CHAPTER 2

REVIEW OF LITERATURE

Natural products serve as a consistent source of drug lead with diverse chemical entities being derived from plants (Newman and Cragg, 2006), microbes (Zhang et al., 2005), invertebrates (McCarthy and Pomponi, 2004), etc. Most of the drugs in current use are natural products or natural product based. Microbes respond to their environment quickly and compete for defense and survival by production of unique secondary metabolites, which have been shown to possess biotechnological or pharmaceutical applications. The marine realm which covers 70% of the earth’s surface provides the largest inhabitation for living organisms, particularly microbes among the major habitats of the biosphere. Marine organisms have been shown to produce numerous novel compounds with multiple pharmacological properties (Fuesetani, 2000; Prosch et al., 2002; Haefner, 2003; Blunt et al., 2004). Marine species comprises approximately a half of the total global biodiversity and hence the sea offers an enormous resource for novel compounds (de Vries and Beart, 1995), and it has been classified as the largest remaining reservoir of natural molecules to be evaluated for drug activity (Gerwick, 1987). In recent years, many bioactive compounds have been extracted from various marine animals like tunicates, sponges, soft corals, sea kares, nudibranchs, bryozoans, sea slugs and marine organisms (Donia and Hamann, 2003; Haefner, 2003). The search for new metabolites from marine organisms has resulted in the isolation of more or less 30,000 metabolites, many of which are endowed with pharmacodynamic properties. The pie graph showing the distribution of organisms belonging to different phylum in marine environment that produce bioactive compounds of therapeutic importance...
is shown in Fig 1. Sponges often have associated symbiotic microbial populations (Lee et al., 2001; Richelle-Maurer et al., 2003). Symbionts include archaea, bacteria, cyanobacteria, and microalgae. In some cases, these microorganisms and not sponge cells are the likely source of the secondary metabolites of interest (Bewley and Faulkner, 1998; Lee et al., 2001; Proksch et al., 2002).

![Pie chart showing distribution of marine natural products by Phylum, 2002 (Blunt et al., 2004)](chart.png)

**Fig 1 Distribution of marine natural products by Phylum, 2002 (Blunt et al., 2004)**

The anti-inflammatory activity of a new sphingosine derivative and cembrenoid diterpene (lobohedleolide) isolated from the soft corals of Sinularia crassa and Lobophytum sp. respectively, collected on the coasts of Andaman and Nicobar Islands was reported by Radhika et al., (2005).

### 2.1 Microbes in Marine ecosystems

Marine microbes thrive not only on the surface of the sea but also in the lower and abyssal depths, offshore to coastal regions. Microbial life is widely distributed although they are rarely conspicuous; but Whitman et al., (1998) have reported that about half the biomass of earth is microbial and is found in all
ecosystems. Marine microbes live in a biologically competitive environment with unique conditions of salinity, pH, temperature, pressure, oxygen, light and nutrients. Hence the marine microbial metabolites exhibit wide diversity both in chemical composition and biological activities compared to terrestrial bacteria (Blunt et al., 2004; Berdy 2005). Serious attempts to tap the vast potential of marine organisms as sources of bioactive metabolites that may be directly utilized as drugs or serve as lead structures for drug development started in the late 1960s. Realization of the tremendous biological reserve of marine microbes has resulted in increasing interest in the exploitation of microbes in search of new chemical entities.

2.1.1 Mangrove ecosystem

Mangroves are unique inter-tidal ecosystems, which support the growth of genetically diverse groups of aquatic and terrestrial organisms. This ecosystem situated at the inter-phase between the terrestrial and marine environment help to sustain a rich and diverse group of microorganisms. Holguin et al., (2001) reported the presence of different groups of bacteria that get nourished by detritus which in turn help the mangrove ecosystem in different ways. The mangrove environment has become a potent source for the isolation of antibiotic-producing actinomycetes and marine fungal diversity is also best-studied in the mangrove ecosystem (Hyde et al., 2000; Cheng et al., 2009).

