moderate bioprocesses using mesophiles. One important advantage is that high temperature prevents the growth of animal pathogens, which is especially important in bioprocesses using crude biomass. *Geobacillus* spp. are also for thermoadaptation-directed enzyme evolution, which can generate enzyme variants that are more thermostable and retain high catalytic activities at moderate temperatures.

Among the *Geobacillus* spp. identified to date, one of the strains most studied is *Geobacillus kaustophilus* HTA426. This strain was isolated from deep-sea sediment in the Mariana Trench [23] and was the first of the *Geobacillus* spp. to undergo whole-genome sequencing [24]. Moreover, several genetic tools have been established [21, 25–31], thereby making *G.*

kaustophilus HTA426 a model or pilot strain of the genus *Geobacillus*. To provide insight into *Geobacillus* spp., this chapter describes their microbial properties, genomic features, bacteriophages, thermostable proteins, genetic tools, high-temperature bioprocesses, and thermoadaptation-directed enzyme evolution with particular focus on *G. kaustophilus* HTA426.

Table 1. Examples of *Geobacillus* habitats

Geobacillus spp.	Source	Reference
G. debilis DSM 16016	Cool soil in Northern Ireland	[16]
G. jurassicus DSM 15726	High temperature oilfield in China	[32]
G. kaustophilus A1	Geothermal field in Monterotondo	[33]
G. kaustophilus DSM 7263	Pasteurized milk	[34]
G. kaustophilus HTA426	Sediments of the Mariana Trench	[23]
G. kaustophilus TERI NSM	Oil contaminated soil	[35]
G. lituanicus DSM 15325	High temperature oilfield in Lithuania	[36]
G. subterraneus DSM 13552	High temperature formation waters	[34]
G. stearothermophilus BR219	Tittabawassee River sediment	[37]
G. stearothermophilus strain 10	Hot springs in USA	[34]
G. stearothermophilus XL-65-6	Rotting wood in Florida	[34]
G. thermocatenulatus DSM 730	Thermal bore-hole pipe	[34]
G. thermodenitrificans DSM 465	Sugar beet juice	[34]
G. thermodenitrificans NG80-2	Deep-subsurface oil reservoir	[38]
G. thermoglucosidasius DSM 2542	Soil in Japan	[34]
G. thermoglucosidasius W-2	Deep-subsurface oil reservoir	[39]
G. thermoleovorans CCB_US3_UF5	Hot spring in Malaysia	[40]
G. thermoleovorans DSM 5366	Soil near Bethlehem	[34]
G. thermoleovorans DSM 15393	Geothermal volcanic environment	[41]
G. uzenensis DSM 13551	High temperature formation waters	[34]
G. zalihae DSM 18318	Palm oil mill effluent in Malaysia	[42]
Geobacillus sp. 12AMOR1	Deep-sea hot sediment	[43]
Geobacillus sp. ASR4	Agricultural soil in Taiwan	[44]
Geobacillus sp. DC3	Homestake gold mine	[45]
Geobacillus sp. EPT3	Deep-sea hydrothermal field	[46]
Geobacillus sp. GWE1	Sterilization ovens	[47]
Geobacillus sp. Sah69	Saharan soil	[48]
Geobacillus sp. SBS-4S	Hot spring in Pakistan	[49]
Geobacillus sp. SH-1	Deep oil well in China	[50]
Geobacillus sp. T1	Soil in Iran	[51]
Geobacillus sp. Y412MC52	Hot spring in USA	[52]
Geobacillus sp. ZY-10	High temperature oilfield	[53]

MICROBIAL PROPERTIES

On March 2, 1996, a submersible vehicle collected mud samples from the bottom of the Challenger Deep in the Mariana Trench (10,897 m in depth). *G. kaustophilus* HTA426 was isolated by Takami and colleagues of the Japan Agency for Marine-Earth Science and Technology (Yokosuka, Kanagawa, Japan) from the sample [23]. According to their reports [23, 54], the strain is a rod-shaped, endospore-forming thermophile that grows at a pH range of 4.5–8.0 and a temperature range of 42–74°C (optimally at 60°C). Its main isoprenoid quinone is menaquinone-7, as in related species with thermophilic phenotypes, and its main cellular fatty acids are iso-15:0, iso-16:0, iso-17:0, and anteiso-17:0, as observed for *G. stearothermophilus* ATCC 12980. Growth proceeds even in the presence of 3% NaCl, but not under anaerobic conditions or a hydrostatic pressure > 30 MPa. Thus, *G. kaustophilus* HTA426 is defined as an aerobic, moderate thermophilic, halotolerant, and endospore-forming bacillus.

Colleagues and I further characterized the microbial properties of *G. kaustophilus* HTA426. The strain was obtained from the RIKEN BioResource Center (Tsukuba, Ibaraki, Japan; JCM 12893). Cells were cultured using lysogeny broth (LB; Miller) and minimal medium if not otherwise specified. Minimal medium contained 0.3 g l^{-1} K_2SO_4, 2.5 g l^{-1} $Na_2HPO_4 \cdot 12H_2O$, 1 g l^{-1} NH_4Cl, 0.1% (vol/vol) trace element solution [55], 0.4 g l^{-1} $MgSO_4$, 3 mg l^{-1} $MnCl_2 \cdot 4H_2O$, 5 mg l^{-1} $CaCl_2 \cdot 2H_2O$, 7 mg l^{-1} $FeCl_3 \cdot 6H_2O$, and 10 mM Tris-HCl (pH 7.5). Cells were stained using a Gram-staining kit (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), and observed using an optical microscope (CX41; Olimpus Corp., Tokyo, Japan). Endospore formation was examined at 60°C for 7 days on LB plates and analyzed using Wirtz's Endospore Stain Kit (Muto Pure Chemicals Co., Ltd., Tokyo, Japan). To analyze growth rates, cells were inoculated into LB medium (20 ml) in an Erlenmeyer flask (100 ml) with a silicon plug. The flask was incubated with rotary shaking at 180 revolutions per minute, and optical density at 600 nm was monitored using an OD-MonitorA instrument (Taitec Corp., Saitama, Japan). To examine the intracellular glycosidases, *G. kaustophilus* HTA426 was cultured at 60°C in LB medium and homogenized by sonication followed by centrifugation to prepare a crude extract. The extract was incubated at 60°C in 50 mM sodium phosphate (pH 6.0) with 2 mM *p*-nitrophenyl (pNP) glycosides. Subsequently, the amount of *p*-nitrophenol released from the substrate was determined by measuring the absorbance at 405 nm.

Microscopic analysis confirmed that *G. kaustophilus* HTA426 formed Gram-positive, rod-shaped, and partially chained cells (Figure 1). *G. kaustophilus* HTA426 did not produce detectable endospores, in disagreement with a previous observation [54]. One possible explanation for this is that successive cultures cause genetic and/or expression alterations that repress endospore formation.

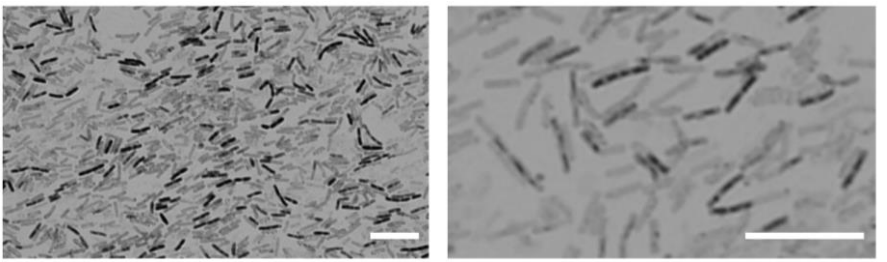

Figure 1. Cell morphology of *Geobacillus kaustophilus* HTA426. Cells were stained using a Gram-staining kit (Becton, Dickinson and Company). Scale bars indicate 10 μm.

Table 2 summarizes the growth rate and doubling time of *G. kaustophilus* HTA426 in LB and minimal media. In LB medium, the most rapid growth was observed at 65°C with a doubling time of 15.3 min, which is comparable to the optimal growth of *Escherichia coli* and *Bacillus subtilis*. Rapid growth at 65°C was also observed in minimal medium with D-glucose. However, in minimal medium with *myo*-inositol, *G. kaustophilus* HTA426 grew most efficiently at 55°C and even grew substantially at 35°C. This observation suggests that the optimum growth temperatures of *G. kaustophilus* HTA426 is dependent on the nutrients present, probably because of differencies in the catalytic properties of enzymes involved in catabolism, and that *Geobacillus* spp. may propagate under much more diverse conditions than those tested in laboratories to date.

G. kaustophilus HTA426 grew in LB medium containing 4% NaCl (Figure 2A) and in seawater supplemented with Casamino Acids (1 g l^{-1}). These observations confirm its halotolerance, implying that the strain can propagate in the sea. Although *G. kaustophilus* HTA426 is not balophilic or pressure resistant despite its origin under high pressure (about 100 MPa), this can be explained by the hypothesis that *G. kaustophilus* HTA426 exists as endospores in deep-sea sediment but propagates in shallow waters. *G. kaustophilus* HTA426 was capable of utilizing various carbohydrates as carbon sources (Figure 2B). Reflecting the fact that various glycosides serve as carbon sources for *G. kaustophilus* HTA426, many pNP glycosides (especially

pNP-α-D-galactopyranoside and pNP-α-D-glucopyranoside) were efficiently hydrolyzed (Figure 3).

Table 2. Effects of media on the growth temperatures of *Geobacillus kaustophilus* HTA426

Temperature	Growth rate (min^{-1})			Doubling time (min)		
	LB	Glucose	Inositol	LB	Glucose	Inositol
35°C			0.0016			625
37°C		0.0023	0.0025		435	400
40°C		0.0045	0.0047		222	213
45°C	0.0146	0.0117	0.0139	68	85	72
50°C	0.0315	0.0259	0.0219	32	39	46
55°C	0.0441	0.0275	0.0360	23	36	28
60°C	0.0566	0.0328	0.0267	18	30	37
65°C	0.0653	0.0360	0.0258	15	28	39
70°C	0.0504	0.0244	0.0181	20	41	55

LB, lysogeny browth; Glucose, minimal medium containing D-glucose (10 g l^{-1}); Inositol, minimal medium containing *myo*-inositol (10 g l^{-1}). Minimal medium additionally contained Casamino Acids (1 g l^{-1}).

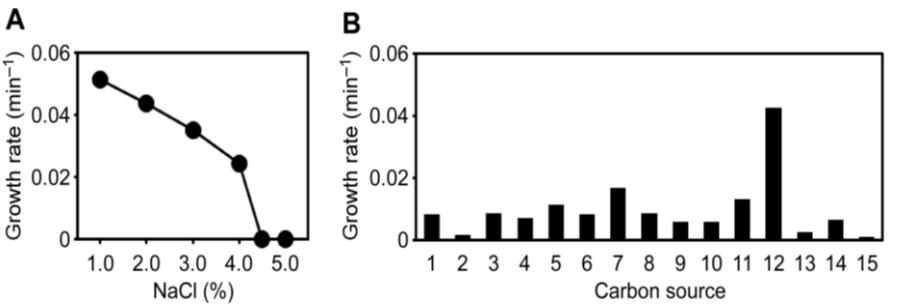

Figure 2. Growth properties of *Geobacillus kaustophilus* HTA426. (**A**) Cells were cultured in lysogeny broth supplemented with NaCl. (**B**) Minimal medium supplemented with the following carbon source (10 g l^{-1}): 1, D-galactose; 2, D-galactitol; 3, D-glucose; 4, *myo*-inositol; 5, D-mannose; 6, L-arabinose; 7, D-xylose; 8, cellobiose; 9, maltose; 10, sucrose; 11, soluble starch; 12, Casamino Acids; 13, ethanol; 14, glycerol; and 15, sodium acetate. The following compounds served as negligible carbon sources: D-fructose, D-sorbitol, D-xylitol, melibiose, lactose, acetone, methanol, isopropanol, *n*-butanol, polyethylene glycol 6000, and urea.

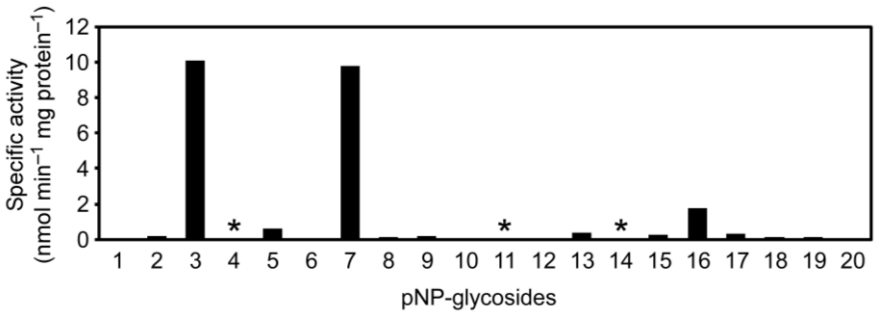

Figure 3. *p*-Nitrophenyl glycoside-hydrolyzing activity in *Geobacillus kaustophilus* HTA426. 1, *p*-nitrophenyl (pNP)-α-L-arabinopyranoside; 2, pNP-β-L-arabinopyranoside; 3, pNP-α-D-galactopyranoside; 4, pNP-β-D-galactopyranoside; 5, pNP-*N*-acetyl-β-D-galactosaminide; 6, pNP-β-D-galacturonide; 7, pNP-α-D-glucopyranoside; 8, pNP-β-D-glucopyranoside; 9, pNP-*N*-acetyl-β-D-glucosaminide; 10, pNP-β-D-glucuronide; 11, pNP-β-D-cellobioside; 12, pNP-β-L-fucopyranoside; 13, pNP-β-D-fucopyranoside; 14, pNP-β-D-lactoside; 15, pNP-β-D-maltoside; 16, pNP-α-D-mannopyranoside; 17, pNP-β-D-mannopyranoside; 18, pNP-α-L-rhamnopyranoside; 19, pNP-α-D-xylopyranoside; and 20, pNP-β-D-xylopyranoside. Asterisk indicates a specific activity of < 0.05 nmol min^{-1} mg $protein^{-1}$.

GENOMIC FEATURES

In recent years, an increasing number of genomic sequences of *Geobacillus* spp. have been registered: complete or draft sequences have now been published for > 57 strains (Figure 4). *G. kaustophilus* HTA426 is the first strain mong *Geobacillus* spp. for which the complete genomic sequence was determined [24]. The strain has a circular chromosome (3.54 Mb) and a large circular plasmid (47.9 kb), which have GC contents of 52.1% and 44.2%, respectively. These values are comparable to those of other *Geobacillus* spp., but are higher than those of mesophilic *Bacillus*-related strains (Table 3). This high GC content may contribute to the thermophilic traits of *Geobacillus* spp., because a high GC content allows stable base-pair formation in the DNA. *G. kaustophilus* HTA426 shares approximately half of the orthologous genes of *Bacillus*-related strains [24]. Moreover, the genome contains > 21 putative phage-associated genes and > 70 possible transposable elements [24], both of which outnumber those of other *Bacillus*-related strains. These observations imply that *G. kaustophilus* HTA426 has acquired many genes via horizontal gene transfer and/or from phages. Bezuidt et al. [22] performed a genome comparison of 29 *Geobacillus* spp., whcih suggested that horizontal gene

transfer was a driving force for the evolution of *Geobacillus* from *Bacillus*, with genetic contributions from other phylogenetically distant taxa.

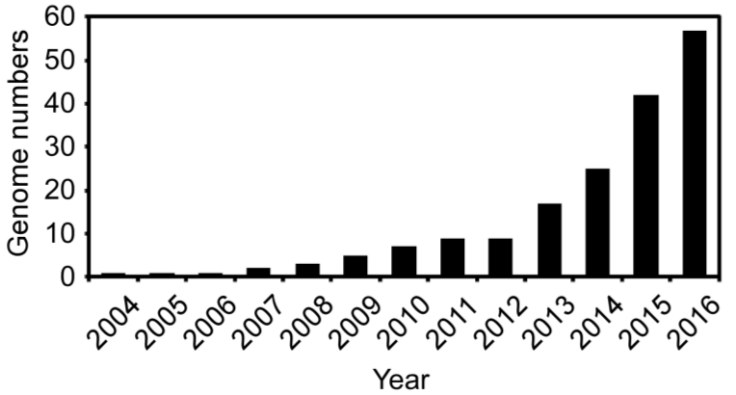

Figure 4. The number of *Geobacillus* genomes published in the GenBank database (https://www. ncbi. nlm.nih. gov/genome). Data were collected on October 17, 2016.

Table 3. Genomic sequences of *Bacillus*-related genera

Genus	Published	Size (Mb)	GC content (%)	Total genes
Bacillus	749	4.87 ± 0.94	39.8 ± 5.2	4,939 ± 1,003
Alicyclobacillus	11	3.51 ± 0.69	55.0 ± 7.2	3,401 ± 619
Amphibacillus	2	3.21 ± 0.90	36.5 ± 1.1	2,888 ± 814
Aneurinibacillus	10	5.61 ± 0.94	43.6 ± 0.8	1,937 ± 1,879
Anoxybacillus	26	3.08 ± 0.89	42.2 ± 1.2	3,158 ± 849
Brevibacillus	27	5.60 ± 0.69	48.7 ± 5.5	5,094 ± 1,190
Filobacillus	0			
Geobacillus	57	3.42 ± 0.29	51.4 ± 2.5	3,439 ± 294
Gracilibacillus	5	3.97 ± 0.63	37.3 ± 1.6	3,799 ± 521
Halobacillus	8	3.96 ± 0.17	43.6 ± 2.7	3,974 ± 110
Oceanobacillus	10	3.99 ± 0.58	37.5 ± 2.0	3,902 ± 497
Paenibacillus	136	6.37 ± 1.04	47.5 ± 5.6	5,672 ± 810
Salibacillus	0			
Thermobacillus	1	4.21	60.6	3,878
Ureibacillus	1	2.85	35.6	2,726
Virgibacillus	13	4.57 ± 1.34	39.5 ± 4.4	4,430 ± 1,323

Data were collected on October 17, 2016 from the GenBank database (https://www. ncbi. nlm.nih. gov/genome). Data are presented as the mean ± standard deviation.

According to the GenBank database (https://www. ncbi. nlm.nih. gov/genome), the chromosome of *G. kaustophilus* HTA426 contains genes for 3,301 proteins, 27 rRNAs, 87 tRNAs, and 97 pseudogenes, whereas the pHTA426 plasmid contains 43 protein genes and two pseudogenes. The genome size and gene numbers are average among *Geobacillus* spp., but are somewhat smaller than several *Bacillus*-related strains (Table 3). The genome contains genes that are potentially involved in the stabilization of nucleic acids at high temperatures, such as polyamine synthase, tRNA methyltransferase, and rRNA methyltransferase. *G. kaustophilus* DNA contains N^6-methyladenine but not methylcytosines [26]. N^6-methyladenine is attributable to DNA methyltransferases involved in restriction–modification systems. Indeed, biochemical characterization suggested that two Type-I systems (encoded by *GK1380–GK1382* and *GK0343–GK0346* on the chromosome), one Type-II system (encoded by *GKP08–GKP09* on the pHTA426 plasmid), and Type-IV systems (encoded by *GK1378*, *GK1379*, and *GK1390* on the chromosome) are active in *G. kaustophilus* HTA426 [26, 28]. Reflecting the broad range of glycosides utilized by *G. kaustophilus* HTA426 (Figure 2B), many genes for glycoside hydrolases have been identified (http://www.cazy.org). Among them, three GH1 enzymes encoded by *GK1856*, *GK2337*, and *GK3214* have been biochemically characterized [56]. The products of *GK1856* and *GK3214* efficiently hydrolyze pNP-β-D-glucopyranoside-6-phosphate and pNP-β-D-galactopyranoside-6-phosphate. The *GK1856* gene participates in a cluster with *GK1859–GK1857*, which encodes a phosphoenolpyruvate-dependent phosphotransferase system. Given that the promoter region upstream of the *GK1859–GK1856* cluster was induced in the presence of cellobiose [27], *GK1856* is apparently involved in cellobiose catabolism via this phosphotransferase system.

BACTERIOPHAGES

Table 4 summarizes the bacteriophages isolated from *Geobacillus* cells or from their environments with the ability to infect *Geobacillus* spp. Zhang and colleagues identified the phages DE6, GBSV1, GVE1, and GVE2 from *Geobacillus* spp. in hot environments and have intensively studied their features [57–65]. GBK2 was isolated from a backyard compost pile, and ϕOH2 was isolated from *G. kaustophilus* GBlys in sediment from a hot spring. These phages have been demonstrated to infect *G. kaustophilus* ATCC 8005 [66, 67], and are likely to be specific to *G. kaustophilus*. GVE3 specifically

infects *G. thermoglucosidasius* DSM 2542 [68], in contrast to the other phages.

Table 4. *Geobacillus* phages identified

Phage	Source	Reference
DE6	*Geobacillus* sp. E263 from deep-sea hydrothermal fields	[59]
GBK2	Backyard compost pile in USA	[66]
GBSV1	*Geobacillus* sp. 6k51 from a hot spring	[58, 61]
GVE1	*Geobacillus* sp. E26323 from deep-sea hydrothermal fields	[62]
GVE2	*Geobacillus* sp. E263 from deep-sea hydrothermal fields	[57, 60, 63–65]
GVE3	TMO Renewables Ltd., Guildford, UK	[68]
ϕOH2	*G. kaustophilus* GBlys in sediment from a hot spring	[67]

Table 5. *Geobacillus kaustophilus* HTA426 proteins characterized

Protein	Characterization	Reference
Arabinose isomerase	Enzymatic and crystal structure analyses	[69]
Carbonic anhydrase	Crystallization	[70]
Carboxylesterase	Crystallization	[71]
DnaC	Enzymatic analysis	[72]
DnaC/DnaI complex	Electron microscopic, enzymatic, and crystal structure analyses	[73, 74]
DnaC/ssDNA complex	Crystal structure analysis	[75]
DnaD	Crystal structure analysis	[76]
DnaK/DnaJ complex	Enzymatic and crystal structure analyses	[77]
DnaK/GrpE complex	Enzymatic and crystal structure analyses	[78]
DHNA synthase	Crystallization	[79]
DUF1181-family protein	Crystal structure analysis	[80]
Glycine oxidase	Enzymatic analysis	[81]
Glycosidases	Enzymatic analysis	[56]
Inositol dehydrogenases	Enzymatic analysis	[82]
Isobutyryl-CoA mutase	Enzymatic analysis	[83]
KAPA synthase	Enzymatic analysis	[84]
Ligand-binding oxidase	Enzymatic and crystal structure analyses	[85, 86]
Nitrite reductase	Enzymatic and crystal structure analyses	[87, 88]
Lactonase	Enzymatic and crystal structure analyses	[89–92]
PurM	Enzymatic analysis	[93]
PurN	Crystal structure analysis	[94]
Racemase	Enzymatic analysis	[95]
RuBisCO	Enzymatic and crystal structure analyses	[96]

THERMOSTABLE PROTEINS

G. kaustophilus HTA426 produces various thermostable proteins, which serve as stable enzymatic catalysts. They are also attractive as models for crystal structure analysis, because thermostable proteins have less flexibility and thereby form crystals more efficiently than the unstable proteins of mesophiles. Publication of the genome sequence of *G. kaustophilus* HTA426 has facilitated protein analysis through heterologous protein preparation. Moreover, *G. kaustophilus* proteins are generally produced in *E. coli* without problems such as negligible production or inclusion-body formation. Because of these advantages, *G. kaustophilus* proteins have been widely used for enzymatic and crystal structure analyses (Table 5).

GENETIC TOOLS

Rational genetic modification is generally achieved by introducing exogenous DNA into microbial cells [97], but the process is often troublesome because the most suitable methods and conditions greatly vary between microbes. Consequently, the number of microbes capable of undergoing genetic modification is small; nevertheless, among the *Geobacillus* spp. identified to date, several strains have been demonstrated to uptake exogenous DNA using the electroporation or protoplast methods [98–107].

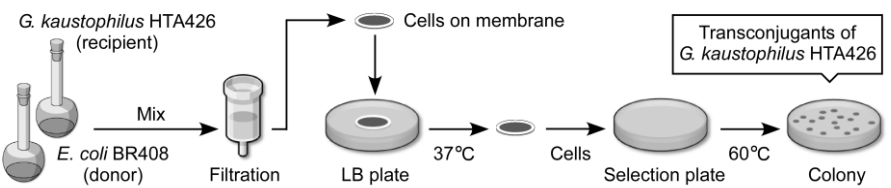

Figure 5. Plasmid transformation of *Geobacillus kaustophilus* HTA426 using conjugative transfer from *Eschrichia coli* BR408. Briefly, *Eschrichia coli* donors and *Geobacillus kaustophilus* recipients were grown in lysogeny broth (LB) and mixed. Cells were collected on a membrane and incubated on LB plates at 37°C for conjugation. The resultant cells were spread on appropriate selection media and incubated at 60°C to isolate transconjugants. This method can theoretically introduce any plasmids that contain the *oriT* region into *G. kaustophilus* HTA426.

Table 6. Central genetic tools available in *Geobacillus kaustophilus* HTA426

Strain or plasmid	Relevant description	Reference
E. coli BR408	Strain IR24 carrying pUB307 and pIR408; F$^-$ Δ(*lacZ*)*r1 e14*$^-$(McrA$^-$) *metB1* Δ*(mcrC-mrr)114*::*IS10* Δ*dam*::*metB* Δ*dcm*::*lacZ*	[26]
E. coli DH5α^{pUB307}	Strain DH5α carrying pUB307; conjugation helper	[28]
E. coli DH5α^{pRK2013}	Strain DH5α carrying pRK2013; conjugation helper	[28]
G. kaustophilus MK72	Derivative of strain HTA426; Δ*pyrF* Δ*pyrR*	[29]
G. kaustophilus MK242	Derivative of strain MK72; Δ*pyrF* Δ*pyrR* Δ*hsdM$_1$S$_1$R$_1$* Δ(*mcrB$_1$-mcrB$_2$-hsdM$_2$S$_2$R$_2$-mrr*) *GK0707*::P$_{gk704}$-*bgaB*	[21]
G. kaustophilus MK244	Derivative of strain MK72; Δ*pyrF* Δ*pyrR* Δ*hsdM$_1$S$_1$R$_1$* Δ(*mcrB$_1$-mcrB$_2$-hsdM$_2$S$_2$R$_2$-mrr*)	[28]
G. kaustophilus MK480	Derivative of strain MK242; Δ*pyrF* Δ*pyrR* Δ*hsdM$_1$S$_1$R$_1$* Δ(*mcrB$_1$-mcrB$_2$-hsdM$_2$S$_2$R$_2$-mrr*) *GK0707*::P$_{gk704}$-*bgaB* Δ(*mutS-mutL*) Δ*mutY* Δ*ung* Δ*mfd*	[21]
Plasmid pIR408	Derivative of pACYCDuet-1; Cm^R, p15A replicon, *GK0343-GK0344*, *GK1380-GK1381*	[26]
Plasmid pUB307	Derivative of IncP-1 plasmid RP1; Km^R, Tet^R, *oriV*, *oriT*, *tra*	[108]
Plasmid pRK2013	Derivative of IncP-1 plasmid RK2; Km^R, ColE1 replicon, *oriT*, *tra*	[109]
Plasmid pSTE33T	Derivative of pSTE33; Amp^R, *TK101*, pUC replicon, pSTK1 replicon, *oriT*	[26]
Plasmid pUCG18T	Derivative of pUCG18; Amp^R, *TK101*, pUC replicon, pBST1 replicon, *oriT*	[26]
Plasmid pGKE25	Derivative of pUC19; Amp^R, pUC replicon, *oriT*, *pyrF*	[28]
Plasmid pGKE70	Derivative of pUC19; Amp^R, *TK101*, pUC replicon, *oriT*, P$_{gk704}$, *trpE* internal region	[21]
Plasmid pGKE73	pUCG18T carring P$_{gk704}$	[28]
Plasmid pGKE75	pGKE73 lacking an *Sph*I site	[30]
Plasmid pGAM46	Derivative of pUC19; Amp^R, pUC replicon, *oriT*, *pyrF*, *GK0707* fragments	[29]
Plasmid pGAM47	pGAM46 carrying P$_{sigA}$	[29]
Plasmid pGAM48	pGAM46 carrying P$_{gk704}$	[29]

Amp^R, Cm^R, Km^R, and Tet^R denote genes that confer ampicillin, chloramphenicol, kanamycin, and tetracycline resistance to *Escherichia coli*, respectively. *TK101* confers kanamycin resistance at high temperatures [102]. *pyrF* encodes orotidine-5'-phosphate decarboxylase of *Geobacillus kaustophilus* HTA426. *oriT* denotes the conjugative transfer origin from pRK2013. P$_{sigA}$ and P$_{gk704}$ denotes promoters functional in *G. kaustophilus* HTA426. *trpE* and *GK0707* encode anthranilate synthase component I and α-amylase of *G. kaustophilus* HTA426, respectively. The MK480 strain lacks genes related to DNA repair (i.e., *mutS*, *mutL*, *mutY*, *ung*, and *mfd*).

Table 6 summarizes the central genetic tools available in *G. kaustophilus* HTA426. Although there are no reports of the genetic modification of *G. kaustophilus* HTA426 using the electroporation or protoplast methods, this strain can be transformed using conjugative plasmid transfer from *E. coli* BR408 (Figure 5).

During conjugation, *G. kaustophilus* HTA426 efficiently accepts plasmids transferred from *E. coli* BR408, which is engineered to produce DNA that circumvents the restriction–modification systems of *G. kaustophilus* HTA426, but does not accept plasmids transferred from other general strains (e.g., *E. coli* DH5α). However, *E. coli* BR408 is unsuitable for DNA manipulation because it harbors the plasmids pIR408 and pUB307 and the genes *recA* and *endA1*, which may cause involuntary plasmid alteration and degradation. Consequently, plasmid transformation in *G. kaustophilus* HTA426 requires three steps: plasmid construction using a general *E. coli* strain; plasmid introduction into *E. coli* BR408; and plasmid transfer from *E. coli* BR408 to *G. kaustophilus* HTA426. To resolve this problem, my colleagues and I constructed two strains (MK242 and MK244; Table 6) from *G. kaustophilus* HTA426. These strains are deficient in all restriction–modification systems and thus accept plasmids from *E. coli* DH5α, allowing direct plasmid transfer from *E. coli* DH5α to *G. kaustophilus* in the presence of the helper *E. coli* strains DH5α^{pUB307} or DH5α^{pRK2013} (Table 7). *G. kaustophilus* MK242 and MK244 are available from the RIKEN BioResource Center (JCM 31151 and JCM 31152, respectively).

Table 7. Comparison of the conjugative transfer efficiency of the pUCG18T plasmid

Donor	Recipient	Conjugation helper	Efficiency
E. coli BR408	*G. kaustophilus* HTA426		$(1.2 \pm 0.5) \times 10^{-3}$
E. coli DH5α	*G. kaustophilus* HTA426	*E. coli* DH5α^{pUB307}	$< 1 \times 10^{-7}$
E. coli DH5α	*G. kaustophilus* HTA426	*E. coli* DH5α^{pRK2013}	$< 1 \times 10^{-7}$
E. coli DH5α	*G. kaustophilus* MK244	*E. coli* DH5α^{pUB307}	$(6.4 \pm 4.3) \times 10^{-4}$
E. coli DH5α	*G. kaustophilus* MK244	*E. coli* DH5α^{pRK2013}	$(2.1 \pm 1.5) \times 10^{-4}$

The donor harboring pUCG18T and the recipient were incubated with or without the conjugation helper. Cells were collected on a membrane and incubated on lysogeny broth plates at 37°C for conjugation. The resultant cells were incubated at 60°C on selection media to analyze the generation efficiency of *G. kaustophilus* transconjugants harboring the pUCG18T plasmid (i.e., the number of transconjugants per total number of recipients). Data are cited from the references [26, 28], which are expressed as the mean ± standard deviation (n = 4).

Conjugation was performed in a mixture that contained an *E. coli* donor and a *G. kaustophilus* recipient without the minute optimization of experimental conditions. Although conjugation generally makes it difficult to distinguish recipient transconjugants from donor cells because both cells share a selectable marker, *G. kaustophilus* is distinguishable from *E. coli* by incubation at high temperatures. Remarkably, conjugative plasmid transfer is effective for plasmid transformation in other *Geobacillus* spp. and thermophilic *Bacillus* spp., including *G. stearothermophilus*, *G. thermoglucosidasius*, *G. thermoleovorans*, *G. uzenensis*, *G. subterraneus*, *B. caldovelox*, *B. caldotenax*, and *B. caldolyticus* [110].

Since the first report in 1982 of plasmid transformation in *G. stearothermophilus* ATCC 12980, many *E. coli–Geobacillus* shuttle plasmids have been developed [107]. Examples include pSTE33 and pUCG18, which were originally constructed for *G. stearothermophilus* and *G. thermoglucosidasius*, respectively [104, 105]. The pSTE33 plasmid contains pUC19 and pSTK1, a cryptic plasmid from *G. stearothermophilus* TK015, along with the *TK101* gene, which confers kanamycin resistance at high temperatures [102]. The pUCG18 plasmid [105] contains pUC18, the *TK101* gene, and a replicon region of the cryptic plasmid pBST1 from *G. stearothermophilus* NRRL 1102. In *G. kaustophilus* HTA426, pSTE33T and pUCG18T derived from pSTE33 and pUCG18, respectively, have been demonstrated to propagate with distinct properties [26]. pUCG18T exhibits a high transformation efficiency, but apparently replicates with a low copy number and low segregational stability [26]. In contrast, pSTE33T stably propagates with a substantial copy number, but is relatively lower in transformation efficiency than pUCG18T. pUCG18T has been further modified to yield pGKE75, which contains multiple cloning sites downstream of the P_{gk704} promoter and directs forcible expression [27]. In addition, pGAM48, pGKE25, and pGKE70 have been constructed as integration vectors. pGAM48 is designed for the marker-free integration of genes under the control of the P_{gk704} promoter at the *GK0707* locus (encoding α-amylase) via two reciprocal crossovers using counterselection (see below). pGKE25 is designed for marker-free gene deletion using counterselection. pGKE70 can integrate genes under the control of the P_{gk704} promoter at the *GK2204* locus (encoding TrpE) via the Campbell-type integration.

Antibiotic-resistance genes are commonly used as selectable markers for genetic modification, but are fewer in thermophiles relative to mesophiles. *G. kaustophilus* HTA426 is intrinsically resistant to spectinomycin [25]. Ampicillin is effective but the ampicillin-resistance gene cannot confer

resistance to ampicillin on *G. kaustophilus* HTA426 even at 45°C [25]. However, genes that confer kanamycin [102], chloramphenicol [30], and thiostrepton [25] resistance have been demonstrated to serve as selectable markers in *G. kaustophilus* HTA426. Among them, the kanamycin-resistance gene is the most practical marker, because it even functions at 70°C, but the thiostrepton- and chloramphenicol-resistance genes are only functional at < 55°C and < 65°C, respectively. In *G. kaustophilus* MK72 and its derivatives (e.g., MK242 and MK244), counterselection using the *pyrF* marker is available because of their deficiency in *pyrF*, which is involved in the *de novo* synthesis of uracil-related metabolites and in the anabolism of 5-fluoroorotate into toxic metabolites in cooperation with the *pyrE* product. Thus, the counterselection system uses uracil prototrophy in forward selection and 5-fluoroorotate resistance in reverse selection (Figure 6). This system is especially useful for marker-free gene integration and deletion using pGAM48 and pGKE25, respectively. Note that reverse selection is performed in the presence of uracil, but uracil causes *pyrE* repression via control of the *pyrR* product in *G. kaustophilus* HTA426, hindering reverse selection. Therefore, *G. kaustophilus* MK72 and its derivatives lack *pyrR* in addition to *pyrF* (Table 6).

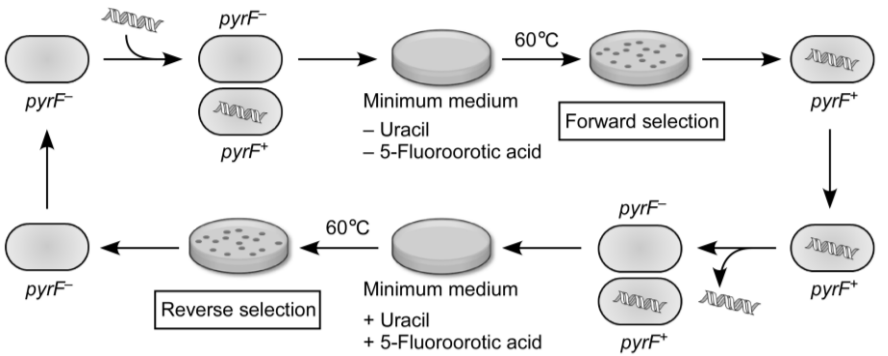

Figure 6. *pyrF*-based counterselection in *pyrF*-deficient microbes. The *pyrF* gene is involved in the *de novo* biosynthesis of pyrimidine-related metabolites and is responsible for the toxicity of 5-fluoroorotic acid. Thus, *pyrF*$^+$ cells can grow on minimal medium without uracil but *pyrF*$^–$ cells cannot, whereas *pyrF*$^–$ cells are resistant to 5-fluoroorotic acid but *pyrF*$^+$ cells are not. This system allows positive selection for both the introduction of a *pyrF* marker into *pyrF*$^–$ cells and the elimination of the marker from *pyrF*$^+$ cells.

HIGH-TEMPERATURE BIOPROCESSES

Geobacillus spp. can be used for microbial bioprocesses performed at high temperatures, which have advantages over moderate bioprocesses [111]. One important advantage is that high temperature prevents the growth of animal pathogens, including all viruses, which are killed or at least prevented from proliferating at temperatures above 65°C. This advantage is especially important in bioprocesses using crude biomass (e.g., sewage, municipal or agricultural waste, and materials from animal farms), because virulent pathogens are common in biomass from the natural environments and may increase during reactions under moderate conditions. Other advantages include inhibiting the growth and metabolism of mesophiles that may hamper processes of interest via involuntary reactions. In addition, high temperature facilitates the easy removal of volatile products (e.g., ethanol and butanol) while decreasing oxygen solubility. Thus, a facultative-anaerobic and alcohol-tolerant strain of *G. thermoglucosidasius* has been studied for high-temperature fermentation to produce ethanol and isobutanol [99, 105, 122]. Moreover, many *Geobacillus* spp. show properties useful for high-temperature bioprocesses involved in bioproduction, bioremediation, and biosorption. Notable properties include the ability to utilize cellulose [51] or hemicelluloses [112]; the ability to produce acetoin and 2,3-butanediol [113]; the ability to accumulate toxic metal ions [114]; the ability to degrade hydrocarbons [115], long-chain alkanes [38, 116–118], herbicides [119], or polyvinyl alcohol [120]; ethanol tolerance [121]; and arsenate resistance [33]. These properties have expanded potential of *Geobacillus* spp. for high-temperature bioprocesses.

G. kaustophilus HTA426 has been studied for the high-temperature degradation of crystal cellulose [27]. Remarkably, an HTA426 derivative that produces a thermostable endoglucanase (PH1171) from the hyperthermophilic archaea *Pyrococcus horikoshii* JCM 9974 can break down filter papers at high temperatures (Figure 7). This strain may be practical for high-temperature bioprocesses that produce soluble sugars from biomass abundant in cellulose. In addition, considering that *G. kaustophilus* HTA426 can utilize various carbohydrates and grow in seawater, this strain may have considerable potential for bioproduction using plant biomass and seawater.

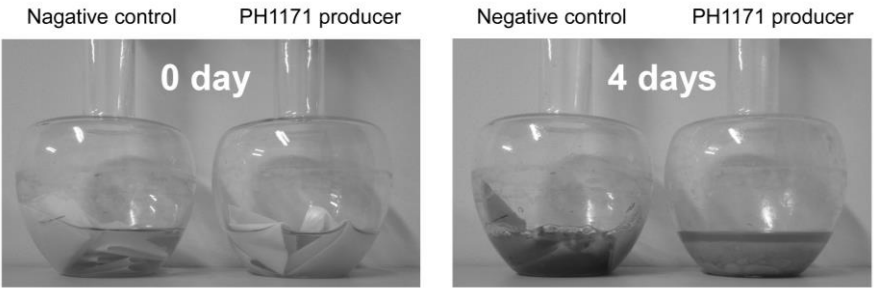

Figure 7. Degradation of cellulose papers using *Geobacillus kaustophilus* that produces the thermostable endoglucanase PH1171. Cells were cultured for 4 days with 0.3% (wt/vol) cellulose papers.

THERMOADAPTATION-DIRECTED ENZYME EVOLUTION

Enzymes catalyze numerous reactions that are difficult for chemical catalysts. However, the enzymes identified in mesophiles often show poor thermostability, which precludes their prolonged storage or use as catalysts, although their catalytic activities are efficient at moderate temperatures. In contrast, enzymes identified in extreme thermophiles show excellent thermostability, but their catalytic activities are poor at moderate temperatures; thus, they are less useful in applications that require efficient catalytic activities at such temperatures (e.g., applications in biosensors and medical diagnostics). This has increased interest in approaches that enhance the thermostability of thermolabile enzymes while maintaining their catalytic activities at moderate temperatures.

The enhancement of thermostability has been achieved using two main approaches. One uses an rational design *in silico* to predict enzyme variants with enhanced thermostability. This approach is low in cost, but requires three-dimensional structural information on the enzyme. The other uses random mutagenesis *in vitro* to construct a mutant library of the enzyme and then screens the library for enzyme variants with enhanced thermostability. This approach is proven, but may require *in vitro* characterization of numerous enzyme variants. In several studies [102, 123–129], thermophiles are used as the library host to facilitate the screening process, because thermophiles can select enzyme variants with enhanced thermostability following incubation at high temperatures when the catalytic activity of the enzyme is detectable *in vivo*. One example is provided by the generation of thermostable kanamycin

nucleotidyltransferase (KNT), which confers kanamycin resistance to the host microbe and is therefore used as a selection marker. KNT encoded by plasmid pUB110 from *Staphylococcus aureus* (KNT_{pUB110}) functions at < 55°C. However, two variants (D80Y and T130K) have been identified from a chemical mutant library of KNT_{pUB110} in *G. stearothermophilus* CU21 by incubation at high temperature in the presence of kanamycin [124]. Intriguingly, the T130K variant has been identified in the pTB19 plasmid from a thermophile [101], suggesting that the gene was generated from KNT_{pUB110} in the thermophile via spontaneous mutation. Moreover, a similar observation was made in laboratory experiments: *G. stearothermophilus* 1174 generated the D80Y/T130K (functioning at 69°C) and D80Y variants from KNT_{pUB110} during culture at high temperatures [102]. The gene for the D80Y/T130K variant is termed *TK101* and is widely used as a selection marker in thermophiles. These observations suggest that *Geobacillus* spp. can be used not only for screening, but also for generating thermostable enzyme variants via spontaneous intracellular mutations. This approach, termed thermoadaptation-directed enzyme evolution, has been intensively studied using *G. kaustophilus* MK480. This strain is an HTA426 derivative that lacks DNA repair genes, thereby serving as an error-prone thermophile. This was first used for the thermoadaptation-directed enzyme evolution of *B. subtilis* PyrF [21]. The experiment was as follows:

i. *G. kaustophilus* MK480, which is auxotrophic for uracil because of a deficiency in the *pyrF* gene, was transformed with the gene encoding *B. subtilis* PyrF (Figure 8).
ii. The resultant strain was originally prototrophic for uracil at 60°C, but was auxotrophic at 65°C, probably because of the thermal denaturation of *B. subtilis* PyrF.
iii. However, the resultant strain produced cells prototrophic for uracil even at 65°C, following successive cultures at 60°C and then 65°C in minimal media without uracil.
iv. The prototrophic cells were isolated on solid minimal media without uracil.
v. Among the colonies grown, eight were analyzed for the sequence of *B. subtilis pyrF*.
vi. Two mutant genes were identified. One carried a C497T mutation responsible for an A166V substitution in the amino-acid sequence of *B. subtilis* PyrF. The other carried a C169A mutation responsible for an L57I substitution in addition to the C497T mutation.

vii. A thermostability assay confirmed that the two variants were more thermostable than *B. subtilis* PyrF.

viii. Similar experiments using *G. kaustophilus* MK242, which is the parent strain of MK480 and has DNA repair genes, produced no cells prototrophic for uracil at 65°C, suggesting that *G. kaustophilus* MK480 is more practical for use in thermoadaptation-directed enzyme evolution than the wild type strains of *Geobacillus* spp.

ix. In addition to *B. subtilis* PyrF, thermoadaptive enzyme variants were successfully generated in *G. kaustophilus* MK480 (Table 8).

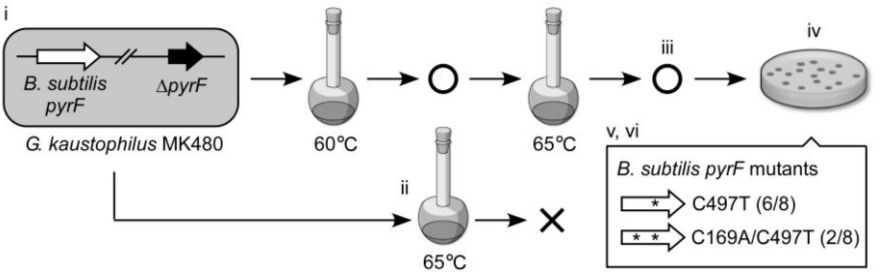

Figure 8. Thermoadaptation-directed enzyme evolution of *Bacillus subtilis* PyrF. *Geobacillus kaustophilus* MK480 was transformed with *Bacillus subtilis pyrF*. The resultant strain was originally auxotrophic at 65°C but became prototrophic following successive cultures at 60°C and then 65°C. The prototrophic cells harbored two *B. subtilis pyrF* mutants.

Table 8. Thermoadaptation-directed enzyme evolution using *Geobacillus kaustophilus* MK480

Enzyme	Outcome	Reference
Bacillus subtilis PyrF	Two enzyme variants A166V and L57I/A166V with 9.7°C- and 10.7°C-enhanced $T_{1/2}$, respectively	[21]
Staphylococcus aureus CAT	An enzyme variant A138T with 4.7°C-enhanced $T_{1/2}$	[30]
Streptomyces azureus TsrR	An enzyme variant H258Y with 4.2°C-enhanced $T_{1/2}$	[25]
pGKE75-*cat* (plasmid)	A plasmid variant that confers chloramphenicol resistance at 65°C	[31]

Thermoadaptation-directed evolution using *G. kaustophilus* MK480 requires no construction of mutant libraries because mutations are generated during successive cultures. This strain also enables *in vivo* enzymatic

characterization at high temperatures to select thermoadaptive enzymes when cell growth is accelerated by the catalytic activity of the enzyme or the activity can be detected by a chromogenic or fluorogenic assay *in vivo*. Moreover, *G. kaustophilus* MK480 has higher mutability than *E. coli* strains engineered for increased mutability (e.g., *E. coli* XL1-Red; Agilent Technologies, Inc., Santa Clara, CA, USA) and can grow at moderate to high temperatures (42–74°C) that are favorable for the thermoadaptation-directed evolution of thermolabile enzymes. Thus, *G. kaustophilus* MK480 is a practical resource for generating thermostable enzyme variants by thermoadaptation-directed evolution, providing new opportunities for the easy generation of thermostable enzymes.

CONCLUSION

Geobacillus spp. were identified during the early stages of thermophile research. Although they are now of limited interest to biology because other genera of thermophiles that can grow at much higher temperatures (> 100°C) have been identified. However, facultative nature of thermophilic properties of *Geobacillus* spp. offers advantages in certain thermophile applications, as exemplified by studies on *G. kaustophilus* HTA426. In addition, abundant genetic tools and diverse microbial properties of *Geobacillus* spp. furtehr expand their potential for various biotechnological applications.

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Chapter 5

GLYCANS PROVIDE MOLECULAR RECOGNITION MOTIFS WHICH REGULATE ENDOPLASMIC PROTEIN FOLDING, TRANSPORT, LYSOSOMAL TARGETING, AND ARE USED BY PATTERN RECOGNITION RECEPTORS IN PATHOGEN SURVEYANCE AND INNATE IMMUNITY

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ABSTRACT

Glycans are ancient highly conserved molecules which occur throughout 500 million years of vertebrate and invertebrate evolution. Glycans represent a significant repository of "recognition" information encoding functional data rivalling that of RNA, DNA and proteins. A diverse range of lectins have evolved to identify glycans and are used to regulate many essential physiological processes including protein folding, endoplasmic transport and secretion and the recognition of pathogenic organisms through molecular pattern signatures arising from pathogen cell wall components. Viruses and pathogenic bacteria have also developed their own glycan binding proteins which recognise cell surface glycans of the host cells and use these as docking modules for infection. A greater understanding of glycan interactive processes in health and disease is expected to deliver useful information relevant not only to tissue homeostasis and functionality but also to the development of preventative measures to combat deleterious glycan-pathogen interactions in disease.

Keywords: glycosaminoglycan/glycan recognition molecules, sulphation motifs, tissue morphogenesis, innate immunity, pattern recognition, glycan binding proteins

INTRODUCTION

The Biodiversity of Glycans and Their Molecular Recognition Motifs as Information Packages

Of the four major repeating “life”-biopolymers in nature - proteins, nucleic acids, lipids and glycans, the latter are the most complex, in terms of assembly into linear and branched structures modified with a number of functional groups (sulphation, phosphorylation) at variable ring positions [1-5]. Glycans in all their forms on cell-associated, pericellular and ECM components represent a significant repository of “recognition data” encoding functional information rivalling that of RNA, DNA and proteins [1-3, 5-8]. Glycans possess tremendous coding capacity and carry significant levels of biological information. The diversity of glycan structure and interactive form is reflected in the number of physiological processes they modulate. Given the ubiquitous presence and varied complexity of cell surface glycans it is not

surprising that they display such a high level of functional diversity. An example is presented where a sophisticated system of glycan binding proteins relies on the structural information conveyed by glycoproteins in the ER/Golgi to monitor the correct folding and export of proteins and is fundamentally important to the viability of higher organisms (Figure 1, 2).