2.1.2 Deep sea

The deep sea is characterized by unique and extreme environment like high pressure, low temperature, lack of light, variable salinity and oxygen concentration. In spite of the vast geographical area of deep sea, scant information is available about the deep sea microorganisms. However, Bull et al., (2000) have reported deep
sea as a good source of novel organisms for microbiologists and biotechnologists. Sterling efforts of Zobell and co-workers, who initiated work on the effect of hydrostatic pressure on bacterial activity (Zobell and Johnson, 1949), have brought improvements in the knowledge of deep-sea biology. The deep-sea habitat remained untouched for a long time. Colquhoun et al., (1998) have isolated a large number of actinomycetes from deep-sea sediments. Actinomycetes species isolated from deep sea are mostly novel with potent sources of antibiotics. Two new genera and species of Ascomycetes and two new Deuteromycetes from 1615 and 5315 m depth were described by Kohlmeyer (1977). Gautschi et al., (2004) isolated Penicillium sp from deep water sediment at 4380 ft and evaluated the cytotoxic activity.

2.1.3 Extreme environments

Any environmental condition that can be perceived as beyond the normal acceptable range is an extreme condition (Satyanarayana et al., 2005). Extreme environments can be found in all ecosystem and surprisingly almost all these environments are colonized by microorganisms well adapted to the extreme conditions and the microorganisms inhabiting such environments are termed as ‘extremophiles’. Highly saline conditions in water and soil exert a strong selective pressure on the biota, favouring the development of halotolerant and halophilic forms. Marine saltern is the common habitat of halophiles and the most common genera are Halococcus and Halobacterium. Reports on fungi living in the extreme environments were rare except the report on the diversity of fungi from the hypersaline Dead Sea environment, which is one of the most extreme environments for microorganisms on the earth (Molitori et al., 2000; Kis-Papo et al., 2003). Archaeoglobus is found in hot sediments of marine hydrothermal vents with optimum growth at 83 °C (Burggraf et al., 1990). A methanogenic bacterium Methanopyrus
capable of growth at 100 °C has also been recorded (Huber, 1989; Huber et al., 1995).

2.2 Screening of marine bacteria for secondary metabolites

Secondary metabolites are compounds with varied and sophisticated chemical structures, produced during the growth of the microbes (Barrios-Gonzalez and Mejia, 1996; Barrios-Gonzalez and Mejia, 2008). The first document on antibiotic-producing marine bacteria was by Rosenfeld and Zobell, (1947). Since then, there are several reports of antibiotic-producing marine bacteria (Hanefeld and Laatsch, 1991; Jensen and Fenical, 1994 and James et al., 1996). It is critical to have efficient system to identify uninteresting or already known antibiotics as early as possible, in order to focus the resources on the important ones. Buss and Butler, (2004) state the importance of the use of secondary assays or counter-screens that help to eliminate the uninteresting biological activities. However, the use of secondary assays at an early stage may yield misleading results, since the extract may contain several active compounds, and they may camouflage each other in the secondary assays. Instead, all the selected extracts from primary screening can be subjected to some type of fractionation; however this technique is expensive and time-consuming. The chromatographic techniques and other separation technologies are employed for retrieving the active compound. Spectroscopic techniques such as UV, FTIR, NMR and MS as used for structure elucidation, are obviously an enormous improvement in the process isolation of active lead (Koehn and Carter, 2005). However, the complexity of the process, which involves not only bioassay guided isolation chemistry, but also re-supply of extract in volume to obtain sufficient pure material, still recognized as the rate limiting step of natural products lead discovery.
Marine bacteria are capable of producing unusual bioactive compounds that are not observed in terrestrial sources (Fenical, 1993; Fenical and Jensen, 1993). Thermo-stable proteases, lipases, esterases, and starch and xylan degrading enzymes have been found in bacterial and archaeal hyperthermophilic marine microorganisms (Bertoldo and Antranikian, 2003). The first marine antibiotic isolated was pentabromopseudilin (Burkholder et al., 1966; Hanessian et al., 1966; Lovell, 1966). Yoshikawa et al., (1997) have isolated Korormycin from a marine Vibrio alginolyticus, which is a specific inhibitor of the enzyme sodium dependent NADH:quinone reductase making this organism halotolerant (Yoshikawa et al., 1999). Alteromonas sp produces isatine, a long-known synthetic product, which showed a reasonable anti fungal activity and protects the eggs of shrimp Palaemon macrodactylus successfully against the pathogenic fungus Lagenidium callinectes (Gil-Turnes et al., 1989). Streptomycetes are especially rich in biologically highly active quinones; Parimycin, C-glycosides bimalomycins and anthraquinones with the rare fridamycin E chromophor, a precursor of the anthracycline antibiotics were isolated from Streptomyces sp P6821 (Maskey et al., 2003). Korormicins have been isolated from a marine Pseudoalteromonas sp. F- 420 (Yoshikawa et al., 2003), which are selective inhibitors of the primary sodium pump. Jeong et al., (2003) have isolated bacillamide from Bacillus sp which is active against dinoflagellate.