Glycan Recognition Motif Interactions with Specific Lectins of the ER/Golgi Direct Protein Folding, Endoplasmic Transport along the Secretory Pathway and Export of Correctly Folded Functional Proteins from the Golgi Apparatus

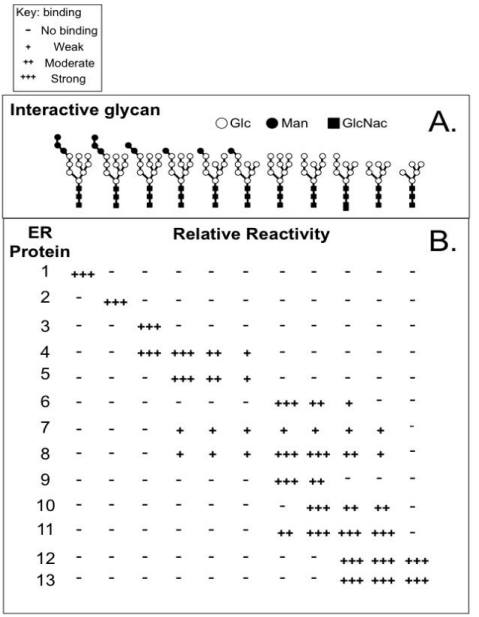
The lectin superfamily so far numbers in excess of one thousand members, a defining feature is their carbohydrate recognition domain (CRD), however by definition lectins have no catalytic enzymatic activity despite the fact that some share significant homology with glycoside hydrolases e.g., EDEM-1, 2, 3 (ER-associated degradation enhancing α-mannosidase like proteins) and α-mannosidases; YKL39, YKL40 and chitinases [9]. Control of protein synthesis and assembly into a correctly folded active protein are vital physiological events essential to the survival of higher organisms. Glycans provide recognition motifs on newly synthesised proteins from the endoplasmic reticulum (ER) which have critical roles to play directing this process in the ER/Golgi through a sophisticated series of specific ER/Golgi lectins [10-15]. The lectins of the ER/Golgi include the proteins malectin [16-18], calnexin/calreticulin [19, 20], MR60/ERGIC-53 (ER-Golgi intermediate compartment 53 kDa protein)[18], VIP-36/VIPL [21, 22], EDEM-1, 2, 3 α-mannosidase like proteins [23]; osteosarcoma-9 (OS-9) [24, 25], XTP3-B/Erlectin [26] which closely monitor and regulate protein folding in the ER/Golgi and transport correctly folded proteins along the secretory pathway while misfolded proteins are transported out of the ER for proteasomal degradation [27-33]. A number of glycosidases (Glucosidase I, II; ER mannosidase 1; Golgi mannosidase 1A, 1B, 1C) act in concert during this process sequentially removing glucose and mannose residues from the glycan side chain (Figure 1). These modifications to the glycan component provide a sophisticated system whereby the trimmed glycan sequentially directs the binding of several different lectins during protein-folding, and endoplasmic transport along the secretory pathway to the Golgi apparatus or of misfolded proteins out of the ER for proteasomal degradation. Some of these lectins are related to the glycosidases that trim the glycan side chain of the glycoproteins

during these processes. EDEM1, EDEM2, EDEM3 (ER-associated degradation enhancing α-mannosidase like proteins) are Type II transmembrane lectins of the glycoside hydrolase family 47 protein group that lack enzymatic activity. They are closely related to the α-mannosidases of the ER-Golgi. Humans have four α-mannosidases, ER mannosidase and Golgi mannosidases 1A, 1B, 1C. ER mannosidase trims Man from $Man_9GlcNAc_2$ on newly synthesised glycoproteins emerging from the ER to form $Man_8GlcNAc_2$, the Golgi mannosidases 1A, 1B and 1C further trim this to $Man_5GlcNAc_2$. EDEM1 identifies misfolded and unfolded proteins and participates in the ER-associated degradation (ERAD) system transporting these to the cytoplasm for proteasomal degradation. EDEM 2 and 3 act along with EDEM1 to accelerate ERAD. The P-type lectin OS-9 assists in the identification of misfolded proteins for disposal by ERAD. Several lectins oversee the process of protein folding and endosomal transport ensuring that only properly folded functional proteins are processed through the Golgi for export. Efficient control of functional protein synthesis is fundamental to the survival of higher organisms and explains their multiple forms and varied tissue locations. Calnexin and Calreticulin are two ER lectins which act as chaperones for the newly synthesised ER proteins and also interact with ERp57 in the ER lumen[19, 20, 34-36](Figure 2 A, B). ERp57 is a protein disulphide isomerase which catalyses the formation of disulphide bonds which stabilise the correctly folded proteins synthesised in the ER/Golgi (Figure 2A, B, C). Another means whereby the quality control of protein production in the ER/Golgi is monitored/controlled is provided by the cation independent and cation dependent mannose-6-phosphate lectin receptors (Figure 2C). The mannose-6-phosphate receptors (MPRs) are P-type lectin transmembrane proteins that target enzymes in the RER/Golgi apparatus [29, 37]. A $Glc_3Man_9GlcNAc_2$ glycan tag is added by oligosaccharyl transferase to monitor conformational refolding steps the protein undergoes into an active configuration prior to transport to the lysosome. This processing is supervised by a number of M-type lectins including malectin, calnexin/calreticulin, ERGIC53, VIP36/VIPL, EDEM1, 2, 3; OS9 and XTP3-B ensuring that only correctly folded proteins are transported along the secretory pathway. M6P receptors bind newly synthesised lysosomal hydrolases in the trans-Golgi delivering these to pre-lysosomal compartments (Figure 2C). Phosphorylation of mannose residues on ER proteins occurs by transfer of N-acetylglucosamine phosphate groups to the C6 position of one or more mannose residues by N-acetyl glucosamine phosphotransferase, followed by removal of GlcNac by a phosphodiesterase leaving a mannose-6-phosphate tag on the protein which is now targeted to

lysosomes following its correct folding and transit along the secretory pathway to the Golgi [38]. The Man-6-P tagged proteins bind to CI-MPR (calcium independent M6P receptor) and CD-MPR (calcium dependent M6P receptor) which monitor protein processing through the ER secretory pathway. The MPR-Man-6-P protein complex is then translocated to an endosome for transport (Figure 2C). The acidic pH in the endosome dissociates the enzyme from M6P tag which is recycled and the active enzyme is transported to the lysosome for export. Around 10-20% of the CI-MPRs are also present on the cell surface where it captures any M6P tagged enzyme which escape this process and recycles these to the RER preventing cellular damage from these enzymes. CI-MPR and CD-MPR share a similar overall structure however they are of widely differing sizes. CD-MPR is 46kDa occurring as a dimer while CI-MPR is ~300kDa and contains 15 P-type CRDs collectively known as M6P receptor homology domains. Both receptors have a large N-terminal extra cytoplasmic domain one transmembrane domain and a short cytoplasmic tail containing multiple sorting signals. CI-MPR and CD-MPR bind to a wide range of N-glycan structures which have important recognition motifs which direct lectin processing steps [31, 39-44].

Glycan Recognition Motifs and Innate Immunity

Many ECM components and cell adhesion molecules display a range of N- and O-linked glycan structures which are recognised by cells and play roles in cell attachment, migration and tissue development. Specific glycan structures on pathogens are also recognised by pattern recognition receptors (PRCs) such as the Toll-like receptor (TLR) family and a multitude of soluble, cell bound and cytosolic glycan binding receptor proteins which have important roles to play in pathogen surveillance in innate and adaptive immunity. Detection of tumors occurs by a similar monitoring system employing PRCs such as Dectin-1 which detect variation in tumor cell glycosylation patterns [7, 45].



Micro-array sample plan

Entry	ER Protein
1	OST, oligosaccharyltransferase
2	GI, Glucosidase I
3	Malectin
4	GII, Glucosidase II
5	CNX/CRT, Calnexin/Calreticulin
6	UGTI, UDP Glucuronosyl transferase I
7	ERGIC-53, ER-Golgi intermediate compartment glycan receptor
8	VIP-36, vesicular integral membrane protein/VIPL, VIP-36 like protein
9	ER Man-1, ER Mannosidase
10	EDEM-1, 2, 3 ER Degradation enhancer
11	Golgi Man 1A, 1B, 1C, Golgi mannosidases
12	OS9, Osteosarcoma amplified 9 ER lectin
13	XTP3-B

Figure 1. Sequential changes in the $Man_9GlcNAc_3Glc_3$ glycan chain which is attached to newly synthesised proteins in the ER by oligosaccharyltransferase and the sequential processing this glycan receives from glucosidases I, II; ER mannosidase-1, and Golgi mannosidases 1A, 1B and 1C which regulates interactions with ER lectins which monitor protein folding and transport of glycoproteins to the Golgi along the secretory pathway (A) and demonstration of ER glycan interactive proteins and their specific glycan interactive partners identified by glycan microarray which regulate protein folding (Malectin) and transport/folding of the glycoprotein along the secretory pathway (CX/CRT). Some lectins identify improperly folded glycoprotein for transport out of the ER (ERGIC53, VIP 36, OS9, XPT3-B) for proteasomal degradation (ERAD) (B). Modified from [27].

Lectin Biodiversity and Innate Immunity

Lectins are sugar-binding proteins of non-immune origin that agglutinate cells and precipitate polysaccharides or glycoproteins. Certain lectins, lack this ability since they interact with sugars on a monovalent basis however most lectins are multivalent. Lectins are distinct from carbohydrate-specific enzymes such as kinases, glycosidases, transferases and transporters, since lectins by definition are devoid of enzymatic activity. Some lectins however share significant homology with glycoside hydrolases e.g., EDEM-1, 2, 3 (ER-associated degradation enhancing α-mannosidase like proteins) and α-mannosidases; YKL39, YKL40 and chitinases [9]. Some lectins may also contain one or more site that interacts with non-carbohydrate ligands. In simple organisms which have not evolved to synthesise the immunoglobulin family and thus do not contain an immune system similar to that found in animals, this lectin system acts as their means of detecting and combatting infection. Carbohydrate-binding sites are often shallow depressions on the surface of glycan binding proteins. In all cases the combining site appears to be performed since in most cases few conformational changes occur upon binding of the lectin to its glycan determinants. Lectins bind carbohydrates through a network of hydrogen bonding, hydrophobic interactions and water bridges (Figure 3). Van der Waals forces, although rather weak, are frequently numerous and contribute significantly to the overall binding. The steric disposition of hydroxyl groups in the sugar rings of saccharides creates hydrophobic patches on the sugar surface that can interact with hydrophobic regions on the protein. Some of these interactive sites are illustrated in the peanut agglutinin (PNA) binding disaccharide and the amino acids involved (Figure 3). The steric conformation and planar disposition of the sugar is clearly an important determinant in these interactions, the biodiversity of glycan structural forms provides a multitude of such conformations which are explored leading to productive associations and explains their large number of glycan interactive ligands [46]. Glycans possess tremendous coding capacity and carry significant levels of biological information. Like-wise lectins exhibit exquisite specificity for oligosaccharide structures on glycoproteins and glycolipids on the cell surface of resident cells in animal tissues and also on invading pathogenic organisms. This ability to distinguish between subtle variations in glycan oligosaccharide structure equips lectins as useful decoders of this glycan-encoded information and for the deciphering of the “glycocode” by cells.

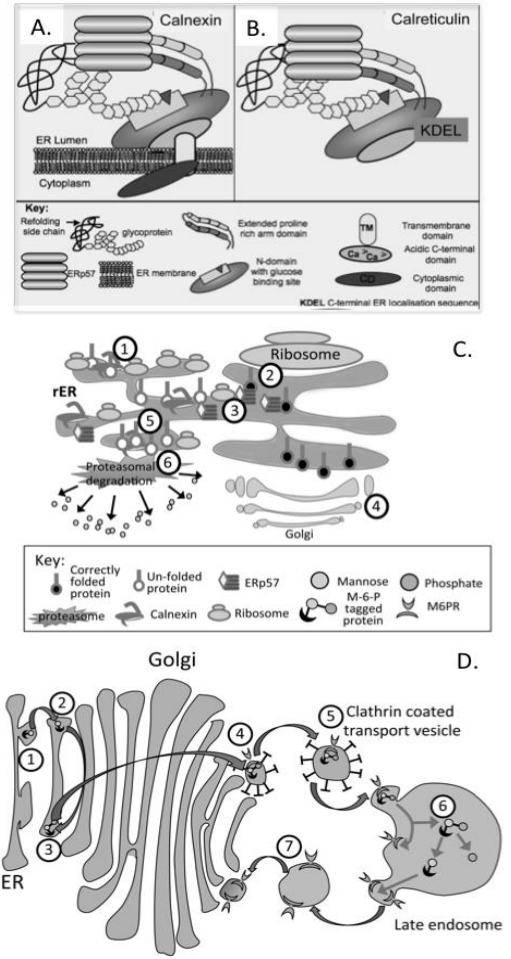


Figure 2. Schematic depiction of the endoplasmic glycan binding receptors (A) Calnexin (CNX) and (B) Calreticulin (CRT) showing their extended proline rich extension arm which interacts with ERp57 and the glycan refolding region of the ER bound protein component and various sub-domains in the C terminal region. Inappropriately folded glycoproteins are transported to the proteasome for disposal (C). Ribosomal and ERp57 driven protein folding (1-3) and protein maturation in the rER and its lysosomal transport to the Golgi (4), inappropriately folded proteins are degraded by the proteasomal apparatus (5, 6). Modified from [27, 31, 33]. In D the scheme shows the steps involved in the labelling of lysosomal hydrolases with mannose-6-phosphate (M6P) (1) and progressive steps in the controlled transport (2, 3) of the M6P labelled lysosomal enzyme through the Golgi apparatus to a clathrin coated lysosome (4) and its interaction with plasma membrane tethered M6P receptor (4) followed by secretion of the endosome (5) and endosomal de-phosphorylation of the M6P labelled enzyme (6). The M6P receptor is subsequently recycled for re-use by lysosomal transport to the Golgi apparatus (7).

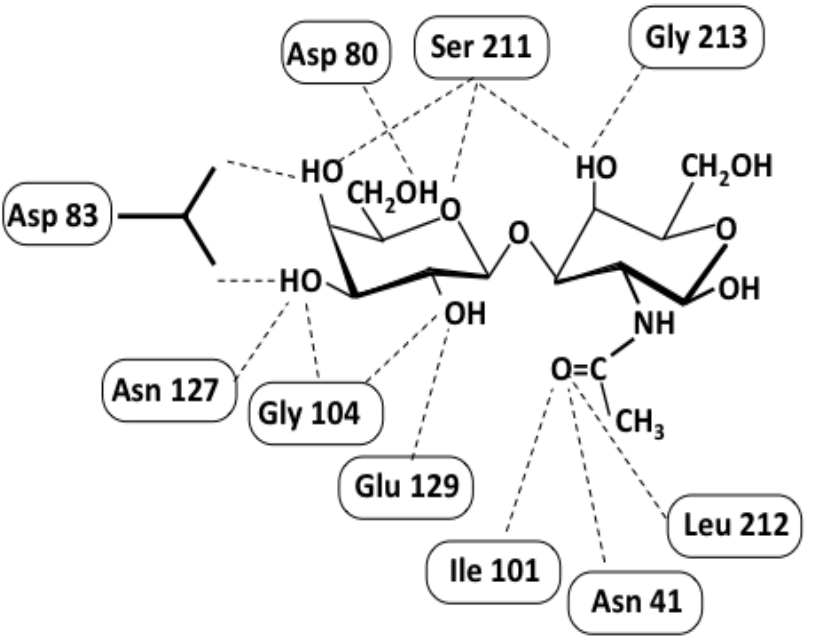


Figure 3. Interactions between specific amino acids in peanut agglutin with molecular determinants on the disaccharide Gal-β(1-3)-GalNAc. Figure re-drawn in a modified form from [269].

Table 1. Classification of Lectin Families

Family name	Sugars identified	* Motifs	Subcellular localisation	Function
Calnexin	Glc_1Man_9		ER	Protein sorting in ER
M-type lectins	Man_8		ER	Degradation of glycoproteins in ER
L-type lectins	various	PS00307 PS00308 PF00139 IPR000985 IPR001220	ER, ERGIC, Golgi	Protein sorting in ER
P-type lectins	Man-6-phosphate	PF00878 PF02157 IPR000479 IPR000296	Secretory pathway	Protein sorting in post-Golgi, ER-associated degradation of glycoprotein degradation,

Table 1. (Continued)

Family name	Sugars identified	* Motifs	Subcellular localisation	Function
C-type lectins	Man, Gal, Fuc	PS50041 PS00615 PF00059 IPR001304	Cell-membrane, extracellular	Cell adhesion (Selectins), glycoprotein clearance, Innate Immunity (Collectins)
Galectins	β-Galactosides	PS00309 PF00337 IPR001079	Cytoplasm, extracellular	Glycan cross-linking in ECM
I-type lectins (SIGLECS)	Sialic acid	PF00047 IPR003006	Cell membrane	Cell adhesion
R-type lectins	variable	PF00652 IPR00772	Golgi, cell-membrane	Enzyme targeting, glycoprotein hormone turnover
F-box lectins	$GlcNAc_2$		Cytoplasm	Degradation of misfolded proteins in ER
Ficolins	GlcNAc, GalNAc		Cell-membrane, extracellular	Innate Immunity
Chitinase-like lectins (chilectins)	Chito-oligosaccharides		Extracellular	Collagen metabolism (YKL-40)
F-type lectins	Oligosaccharides terminated in Fuc		Extracellular	Innate immunity
Intelectins (eglectins)	Gal, Galactofuranose, pentoses		Cell membrane extracellular	Innate Immunity, Fertiization, embryogenesis, host defence molecule in small intestine Humans have 2 intelectins intelectin-1 (endothelial lectin HL-1, intestinal lactoferrin receptor, omentin) and

Family name	Sugars identified	* Motifs	Subcellular localisation	Function
				intelectin-2 (endothelial lectin HL-2).
Misc				
Eel lectins (fucolectin, CRP analogue)	Fucose		serum	*Anguilla anguilla* European freshwater eel is a fucolectin
Frog egg lectin/Jeltraxin			Egg cell membrane	Related to pentraxins CRP and SAP

* PS Motifs are from the ProSite database <http://www.expasy.ch/prosite/>; PF motifs are from the Pfam protein families data base <http:www.sanger.ac.uk/ Software/Pfam/>; IPR Motifs are from the InterPro database<http:www.ebi.ac.uk/ Interpol/>.

Lectins are a diverse group of molecules with soluble, extracellular, cell-bound and intracellular members (Tables 1-4). Lectins are phylogenetically ancient molecules with specific recognition and binding functions for complex carbohydrates of glycoproteins, proteoglycans and glycolipids and their structure is conserved throughout vertebrate and invertebrate evolution indicating their participation in physiological processes essential for life[47-49]. The C-type lectin (CLECT) motif for carbohydrate recognition emerged early during evolution and was so named from the calcium dependance by many of the original lectin members displayed towards glycan binding [9]. The CLECT motif is found in many proteins in organisms as simple as the metazoan sponges, invertebrate urochordate sea squirt *Botryllus schlosseri*, yeast *Saccharomyces cerevisiae*, nematode *Caenorrhabditis elegans* and fruit fly *Drosophila melanogaster* [9]. Marsupial mammals (opossum, *Monodelphis demestica*) and monotremes (platypus, *Ornithorhynchus anatinus*) which diverged from placental mammals 148-190 million and 167-218 million years ago respectively, also possess an NK gene complex and share many orthologous C-type lectin like receptors in their genomes [9, 50]. Mammalian C-type lectin-like receptors share a highly conserved common protein structure [50]. They are type II transmembrane proteins containing short N-terminal cytoplasmic, transmembrane and stalk regions which act as a flexible linker to the ligand binding C-terminal carbohydrate recognition domain (CRD). Six cysteine residues in the CRD are highly conserved and essential for formation of the intrachain disulphide stabilised C-type lectin fold crucial

for ligand binding. The intelectin CRD is novel and not related in sequence to any other known protein domain [51-53]. Intelectins are secreted proteins but in some cases may be stored in secretory granules or anchored to membranes by a GPI linkage. Some intelectins are N-glycosylated and this glycan moeity facilitates cross-talk with other lectins [54].

Lectins have also been categorised into C-type [55], I-type (immunoglobulin)[56], M-type (mannosidase), L-type (legumes)[57], R-type (ricin)[58], P-type (M6P)[59], based on the characteristics of their CRDs (Table 1). Lectins can be further categorised into the soluble collectins [60, 61], intelectins [54], ficolins [62, 63], pentraxins [64] and galectins [65-67] (Table 2, Figure 4). Individual lectin-saccharide interactions are weak but multimeric lectin assemblies increase the avidity for glycans and the resultant agglutination activity prevents further migration of an invading organism, furthermore additional systems such as the Complement Cascade are subsequently activated, targeting the invading pathogens for destruction by dendritic cells, macrophages and cytotoxic T-lymphocytes as part of the innate and adaptive immune response (Figure 4b). The soluble collectin, ficolin, intelectin, pentraxin, mannose binding lectin and the surfactant proteins A, D display similar molecular arrangements based on collagen and fibrinogen fragments but their CRDs have subtly differing properties. These are summarised in Table 2 and Figure 4. C-reactive protein and the serum amyloid P component are both members of the pentraxin family, pentraxin-3 (PTX3) is an interesting molecule which displays a higher mannose glycan motif which allows it to interact with members of the collectin family (Figure 4f). The intelectins (ITLN-1, 2) are a novel lectin family with a CRD which does not display homology with any known protein domain. The ITLNs identify glycans such as galactofuranose and arabinogalactan which are not synthesised by mammalian cells but are components of bacterial cell walls thus the ITLNs are useful for monitoring invading bacterial infection[51, 52]. ITLN-1 also suppresses neuroblastoma and is considered a good marker of mesothelioma suggesting that the ITLNs also detect some abnormal glycan presentations produced by tumor cells [68] (Table 2).

Cell bound lectin binding proteins include Dectin-1 and 2 [69-71], Mincle (macrophage inducible C-type lectin)[72, 73], DC-SIGN (ICAM3-grabbing non-integrin)[74], DNGR-1 (dendritic cell natural killer cell lectin group receptor-1)[75], DEC205/CD205 (dendritic and epithelial cells, 205 kDa)[76], MMR (macrophage mannose receptor)[77-80], MGL (macrophage galactose lectin)[81], CD MPR (cation-dependent mannose-6-phosphate receptor)[82], CI MPR (cation independent mannose-6-phosphate receptor)[83, 84], the

SIGLEC Sialic acid binding lectin family (Figure 5) [85] and P, E, L-selectin (Figure 6), Table 3 [86, 87]. These lectins have roles as pattern recognition receptors which detect molecular signature molecules from bacterial and fungal cell walls or the cell envelope of viruses and along with the Toll-like receptor (TLR) family (Figure 7), cytosolic NOD-like receptor (NLR) family (Figure 8) and specific receptors such as FIBCD-1 (fibrinogen C domain containing 1), a specific chitin receptor, are active participants in the innate immune response ensuring that pathogens do not evade detection and inactivation (Table 2, 3) (Figure 9).

Table 2. Soluble Mammalian Glycan Binding Pattern Recognition Receptor Proteins and their interactive ligands

Protein	Ligands	Properties	Ref
Collectins	GalNAc, GlcNAc, Man, Fuc, Gal	Soluble pattern recognition glycan receptors produced by the liver which form part of the innate immune response. A family of Ca2+ dependent defence lectins	[174-179]
Ficolins (L, M, H)	GlcNAc, mannan Selected acetylated sugars, MASP1-3, PTX3 β1,3-glucans (L-Ficolin), glycoproteins E1 and E2 of hepatitis C virus.	Soluble oligomeric pattern recognition receptors-activate lectin pathway of complement activation. Roles in innate immunity. Ficolin-1, 2, 3 found in man Ficolins are homologous to Collectins structurally and functionally but are directed to different ligands.	[62, 63, 180-183]
Pentraxins	Gal-3-sulphate, GalNAc, GlcA, Man-6-phosphate	Ancient family of soluble glycan binding proteins found extracellularly and in cytosol. Pentraxins have a unique structure which can be evolutionarily traced back to the horse shoe crab. CRP and Amyloid P protein are pentraxin members in humans. Soluble pattern recognition molecules which identify pathogenic bacteria as part of the innate immune system	[64, 184-186]
Mannan binding lectin (MBL)	repetitive mannose and or GlcNAc residues on microbe cell walls and carbohydrates on gp120 of HIV-1, binds to MASP 1-3	Soluble serum pattern recognition receptor which forms part of the innate immune response. Has crucial roles in innate immunity against yeasts by upregulating Complement activation and enhanced uptake of PMNs. Inhibits DC-SIGN mediated uptake and spread of HIV-1	[187-192]
Galectins	β-galactosides such as Galβ1-3GlcNAc, Galβ1-4GlcNAc, β-1, 2-oligomannans	Expressed by macrophages with intra- and extracellular functions. Found in cytosol and nucleus as well as ECM. Cooperates with TLR-2 in	[65-67, 193-196]

Table 2. (Continued)

Protein	Λιγανδσ	Properties	Ref
		the specific detection of β-1, 2 oligomannosides from *Candida albican* cell wall	
Lung surfactant protein A, D (SP-A, SP-D)	LPS on Gram -ve bacteria, glycans in haemagglutinin head region of viruses, mannose, glucose, GlcNAc in yeast and fungal cell wall glycoproteins.	Lung glycan binding proteins, also found in gastric mucosa. SP-D has also been detected in spherical phospholipid deposits in the hippocampus of Dementia, Down's Syndrome and Schizophrenia patients. SP-D binds to invading bacteria and acts as a chemoattractant for monocytes and neutrophils.	[125-128, 197]
Intelectins (ITLN-1, 2) also known as eglectins, X-lectins and intestinal lactoferrin receptor. ITLN1-3 identified in sheep	Bacterial Galactofuranose pentose, arabinogalactans, human lactiferrin	Intelectin ligands are not produced by mammals. ITLN-1 suppresses neuroblastoma growth and metastasis. ITLN-1 is a mesothelioma marker	[52, 54, 119, 198-201]

TLRs are evolutionarily conserved type I transmembrane glycan recognition receptors named after the Drosophila ‘Toll’ protein. Ten human TLRs have been identified, some TLRs can undergo hetero-dimerisation (TRL1/2, TRL2/6) extending TLR interactive capabilities (Figure 7). TLRs recognise highly conserved structural motifs known as pathogen associated microbial patterns (PAMPs) exclusively found on microbial pathogens or danger associated molecular patterns (DAMPs) which are endogenous molecules released from dead and necrotic cells. PAMPs include LPS, peptidoglycan and lipopeptides, teichoic and lipoteichoic acid and lipomannan components of the bacterial cell wall. Bacterial flagellin, bacterial DNA and viral dsRNA also provide molecular signatures for bacteria and viruses detected by the TLRs. Binding of PAMPs or DAMPs to TLRs initiates signalling cascades which leads to transcription factor expression such as AP-1 and NFκB resulting in the production of pro-inflammatory cytokines which direct the adaptive immune response. All ten members of human TLRs have the same basic structure and have an extracellular leucine rich repeat domain involved in ligand identification and a cytoplasmic tail containing a Toll/IL-1 receptor (TIR) domain which directs cell signalling by two distinct pathways.