In our lab, potential marine Pseudomonas sp strains PS3 and PS7 producing anti inflammatory compounds (3S, 8aS)-3-Isobutyl-hexahydro-pyrrolo [1, 2-a] pyrazine-1,4-dione and (3S, 8aS)-3-(4-Hydroxy-benzyl)-hexahydro-pyrrolo [1, 2-a] pyrazine-1,4-dione respectively have been isolated, characterized and structurally elucidated by Rupesh (2007). Secondary metabolites from cultured microbes have been a major source of clinically useful chemotherapeutics. Identification of
compounds that interact specifically with specific molecular targets will be useful in controlling inflammation and cancer.

2.3 Inflammation

Inflammation is a process of body’s defense mechanism which recognizes the danger signals that activate intracellular signaling cascades leading to increased expression of pro inflammatory cytokine genes. Human diseases with acute and chronic inflammation such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, psoriasis, etc., still demand better therapies. Inflammation occurs as a result of complex series of events that follow tissue damage caused by trauma, or by microbial products. It is basically a protective and restorative reaction but due to increased vasopermeability caused by local mediators liberated by damaged tissue, granulocytes actively extravasate and accumulate at the site of injury attracted by leucotactic factors of various origins (lymphocyte products, bacterial metabolites, and fibrin split products). Monocytes and macrophages allured by similar mechanism reach the inflammatory site within few hours and later become predominating cells during healing or in chronic inflammation.

2.3.1 Activation of cells with mitogen

Exposure of immune-related cells to mitogen such as Phytohaemeagglutinin (PHA), Lipopolysaccharide (LPS), etc., results in the intense production of inflammatory cytokines, including TNF-α, IL-1β and other inflammatory mediators, such as arachidonic acid (AA), thereby leading to septic shock in mammals (Sweet and Hume, 1996) LPS, a component of the cell envelope of Gram-negative bacteria, was shown by Glauser et al., (1991) to cause septic shock. Lymphocyte proliferation assay (LPA) is used to assess the proliferation of
peripheral blood mononuclear cells (PBMC) in response to various non specific (mitogen) and specific stimuli (antigen) that trigger a complicated series of events that ultimately leads to cell division, which is measured by the incorporation of \[^{3}\text{H}\] labeled thymidine into newly synthesized DNA. This method reported by Sitz and Birx, (1999) has become a standard surrogate measurement of proliferation.

2.3.2 Mediators of inflammation

Inflammation is a complex phenomenon involving multiple cellular and molecular interactions which must be tightly regulated to avoid different pathology and disorders (Balkwill et al., 2005; Consilvio et al., 2004). Activated mononuclear cells that migrate by the way of PBMC, to bone, muscle, liver and other tissues release inflammatory mediators, including TNF-\(\alpha\), Prostaglandin E2 (PGE2) and interferon gamma (IFN-\(\gamma\)). Nitric oxide (NO) and PGs have been reported to play an important role in inflammation and the role of phospholipase and cyclooxygenase in production of prostaglandin is shown in Fig 2. The discovery of sizeable quantities of PGs, which had been discovered as important mediator involved in inflammatory diseases, fever and pain is usually, considered as the “take-off point” of any serious search for “drugs from the sea”.

Membrane phospholipids are converted into arachidonic acid by phospholipase A\(_2\) (PLA\(_2\)) and the PGs are generated from AA via cyclooxygenase pathway (Funk, 2001). Cyclooxygenases are endogenous enzymes which exist in two isoforms a housekeeping enzymes, COX-1 (Vane et al., 1998) and an inducible enzyme, COX-2, expressed in patho-physiological condition such as inflammation due to pro inflammatory stimulus (Seibert et al., 1994; Needleman and Isakson, 1997; Nantel et al., 1999).
COX-2 is not only elevated in inflammatory conditions but also in many human malignancies (Eberhart et al., 1994). Reports by Shiotani et al., (2001) and Tiano et al., (2002) have shown that pharmacological inhibition of COX-2 reduces the formation of various tumours in animal models. PG is a major cyclooxygenase product at inflammatory sites where it contributes to increase in local blood flow, edema formation, and pain sensitization (Williams and Peck, 1977). Cyclooxygenase is the key enzyme that catalyses the two sequential steps in the biosynthesis of PGs from AA (Smith et al., 1991). COX-2 is regulated by LPS, IL-1β, TNF and active oxygen derivatives according to various cells (Martin-Sanz et al., 1998).

NO, like PG forms an important mediator of inflammatory processes (Moncada, 1999). NO is synthesized by nitric oxide synthase and three NOS
isoforms have been characterised: eNOS and nNOS, calmodulin dependent or constitutive, and calmodulin independent and inducible iNOS (Moncada et al., 1991). Reviews by Nathan, (1992), Moncada and Higgs, (1993) and Beckman and Crew, (1993) have revealed the multiple patho-physiological functions of NO. NO has many actions appropriate for a pro inflammatory agent, like, it is made by numerous cell types in sites of inflammation, it increases blood flow and vascular permeability, cell/tissue destructive abilities, and it may induce cyclooxygenase, cause pain, destroy certain protease inhibitors, and enhance production of IL-1 and TNF, and NADPH oxidase activity in myeloid cells (Magrin et al., 1992, Clancy and Abramson, 1995). NO production may result from the action of several substances including cytokines, immune complexes, bacterial products, and mechanical stress. NO production was determined by measuring the amount of nitrite in the culture media using Griess reagents (Na et al., 2004).

2.3.3 Role of cytokines in inflammation

Cytokines mediates and control immune and inflammatory responses and complex interactions between cytokines, inflammation and the adaptive responses helps in maintaining homeostasis, health, and well-being (Elenkov et al., 2005). Dysregulation of the pro versus anti inflammatory and Th1 versus Th2 cytokine balance results in common human diseases such as atopy/allergy, autoimmunity, chronic infections and sepsis.

The pro inflammatory cytokine TNF-α, controls inflammatory cell populations as well as mediates many of the other aspects of the inflammatory process. TNF-α is a potent inflammatory cytokine that is important for host defense and is expressed at sites of inflammation where it activates resident tissue cells, infiltrating immune cells and endothelial cells in surrounding blood vessels. The
significant role of TNF-α in chronic inflammatory disease is well established (Lin and Yeh, 2005), TNF-α has been implicated in the pathogenesis of many acute and chronic inflammatory conditions, including septic shock, rheumatoid arthritis, and inflammatory bowel disease (Feldmann et al., 1996; Dinarello 1997; van Heel et al., 2002). TNF-α production require tight control to avoid excessive inflammation, tissue damage, and toxicity. Moldawer, (2003) have reported NF-κB as an important inducer of TNF-α transcription, and activation of the p38 MAPK is required for stabilization and efficient translation of TNF-α mRNA.

IL-1 β is thought to be the primary cytokine and the main regulator of T cell proliferation during inflammation and is involved in the initiation and persistence of inflammation. IL-1β is synthesized as 31 kD precursors, proIL-1β which lacks biological effects. The active, mature IL-1β is produced upon cleavage of proIL-1β by a specific IL-1β-converting enzyme (ICE or caspase-1). IL-1β is a typical pro inflammatory cytokine and induces the expression of a variety of genes and the synthesis of several proteins that in turn induce acute and chronic inflammatory changes and can be provoked by infectious agents, products of activated lymphocytes and complement (Dinarello, 1996). TNF-α and IL-1β stimulate the production of secondary cytokine IL-6, which is a major inducer of acute phase proteins. Combination of IL-1β, TNF-α, and/or IFN-γ have often been found to be co-stimulatory (Sozzani et al., 1996; Proost et al., 1996). IFN-γ, the principal Th1 effector cytokine, has been shown to be crucial for the development of inflammation.