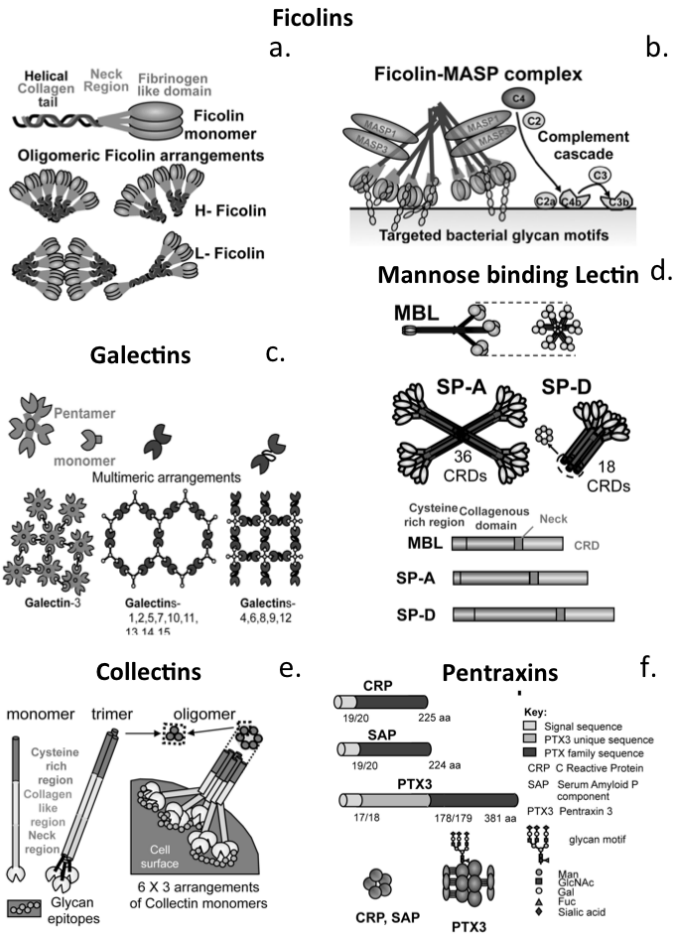


Figure 4. Schematic representations of monomeric and multimeric arrangements of soluble glycan binding lectins which form part of the innate immune response for the detection of pathogenic bacteria through glycan molecular signatures. a. Ficolin with its helical collagenous tail, neck and fibrinogen like carbohydrate recognition domain (CRD) assembled into a trimeric form which is furher assembled into various multimeric arrangements b. Ficolin-macrophage associated serine proteinase (MASP) complexes target specific bacterial cell surface glycan motifs resulting in activation of the complement cascade. c. Examples of Galectin family members and the multimeric arrangements they are assembled into with divalent, trivalent and tetravalent ligands. d. Comparison of the molecular organisation of Mannose Binding Lectin (MBL), SP-A, SP-D. e. Hexameric arrangements of trimeric bundles of the Collectins binding to cell surface glycan motifs. f. Examples of the Pentraxins including C reactive protein (CRP) and serum amyloid P component (SAP) which are assembled into characteristic pentamers. Pentraxin 3 (PTX3) also displays a glycan motif which is interactive with the Collectin family members facilitating unique cross-talk between these classes of lectins.

(i) A MyD88 (Myeloid Differentiation Primary Response 88) pathway which leads to the production of inflammatory cytokines and (ii) a MyD88 independent pathway, resulting in expression of IFN-β and maturation of dendritic cells. The MyD88 dependent pathway is common to all TLRs except TLR3. Upon activation of the TLRs by PAMPs or DAMPs TLRs undergo hetero or homo-dimerisation and recruit adaptor proteins via their TIR cytoplasmic tails. Besides MyD88, adaptor proteins include TIRAP (TIR associated protein), Mal (MyD88 like protein), TRIF (TIR domain adaptor protein inducer of IFN-β) and TRAM (TRIF related adaptor molecule) which co-ordinate cell signalling. Recruitment of MyD88 recruits IRAK1 and IRAK4 (IL-1R-associated kinases 1 and 4) and the former undergoes phosphorylation [88-91]. IRAK1 and IRAK4 leave the MyD88-TLR complex to associate with TRAF6 (Tumor Necrosis Factor receptor-associated factor-6); Bcl10 (B-Cell Lymphoma/leukemia 10) and MALT1 (mucosa-associated lymphoid tissue-1) oligomers, bind to TRAF6 and promote its ubiquitination and formation of a TAB2 (Transforming growth factor β-activated protein kinase 1 (TAK1)-binding protein 2)/TAB3/TAK1(TGF-β activated kinase 1) complex [92]. This activates TAK1 which complexes with IKK (nuclear factor κB kinase)/NEMO (NF-κB essential modulator), phosphorylates IkB and localises NFkB in the nucleus. Activation of NFkB subsequently triggers TNFα, IL-1 and IL-12 pro-inflammatory cytokine production. Specific TLRs induce differential signalling responses by recruiting different combinations of adaptor proteins. e.g., TLR 2 and TLR4 signalling requires the TIRAP/Mal (MyD88 adapter-like) adapters and the MyD88 dependant pathway [93]. TLR3 induces IFN-β production in response to dsRNA using the MyD88 independent pathway and the adaptors TRIF/TICAM-1. TLR4 uses the TRAM/TICAM-2 adaptors in the MyD88 independent pathway to induce cell signalling. Binding of viral nucleic acids to TLR3, TRL7, TLR8, TLR9 induces IFN production and interferon regulatory factors (IRFs) with crucial roles to play in antiviral defence, cell growth and immune regulation. IRF-3, 5, 7 are direct transducers of virally mediated TLR signalling.

The Siglecs are a family of sialic acid binding Ig-like type-I lectins which contain an N-terminal V set Ig domain mediating sialic acid binding and variable numbers of C2 set Ig domains [94, 95] (Figure 5). This lectin family was uncovered from initial studies on sialoadhesin (Siglec-1/CD169) [96] a macrophage lectin-like adhesion molecule and CD22 (Siglec-2) a B cell Ig superfamily member [97]. CD169 and CD22 both mediated cell-cell interactions via sialic acid cell surface glycan determinants, cloning of

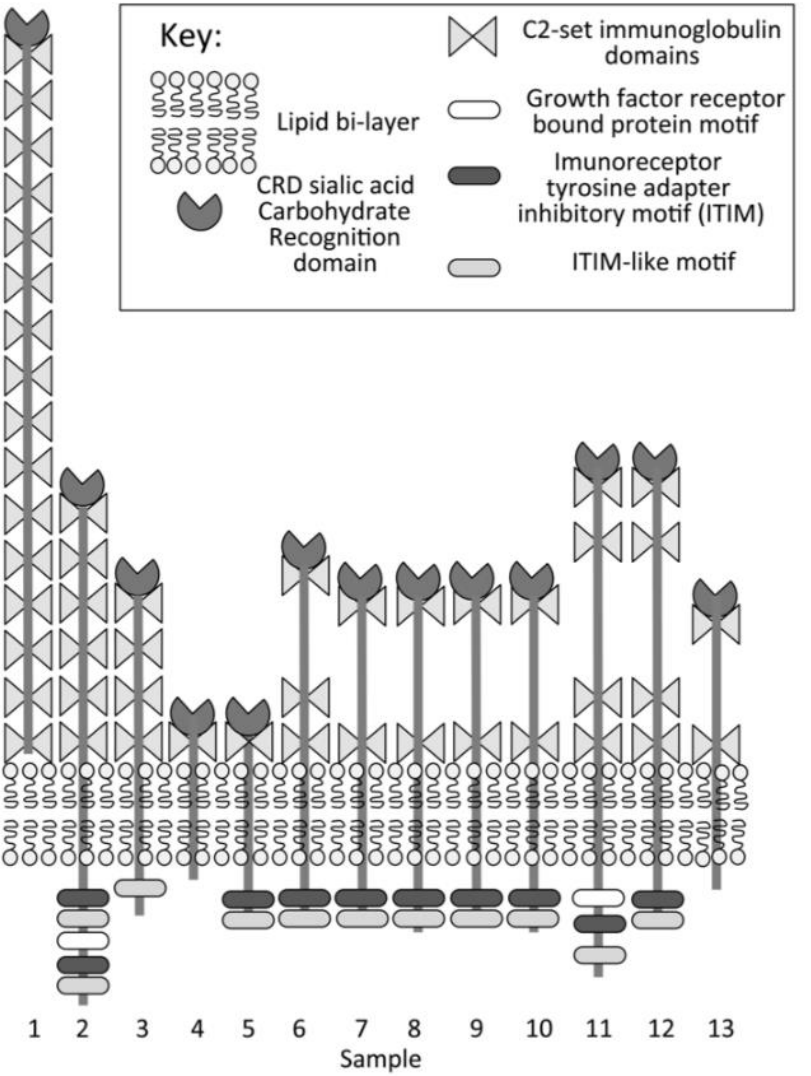


Figure 5. Schematic depiction of the biodiversity in molecular organisation of the cell membrane bound sialic acid binding Ig-like type-I lectin (SIGLEC) receptor family and Sialoadhesin. Alternative CD names for SIGLEC members are also indicated in some cases.

Samples. 1. Sialoadhesin (CD169). 2. Siglec 2 (CD22). 3. Siglec 4 (MAG, myelin associated glycoprotein). 4. Siglec 15. 5. Siglec 3 (CD33). 6. Siglec 5 (CD170). 7. Siglec 6 (CD327). 8. Siglec 7 (CD328). 9. Siglec 8. 10. Siglec 9 (CD 329). 11. Siglec 10. 12. Siglec 11 13. Siglec 14.

sialoadhesin demonstrated extensive similarities in sequence homology with CD22 it also became apparent that two CD-22 related proteins, mylein associated glycoprotein (MAG/Siglec-4) and CD33 (Siglec-3) were also members of the Siglec family but it had yet to be shown that these proteins interacted with sialic acids. The Siglec protein family now contains 13 members (Figure 5). These contain similar structural motifs such as their N terminal CRD but variable numbers of C2 set Ig domains and cytoplasmic effector domain organisation (Figure 5) [94, 97].

Table 3. Cell Membrane Bound Mammalian Pattern Recognition Receptors and their interactive ligands

Protein	Ligands/molecular signature identified	Properties	Ref
Toll-like receptors (TLR1-10) TLR1 TLR2 TLR3 TLR4 TLR5 TLR6 TLR7 TLR8 TLR9 TLR10	Gram +ve bacterial and fungal lipopeptides Lipoteichoic acid, peptidoglycan, bacterial lipoproteins. Viral ds RNA Gram -ve Enterobacterial LPS, Gram +ve bacterial cell wall lipoteichoic acid. Bacterial Flagellin Mycoplasmal lipopeptide Viral ssRNA Viral ssRNA Non-methylated DNA Not known	A family of ten early pattern recognition receptors which make an important contribution to the innate immune response. TLRs are single-pass type I transmembrane spanning catalytic receptors containing multiple extracellular leucine rich repeats (LRRs) which have roles in ligand interaction. TLR1, 2, 4, 5, 6 are cell surface receptors whereas TLR3, 7, 8, 9 are found associated with endosomes. TLRs interact with other pattern recognition receptors providing cross-talk which co-ordinates the innate immune response.	[99, 202-205]
Dectin-1	β-glucan receptor for cell wall components from yeast and fungi	Type II C-type lectin receptor (CLR) with roles in antifungal innate immunity. Positive identification of its binding ligands induces phagocytosis and activation of Src and Syk	[45, 50, 204]

Protein	Ligands/molecular signature identified	Properties	Ref
		kinases leading to NFkB activation, a reactive oxygen species (ROS) burst and secretion of proinflammatory cytokines. ROS has a microbicidal role in the phagasome. Dectin-1 collaborates with TLR2 signalling and can modulate NFAT induced cytokine expression through the Ca2+ calcineurin-NFAT pathway.	
Dectin-2 (Dendritic cell associated C type lectin-2, CLEC6A)	Binds high mannose type carbohydrates and is a receptor for α-mannans.	Type II CLR. Links pathogen recognition to adaptive immunity. Dectin-2 is the major receptor which combats fungal infection. Like Dectin-1 upon activation, Dectin-2 triggers ROS, and NLRP3 inflammasone activation to combat infection.	[70, 71, 100, 101, 206, 207]
MINCLE (Macrophage inducible C-type lectin)	α-mannose	Mincle is a multi-task danger receptor which recognises a wide range of ligands expressed in damaged cells, fungi, yeast and mycobacteria. Binds fungal α-mannose, the mycobacerial lipid trehalose-6, 6'-dimycolate, the major immunostimulatory product of *M. tuberculosis*, recognises DAMPs such as splicesome associated protein SAP 130 produced by necrotic cells. Interacts with Fc receptor γ chain triggering SYK signalling NFkB activation,	[72, 73, 102, 208, 209]

Table 3. (Continued)

Protein	Ligands/molecular signature identified	Properties	Ref
		mobilisation of intracellular $Ca2^{+}$ and the Calcineurin-NFAT pathway.	
DC-SIGN (ICAM3-grabbing non-integrin)	Binds high Mannose, GlcNAc, Fuc, $Lewis^{X}$ and carbohydrates in viral glycoprotein coats of HIV-1, HCV, Dengue, CMV, measles, Ebola; and *M. tuberculosis, M leprae, C. albicans*	Type II CLR expressed by dendritic cells (DCs), macrophages, activated B cells and immature monocyte derived DCs. Modulates adaptive immunity of DCs to bacterial, fungal and viral pathogens. Modulates NFkB signalling of TLRs. Interaction of DC-SIGN tetramer with glycans of viral coats triggers production of the serine and threonine kinase Raf-1 and acetylation of NFkB p65 and elevated and prolonged expression of IL-10 producing an increased anti-inflammatory cytokine response. Binding of DC-SIGN to Ebola and HIV cell envelope enhances infection of T cells and other cells of the immune system. DC-SIGN antagonists prevent binding of native DC-SIGN ligands and are being evaluated as novel anti-infectives for HIV-1 and Ebola.	[103, 129, 130, 132, 133]
DNGR-1 (Dendritic cell Natural killer cell lectin group receptor-1)	Actin microfilaments	DNGR-1 is a type II CLR, it displays a restricted expression in dendritic NK cells, binds to damaged or dead cells through exposed actin filaments.	[75, 111, 112]

Protein	Ligands/molecular signature identified	Properties	Ref
DEC 205	Despite sharing significant sequence homology with MMR the glycan ligands which DEC 205 identifies are currently not known.	DEC 205 is a type I transmembrane endocytic pattern recognition receptor of dendritic, B cells and leucocytes and is one of a group of 4 multilectins which includes MMR. DEC 205 shares significant homology with MMR. DEC205 has 10 CRDs, MMR has 8 CRDs. DEC 205 transports CpG oligonucleotides for endosomal degradation and is involved in antigen processing.	[76, 210, 211]
Macrophage Mannose receptor (MMR)	GalNAc-4-sulphate Preferentially recognises O-linked oligomannose	Type I transmembrane protein expressed by macrophages and dendritic cells. Contains an N-terminal R-type CRD named after the castor bean protein ricin in addition to 8-10 C-type CRDs. The R type CRD interacts with 4-O-sulphated GalNAc on glycoproteins.	[77-79, 113]
M6P receptors (M6PR) (i) CD-M6PR (cation dependent M6PR) (ii) CI-M6PR (cation independent M6PR)	Mannose-6-phosphate, TGFβ1 precursor, Granzyme B, IGF-II, uPAR, plasminogen	M6P labelling of glycoproteins in the Golgi /ER traffics them to endosomes and eventually membrane vesicles. The acidic environment in the endosome dissociates the M6PR which gets recycled. uPAR and plasminogen substrates may have roles in the activation of TGF-β1.	[29, 213-216]
SIGLECs Sialic acid binding immunoglobulin type lectins	Terminal sialic acid	The I type lectins bind carbohydrate motifs through Immunoglobulin domains (I-type CRDs). The Siglec family are type I transmembrane proteins which contain an N	[94, 217]

Table 3. (Continued)

Protein	Ligands/molecular signature identified	Properties	Ref
		terminal V set immunoglobulin domain which acts a s a sialic acid binding CRD. The Siglecs have been classified into CD33-like (Siglecs 3, 5-12) and Siglec 1, 2, 4 (sialoadhesin, CD22 and myelin associated glycoprotein /MAG). Sialadhesin is the largest Siglec with 16 C-set domains, CD22 has 6 C set domains and MAG has 4 C set domains.	
Selectins (CD62)	Fucosylated and sialylated glycoprotein ligands. E-Selectin : sialyl Lewis ^{X} mucin-like cell surface glycan. L-Selectin: sialyl 6-sulfo Lewis ^{X}. P-Selectin: PSGL-1 carrying sialyl Lewis ^{X} and N-terminal sulphated Tyr.	Carbohydrate binding molecules to fucosylated and sialylated glycoprotein ligands on endothelial cells (E), leucocytes (L) and platelets (P). The selectins have roles in the trafficking cells in the innate immune response and in inflammation, T-lymphocyte regulation, platelet activation and leucocyte extravasation.	[86, 87, 98, 218, 219]
FIBCD1 (Fibrinogen C domain containing 1 HC gp39 YKL40, YKL-39	$(GlcNAc)_n$, chitosan, Chitin fragments/particles < 40μm in size but not polymeric insoluble chitin, chitosan	Chitin is recognised by chitinases as a substrate, a number of mammalian chitinase-like but catalytically inactive (glycosyl hydrolase family 18) proteins bind chitin and play an active role in the inflammatory response it ellicits, participating in the innate surveilance of this molecule. Several chitin and chitosan (the deacetylated form of chitin) receptors and binding proteins have been identified.	[114-116]

Protein	Ligands/molecular signature identified	Properties	Ref
		FIBCD1 is a conserved homotrimeric 55kDa type II transmembrane protein expressed in the gastrointestinal tract. HC gp39/YKL40,YKL-39 are expressed by chondrocytes. Responses are generally greater with small particles of chitin/chitosan < 40μm in size but polymeric insoluble chitin is not identified.	

The selectin family interact with a heavily N- and O- glycosylated mucin-like molecule expressed in the endothelium during chronic inflammation (Figure 6). P-selectin glycoprotein ligand (PSGL-1) is a disulphide stabilised dimer which has a small cytoplasmic domain and large extracellular domain heavily substituted with N- and O- linked oligosaccharides. Of particular interest is a region towards the amino terminus of one of these chains which contains a sialyl Lewis^X motif adjacent to an acidic amino acid sequence containing a number of sulphated tyrosine residues. (Figure 6). PSGL-1 interacts with E, L, P-selectin molecules expressed by endothelial cells, leucocytes and platelets. Binding with L-selectin facilitates leucocyte homing in acute inflammation, leucocyte rolling on the endothelium and to extravasation of leucocytes into the adjacent tissues. PGSL-1 is a good example of GAG/glycan recognition motifs in action which direct a cellular response [86, 87, 98].

A diverse series of cell membrane bound lectins have been identified which display PAMP and DAMP receptor activities and participate in the innate immune response. Table 3 summarises the properties of this diverse range of proteins and Figure 9 their molecular organisation. As already mentioned the TLR family have important roles to play as PAMP and DAMP receptors and can also participate in cross-talk with other lectin receptors [90, 91, 99]. Dectin 1 is a β-glucan receptor [69] and Dectin-2 an α-mannan receptor[100, 101] (Figure 10), both of these glycans are found as integral components in bacterial and fungal cell walls [70] (Figure 11). Mincle (macrophage inducible C-type lectin) is a multi-task DAMP receptor and recognises a range of signature molecules from damaged bacterial and fungal cell walls, yeast and mycobacteria [73, 102] (Figure 11). DC-SIGN (ICAM3-

grabbing non integrin) is a lectin produced by dendritic cells and macrophages and modulates adaptive immunity through the signature molecules it detects (Figure 10). DC-SIGN detects high mannose, GlcNAc, Fuc, Lewis^x antigen as well as carbohydrate components from the glycoprotein viral coats of HIV-1 [103], HCV [104], Dengue [105], CMV [106], Measles, Ebola [107] and cell walls of the pathogenic bacteria *M. Tuberculosis*, *M. leprae* and *C. albicans* and upregulates TLR induced expression of NFκB and an elevated anti-inflammatory cytokine response. Binding of DC-SIGN to the Ebola and HIV-1 cell envelope can result in viral infection of immune cells [103, 107]. Bacteria, fungi and viruses have also developed cell surface lectin receptors which can interact with mammalian cell surface GAGs and glycans and these can act as docking modules for the invading organism [108-110]. HS is a frequently identified GAG however DS and individual proteoglycan species have even been identified as binding modules on host cells. Once the organism has gained access to the host cell then TLR-3,-7,-8 and -9 may internalise the pathogen within endosomes in an effort at containment however this may inadvertently lead to their evasion from immune detection (Figure 7). DNGR-1 (dendritic cell natural killer cell lectin group receptor-1) is a danger receptor with restricted expression in dendritic NK cells which binds to damaged or dead cells through exposed actin filaments [75, 111, 112] (Figure 9). DEC-205 is a large endocytic multilectin lectin displaying 10 CRDs and sharing significant homology with macrophage mannose receptor (MMR) however its ligands have yet to be fully characterized [78-80, 113]. MMR is a large transmembrane protein containing 8-10 C-type CRDs plus an N-terminal R-type CRD and preferentially identifies oligomannose residues and GalNAc-4-sulphate. Macrophages also express galactose lectin (MGL) which identifies α and β linked GalNAc on glycoproteins/glycolipids and LPS [81]. FIBCD1 (fibrinogen C domain containing 1) is a specific chitin receptor but does not bind polymeric insoluble particles of chitin >40μm in size [114-116]. It is a chitinase-like catalytically inactive member of the glycosyl hydrolase family 18. YKL39 and YKL-40 are also chitin/chotosan receptors expressed by chondrocytes. Figure 9 and Table 3 summarise the salient features of the membrane bound glycan binding pattern recognition receptors.

Table 4. Cytosolic and ER associated Mammalian Pattern Recognition Receptor Proteins and their interactive ligands

Protein	Ligands	Properties	Ref
Nucleotide binding oligomerization domain (NOD)-like receptor (NLR) family pattern recognition receptors	peptidoglycan	NODs consist of 3 major functional domains (i) C terminal LRR domain, (ii) Central Oligomerization domain (NACHT) and (iii) Variable effector domain containing a Pyrin motif (PYD), caspase recruitment domain (CARD) or Baculovirus IAP repeat (BIR). Nine NODs have been identified and four gene families NLRA, NLRB, NLRC, NLRP	[220-222]
Malectin	Glc_2-N-glycan, maltose and related oligosaccharides, high mannose oligosaccharidesdisplaying terminal glucose residues.	Malectin is a conserved ER membrane lectin that recognises glycopolypeptides associating with glycoproteins in the ER prior to their entry into the Calnexin Chaperone system. The Malectin system is initiated as a backup quality control system under times of ER stress.	[16, 17, 223-225]
Calnexin (CNX) Calreticulin (CRT)	High mannose in the structure $Glc_1Man_9GlcNAc_2$	CNX and CRT act as molecular chaperones assisting protein folding of glycoproteins in the ER and cooperate with ERp57 to promote protein folding. CNX and CRT retain unfolded or unassembled N-linked ER glycoproteins.	[19, 20, 34, 226-228]
ERGIC53 ER-Golgi intermediate compartment glycan receptor	High mannose	Type-1 transmembrane protein cargo receptor with a large luminal domain containing an L-type CRD and a short cytoplasmic domain. Localises mainly to the ER and ER-Golgi intermediate compartment. interacts with a luminal interaction partner multiple coagulation factor deficiency protein 2 (MCFD2) which aids in the recruitment of glycoproteins however ERGIC 53 can also act independently.	[18, 229, 230]
VIP36 Vesicular integral membrane protein of 36kDa	GlcNAc, high mannose	36kDa pattern recognition glycan receptor and type I transmembrane protein with a single L-type CRD which cycles between the ER and Golgi, displays highest binding to high mannose N-linked glycans which are attached to correctly folded glycoproteins and which have been processed by ER glycosidases. Glycan	[21, 231, 232]

Table 4. (Continued)

Protein	Ligands	Properties	Ref
		binding is Ca2+ dependent. ERGIC 53 and VIP36 share similar glycan binding profiles but their pH dependance for binding differs. VIP36 displays optimal binding at pH 6.5 and operates later in the secretory pathway than ERGIC53 trafficking proteins from the ERGIC to cis Golgi.	
VIPL (VIP36-like) membrane protein	GlcNAc, high mannose	Conserved through evolution. ER export receptor. Bears homology with ERGIC53. Very similar to VIP36 and may have arose from gene duplication.	[22, 233]
EDEM1, 2, 3 (ER-associated degradation enhancing α-mannosidase like proteins)	High mannose, $Man_8GlcNAc_2$	Type II transmembrane M-type lectins of the glycoside hydrolase family 47 protein group but lack enzymatic activity. They are closely related to the ER-Golgi α-mannosidases. Humans have 4 α-mannosidases, ER mannosidase and Golgi mannosidases 1A, 1B, 1C. EDEM1 identifies misfolded proteins and participates in the ER-associated degradation (ERAD) system transporting these to the cytoplasm for proteasomal degradation. EDEM 2 and 3 assist EDEM1 in ERAD.	[234-236]
OS9 Osteosarcoma amplified 9, ER lectin	GlcNAc, high mannose, $Man_5GlcNAc_2$	P-type lectin. Functions in ER quality control and ER associated degradation of misfolded glycoproteins in ERAD.	[24]
XTP3-B ER lectin 1	$Man_9GlcNAc_2$ glycan	May form a complex with OS-9. Bears one MRH mannose-6-phosphate receptor homology domain.	[26, 237, 238]
M6P receptors (M6PR) (i) CD-M6PR (cation dependent M6PR) (ii) CI-M6PR (cation dependent M6PR)	Mannose-6-phosphate	P-type lectins, P refers to the phosphate group on M6P. M6P labelling of glycoprotein hydrolases in the Golgi /ER facilitates their trafficking to endosomes and eventually membrane vesicles. The acidic environment in the endosome dissociates the M6PR which is recycled.	[37, 239]

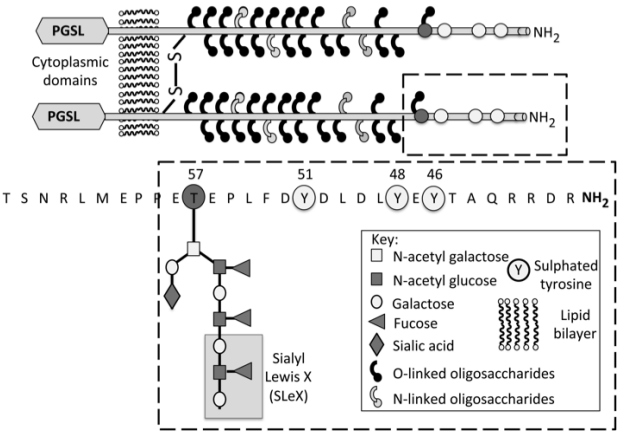


Figure 6. Schematic of the transmembrane disulphide stabilised dimeric P-selectin glycoprotein ligand-1 (PSGL-1) and its glycan motifs which are important in P-selectin binding. Three amino terminal sulphated tyrosine residues on positions 51, 48 and 46 are also characteristic features of this molecule. Optimal binding of P- and L-selectin to PSGL-1 requires a precise order of N-terminal amino acids bearing an O-glycan with a terminal sialyl Lewis^X motif adjacent to an acidic rich amino acid sequence containing sulphated tyrosine residues.