IL-10 is a Th2 cytokine also called as a cytokine synthesis inhibitory factor inhibits the LPS induced synthesis of a number of cytokines implicated in the regulation of T cells, including IL-1γ, IL-6, IL-8 and TNF-alpha (Firentino et al., 1991). Elevated plasma IL-10 levels are detected in septicemia (Marchant et al.,
1994) and the protective effects of IL-10 against sepsis, morbidity and mortality are well recognized, correlating in many cases with the inhibition of TNF-α production (Marchant et al., 1994; Pajkrt et al., 1997; Cartmell et al., 2003). IL-10 as immunosuppressive and anti-inflammatory cytokine has been well explained by Pestka et al., (2004) and inhibition of production of pro-inflammatory cytokines, including TNF-α, IL-6, and IL-12 were reported by Moore et al., (2001). Reverse-transcriptase-polymerase chain reaction (RT-PCR) has become one of the most widely applied techniques in biomedical research. The ease with which the technique permits specific mRNA to be detected and quantified has been a major asset in the molecular investigation of disease pathogenesis. Disease related imbalances in the expression of specific mRNAs can be sensitively and quantitatively determined by RT-PCR. Lee et al. (2008) found that CT20126 inhibited the expression iNOS, COX-2, cytokines like TNF-α and IL-1β and chemokines, which are NF-kB regulated inflammation-associated genes, in immune activated primary macrophages and human synoviocytes in vitro as well as in vivo animal models. NF-kB is an important transcription factor for the induction of various inflammation associated genes including iNOS, cytokines, and chemokines in response to LPS and TNF-α (Tak and Firestein, 2001; Ahmed et al., 2006).

2.3.4 MAP kinases in inflammation

Immune responses involve a number of cell types that functions as initiators, regulators, and effectors. These cells interact with and cross-regulate each other, and the target cells respond using signal transduction pathways to mediate gene expression and immune function. Many of the mediators of inflammation are regulated by MAPK pathways and downstream transcription factors. There are three major groups of MAP kinases in mammalian cells, viz ERK (Schaeff and Weber, 1999), the p38 MAP kinases (Han and Ulevitch, 1999) and the JNK/SAPK (Davis
In general, ERK are activated by growth factors and hormones, whereas both JNK and p38 MAPK are activated by environmental stress and inflammatory cytokines. The p38 MAP kinases are a family of serine/threonine protein kinases that play important roles in cellular responses to external stress signals. In general, p38 MAPK is activated by many stimuli, including cytokines, hormones, ligands for G protein-coupled receptors, and stresses such as osmotic shock and heat shock and elevated levels of these cytokines are associated with several chronic inflammatory diseases. p38 MAPK inhibitors have been shown to be efficacious in several disease models, including those for asthma and chronic obstructive pulmonary disease (COPD) (Adams et al., 2001).

### 2.4 Cancer

Cancer is a hyper proliferative disorder that involves morphological cellular transformation, dysregulation of apoptosis, uncontrolled cellular proliferation, invasion, angiogenesis, and metastasis (Hanahan and Weinberg, 2000). It has been established that cancer can be promoted and/or exacerbated by inflammation and infections. Lin and Karin, (2007) elaborated the link between cancer and inflammation. COX-2 has been shown to be associated in a wide variety of cancers, including that of the colon (Fournier and Gordon, 2000), lung (Hida et al., 1998), and breast (Harris et al., 2000) cancers. Celecoxib, a specific inhibitor of COX-2, has been shown to suppress colon carcinogenesis in animals (Reddy et al., 2000). Colorectal cancer represents a major public health problem accounting for over 1 million cases and about half a million deaths worldwide (Chau and Cunningham, 2006). Survival from colon cancer has been found to vary demographically and estimated to be 65 % in North America, 54 % in Western Europe, 34 % in Eastern Europe, and 30 % in India (Parkin et al., 2005).
The uncontrolled cell proliferation in malignancy could be due to an increased proliferation or decreased cell death. A block in apoptosis has been implicated in cancer (Barr and Tomei, 1994). Since cancer cells often have an immature phenotype representing a block in the normal differentiation pathway, restoration of the normal differentiation program in cancer cells appears to activate an apoptotic mechanism similar to the normal physiological process (Ohasi et al., 1992). The proposition that apoptosis is a discrete phenomenon that is fundamentally different from degenerative cell death or necrosis, is based on its morphology, bio-chemistry and incidence. A variety of anti cancer drugs have been shown to induce extensive apoptosis in rapidly proliferating normal cell population, lymphoid tissues, and tumors (Kerr et al., 1994; Chintamani et al., 2004).

2.4.1 Programmed cell death

Apoptosis or programmed cell death is a critical determinant of tissue mass homeostasis and may play a role in carcinogenesis. Apoptotic cell death occurs in two phases: an initial commitment phase followed by an execution phase which is characterized by a series of changes including cell shrinkage, plasma membrane alterations, and condensation and fragmentation of chromatin (Earnshaw, 1995). In the nucleus, chromatin is disrupted and endonucleases are activated, resulting in cleavage of DNA. Apoptotic death is known to involve a cascade of proteolytic events driven mainly by activation of family of aspartate-specific cysteine proteases (Martin and Green, 1995) such as caspase-3, the major executioner of apoptosis. Studies by Boldin et al., (1996), Muzio et al., (1996) and Sun et al., (1999), suggests that the activator caspase-8 is an integral component of the cell death-inducing mechanism and in receptor-mediated apoptosis, activation of caspase-8
represents a commitment point to cell death. Dimerization of the initiator caspases was proposed to be the driving force for their activation (Renatus et al., 2001; Boatright and Salvesan, 2003; Donepudi et al., 2003). Janicke et al., (1998) have shown that caspase-3 is required for certain distinctive biochemical and morphological changes during apoptosis and they have also demonstrated that caspase-3 is essential for fragmentation of MCF-7 cell chromosomal DNA during TNF induced apoptosis. Reyes et al., (2006) have reported caspase dependent DNA fragmentation leading to apoptosis of HT 29 cells. Agents that suppress the proliferation of malignant cells, by inducing apoptosis may represent a useful mechanistic approach to both chemoprevention and chemotherapy of cancer.

Marine natural products have been prominently featured in the area of cancer research mainly because of underlying preponderance of anti tumor agents produced by marine organisms. There are many compounds such as didemnin B (Rinehart et al., 1981), bryostatin 1 (Pettit et al., 1982), jaspamide (Crews et al., 1986), dolastatin 10 (Pettit et al., 1987), ecteinascidin 743 (Rinehart et al., 1990), curacin A (Gerwick et al., 1994) and thiocoraline (Baz et al., 1997), which were discovered using traditional screening methods.

2.5 Docking

Three-dimensional molecular structure is one of the foundations of structure-based drug design. Molecular docking and virtual screening based on molecular docking have become an integral part of many modern structure based drug discovery efforts. Docking is a term used for computational schemes that attempts to find the “best” matching between two molecules: a receptor and a ligand. The molecular docking can be defined as the prediction of correct bound association
between two molecules whose atomic co-ordinates are known. Molecular docking involves the prediction of ligand, conformation and orientation within the active site of the molecular target. Docking program has two key parts— a search for conformational degrees of freedom and the evaluation or scoring function. The search algorithm finds the potential energy landscape with sufficient details to explore the global energy minimum. In rigid docking, the search algorithm explores the different positions of the ligand in the receptor active site by translational and rotational degrees of freedom whereas in addition torsional degree of freedom is also included in flexible ligand docking. The scoring function gives importance to the experimentally determined complex which assesses both the steric and the chemical complementarities (Brooijmans and Kuntz, 2003). The fundamental goal of protein docking is to determine whether two molecules interact and if so, the orientation that maximizes this interaction as well as minimizing the total energy of the resulting complex.

2.5.1 Types of docking:

Molecular docking process can be broadly categorized into three different types:

2.5.1.1 Protein-Protein docking:

In these docking situations, the protein molecules are usually considered rigid bodies. Thus, one can approximate the problem with 6 degrees of freedom: the 3 possible translations and rotations in the x, y and z axes. To further reduce the search space of the problem, steric constraints (i.e. no overlap in van der Waal envelope, geometric constraints) are applied. Then energies of the resulting binding
confirmations are examined. In essence this type of docking consists of constructing
the 6 dimensional confirmation spaces of all geometrically/physically allowable
configurations, then finding the best configuration by comparing energy scores of
the resulting binding complexes.