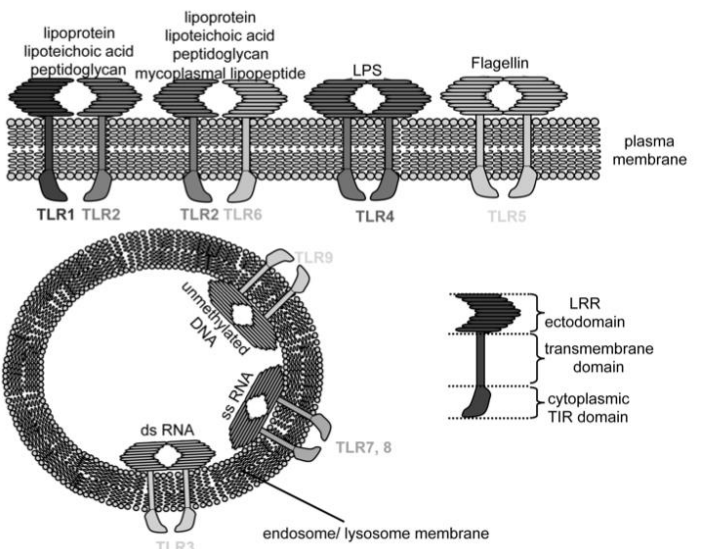


Figure 7. Schematic depiction of the Toll-like receptor (TLR) family members and their ligands, molecular signatures of bacterial and viral invasion of a host cell. TLR1/2 and 2/6 act in combination to effect ligand binding. TLR1, 2, 4, 5, 6 are cell surface pattern recognition receptors while TLR3, 7-9 are intracellular endosomal receptors which both play important roles in immune surveyance for pathogenic bacteria and viruses.

Cytosolic glycan binding pattern recognition proteins provide an additional level of protection from pathogenic organisms by identifying them through their non-self molecular signature molecules and this can initiate cell signalling events aimed at their destruction [41, 117, 118]. The nucleotide binding oligomerization domain (NOD)-like receptor (NLR) family has 9 members (Figure 8). All except NOD-like receptor protein (NLRP)-10 contain ten leucine rich repeat (LRR) domains which have roles in ligand interaction NAD/Nacht, Pyrin and Caspase recruitment (CARD) effector domains have roles in cell signalling initiated by the NOD-like receptors upon recognition of peptidoglycan (Figure 7). An appraisal of all of the membrane bound pattern recognition receptors presented in Figure 9 and the non-self carbohydrate derived recognition signals they identify shows that TLRs identify peptidoglycan and LPS, while DC-SIGN, Dectin-2 MMR, and Mincle are mannose/mannan receptors. Dectin-1 identifies β-glucan of the bacterial cell wall and FIBCD-1, chitin of fungal cell walls (Figure 9C).

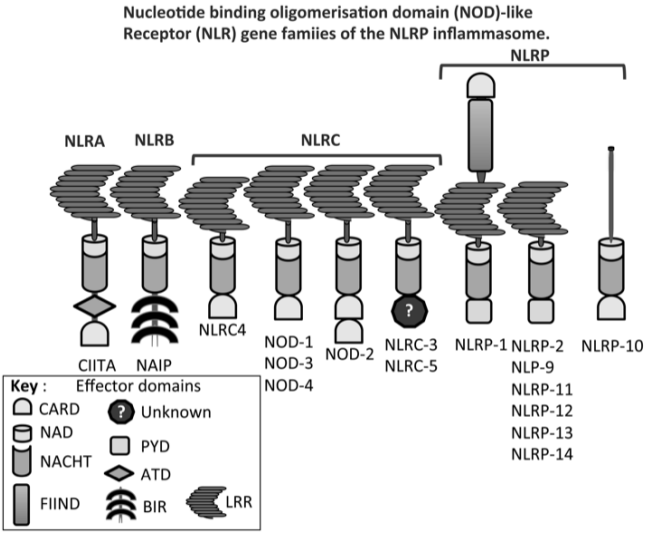


Figure 8. Molecular organisation of the nucleotide binding oligomerisation domain (NOD)-like receptor (NLR) cytosolic pattern recognition receptor family which detect peptidoglycan. All members except NLRP-10 have leucine rich repeat (LRR) domains which have roles in ligand binding and variable arrangements of effector domain motifs which facilitate cell signalling. Key to symbols and abbreviations used: CARD, Caspase recruitment domain; NAD-associated domain; FIIND, Function to Find domain; PYD, Pyrin domain; ATD, Acid transactivation domain; BIR, *Baculovirus* IAP repeat. LRR, leucine rich repeat domain. CIITA, MHC Class II transcription activator. NAIP, NLR family, apoptosis inhibitory protein. Modified from [117, 118, 270].

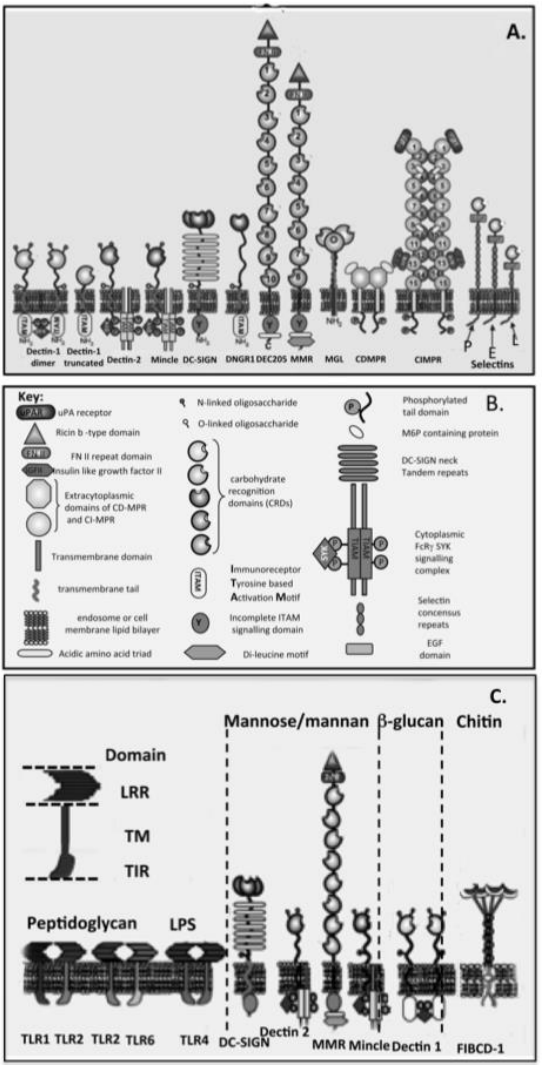


Figure 9. Schematic depiction of the biodiversity of cell membrane bound pattern recognition receptors (A) which have roles in immune surveyance of pathogenic organisms through the glycan motifs they identify as part of the molecular signature of such organisms. Key of Symbols (B).

Abbreviations not covered in the key, DC-SIGN, dendritic cell specific ICAM3 grabbing non integrin; DNGR, Dendritic cell NK lectin group receptor-1; DEC 205, homologous to MMR; MMR, macrophage mannose receptor; MGL, macrophage galactose lectin; CD-MPR; cation dependent mannose-6-phosphate receptor; CI-MPR, cation independent mannose 6 phosphate receptor.

C. Pattern recognition receptors which recognise Fungal cell wall components.

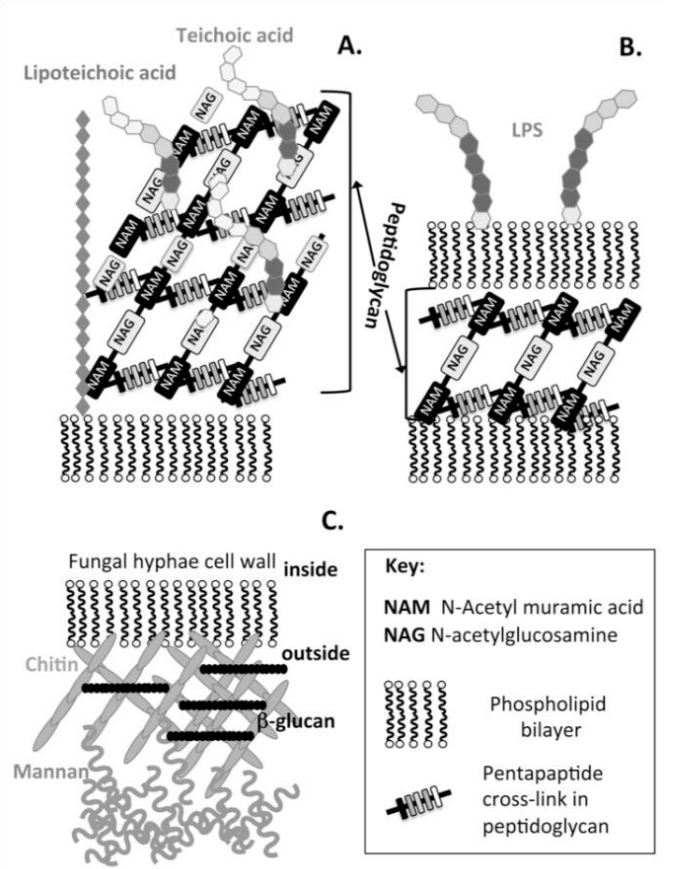


Figure 10. Cell surface pattern recognition components of gram +ve (A) and gram -ve bacteria (B) and fungi. (C). The Toll like receptors 1, 2, 4 and 6 detect peptidoglycan and lipoteichoic acid from Gram +ve bacteria and lipopolysaccharide (LPS) from Gram -ve bacterial cell walls. DC-SIGN (Dendritic cell specific ICAM-3 grabbing non-integrin) binds to high mannose and fucose containing glycans in bacterial cell walls and identifies many viruses through high mannose glycans in their cell envelopes eg gp120 of HIV-1 also detects, HCV, Dengue, CMV, Measles, Ebola as well as *Leishmania* sp, *M. tuberculosis*, *M. leprae* and *C. albicans*. Dectin-2 binds high mannose residues and is considered an α-mannan receptor. MMR (macrophage mannose receptor) is expressed by macrophages and immature dendritic cells, it contains 8 CRDs which bind terminal mannose, N-acetyl glucosamine and fucose residues in glycan components. Mincle binds Fungal α-mannose and mycobacterial glycolipids. Dectin-1 is a fungal β-glucan receptor. FIBCD1 (fibrinogen C domain containing 1) is a specific chitin receptor. Chitin is layed down as rods in the fungal cell wall adjacent to the cell wall plasma membrane. The chitin provides tensile strength to the fungal cell wall and is further stabilised by β-glucan cross-links. Mannan is also assembled in the outermost regions of the fungal hyphal cell wall.

Bacterial and Fungal Cell Wall Components Identified by Pattern Recognition Receptors

Peptidoglycan is a polymer composed of the repeating monosaccharides N-acetyl muramic acid and GlcNAc arranged in linear arrays crosslinked by pentapaptide arrangements of *D* and *L*-amino acid, lipoteichoic acid and teichoic acid are also found attached to peptidoglycan in the Gram +ve bacterial cell wall. Peptidoglycan is a major component of Gram +ve bacterial cell walls but has a lower content in Gram -ve bacterial cell walls (Figure 10A, B, C). Peptidoglycan is attached to the bacterial cell membrane, in Gram +ve bacteria there is a single cell membrane, in Gram -ve bacteria there is an additional outer cell membrane to which is attached lipopolysaccharide (LPS). LPS has a peptide core to which are attached O-antigen monosaccharide (Mannose, Rhamnose, Galactose) externally and a disaccharide diphosphate lipid A fatty acids internally (Figure 10). The fungal cell wall also contains a lipid bilayer cell membrane to which is attached rod-like structural arrangements of insoluble chitin which provides mechanical stability, β-glucan cross-links further stabilise the chitin arrays. Polymeric mannose assembled into a mannan mesh-like arrangements containing some mannose-proteins are found attached to the chitin layer (Figure 10). All of the constituent components of the bacterial and fungal cell walls are foreign to the mammalian glycome and when released upon cell wall fragmentation can ellicit a response from the mammalian pattern recognition receptors and innate immune response [41, 89, 119] (Figure 10).

Interactions of Viruses and Bacterial Pathogens with Host Cell glycans and Glycan Binding Proteins Leading to Host Cell Infection: Advances in Methodologies to Better Understand the Infective Process with a View to Development of Therapeutic Intervention Measures

A large number of viruses and bacterial pathogens bind cell surface sialic acid and glycosaminoglycans (GAGs) during their initial steps in the infection of host cells [110, 120-123]. Glycan binding proteins on the cell membrane of host cells can also act as binding modules. The interaction between lectins and viral glycoproteins may lead to a number of differing responses (i) Lectins such as MBL or SP-A, SP-D function as pattern recognition receptors that bind a number of viruses and in so doing activate anti-viral processes which

form part of the innate immune response which combats infection [124-128]. This includes agglutination activity, activation of the complement cascade and recruitment of macrophages and dendritic cells which inactivate the viruses. (ii) Other lectins such as DC-SIGN or the MPRs present recruitment motifs which identify components of viral cell envelopes leading to attachment of viral particles to the cell membrane enhancing viral entry [103, 105-107, 129-133]. (iii) Some intracellular receptors such as Calnexin or ERGIC-53 may interact with viral encoded proteins and facilitate viral replication or assembly in the ER. Furthermore, some lectins may promote the inhibition of viral infection with one virus but may actually facilitate infection with another eg galectin-1 interacts with NiV and IAV reducing their infectivity however it also enhances gp120-CD4 interactions and facilitates HIV entry into the host cells (Table 5). A better understanding of this infective process is mandatory in order to combat such encounters which are becoming ever more prominent and problematic in modern times. The complexity of the viral interactive glycan structure is compounded by the ability of this virus to alter such glycan presentations in a rapid time-scale and herein lies the difficulty in combatting such infections either with the use of appropriate medications or by the native immune system. Of the 4 major "life" molecules-proteins, carbohydrates, lipids and nucleic acids, carbohydrates (glycans) are the least well understood. Our knowledge of the roles of these glycans in these infective processes is incomplete but is rapidly improving. Armed with this information it may be possible to combat such infections more effectively in the future.

Glycans are difficult to work with since unlike nucleic acids and proteins, they cannot be cloned and sequenced and the wide range of commercial biological tools required in their analysis is not as extensively available as they are in nucleic acid and protein analysis. Thus, until recently, compared to other fields, information about glycan 3D structure and conformational dynamic correlative structure-function information has lagged behind. Advances in the analysis of viral structure by new generation NMR spectroscopy [134, 135], optical reconstruction super high resolution microscopy [136-140] and development of glycan microarray technology [141-143] has improved our understanding of the infective properties of viruses and bacterial pathogens. To gain entry into a host, viruses and bacterial pathogens must first attach to one or several cell-surface receptors. Many viruses also attach to cell-surface GAGs to initiate this infective process, [120, 122, 134, 135, 144, 145]. HS, and DS can both facilitate attachment of viruses and pathogenic bacteria to host cells. In a few cases the actual proteoglycan facilitating binding has been identified. This includes members of the syndecan and glypican HS-

proteoglycan families. *Borrelia burgdorferi* the Lyme disease spirochaete binds to the small leucine repeat proteoglycan decorin to infect the host cell [146]. Host cell glycan binding lectin receptors have varied roles in the identification of pathogens as part of the innate immune response but these can also provide docking modules for the pathogen leading to infection of the host cell (Table 4). DC-SIGN is one such cell membrane bound glycan binding pattern recognition receptor which is utilised by a number of viruses and pathogenic bacteria to gain access to host cells and work is now focussing on agonists to delay or prevent this interaction to counter host cell infection.

A number of pathogenic bacteria bind to cell surface HS/DS as part of the infection process including *Bordotella pertussis, Chlamidia trochomatis, Neisseria gonorrhoea, Mycobacterium tuberculosis* [108]; *Listeria monocytogenes* [147]; *Pseudomonas aeruginosa, Enterococci faecalis* [148]; *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus hemolyticus* [109]; *Streptococcus pyogenes* [149]; *Chlamidia pneumonia* [150]. Many viruses also require to bind to the cell surface of the host cell as an initial step of the infective process and HS is a common binding motif which is utilised, including Parainfluenza type 3 [151], Herpes Simplex [134, 144], Denge Fever Virus Type 2, Yellow fever Virus [152]; Papilloma virus Type 11[153]; Hepatitis B [154], Japanese Encephalitis Virus, Murray Valley Encephalitis Virus [155]; Rous Sarcoma Virus [156], Ebola haemorrhagic Filovirus, Marburg Filovirus [157]. Glycan binding proteins on the cell surface can both combat and enhance viral infection (Table 4) [123, 158].

An impressive number of virus-glycan structures have been determined, however relatively little is known about the affinity between a virus and its cognate receptor or the densities and distributions of interactive glycans on cell surfaces of the host cells. Development of TRICEPS immunization technology now allows identification of these cell surface receptors and their ligands on living cells [159, 160] and quantitation of the interactive process by mass spectrometry [159]. Advances in the biosynthesis of GAG oligosaccharides [161-164] and GAG microarray technology [39, 165-168], and NMR spectroscopy [134, 169] have also helped to define at the atomic level the structure of virus-glycan interactions. X-ray crystallography has further led to a greater understanding of how viruses bind to glycans providing essential information of value in combatting viral infection and in the formulation of therapeutic treatment measures. DARPins (designed ankyrin repeat proteins) are one such measure which have been developed into a robust

Table 5. Glycan binding proteins can both combat and enhance viral infection, data modified from [158]

Lectin Family	Lectin	Virus	↓ ↑ enhances infection	References
	MBL	HIV	↓	[240]
		HBV	↓	[241, 242]
		HCV	↓	[243]
		WNV	↓	[244]
		DENV	↓	[130]
		Ebola	↓	[130]
		MARV	↓	[130]
C-type lectin	SP-A	IAV	↓	[177]
		RSV	↓	[124, 174]
	SP-D	IAV	↓	[175, 177, 179, 245-247]
		RSV	↓	[175]
	DC-SIGN	HIV	↑	[103]
		CMV	↑	[106]
		DENV	↑	[105, 106]
		WNV	↑	[105, 106]
		MARV	↑	[107, 130, 248]
		HCV	↑	[107]
		Ebola	↑	[132, 196, 248]
		SARS	↑	[132, 248, 249]
Galectins	Galectin-1	NiV	↓	[250]
		IAV	↓	[251]
		HIV	↑	[252-255]
	Galectin-3	HSV	↑	[256]
		MVM	↑	[257]
Calnexins	CRT/CNX	UUKV	↑	[258]
		SARS	↑	[259]
		HBV	↑	[260]
		HIV	↑	[261, 262]
		Rotavirus	↑	[263-265]
		Hantavirus	↑	[266]
P-type lectins	CI-MPR	HSV	↑	[267]
	CD-MPR	VZV	↑	[268]
L-type lectins	ERGIC-53	Arenavirus,	↑	
		Hantavirus,	↑	
		Coronavirus,	↑	[230]
		Filovirus,	↑	
		Orthomyxovirus	↑	

Abbreviations: MBL, mannose binding lectin; SP-A, D, surfactant proteins A, D; DC-SIGN, dendritic cell specific ICAM-3 grabbing non-integrin; CRT, calreticulin; CNX, Calnexin; CI-MPR, Cation independent mannose-6-phosphate receptor; CD-MPR, Cation dependent mannose-6-phosphate receptor; ERGIC-53, endoplasmic reticulum Golgi intermediate compartment glycan receptor; HIV, human immunodeficiency virus; HBV, Hepatitis B virus: HCV, Hepatitis C virus: WNV, West Nile virus; DENV, Dengue virus; MARV, Marburg virus; IAV, influenza A virus; RSV, Respiratory synctial virus; CMV, cytomegalo virus; SARS, severe acute respiratory syndrome coronavirus; NiV, Nipah virus; UUKV, Uukuniemi virus; SeV, Sendai virus; VZV, Varicella zoster virus.

and versatile scaffold for binding proteins [170] and used in tumour and viral targeting and quantitative immunohistochemistry [171]. The lectins Griffthsin (GRFT) isolated from the red algae *Griffithsia sp* of coastal waters in New Zealand, Cyanovirin-N (CV-N) from the blue-green algae *Nostoc ellipsosporum* and Scytovirin (SVN) from *Scytonema varium* have been shown to inhibit HIV entry in-vitro by binding to multiple mannose rich glycans on the HIV-1 viral envelope preventing the establishment of productive associations with host cells and resultant HIV-1 infection [172]. Recombinant GRFT has been produced in the tobacco-like plant *Nicotiana benthamiana* [173]. GRFT, CV-N and SVN await testing in clinical trials and the specific glycan structures these lectins bind to await full characterisation however initial findings warrant further studues. Furthermore, these compounds can be readily produced and are inexpensive. Commensal *lactobacilli* can also be engineered to produce these compounds in-situ to establish an environment in the vaginal mucosa hostile to HIV-1. It is to be hoped that such studies will provide effective means of inhibiting the attachment of specific viruses and pathogens to host cells to prevent the early stages of infection to provide effective therapeutic interventions. Viral infections are becoming increasingly prevalent and improved treatments still need to be developed in most cases.

CONCLUSION

1. The biodiversity of glycan structural form represents an important repository of recognition information in strategic molecules which regulate many essential physiological processes. This may explain why glycans are such ancient molecules and their structure are conserved throughout vertebrate and invertebrate evolution.
2. A greater understanding of how effector molecules interact with cell surface glycans in terms of quantitative responses at the atomic level will facilitate how best to promote such responses in repair biology or prevent such interactions in adverse infection based interactions involving viruses and pathogenic bacteria.
3. With the improved technology which is now coming on-line, deciphering the “glyco-code” is becoming a technical reality and with time this information should become available to manipulate cells in-situ and improve repair biology by acting as chemoattractants for progenitor cells to defect sites, improved development of specific activated stem cell

lineages for repair, or regulation of cellular proliferation, differentiation and matrix synthesis. Prevention of viral infections is also a high research priority.

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BIOGRAPHICAL SKETCH

Dr. James Melrose

Dr. Melrose undertook his PhD studies at Heriot Watt University, Edinburgh, Scotland where he identified the fungal microflora of a number of cereals and characterized their glycosidase degradative enzyme systems of relevance in the brewing process and identified *Aspergillus fumigatus* as a dominant spoilage organism during malt production. He also characterized some capsular fungal polysaccharides during their infective stages of cereal germination. In 1983 Dr Melrose migrated to Australia and re-trained in musculoskeletal disorders. He is currently Honorary Senior Research Associate, Raymond Purves Bone and Joint Laboratory, Kolling Institute of Medical Research, of The University of Sydney at Royal North Shore Hospital, St. Leonards, NSW 2065, Australia and a member of Sydney Medical School, Northern. He is also Adjunct Professor, Graduate School of Biomedical Engineering, Faculty of Engineering, University of New South

Wales, Kensington 2052, Australia. His major research interests currently centre around tensional and weight bearing musculoskeletal tissues including intervertebral disc, articular cartilage, fibrocartilaginous meniscus, and tendon and the matrix glycan bearing molecules which convey tissue function. He has extensively investigated these matrix components and evaluated their functional properties in health and disease over the last 35 years. He has also developed skills to isolate cells from these tissues and appropriate methods for their long-term culture maintaining a normal cellular phenotype and has developed biochemical and immunological methodology to examine connective tissue cellular metabolism in health and disease. The candidate also has 25+ years experience of animal models used to initiate degeneration mimicking human clinical conditions in meniscus, articular cartilage, tendon and intervertebral disc. Most recently he has been using adult stem cells in an experimental model of intervertebral disc degeneration to develop therapeutic methods for its treatment. He has been Project leader on seven NHMRC (Australia) funded Project Grants from 1999-2013 and eight externally funded projects. Prof Melrose has published 12 book chapters 117 peer reviewed research papers and has 10 additional papers currently submitted for publication and under review. He has been Associate Editor European Spine Journal since 2007 is an editorial board member of Current Proteomics and Open Orthopaedics journal and also reviews for 25 scientific journals and 10 National and International Granting Agencies. He has supervised 3 PhD candidates to successful completion of candidature, examined 9 PhD theses. His future work on glycans is aimed at understanding the intricacies of these molecules as information modules and how these direct cellular metabolism in connective tissue development and in health and disease processes.

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Chapter 6

THE EFFECTS OF DEHYDROEPIANDROSTERONE (DHEA) ON DIABETES MELLITUS, OBESITY, AND ATHEROSCLEROSIS

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ABSTRACT

Dehydroepiandrosterone (DHEA) and its sulfated ester (DHEA-S) are the most abundant adrenal steroids in human blood. Peak levels of DHEA and DHEA-S occur around the age of twenty and decrease gradually thereafter. DHEA has been reported to have beneficial effects on diabetes mellitus, obesity, and atherosclerosis. Meta-analysis of DHEA supplementation to elderly men or women showed no effects on blood glucose and total cholesterol levels. However, DHEA supplementation to patients with type 2 diabetes has not been fully elucidated. Type 2 diabetes is characterized by an impaired capacity to secrete insulin, insulin resistance, or both. It has been reported in animal models that DHEA and DHEA-S increase not only insulin secretion of the pancreas but also insulin sensitivity of the liver, adipose tissue and muscle. We investigated the effect of DHEA on glucose metabolism in animal models and reported that DHEA decreased liver gluconeogenesis.

Recently, we reported the effect of DHEA on liver and muscle by using insulin receptor substrate 1 and 2 (IRS1 and IRS2)-deficient mice. DHEA increased insulin-stimulated Akt phosphorylation in the liver of C57BL6 IRS1- and IRS2-deficient mice fed a high fat diet, suggesting that the increase in Akt signaling induced by DHEA is sufficient in the presence of IRS1 or IRS2. Here, we introduce the effect of DHEA on the liver, muscle, adipose tissue, and insulin secretion in diabetes mellitus and its effect on obesity and atherosclerosis in human and animal models.

INTRODUCTION

Dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEA-S) are secreted from the adrenal cortex and they are the most abundant adrenal steroids. DHEA is synthesized from pregnenolone and is further metabolized to androstenedione, testosterone, and estrogens. In humans, their levels peak at the age of 20 and decrease gradually with age. Interestingly, in the Baltimore Longitudinal Study of Aging, longevity was associated with high DHEA-S concentration [1]. However, the physiological role of the decline of DHEA concentration after the twenties is not fully elucidated. DHEA has also been reported to have beneficial effects in diseases such as diabetes mellitus, atherosclerosis, malignancy, osteoporosis, and collagen disease [2–7]. In this chapter, we present our data as we discuss the effect of DHEA on diabetes, obesity, and atherosclerosis.

EFFECTS OF DHEA ON DIABETES MELLITUS

1) Human Studies

In 1964, it was reported that urine DHEA levels were low or not detected in obese patients with diabetes [8]. Patients with type 2 diabetes whose serum insulin levels were high had significantly lower DHEA and DHEA-S serum levels than normal subjects and patients with type 2 diabetes whose serum insulin levels were not high [9]. It is known that polycystic ovary syndrome (PCOS) patients often have hyperinsulinemia and insulin resistance. Buffington et al. reported that this insulin sensitivity was associated with the ratio of DHEA to testosterone in obese PCOS women with high testosterone levels, and in obese patients suffering from adult onset adrenal hyperplasia

with high DHEA concentration [10]. Thus, DHEA or DHEA/testosterone is associated with obesity and obesity with diabetes.

In the meta-analysis of DHEA supplementation in elderly men, DHEA supplementation decreased fat mass [11]. However, DHEA had no effects on total cholesterol (TC), glycaemia, and insulin, compared to a placebo group. This effect is dependent on DHEA conversion into its metabolites such as androgens or estrogens. In the meta-analysis of DHEA supplementation in postmenopausal women, DHEA had no effects on TC, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL), triglycerides, serum glucose, weight, body mass index, or bone mineral density [12].

The change of insulin sensitivity by DHEA supplementation varies between reports. In a randomized controlled trial (RCT) of men without diabetes, the administration of DHEA (25 mg/day for 12 weeks) did not decrease plasma glucose; however, steady state plasma glucose using octreotide acetate was decreased by DHEA [13]. This result indicates that DHEA supplementation improved insulin sensitivity. Another report states that 50 mg/day of DHEA supplementation in elder subjects did not change the area under the curve (AUC) of glucose, as measured by an oral glucose tolerance test (OGTT), and decreased the AUC of insulin by OGTT after 6 months [14]. However, a different report states that 50 mg/day of DHEA supplementation for elder subjects with low serum DHEA-S concentrations did not change AUC of glucose and insulin by OGTT after 12 months [15].

In 10 patients with type 2 diabetes whose mean BMI was 25, DHEA supplementation (50 mg/day for 12 weeks) did not change plasma glucose, HbA1c, HOMA index, or BMI [16]. However, DHEA supplementation for patients with type 2 diabetes has not been completely elucidated. These contrasting reports highlight the need for further studies of patients with type 2 diabetes.

2) Animal Studies

Type 2 diabetes is characterized by an impaired capacity to secrete insulin, insulin resistance, or both. Several studies have been conducted utilizing db/db mice. These mice become obese, hyperglycemic, and hyperinsulinemic, and are used to represent type 2 diabetes. In 1982, Coleman et al. reported that administration of DHEA to db/db mice improved hyperglycemia, increased insulin sensitivity by OGTT, and preserved β-cell structure and function [2].

As shown in Figure 1, other reports also indicated that DHEA and DHEA-S increase not only insulin secretion but also insulin sensitivity in animal models [17–21]. Next, we describe the effects of DHEA on the liver, muscle, adipose tissue, and insulin secretion.

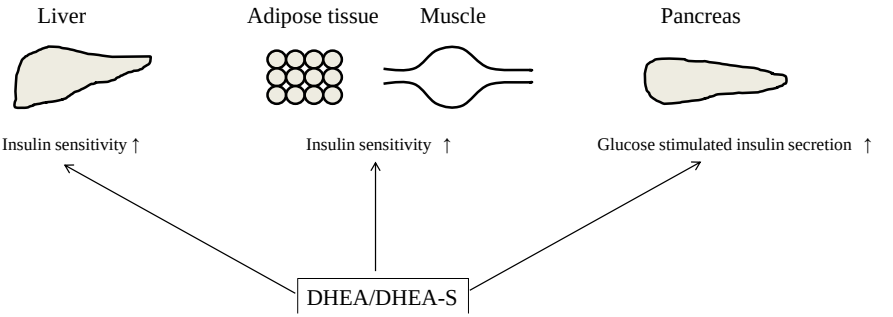

Figure 1. Effect of DHEA/DHEA-S on diabetes mellitus in animal models.

A) Effect of DHEA on Liver

McIntosh et al. reported that DHEA administration decreases hepatic glucose production in isolated hepatocytes from prediabetic male BHE/cdb rats [22]. Since no reports have investigated the anti-diabetic effect of DHEA in db/db mice since Coleman et al. reported this effect, we evaluated the effect of DHEA on glucose metabolism in the liver and muscle of db/db mice in 1999. We reported that administration of DHEA suppresses the increased activity of the hepatic gluconeogenic enzyme glucose-6-phosphatase (G6Pase) in db/db mice [23]. We also reported that administration of DHEA suppresses the increased mRNA expression of the hepatic G6Pase and hepatic glucose production in db/db mice, compared to in heterogeneous db/+m mice [24, 25]. Additionally, we reported that DHEA suppresses the activity, protein expression, and gene expression of G6Pase and enhances 2-deoxyglucose uptake in HepG2 cells [26]. Therefore, administration of DHEA is considered to decrease gluconeogenesis in the liver.

Our investigation also looked at insulin signaling in the liver using phosphoinositide 3 kinase (PI3K) p85 $\alpha^{-/-}$ mice [27]. Jacob et al. reported that DHEA administration increases pAkt/Akt in the liver of rats [28]. Campell et al. reported that the PI3K/Akt pathway is activated in the liver and that DHEA increases the tyrosine phosphorylation of insulin-induced insulin receptor substrates 1 and 2 (IRS1 and IRS2) in the liver of Wistar rats [29]. We also investigated the changes in Akt phosphorylation in the liver and muscle, and PKCζ in the muscle using C57BL6, IRS1$^{-/-}$, and IRS2$^{-/-}$ mice fed a high-fat

diet (HFD) [30]. The administration of DHEA increased Akt phosphorylation in the liver of C57BL6, IRS1$^{-/-}$, and IRS2$^{-/-}$ mice fed a HFD, suggesting that the increase in Akt signaling induced by DHEA is sufficient in the presence of IRS1 or IRS2. Previously, we had reported that DHEA suppressed the activity and mRNA expression of G6Pase in the liver [24–26]. As the deletion of Akt2 increases gluconeogenesis [31], the administration of DHEA may suppress gluconeogenesis by increasing Akt phosphorylation. Similarly, Kang et al. reported the effect of DHEA on the liver in rats treated with HFD. The administration of DHEA increased hepatic PI3K and Akt mRNA expressions, hepatic glycolytic enzyme PFK-2 activity, and hepatic glycogen content. According to them, DHEA may activate the PI3K/Akt-PFK-2 signal pathway [32].

B) Effect of DHEA on Muscle and Adipose Tissue

Our group previously reported that DHEA ameliorates insulin sensitivity in older rats by using the glucose clump test [33]. Kimura et al. also showed, by using the glucose clump test, that the administration of DHEA to obese Zucker fatty rats increased insulin sensitivity and that this effect was independent of secondary weight reduction by DHEA [34].

We also reported that DHEA increased the glycolytic enzyme hexokinase (HK)+glucokinase (GK) and phosphofructokinase (PFK) activities in the muscle of db/db mice [23]. Regarding insulin signaling of animal models, it is reported that an acute treatment with DHEA relieved the impaired activation of Akt and the protein kinase C ζ/λ (PKCζ/λ)-GLUT4 pathway in the skeletal muscle of streptozotocin-induced diabetic rats [35]. DHEA activated HK and PFK activity, phosphorylation of Akt and PKC ζ/λ and GLUT4 protein expression in cultured skeletal muscle cells from SD rats [36]. Jahn et al. reported that DHEA administration for 5 weeks decreases Akt levels in the muscle of Wistar rats, but does not influence Akt phosphorylation in the muscle of streptozotocin-induced diabetic rats [37]. Campbell et al. reported that administering DHEA for a week increases PKCζ/λ phosphorylation in the muscle, though it does not increase the activity of the Akt phosphorylation, and increases the insulin-induced tyrosine phosphorylation of only IRS1 in the muscle of rats [30]. In our study, the administration of DHEA did not increase Akt and PKCζ phosphorylation in the muscle of C57BL6 IRS1$^{-/-}$, and IRS2$^{-/-}$ mice fed with high fat diets. We also found that PKCζ phosphorylation was reduced in IRS1$^{-/-}$ mice but not in IRS2$^{-/-}$ mice. Therefore, the role of IRS2 in the muscle might be less important for the effect of DHEA on glucose

metabolism. However, further studies needed to be performed to confirm this finding.

In regards to adipose tissue, DHEA influences the insulin signaling pathway by increasing tyrosine phosphorylation of IRS1 and IRS2, and stimulates IRS1 and IRS2-associated PI3K activity in 3T3-L1 adipocytes, but has no influence on either insulin receptor or Akt phosphorylation [38]. Activation of PI3K and PKCζ in adipocytes was significantly elevated in DHEA-treated Otsuka Long-Evans Tokushima Fatty (OLETF) rats [39].

C) Effect of DHEA on Insulin Secretion

DHEA is reported to enhance glucose stimulated insulin secretion when administered *in vivo* to rats or *in vitro* to β cell lines [17]. Pancreatic islets from DHEA-treated rats showed an increased β cell mass accompanied by increased Akt1 protein level but reduced insulin receptor (IR), IRS1, IRS2 levels, and enhanced glucose-stimulated insulin secretion [40]. Indeed, the plasma insulin levels were increased in DHEA-treated db/db mice compared to in control db/db mice in our previous report [23]. Thus, DHEA has beneficial effects on the β cell function in animal models.

EFFECTS OF DHEA ON OBESITY

As described above, DHEA supplementation decreased the fat mass in elder men in a meta-analysis [11]. DHEA decreases the body weight-gain in pair-fed obese rats [34]. We also found that DHEA administration decreased the body weight-gain in C57BL6 IRS1$^{-/-}$, and IRS2$^{-/-}$ mice. The anti-obesity effects of DHEA may be mediated by futile substrate cycling in hepatocytes, as reported previously [22].

Ashida et al. identified that DHEA induced dual specificity protein (DDSP) and that this protein regulated 38 mitogen-activated protein kinases (MAPKs) [41]. Indeed, using DDSP-Tg mice showed that DDSP prevents diet-induced obesity and genetic obesity (db/db mice); the anti-obesity effect of DHEA may be induced by DDSP [42].

EFFECT OF DHEA ON ATHEROSCLEROSIS

In the meta-analysis of DHEA supplementation in elderly men and women, no significant effect of DHEA on serum TC was detected [11].

However, in the RCT, Kawano et al. also reported that DHEA supplementation improved flow-mediated vasodilation and decreased plasma PAI-1 levels [13]. Weiss et al. reported the results of 50 mg/day of DHEA supplementation for a year [43]; their results showed a decrease in the carotid augmentation index and the pulse wave velocity of the DHEA-treated group. Yamakawa et al. reported the effect of DHEA on atherosclerosis in apolipoprotein E-deficient mice [4]. Although the plasma cholesterol and triglyceride levels were not decreased by DHEA, atherosclerotic lesions in the aortic sinus showed a reduction in areas with DHEA treatment versus those in control mice. The inhibition of macrophage infiltration was observed in DHEA-treated mice.

IS THE EFFECT OF DHEA EXERTED BY DHEA OR ITS METABOLITES?

DHEA is synthesized from pregnenolone and is further metabolized to androstenedione, testosterone, and estrogens. Therefore, the action of DHEA is considered an effect of DHEA itself or of its metabolites. The receptor of DHEA itself is not known. As described above, the effect of DHEA on reduction of fat mass is considered to be due to its metabolites [11]. Dihydrotestosterone (DHT) converted from DHEA may increase phosphorylation of Akt and PKC ζ/λ in cultured skeletal muscle cells from SD rats, using DHT inhibitor [36].

To determine whether DHEA or its metabolites are responsible for improving hyperglycemia, we administered androstenedione, which is considered to be metabolized from DHEA *in vivo*. Androstenedione did not increase Akt phosphorylation in the liver of C57BL6 mice, suggesting that the increase in Akt signaling in the liver was due to DHEA or DHEA-S [30]. In our previous report, the administration of androstenedione to db/db mice lowered blood glucose slightly as compared to the administration of DHEA. This also suggests that the hypoglycemic effect of DHEA is due to DHEA or DHEA-S [23]. Therefore, DHEA may exert its effect by both DHEA/DHEA-S itself and its metabolites.

CONCLUSION

We described the effect of DHEA on diabetes, obesity, and atherosclerosis in human and animal models. In the meta-analysis of DHEA supplementation in elder men, DHEA showed an effect of fat mass reduction. In the meta-analysis of DHEA supplementation in elder women, DHEA showed no effect on metabolic markers. However, few reports have investigated DHEA supplementation in type 2 diabetes. Further studies are needed for investigating the effect of DHEA on diabetes, obesity, and atherosclerosis in human and animal models, including whether DHEA itself or its metabolites produce these effects.

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Chapter 7

JUVENILE IDIOPATHIC ARTHRITIS: NEW HOPES FOR AN OLD MALADY

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ABSTRACT

Juvenile idiopathic arthritis (JIA), a term referring to a group of disorders characterized by chronic arthritis, is the most common chronic rheumatic illness in children and is a significant cause of short- and long-term disability. There has been a lot of development in the understanding of the disease pathogenesis and etiology in the last decade. Recently, the introduction of biological agents in the management of JIA has changed the approach and outcome in these children. This article discusses the various classifications, clinical features, diagnosis, and management options for JIA.

Keywords: juvenile idiopathic arthritis, children, review

INTRODUCTION

Juvenile idiopathic arthritis, juvenile chronic arthritis and juvenile rheumatoid arthritis are terms used synonymously to describe a heterogeneous group of disorders characterized by chronic synovitis in children. Juvenile

idiopathic arthritis (JIA) is the most common paediatric rheumatic disease with significant long term morbidity and mortality [1]. Collectively, any arthritis in a child less than 16 years of age of more than 6 weeks duration should be considered as chronic arthritis. JIA is not a single disease rather, it is a group of disorders of unknown etiology, which are manifested by chronic joint inflammation [2].

EPIDEMIOLOGY AND PREVALENCE

The true incidence of JIA is not known. Overall prevalence of JIA is estimated to be from 30 - 150 per 100,000 children. In the United States and Canada there are an estimated 30,000 to 60,000 children and adolescents with juvenile idiopathic arthritis. There is limited local data available about the disease prevalence [3].

ETIOLOGY AND PATHOGENESIS

The exact etiology of JIA remains uncertain but it is believed that the etiology of JIA is multi-factorial. The absence of a full understanding of the disease pathogenesis for JIA hinders the development of truly effective treatment approaches. Substantial evidence suggests that the disease involves both autoimmune and genetic factors in varying combinations. It assumes that T lymphocytes have an essential role in releasing pro-inflammatory cytokines like interleukin [IL]-6, IL-1 and tumor necrosis factor–alpha [TNF-α]. A disordered interaction between type 1 and type 2 T-helper cells has also been postulated [4]. There is evidence for irregularities in the humoral immune system including the presence of antinuclear antibodies and other auto-antibodies, elevated levels of serum immunoglobulins in active disease and the presence of circulating immune complexes in patients with JIA [5]. The exact events which trigger or propagate an arthritic disease have yet to be defined.

PATHOLOGY

JIA is considered a chronic, non-suppurative inflammation of the synovium that is characterized by infiltration of B-lymphocyte and invasion of

macrophages and T-cell that is associated with the release of cytokines, which induces synovial proliferation. Affected synovial tissues are edematous, hyperemic and infiltrated with lymphocytes and plasma cells. Secretion of increased amount of joint fluid results in effusion [6].

CLASSIFICATION

Different classification systems have been evolved across the globe. Until recently American College of Rheumatology's (ACR) classification was the most widely used. Another classification proposed by the European League Against Rheumatism (EULAR) uses the term JCA. This classification is based on characteristics at onset. These have been replaced by a new classification, developed by the International League of Association for Rheumatology (ILAR), which is now acceptable worldwide [7-8]. According to this classification, JIA has been divided into eight different categories. This new classification of JIA is compared with the old JRA and JCA in (Table 1). This article uses the ILAR classification.

Table 1. Comparison of classification criteria for chronic arthritis in childhood

ACR (JIA)	EULAR (JCA)	ILAR (JIA)
Systemic arthritis	Systemic arthritis	Systemic arthritis
Pauciarticular arthritis	Pauciarticular arthritis	Oligoarthritis Persistent Extended
Polyarticular arthritis	Polyarticular arthritis JIA (RF-positive) Spondyloarthropathies	Polyarthritis RF-positive RF-negative Psoriatic arthritis Enthesitis related arthritis Undifferentiated arthritis

CLINICAL MANIFESTATIONS

Polyarticular type of JIA affects around one third of children with JIA. It involves] more than 4 joints and any joint of the body can be affected. The

disease begins at any age during childhood but majority of the cases begin below five years of age. Girls are affected more than boys. Onset of arthritis is insidious with gradual development of joint stiffness, swelling and limitation of movements. Polyarticular JIA is further divided into 2 subgroups based on the presence or absence of rheumatoid factor [9]. Rheumatoid factor negative usually has a benign course. These patients show good response to therapy. Rheumatoid factor positive typically begins in later childhood and is characterized by severe arthritis with frequent appearance of rheumatoid nodules [10]. Response to therapy in these children is guarded and may progress to adult rheumatoid arthritis. Deformities of the joints e.g., spindle, swan neck and boutonniere deformities are more common in severe type of polyarticular JIA (Figure 1).

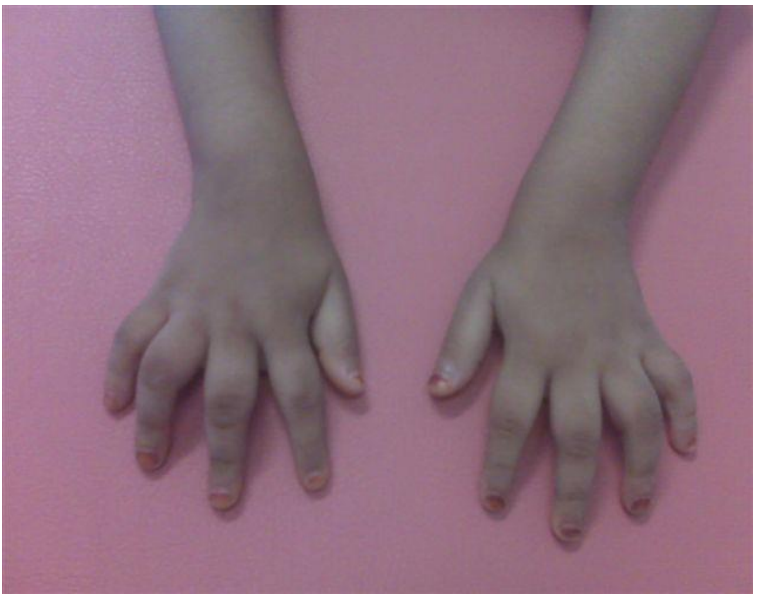

Figure 1. Swelling of the wrist joints and spindle deformities in a child with polyarticular type of JIA.

Oligoarticular type of JIA is the most common type and affects almost half of the cases of JIA. Four or fewer than four joints are involved in the first six months of the disease [2, 9]. Large joints (knee, elbow, ankle and wrist) are involved in majority of the cases (Figure 2). Girls are affected more than boys (4:1). Oligoarticular JIA is further divided into two subgroups that are characterized by the course of the joint disease after the first 6 months:

persistent (affecting no more than four joints throughout the disease) and extended (affecting more than four joints after six month of onset). 40% to 50% of patients have ANA-positive (Anti Nuclear Antibodies). Children with ANA-positive are at the greatest risk of developing uveitis. The children with oligoarticular extended subgroup have a poor outcome and the disease may progress into polyarticular and erosive arthritis.

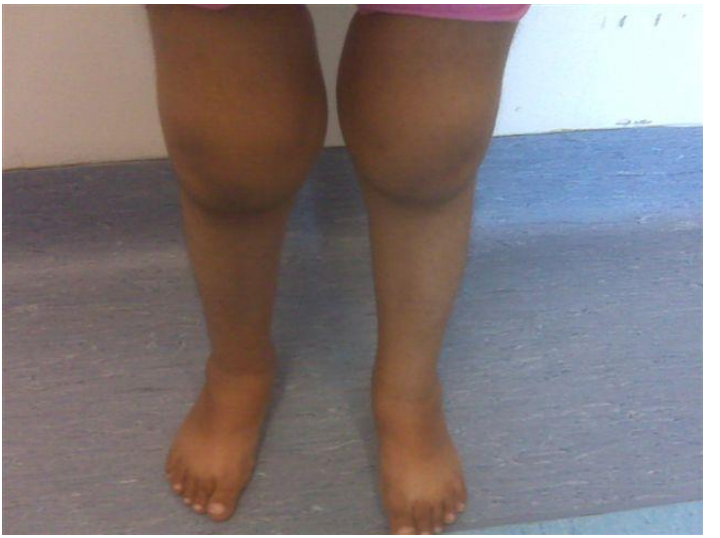

Figure 2. Swelling of the knee joints with synovitis in a child with oligoarticular type of JIA.

Systemic onset JIA is a less frequent type and occurs in 10-15% of all cases of JIA. The disease occurs at any age, with no peak and both genders are equally involved. Patient often presents with high grade fever and classic salmon-pink, evanescent, macular rash [2]. Rashes are transient, blanching and disappear as the fever settles. Other systemic features include generalized lymphadenopathy, hepatosplenomegaly, anemia and myalgia. Serositis including pericardial or pleural effusion can present with shortness of breath in active disease. Arthritis may develop late in the illness with variable joint involvement.

Psoriatic arthritis is a rarer variety of JIA. The child, in addition to psoriasis, will either have arthritis, tenosynovitis, sacroilitis, dactylitis or onycholysis. Most of these children have positive family history of psoriasis or positive for HLA B27 [11]. The arthritis can precede the rash by many years

or vice versa. Children with psoriatic arthritis have chronic course with joint deformities and show poor response to treatment.

Enthesitis-related arthritis, often called spondyloarthropathy, is characterized by inflammation of tendons and ligaments, at the area of insertion into bones (enthesis). This type of arthritis often affects the spine, hips and occurs mainly in older boys and adolescents. Pain, swelling and tenderness at the enthesis are the most common manifestation. In addition to the presence of arthritis or enthesitis children tend to have any two of the following features: inflammatory lumbosacral pain, sacroiliac tenderness, presence of HLA-B27, onset of arthritis in a male 6 years old or older, acute symptomatic anterior uveitis, presence in a first-degree relative of ankylosing spondylitis, enthesitis-related arthritis, inflammatory bowel disease with sacroiliitis or reactive arthritis [12].

Undifferentiated JIA is labeled when patient either clinically does not fulfill any of the above the criteria or fulfills the criteria for more than one category.