2.5.1.2 Protein-Ligand docking:

This type of docking is much more complex because of the flexibility of
the ligand. The ligand introduces this complexity because of rotatable bonds that
exist within the molecule. As the name suggests, these bonds allow parts of the
molecule to rotate, thus increasing the dimensionality of the problem with each
rotatable bond. To model the complex protein-ligand docking, it is usually common
to either reduce the flexibility of the ligand or search the conformational space using
either Monte Carlo methods or molecular dynamics.

2.5.1.3 Blind docking

Blind docking was introduced for the detection of possible binding sites
and modes of peptide ligands by scanning the entire surface of protein targets. Blind
docking can be used for unbiased mapping of the binding patterns of drug candidates
using AUTODOCK (Hetenyi and van der Spoel, 2006)

2.5.2 Computational receptor docking studies

Docking simulations are widely used for lead enhancement, using models
to analyze the atomic interactions between inhibitors and target molecules. A
detailed three- dimensional 3D picture of interactions between the ligands and the
active sites of COX-1, COX-2 and p38 MAPK would help us to determine the mechanism of action.

2.5.3 Structure of COX-1 and COX-2

Molecular cloning and sequencing of pure preparations of COX-1 enzyme from sheep and bovine seminal vesicles by different laboratories elucidated the complete primary structure of COX-1 enzyme (Dewitt & Smith, 1988; Merlie et al., 1988; Yokoyama et al., 1988; Yokoyama and Tanabe, 1989). The predicted amino acid sequence of COX-2 cloned in chicken and mammals showed to possess approximately 60% amino acid identity with COX-1 (Simmons et al., 1991). COX-1 and COX-2 were found to be approximately 600 amino acid in size in all the species. Mammalian COX sequences are represented in Fig 3 and the domains and residues essential for their function are designated.

COX-1 has four distinct domains viz., amino terminal signal peptide, dimerization, membrane binding and catalytic domains (Fig 4). Crystallographic structure of COX-2 show striking similarity with COX-1 (Kurumbail et al., 1996, Bayley et al., 1999). The structures of both the enzymes COX-1 and COX-2 predict that both the enzymes are located in the lumen of the nuclear envelope and endoplasmic reticulum (Simmons et al., 2004).
### Amino acid sequences of human COX enzyme isoforms

Signal peptide sequences, potential glycosylation sites and some important residues are in bold. Dimerization and membrane binding domains are denoted with a heavy underline. All sequence downstream to dimerization domain 2 constitutes catalytic domain. (Adopted from Simmon et al., 2004)

#### 2.5.3.1 Amino-Terminal Signal Peptide

Nascent COX-1 and COX-2 polypeptides are directed into the lumen of the endoplasmic reticulum by amino-terminal signal peptides. The signal peptide for COX-1 is always 22 to 26 amino acids in length with a large hydrophobic core comprised of four or more leucines or isoleucines (Fig 3). COX-2 signal peptide is 17 amino acids long in all species and appears to be less hydrophobic. Immediately

<table>
<thead>
<tr>
<th>COX-1 Sequence</th>
<th>COX-2 Sequence</th>
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<tbody>
<tr>
<td>MRRLLLLL VRKSLFQK</td>
<td>MRRLLLLL VRKSLFQK</td>
</tr>
<tr>
<td>SGLVLVADP GAPTTPVNC</td>
<td>SGLVLVADP GAPTTPVNC</td>
</tr>
<tr>
<td>WYRCQGCH FVFRGLVQD</td>
<td>WYRCQGCH FVFRGLVQD</td>
</tr>
<tr>
<td>DCTRVQGSGP</td>
<td>DCTRVQGSGP</td>
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**Fig 3** Amino acid sequences of human COX enzyme isoforms.
following the signal peptide in COX-1 are eight amino acids that are not found in COX-2 (Fig. 3). The function of this sequence is unknown.