DIAGNOSIS

Juvenile Idiopathic Arthritis is a clinical diagnosis and role of laboratory parameters are of little help. However, investigations can help to ascertain the long term outcome and response to the therapy [13]. In 1997, International League of Associations for Rheumatology (ILAR) in Durban developed standard criteria for diagnosis of Juvenile Idiopathic Arthritis, which was further modified in 2001 [7]. According to the criteria, JIA can be diagnosed definitively if there is a presence of objective signs of arthritis (presence of swelling of the joint or two or more of the following: limitation of motion, tenderness, pain with motion, or joint warmth) in at least one joint for more than 6 weeks in a child younger than 16 years of age, after other types of childhood arthritis have been excluded. JIA should be further classified into different types according to following criteria (Table 2).

Blood tests that can support the diagnosis and assist to identify the disease activity include complete blood picture, Erythrocyte sedimentation rate, C reactive proteins and serum ferritin. The elevated level of these acute phase reactants indicates disease activity [14]. A serological test for rheumatoid factor is non-specific and can be raised in many conditions other than JIA as well. Absence of rheumatoid factor (RF) does not exclude the disease but seropositive cases, especially in polyarticular types, are one of the predictive

factors of poor response to the therapy. Similarly, 40-50% of cases of oligoarticular type have positive ANA and are more prone to develop eye complications like uveitis or extended disease [2]. There is no association between the raised titers and disease activity [9].

Table 2. ILAR Criteria for diagnosis of JIA

	ILAR Criteria for diagnosing JIA
Systemic arthritis	Arthritis with/preceded by fever for at least 2 weeks and one/more of: evanescent erythematous rash, generalized lymphadenopathy, hepato/splenomegaly and serositis.
Oligoarthritis • Persistent • Extended	Arthritis of one to four joints during the first 6 months of disease. Affects no more than four joints throughout the disease course. Affects more than four joints after the first 6 months.
Polyarthritis (RF-negative) Polyarthritis (RF-positive)	Affects five or more joints in the first 6 months of disease. Tests for RF are negative. Affects five or more joints in the first 6 months of disease. Tests for RF are positive on two occasions at least 3 months apart.
Enthesitis-related arthritis	Arthritis and/or enthesitis with at least two of the following: i) sacroiliac joint tenderness with or without inflammatory lubosacral pain, ii) human leukocyte antigen (HLA) B27- positive, iii) family of medically confirmed HLA B27-associated disease history in a first degree relative, iv) onset of arthritis in a male over 6 years of age; v) acute (symptomatic) anterior uveitis.
Psoriatic arthritis	Arthritis and psoriasis or arthritis and at least two of: dactylitis, nail pitting, onycholysis, family history of psoriasis in at least one first-degree relative.
Other	Arthritis of unknown cause persisting for at least 6 weeks that either does not fulfill criteria for any categories or fulfills criteria for more than one category.

Modified from Goldmuntz E. A., White P. H. Juvenile Idiopathic Arthritis: A Review for the Pediatrician. Pediatrics in Review 2006; 27; e24.

Radiological examination has little value in the diagnosis of JIA and is mainly required when few joints are involved and there is a high suspicion of septic arthritis. Radiological changes range from soft tissue swelling and narrowing of joint spaces to bony erosion, intra-articular bony ankylosis and joint subluxation in advance cases (Figure 3) [15]. Generalized osteopenia is a

common finding in majority of the patients. Ultrasonography has relatively better modality in detection of cartilage erosions and effusions and also in identifying synovial thickness and effusion volumes. Computed tomography and magnetic resonance can demonstrate better assessment of disease activity by visualization of synovial thickening and cartilaginous erosion [16].

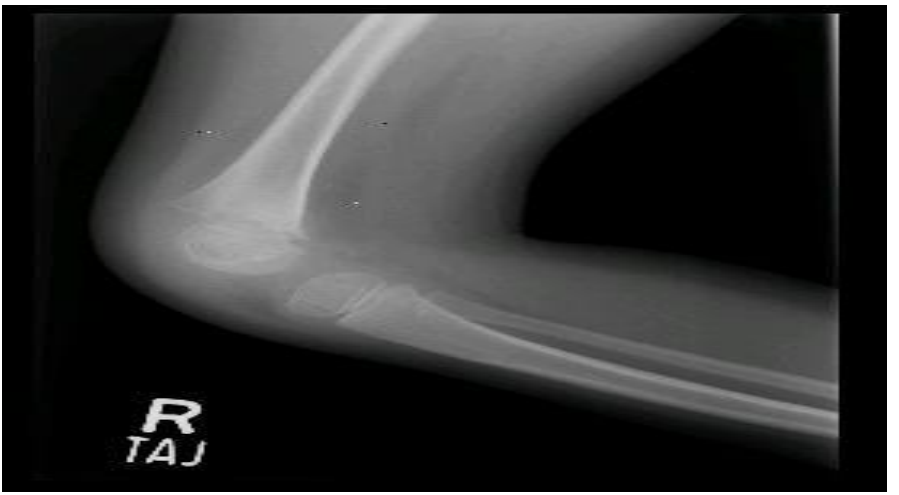

Figure 3. Plain radiograph of elbow joint showing osteopenia and erosion.

DIFFERENTIAL DIAGNOSIS

The clinical picture of JIA mimics many other conditions involving joints. Rheumatic fever and reactive arthritis are sometimes confused with polyarticular type of JIA. Similarly pauci-arthritis is wrongly diagnosed as septic arthritis in rare circumstances. Kawasaki disease, systemic lupus erythematosus, leukemia and pyrexia of unknown origin should be considered in the differential diagnosis of systemic onset JIA.

MANAGEMENT OF JIA

Juvenile idiopathic arthritis is a chronic illness, and there is growing importance given to a holistic approach to care of patients with JIA. A multidisciplinary team of specialized health care workers with specific

expertise in pediatric rheumatology, trained nurse and physiotherapist and occupational therapist, nutritionist and clinical pharmacist has become essential to manage complex childhood rheumatic disease [17].

The treatment should address all areas of normal growth, social development, physical functioning as well as pharmacological treatment to limit chronic joint pain, inflammation, and damage. The goals of any treatment program for juvenile arthritis are to

i. Control inflammation
ii. Relieve pain
iii. Prevent joint damage
iv. Maximize functional abilities

With the understanding that JIA has a potential for significant morbidity, the pharmacologic treatment of the articular and extra-articular manifestations should become more aggressive and initiated earlier in the course of disease for a good outcome [18].

Non-steroidal anti-inflammatory drugs (NSAIDs) traditionally have been the preferred first line drugs for relief of joint symptoms. Most NSAIDs inhibit both the constitutive form of cyclo-oxygenase (COX-1) as well as the inducible form of enzyme (COX-2) that are involved in the inflammatory response [2]. Only ibuprofen, naproxen, indomethacin, diclofenac sodium have been approved by the Food and Drug Administration (FDA) for use in children. Aspirin is no longer the first choice for treatment of JIA due to the increased toxicity and risk of development of Reye syndrome. In cases of mild arthritis, NSAID therapy alone may be sufficient. The average time to symptomatic improvement with a particular NSAID is four weeks, but up to 25% of children do not demonstrate clinical improvement until 8 to 12 weeks of treatment. Most children tolerate NSAIDs well, compared to adults [19]. The most common adverse effects are abdominal pain, rash, headache, dizziness, and anorexia. Taking NSAIDs with antacids, histamine-2 blockers, or proton pump inhibitors can minimize the gastrointestinal toxicity.

DISEASE MODIFYING ANTIRHEUMATIC DRUGS

A second line drug, often referred to as a disease modifying anti-rheumatic drug (DMARD), should be started as soon as it is apparent that NSAIDs alone are not controlling the joint inflammation. Further, chronic

joint changes may occur if arthritis remains active while waiting for benefits from NSAIDs [20]. Thus, the initiation of DMARDs, including immunomodulating drugs, biologic, and anti-inflammatory agents should not be delayed excessively.

Methotrexate — Of the DMARDs, methotrexate has been the standard therapy for children with polyarticular disease, extended oligoarticular and uncontrolled systemic onset disease. Methotrexate is the most widely prescribed DMARD for JIA and has been used for more than 10 years, resulting in better clinical outcomes. The clinical response to methotrexate may not be apparent for 4 to 6 weeks after therapy has been initiated and may not be maximal until 3 to 6 months of treatment. Potential side effects include leukopenia or thrombocytopenia, stomatitis, alopecia, rash, elevation of hepatic transaminases. It can be used as oral or subcutaneous route at a dose of 10 to 15 mg/m^2 body surface area per week [21]. Folic acid (1 mg daily) is administered to decrease the frequency and severity of adverse effects.

Sulfasalazine—Sulfasalazine, the disease modifying anti-rheumatic drug that suppresses the rheumatic process, has been shown to be beneficial for many children with JIA. Used as a second anti-inflammatory drug in children with persisting oligoarticular and polyarticular disease [22]. Its use may be considered as an intermediate step prior to adding immunosuppressive drugs such as Methotrexate. Dosage: 30 - 50 mg/kg/day divided into 3 to 4 doses. Adverse events include anorexia, headache, vomiting, gastritis, pancytopenia, hemolytic anemia.

Hydroxychloroquine—Suppresses the rheumatic process by unknown mechanism. In addition to joint symptoms it also improves the extra articular manifestations [23]. Hydroxychloroquine can be used in selected cases of JIA in a 6.5 mg/kg/day as a single daily dose. Common side effects includes uveitis, gastro-intestinal disturbance, aplastic anemia, hepatic and renal toxicity.

Corticosteroids—Steroid acts by decreasing inflammation by reversing increased capillary permeability and suppressing polymorpho-nuclear activity. It also stabilizes lysosomal membranes and suppresses the lymphocytes and antibody production. Because of the various adverse effects and the availability of better treatment options, use of corticosteroids is indicated only in limited conditions [24]. Intra-articular corticosteroids is recommended for children with oligoarticular arthritis or in selected joints of polyarticular variety. Triamcinolone hexacetonide is the drug of choice; its use has resulted in a significant improvement in joint swelling and function at six months [25]. Low-dose oral steroids are used for short periods of time for children who

have severe polyarticular disease with functional impairment to provide bridging and symptomatic relief while other agents initiate a response. Corticosteroids are not used for prolonged period as long-term use of these agents' results in significant morbidity. The toxicities of corticosteroids include immunosuppression, adrenal suppression, hypertension, diabetes, cataracts, osteoporosis, avascular necrosis, mood swings, cushingoid features, and obesity.

Leflunomide (Adira)—Leflunomide is a DMARD that can be used as an alternative in children who fail to respond to Methotrexate. It is a pyramidine synthesis inhibitor, possesses immunomodulating activity and acts via inhibition of T-cell pyrimidine biosynthesis. It significantly reduces inflammation, including proteoglycan-induced progressive polyarthritis and adjuvant arthritis [26]. Preclinical studies have shown analgesic, antipyretic, and histamine-blocking activities of leflunomide. Adverse effects include alopecia, hepatotoxicity, rash, GI upset.

BIOLOGICAL THERAPY

Introduction of biological agents in the treatment of JIA has ushered in a new era of management of JIA [27]. A few of these agents are approved by FDA for the use in children with JIA, but many are still under consideration and not approved due to limited pediatric studies

Etanercept (ENBREL): a cytokine antagonist, soluble tumor necrosis factor (TNF) p75 receptor fusion protein that binds to and inactivates TNF. It was the first biologic to be approved by FDA in 2002 for the treatment of resistant active polyarticular JIA [28]. It is usually indicated when other DMARDS, mainly Methotrexate, prove unsuccessful in taking the child into remission [29].

Other anti-TNF-alpha biologics, such as Infliximab, anti-IL-6 and Adalimumab have been used in different trials in children with refractory JIA, with improved response. A few anecdotal reports indicate good responses to the use of Anakinra or IL-1 receptor antagonist in JIA [30]. Summary of recently studied biological therapies with their mode of actions and indications are mentioned in Table 3 [31].

Immunoglobulin infusion and an autologous stem cell transplant (ASCT) can be considered in situations where all above mentioned therapies fail to make the child into remission.

Table 3. Summary of biological therapies recently studied in JIA [31]

Drug	Action	Dose	Indications
Etanercept (Enbrel)	Soluble tumor necrosis factor (TNF) p75 receptor fusion protein that binds to and inactivates TNF	0.4mg/kg S/C twice weekly	Approved in 2002 by FDA for the treatment resistant active polyarticular JIA
Infliximab (Ramicade)	Chimeric human/mouse monoclonal antibody that binds to soluble TNF and its membrane bound precursor neutralizing its action	3-6 mg/kg IV at 0, 2,4, then 8 weekly	Persistent polyarthritis despite MTX (Not yet approved)
Adalimumab (Humira)	A humanized immunoglobulin G1 monoclonal antibody which binds to TNF	24mg/m^2 S/C	Polyarticular JIA active disease despite MTX (FDA approved in 2008)
Abatacept (Orencia)	Biological response modifier that demonstrates anti-inflammatory affects by down regulating T cell activation by blocking CD28.	10 mg/kg IV inf. administer on days 1, 15, 29, then q4wk	Active polyarticular disease failed to respond anti-TNF therapy,
Tocilizumab (Actemra)	A humanized anti-human interleukin 6 (IL-6) receptor monoclonal antibody, blocks the activity of IL-6	8 mg/kg IV every 2 weeks	So JIA resistant to anti-TNF therapy (Approved in Japan in 2008)
Anakinra (Kineret)	Anakinra is an IL-1 receptor antagonist (IL-1 RA), competitively binds to both type I and type II interleukin-1 receptors	1-2mg/kg S/C daily (max 100/day)	So JIA resistant to conventional therapy (approved only for SoJIA)

Adapted from Education and Practice, Archives of Disease in Childhood; October 2009, Vol.94 Issue 5.

PHYSICAL AND OCCUPATIONAL THERAPY

Physical therapy can help in reducing pain, maintaining muscle tone, improving mobility and preventing permanent handicaps. Special accommodations with schools may be needed to adjust children with limitations due to their chronic illness. Bed rest is not necessary for the patients with JIA; children are encouraged to be as mobile as possible. In fact, the more active the patient the better the long-term prognosis is. A consistent physical therapy program, with attention to stretching exercises, pain

modalities, joint protection, and home exercises, can help to keep these patients mobile.

Surgical Intervention

Surgical therapy has little role in the management in children with JIA. Nevertheless, surgical intervention may be beneficial in certain situations, such as in children with persistent leg length discrepancy, joint contractures, or dislocations. Potential corrective procedures include synovectomy, soft tissue release, epiphysiodesis (growth plate fusion), total arthroplasty (joint replacement), and arthrodesis (joint fusion) [32].

REMISSION

American college of rheumatology (ACR) have formulated the criteria for complete remission in children with JIA, which includes: absence of joint pain, synovitis and fatigue, there should be no morning stiffness or radiological evidence of progression of joint damage, levels of inflammatory markers includes erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) revert to normal [33].

Prognosis

Outcome of JIA depends on multiple factors, mainly type of JIA, gender, age of onset, sero-positivity, response to initial therapy etc. Table 4 summarizes the predictors of poor outcome. Overall mortality is 0.4% to 2% (greater risk with systemic JIA than with polyarticular JIA) [34].

Table 4. Bad prognostic indicators

• Delayed remission
• No response to NSAID after 8 months
• Having active disease after 5 years of onset
• Systemic onset/polyarticular
• Presence or uveitis
• Develop amyloidosis
• Progressive destructive arthritis
• Radiographic joint damage within 2 years

CONCLUSION

As more biological agents become available over the next decade, there may be dramatic changes in our approach to the management of JIA. Results of ongoing studies of these biological agents in pediatrics are still awaited [35]. We must resist the assumption that these idiopathic conditions will remain unexplained. Newer therapies will hopefully prevent the patient from becoming wheel chair bound.

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Chapter 8

SURGICAL APPROACHES IN KNEE ARTHROPLASTY

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ABSTRACT

Arthroplasty remains the gold standard for end stage arthritis. Excellent long term results can be expected from the well-executed knee arthroplasty and adequate exposure is key to this success. Of the several approaches to the knee, there is no clear consensus as to the best approach. The medial parapatellar approach is by far the most frequently used, in particular in the setting of a primary knee arthroplasty. While most arthroplasty surgeons choose one approach as standard for most of their patients, a working knowledge of other approaches is important as a standard approach may not be suitable for all patients.

Keywords: knee arthroplasty, approaches, exposures

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INTRODUCTION

While several new techniques have been developed to preserve the knee joint, Arthroplasty remains the gold standard for end stage arthritis. Excellent long term results can be expected from the well-executed knee arthroplasty and adequate exposure is the key to this success. Several approaches exist from the most common medial parapatellar to the minimally invasive approaches. While most arthroplasty surgeons choose one approach as standard for most of their patients, a working knowledge of other approaches is important as a standard approach may not be suitable for all patients. In this chapter we discuss several approaches to the knee with their relevant advantages and disadvantages.

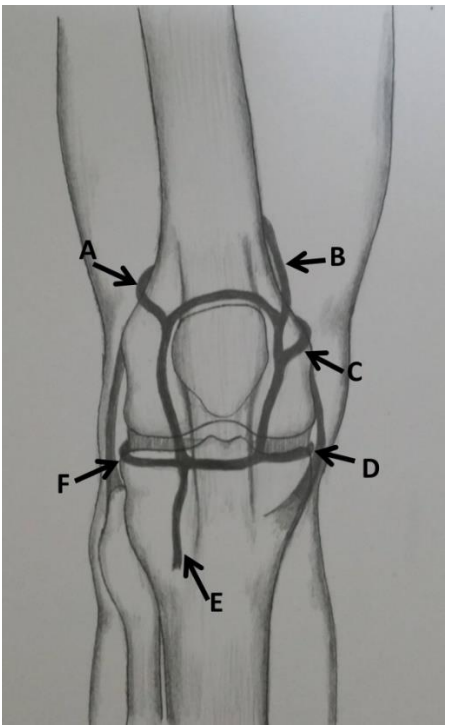

Figure 1. Schematic depicting the arterial anatomy around the patella. A. Lateral superior genicular artery B. Descending genicular branch of the Lateral femoral circumflex artery. C. Medial superior genicular artery D. Medial Inferior genicular artery. E. Anterior tibial recurrent artery. F. Lateral Inferior genicular artery.

SKIN INCISION

There are several factors to be considered prior to placing an incision over the knee joint. The knee is a superficial joint with the skin and a very thin layer of subcutaneous tissue overlying the bony prominences of the patella and the tibial tuberosity. Furthermore it is a joint with a significant range of motion.

It is important to have an understanding of the vascular anatomy of the skin around the knee to avoid wound complications, in particular in the presence of previous incisions. The skin over the anterior aspect of the knee is supplied by an anastomotic ring around the patella. The descending branch of the lateral femoral circumflex artery, the genicular branches of the popliteal artery and the anterior tibial recurrent artery contribute to this anastomosis (Figure 1). These vessels send perforators that penetrate the fascia in a deep to superficial fashion to supply the skin. Excessive subcutaneous dissection superficial to the fascia can compromise the blood supply and should be avoided [1]. Most of these perforators arise from the medial side of the joint. The overlying skin derives its blood supply predominantly from the medial genicular vessels placing the lateral incision at greater risk of necrosis [2, 3].

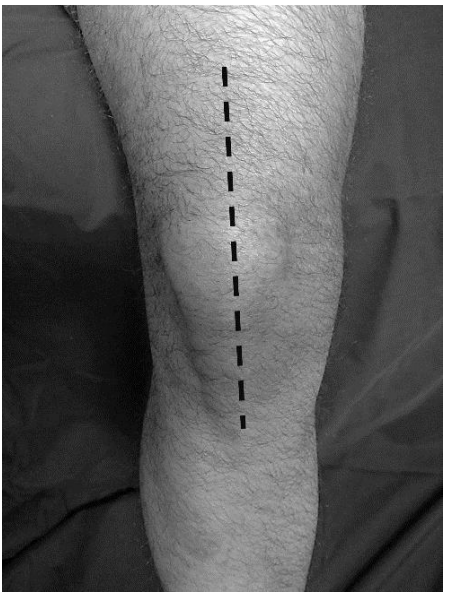

Figure 2. Photograph of the knee demonstrating the anterior midline incision.

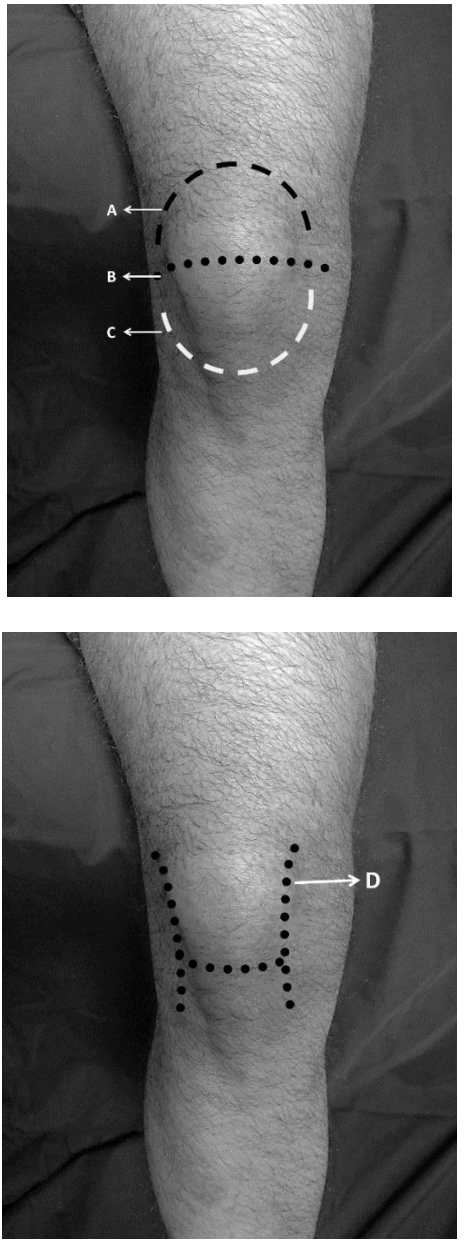

Figure 3. A. Putti incision. B. Transverse incision. C. Textor's incision D. "H" shaped Ollier's incision.

The most common incision is the anterior midline incision. With the knee flexed to 90˚, the incision begins approximately 5cm proximal to the patella and is carried distally over the patella up to the tibial tuberosity in a straight line (Figure 2). The extent of the incision will vary depending on the size of the patient. It is advisable to place the distal extent of the incision medial to the tibial tuberosity to avoid the bony prominence. The transverse incision is not extensile and provides a limited exposure to the knee joint and is to be avoided. Several other incisions have been described (Figures 3) which are associated with complications from vascular compromise and are largely of historical interest [4].

MEDIAL PARAPATELLAR APPROACH

The earliest description of the medial parapatellar approach has been credited to Von Langanbeck [5] and there have been several variations since. With the knee flexed and a midline skin incision, the arthrotomy is typically carried in a proximal to distal fashion. Proximally the medial third of the quadriceps tendon is incised longitudinally along its border with vastus medialis. At the level of the patella the arthrotomy is carried around the medial border of patella leaving a 5mm cuff of tissue on the patella to facilitate closure. The arthrotomy is carried distally medial to the patella tendon up to the level of the tibial tuberosity (Figure 4). The patella is then everted or laterally subluxated to complete the exposure. Additionally Hoffa's infrapatellar fat pad can be excised to increase exposure.

Everting the patella can reduce the intraosseous blood supply of the patella to a significantly greater degree than lateral retraction [6]. Several prospective studies have demonstrated that lateral subluxation is safe, provides earlier return of straight leg raise, a reduced length of stay, and improved range of motion when compared to everting the patella [7, 8]. Excision of the infrapatellar fat pad while routinely performed has the potential to disrupt patellar blood flow, in particular the inferior portion of the patellar anastomotic ring [9, 10] Studies have shown up to 50% reduction in patellar blood flow following complete infrapatellar fat pad excision [10, 11], however no correlation has been found between fat pad excision and avascular necrosis of the patella [12]. Complete excision of the infrapatellar fat pad is also associated with a higher incidence of postoperative patella baja [13]. It is

recommended to retract the fat pad where possible and to only partially excise it where necessary.

There are several variations of the medial parapatellar arthrotomy. Insall [14] described a more midline approach. Proximally the medial third of the quadriceps tendon is incised longitudinally down to the level of the patella. At this level the capsule over the medial third of the patella is peeled off is a subperiosteal fashion. Distally the capsule is entered just medial to the patellar tendon to complete the arthrotomy. This approach can result in post-operative subluxation, fragmentation and avascular necrosis of the patella [15, 16]. In the Von Langanbeck approach the proximal arthrotomy is carried through the fibers of the vastus medialis rather than the quadriceps tendon. This approach may disrupt postoperative muscle function leading to increased risk of patellar dislocation and therefore not recommended.

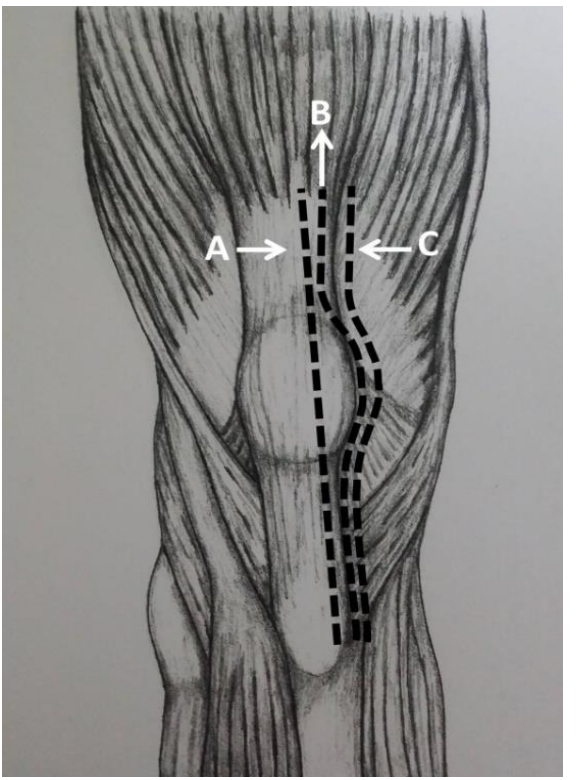

Figure 4. A. Insall approach B. Medial Paraptellar approach C. Von Langanbeck approach.

SUBVASTUS APPROACH

The earliest description of a subvastus approach was by Erkes in 1929 [17]. The inferior border of vastus medialis is identified and bluntly dissected free from the intermuscular septum. The muscle fibres are retracted proximally and medially to expose the underlying capsule. A transverse arthrotomy is performed at this level beginning medially up to the medial border of the patella. The arthrotomy is carried around the patella distally in a similar fashion to the medial parapatellar approach (Figure 5). The proponents of this approach argue that it is a more anatomic approach leading to less blood loss and the preservation of quadriceps tendon leads to quicker recovery of quadriceps strength [18, 19]. This approach can result in a longer operative time and can be difficult in the larger or more muscular patient. Structures of Hunters canal are at risk of injury if the arthrotomy is carried too far proximally. Other relative contraindications to the use of this approach include patella baja, a very stiff knee with less than 50˚ range of motion and those requiring revision arthroplasty [20].

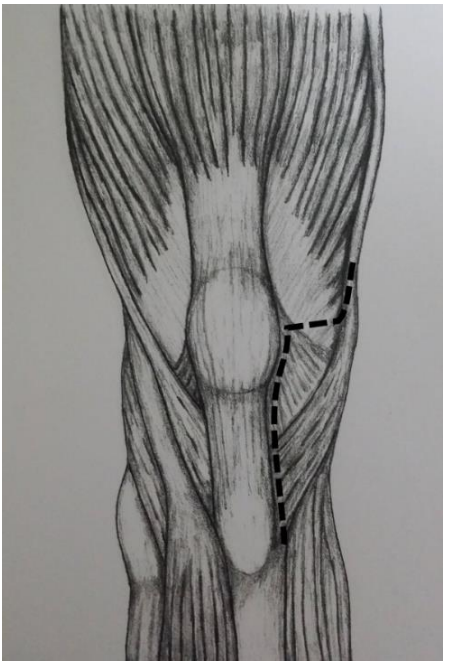

Figure 5. Subvastus approach.

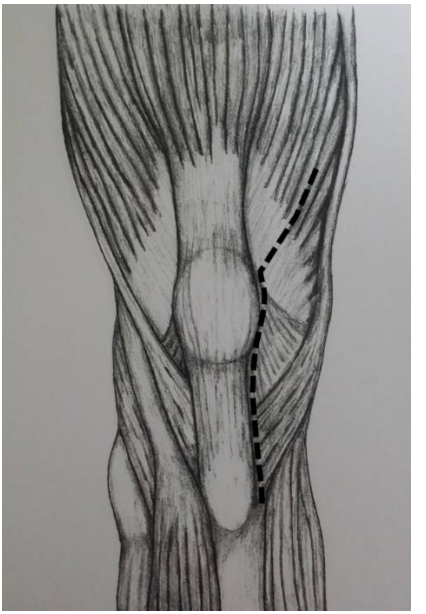

Figure 6. Midvastus approach.

MIDVASTUS APPROACH

The midvastus approach was described by Engh in 1997 [21]. Using a midline incision the knee is exposed and the inferior border of the vastus medialis is identified. At the level of the superior pole of the patella the vastus medialis muscle is bluntly split with a finger in a proximal and medial direction for 4cm, staying in line with the muscle fibres. The underlying capsule is incised leaving the quadriceps tendon and the insertion of the vastus medialis obliquus uninterrupted. The arthrotomy is carried around the patella distally similar to the medial parapatellar approach (Figure 6). The exposure is better that the subvastus approach. Similar to the subvastus approach, the sparing of the extensor mechanism can lead to quicker recovery of quadriceps strength. The medial superior genicular artery is at risk [22] and can potentially compromise the blood flow to the patella; however studies have shown no difference in patellar vascular compromise between midvasus and the medial parapatellar approach [23]. The splitting of the vastus medialis fibers may lead to denervation of the muscle [24]. Other disadvantages include difficulty with everting the patella laterally and a longer operative time

compared to the standard medial parapatellar approach [25] Similar to the subvastus approach, relative contraindications for the use of this approach include large or muscular patients, a very stiff knee and a patella baja [21].

TRIVECTOR APPROACH

The Trivector arthrotomy begins distally at the level of the tibial tubercle similar to the medial parapatellar approach up to the level of the superior pole of the patella. Here there are two divergent limbs to the incision. The medial limb mirrors that of a mid vastus approach extending 2cm into the vastus medialis muscle and in line with its fibers. The lateral limb ascends 2cm along the medial border of the quadriceps tendon. The patella is then everted or subluxated laterally to expose the joint. The exposure is better than the subvastus approach. Another advantage is a quicker return of quadriceps strength compared to the standard medial parapatellar approach [26].

LATERAL PARAPATELLAR APPROACH

The lateral parapatellar approach was first described by Kocher [4] but later popularised by Keblish [27] in 1991. This is a less frequently used approach. It is used primarily in the setting of a significant fixed valgus deformity of the knee. Proximally the arthrotomy divides the lateral third of the quadriceps tendon longitudinally down to the patella. At the level of the patella the arthrotomy is carried laterally around the patella leaving a 5mm cuff of tissue on the patella to facilitate closure. The arthrotomy is continued distally lateral to the patella tendon up to the level of the tibial tuberosity (Figure 7). Everting the patella and its medial subluxation is significantly more difficult with this approach, placing the patella tendon at greater risk of avulsion from the tibial tuberosity. While this approach can be extensile, extensive dissection risks devascularising skin and patella. It is a technically demanding approach and is relatively contraindicated in the fixed varus knee. A tibial tubercle osteotomy may be considered in cases where the exposure remains inadequate.

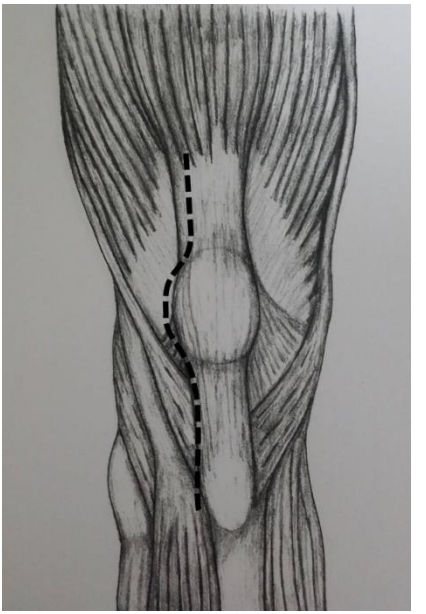

Figure 7. Lateral parapatellar approach.

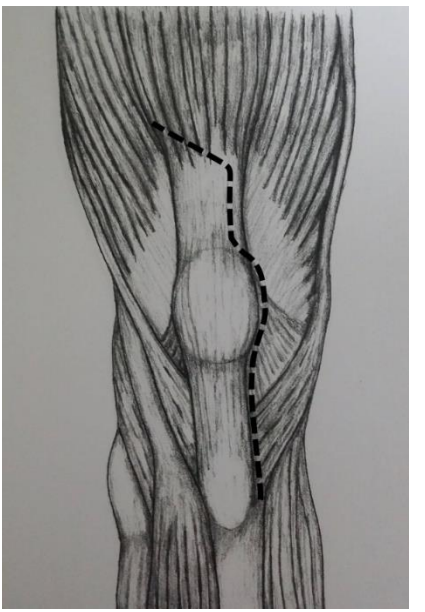

Figure 8. The Quadriceps snip.

QUADRICEPS SNIP

The quadriceps snip was first described by Insall [28]. The medial parapatellar arthrotomy is carried proximally up to the proximal extent of the quadriceps tendon. Here an oblique 45˚ incision is made laterally through the quadriceps tendon along the line of the muscle fibres of vastus lateralis (Figure 8). The patella is then everted and the tibia externally rotated to expose the joint. This is a useful approach to the stiff knee where the standard medial parapatellar approach has proved inadequate.

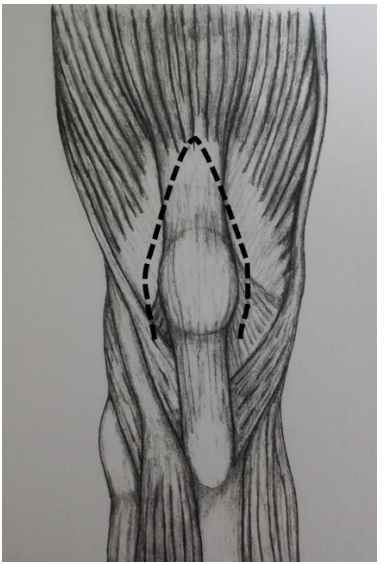

Figure 9. The Quadriceps turndown.

QUADRICEPS TURNDOWN

The quadriceps turndown was first described by Coonse and Adams [29]. They described their experience of one case where they had performed an inverted "V" shaped incision of the quadriceps tendon with the apex of the V at the proximal extent of the quadriceps tendon and the 2 prongs of the "V" curved on either side of the patella (Figure 9). Insall [28] later described a modified version, the "patella turndown approach" where a medial para patellar arthrotomy was carried proximally to the proximal extent of the

quadriceps tendon. Here a further incision is carried distally at a 45^0 angle laterally along the margin of vastus lateralis (Figure 10). This modified version has the advantage of being an option when a medial parapatellar arthrotomy has proven inadequate in the exposure of a stiff knee. While the exposure is excellent these approaches require a more gradual post-operative mobilisation. The lateral superior genicular artery is at risk with this approach and care should be taken to preserve it. An extensor lag and avascular necrosis of the patella have been reported as complications [30, 31].

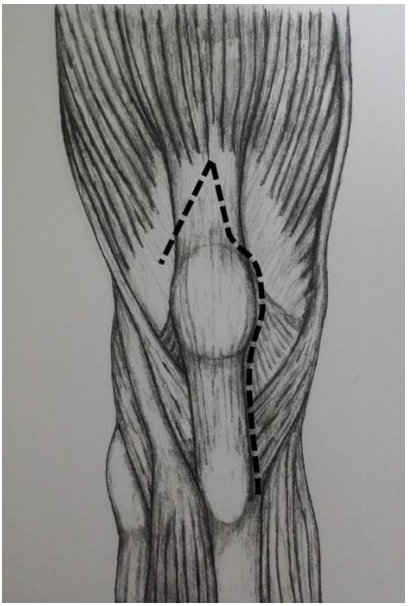

Figure 10. The Patella turndown.

TIBIAL TUBERCLE OSTEOTOMY

The tibial tubercle osteotomy for total knee arthroplasty was first described by Dolin [32] in 1983. It is primarily indicated when the standard medial parapatellar approach and perhaps a quadriceps snip have failed to provide adequate exposure of the joint [33]. The standard medial parapatellar arthrotomy is performed and carried distally 8-10cm beyond the tibial tuberosity to accommodate the osteotomy. Once the patella tendon and tibial tuberosity have been clearly defined, an oscillating saw is used to create the

osteotomy to the desired length and thickness (Figure 11). Dolin described a 4.5cm long osteotomy that was fixed with a screw. Whiteside [34] modified the approach by increasing the size of the osteotomy to 8-10cm with the muscle and periosteal attachments left intact and held it with 2 to 3 cerclage wires instead of screws. The approach has a large learning curve but can provide excellent exposure to the ankylosed knee and advancing the tibial tubercle proximally can help correct a patella baja or an elevated joint line. It is advisable to pre drill the holes for the screws or cerclage wires to prevent proximal migration of the tibial tubercle. While excellent exposure is achieved, Dolin reported a 23% complication rate with an 11% non-union rate. Other notable complications include fractures of the proximal tibia and the tibial tubercle and skin necrosis have been reported [35, 36]. The tibial tubercle osteotomy is contraindicated in the presence of osteoporotic bone, significant scarring of the extensor mechanism and large proximal tibial bone defects [35]. The tibial tubercle osteotomy can be combined with a lateral parapatellar approach in the setting of a stiff knee with a fixed valgus deformity [38]. While outcomes have been shown to be similar to the quadriceps turndown approach, a higher number of patients reported difficulty with kneeling and stooping, however tibial tubercle osteotomy results in less extensor lag post operatively [39, 40].

Figure 11. Tibia tubercle osteotomy. Dotted line depicts a quadriceps snip.

Table 1. The advantages and disadvantages of the various approaches to the knee

Approach	Advantages	Disadvantages
Medial parapatellar approach	• Relatively safe and easy approach • Excellent exposure • Extensile	• Disruption of blood supply to the patella • Disruption of the extensor mechanism
Subvastus approach	• A more anatomic approach • Quadriceps tendon and vastus medialis not disrupted. • Less blood loss • Earlier return of quadriceps strength • Improved patellar tracking	• Difficulty everting or laterally subluxating patella. • Not suitable for the larger or more muscular patient. • Injury to structures of Hunters canal. • Less extensile
Midvastus approach	• Quadriceps tendon and vastus medialis obliqqus insertion not disrupted. • Earlier return of quadriceps strength	• Difficulty everting or laterally subluxating patella. • Not suitable for the larger or more muscular patient. • Denervation of vastus medialis. • Less extensile
Trivector approach	• Better exposure • Earlier return of quadriceps strength	• Longer operative time • Disruption of vastus medialis. • Disruption of blood supply to patella • Greater blood loss • Less extensile
Lateral parapatellar approach	• Useful in knees with significant fixed valgus +/- fixed flexion deformity.	• Technically demanding. • Difficult eversion or medial subluxation of patella. • Not suitable in fixed varus knees • Less extensile leading to vascular compromise to skin and patella
Quadriceps turndown	• Excellent exposure	• Complete disruption of quadriceps tendon. • Disruption of blood supply to patella • Prolonged rehabilitation • Post-operative extensor lag
Tibial tubercle osteotomy	• Excellent exposure • Tibial tuberosity can be repositioned	• Contraindicated in osteoporotic bone. • Non-union of osteotomy site • Proximal migration of tibial tuberosity • Fracture of tibial tubercle and proximal tibia • Prolonged rehabilitation

CONCLUSION

The planning of every total knee arthroplasty begins with the incision and approach. The choice of approach is usually dictated by the surgeons experience and training but also can be dictated by the knee to be approached. While there is no clear consensus as to the best approach, the medial parapatellar approach is by far the most frequently used, in particular in the setting of a primary knee arthroplasty. In the more complex cases, a working knowledge of the various approaches is important for the execution of a successful knee arthroplasty. Table 1 summarised the advantages and disadvantages of the various approaches.

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Chapter 9

ENHANCEMENT OF ANTIOXIDANT SYSTEM BY LIPOIC ACID IN EXPERIMENTAL LUNG TUMORIGENESIS

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ABSTRACT

Modulatory role of lipoic acid on oxidative stress produced during benzo(a)pyrene (B(a)P) induced lung cancer was studied in mice. Administration of B(a)P to mice caused a significant increase in oxidative stress indicated by abnormal changes in lipid peroxidation, enzymic antioxidants (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase) and non-enzymic antioxidants (reduced glutathione, vitamin C, vitamin E and vitamin A). Treatment with lipoic acid prevented all the above abnormal changes and

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restored cellular normalcy suggesting the protective role of lipoic acid in curbing the oxidative stress produced in B(a)P induced lung carcinogenesis in mice.

Keywords: Lipoic acid, Benzo(a)pyrene, lipid peroxidation, antioxidants

1. INTRODUCTION

Lung cancer remains a major cause of death from cancer in the world, causing more than one million deaths each year [1]. Smoking is the main risk factor, to which 90% of lung cancer cases are attributable [2]. Benzo(a)pyrene (B(a)P) is a highly carcinogenic polycyclic aromatic hydrocarbon (PAH) present in tobacco smoke that is involved in the aetiology of lung cancer [3, 4]. B(a)P is metabolically activated into benzo(a)pyrene 7,8-diol-9,10-epoxide (BPDE) which reacts with DNA predominantly to form an adduct and progression of the disease [5].

Lipoic acid (LA) is a naturally occurring compound, which is an essential component of oxidative metabolism, participating as protein bound lipoamide in alpha keto acid dehydrogenase complexes in mitochondria. Role of lipoic acid (LA) a mitochondrial “metavitamin” in intermediary metabolism may not be confined to its cofactor role but may well extend to certain reactions in lipid biosynthesis where it replaces coenzyme A for activating fatty acids prior to acylation and has been established as a universal antioxidant due to its high singlet oxygen quenching constant, is reported to decrease lipid peroxidation [6]. Supplementation of lipoic acid leads to its reduction to produce the more active antioxidant molecule dihydrolipoic acid [7]. The present study is an attempt to explore the antioxidant potential of LA in ameliorating the oxidative stress produced during lung carcinogenesis.

2. MATERIALS AND METHODS

2.1. Materials

Benzo(a)pyrene and Lipoic acid were purchased from Sigma chemicals, St. Louis, USA. All other chemicals were of analytical grade procured from local commercial sources.

2.2. Animal Model

Healthy male Swiss albino mice weighing 20-25g (8-10 weeks old) obtained from Veterinary College, Chennai, were used throughout the experiment. The animals were housed under conditions of controlled temperature (26 ± 2oC) with 12-hr day/night cycle. They were fed standard rat/mice pellet diet (M/s. Hindustan Lever Ltd., Mumbai) under the trade name Amrut rat/mice feed and were given access to water ad libitum.

2.3. Experimental Design

Experimental animals were divided into four groups of six mice each as follows. Group I (control) received olive oil throughout the course of the experiment. Group II (B(a)P) were treated with benzo(a)pyrene (50 mg/kg b.wt. dissolved in olive oil) orally twice a week for four successive weeks. Group III (LA) received lipoic acid (10 mg/kg b.wt. dissolved in olive oil) intraperitoneally once in a week for 14 weeks to assess the cytotoxicity (if any) induced by LA. Group IV (B(a)P + LA) received B(a)P (as in Group II) along with lipoic acid (10 mg/kg b.wt dissolved in olive oil) intraperitoneally. LA treatment was started one week prior to the first dose of B(a)P administration and continued for 14 weeks.

At the end of the experimental period (14th week), animals were sacrificed by cervical decapitation under ether anesthesia and lungs were excised immediately and washed with ice-cold saline. A 10% homogenate of the washed tissue (lung) was prepared in 0.01 M phosphate buffer (pH 7.4). The homogenate was centrifuged at a speed of 12,000 x g for 30 min in a refrigerated high-speed centrifuge at 4ºC. The following biochemical estimations were carried out in the supernatant.

Biochemical Analysis

Protein estimation was carried out [8]. LPO was assayed by the method in which the malondialdehyde (MDA) released served as the index [9]. Superoxide dismutase (SOD) and catalase (CAT) were assayed [10, 11]. The activities of glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferase were also was determined. [12-14]. Reduced glutathione (GSH), vitamin E, vitamin C and vitamin A were assayed [15-18].

Data analysis

All data were expressed as mean ± S.D for six rats The results were computed statistically (SPSS Software Package) using one-way ANOVA. Post-hoc testing was performed for inter comparisons using the LSD. ($P < 0.05$) was considered significant.

3. RESULTS

Table 1 depicts the effect of B(a)P and LA on lung LPO and enzymic antioxidant status. Significant ($P < 0.05$) increase in LPO with concomitant decrease in the activities of enzymic antioxidants SOD, CAT, GPx, GR and GST were observed in the B(a)P administered group (Group II). LA pretreatment resulted in a free radical quenching effect and thereby significantly ($P < 0.05$) decreasing LPO and reinstating the enzymic antioxidant activities to near normalcy in Group IV animals.

Table 2 shows the levels of non-enzymic antioxidants GSH, vitamin C, vitamin E and vitamin A in control and experimental group of animals. B(a)P treatment markedly ($P < 0.05$) reduced the levels of GSH, vitamin C, vitamin E and vitamin A in Group II animals that were significantly ($P < 0.05$) restored to near normalcy on LA pretreatment in Group IV animals.

Table 1. Effect of benzo(a)pyrene and lipoic acid on LPO and enzymic antioxidants in lung tissue

Parameters	Group I (Control)	Group II (B(a)P)	Group III (LA)	Group IV (B(a)P + LA)
LPO	54 ± 6.3	97 ± 9.5[a]	53 ± 5.3	61 ± 6.1[b]
SOD	6.20 ± 0.59	3.80 ± 0.38[a]	6.21 ± 0.59	5.73 ± 0.61[b]
CAT	140 ± 13.7	89 ± 8.67[a]	139 ± 13.5	127 ± 13.1[b]
GPx	5.86 ± 0.58	1.97 ± 0.19[a]	5.85 ± 0.59	5.40 ± 0.18[b]
GR	4.36 ± 0.43	1.26 ± 0.13[a]	4.38 ± 0.43	4.05 ± 0.08[b]
GST	47.34 ± 0.48	7.89 ± 0.79[a]	46.78 ± 0.46	41.10 ± 0.68[b]

Each value is expressed as mean ± S.D for six mice in each group.

LPO – nmoles of MDA released per mg protein; SOD – units/mg protein. (One unit is equal to the amount of enzyme required to inhibit autoxidation of pyrogallol by 50%); CAT - μg of H_2O_2 consumed/min/mg protein; GPx - μg of reduced GSH utilized/min/mg protein; GR - nmoles of NADPH oxidized/min/mg protein; GST - nmoles of CDNB - GSH conjugate formed/min/mg protein;

Statistical significance at $P<0.05$, as compared with [a]Group I, [b]Group II.

Table 2. Effect of benzo(a)pyrene and lipoic acid on non-enzymic antioxidants in lung tissue

Parameters	Group I (Control)	Group II (B(a)P)	Group III (LA)	Group IV (B(a)P + LA)
GSH	0.73 ± 0.07	0.39 ± 0.03 [a]	0.72 ± 0.07	0.65 ± 0.06 [b]
Vitamin C	0.54 ± 0.05	0.11 ± 0.01 [a]	0.52 ± 0.05	0.47 ± 0.05 [b]
Vitamin E	0.60 ± 0.06	0.22 ± 0.02 [a]	0.61 ± 0.05	0.52 ± 0.05 [b]
Vitamin A	0.51 ± 0.05	0.17 ± 0.01 [a]	0.51 ± 0.05	0.43 ± 0.04 [b]

Each value is expressed as mean ± S.D for six mice in each group.

GSH – μg/mg protein; vitamin C – μg/mg protein; vitamin E – μg/mg protein; vitamin A – μg/mg protein.

Statistical significance at $P<0.05$, as compared with [a]Group I, [b]Group II.

Discussion

Modulation of various cellular molecular pathways by reactive free radicals mediated LPO generated during cytochrome P450 dependent metabolism of B(a)P has been implicated during the pathogenesis of lung carcinogenesis [19]. Our results were in agreement with the above findings where we observed an increase in LPO in B(a)P induced lung cancer animals (Group II). Treatment with LA reduced LPO induced by B(a)P in group IV animals suggesting its antioxidant potential.

Body cells are continuously targeted by ROS and other chemical carcinogens and antioxidants are very important in fighting against these toxic components [20]. SOD, which converts superoxide radicals to hydrogen peroxide, is widely distributed in cells having oxidative metabolism and is thought to protect such cells against the toxic effects of superoxide anion [21]. CAT is a heme protein that catalyzes the direct degradation of hydrogen peroxide to water. It protects the cellular constituents against oxidative damage [22]. GPx catalyzes the reduction of hydrogen peroxide and hydroperoxide to non-toxic products and scavenges the highly reactive lipid peroxides in the aqueous phase of cell membranes [23]. GST is a group of multifunctional proteins that perform tasks ranging from catalyzing the detoxification of electrophilic compounds to protection against peroxidative damage [24]. GR plays a major role in regenerating GSH from GSSG, thus maintaining the balance between the redox couple [25].

Activities of the antioxidant enzymes SOD, CAT, GPx, GST and GR were observed to be lowered in B(a)P treated animals (Group II). On LA pretreatment, the activities of these enzymes were markedly elevated in Group IV animals. This may be due to the direct reaction of LA with superoxide, hydroxyl and alkoxyl radicals, which in turn reduces free radical formation and oxidative damage during lung carcinogenesis.

GSH plays a unique role in detoxification of xenobiotic compounds, in the antioxidation of ROS and free radicals [26]. Decreased GSH levels signify increased oxidative stress. In our present study, we have observed a decline in GSH levels in lung cancer bearing animals (Group II), which may be due to the excess utilization of this antioxidant for tumor cell proliferation. LA pretreatment resulted in the increased availability of reducing equivalents possibly due to improved glucose-6-phosphate dehydrogenase activity thereby significantly increasing the activity of glutathione reductase with subsequent regeneration of reduced glutathione pool.

Vitamin C an important antioxidant acts in tissues, involving ROS in aqueous phase and it has been reported that the tissue concentration of vitamin C is a good indicator of oxidative stress [27]. Vitamin E is a principal lipid soluble antioxidant in cell membranes that protects critical cellular structures against oxidative damage [28]. Vitamin A as an antioxidant is involved in counteracting free radicals and is known to help in the repair of damaged tissues [1]. Vitamin C, E and A levels were found to be reduced in B(a)P administered groups (Group II) suggesting an increase in LPO in these animals. The levels of these vitamins in mice challenged with B(a)P were almost normalized upon LA pretreatment.

Thus the results of the present study undoubtedly revealed the role of LA as a potent antioxidant that prevented oxidative stress and in turn paved way for its protective role against B(a)P induced lung carcinogenesis.

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