### 2.5.2.2 Dimerization Domain

COX-1 and COX-2 dimers are held together via hydrophobic interactions, hydrogen bonding, and salt bridges between the dimerization domains of each monomer. Heterodimerization of COX-1 and COX-2 subunits do not occur. The dimerization domain is encoded by approximately 50 amino acids near the amino terminus of the proteolytically processed protein (Fig 4). Three disulfide bonds hold this domain together in a structure reminiscent of epidermal growth factor. A fourth disulfide bond links the dimerization domain with the globular catalytic domain. The presence of disulfide bonds, which require an oxidizing environment, is consistent with the location of COX inside the lumen of the nuclear envelope, ER, or Golgi, which have redox states that are significantly more oxidized than cytosol.

![Diagram of functional domains of COX-1 and COX-2](image)

Fig 4 Diagram of functional domains of COX-1 and COX-2
2.5.3.3 Membrane Binding Domain

COX isozymes associate with the intra luminal surface of microsomal membranes in an unusual fashion. COX isozymes contain a tandem series of four amphipathic helices, which creates a hydrophobic surface that penetrates into the upper portion of the luminal side of the hydrophobic core of the lipid bilayer. These helices are encoded by approximately 50 amino acids found immediately to carboxy-terminal of the dimerization domain (Fig 3). The helices allow COX dimers to float on the surface of the lumen of the endoplasmic recticulum/nuclear envelope, with the majority of the protein protruding into the luminal space of these compartments. The membrane binding domain also forms the mouth of a narrow hydrophobic channel that is the cyclooxygenase active site.

2.5.3.4 Catalytic Domain

Carboxy-terminal to the membrane binding domain in COX primary structures is the catalytic domain, which comprises 80% (approximately 480 amino acids) of the protein and contains two distinct enzymatic active sites.

a. Peroxidase Active Site: The catalytic domain is globular with two distinct intertwining lobes. The interface of these lobes creates a shallow cleft on the upper surface of the enzyme, where the peroxidase active site is located and where heme is bound.

b. Cyclooxygenase Active Site: The cyclooxygenase active site is a long, narrow, dead-end channel of largely hydrophobic character whose entrance is framed by the four amphipathic helices of the membrane binding domain. The channel extends approximately 25 Å into the globular catalytic domain and is on average about 8 Å wide (Picot et al., 1994). However, significant narrowing of the
channel is observed where arginine 120, one of only two ionic residues found in the COX active site, protrudes into the channel and forms a hydrogen bonded network with glutamate 524 and tyrosine 355. Arg 120 is essential for binding substrates and carboxylate-containing NSAIDs in COX-1. In contrast, this residue is unessential in binding substrate in COX-2 (Rieke et al., 1999).

A crucial structural difference between the active sites of COX-1 and COX-2 is a substitution of isoleucine 523 in COX-1 for a valine in COX-2 (Fig 3). This single difference opens a hydrophobic out pocketing in COX-2 that can be accessed by some COX-2-selective drugs (Kurumbail et al., 1996). The evolutionary conservation of an enlarged cyclooxygenase active site in COX-2 relative to COX-1 may be essential to the recognition of bulkier substrates by COX-2.

2.5.4 Synthetic cyclooxygenase inhibitors, NSAIDs

NSAIDs are widely used for reducing pain and swelling associated with inflammation. The known NSAIDs can be broadly classified into four types as follows.

2.5.4.1 Irreversible inhibitors of COX-1 and COX-2 (aspirin)

Of the NSAIDs in medical use, only aspirin is a covalent modifier of COX-1 and COX-2. Like other NSAIDs, aspirin diffuses into the COX active site through the mouth of the channel and traverses up the channel to the constriction point formed by Arg 120, Tyr 355, and Glu 524. At this point in the channel, the carboxyl of aspirin forms a weak ionic bond with the side chain of Arg 120, positioning aspirin only 5 Å below Ser 530 and in the correct orientation for transacetylation (Leu et al., 1995).
2.5.4.2 Reversible, competitive inhibitors of both isoforms (mefenamate and ibuprofen)

Other NSAIDs besides aspirin inhibit COX-1 and COX-2 by competing with AA for binding in the COX active site. However, NSAIDs significantly differ from each other in whether they bind the COX active site in a time-dependent or independent fashion.

2.5.4.3 Slow, time dependent inhibitors of COX-1 and 2 (flurbiprofen and indomethacin)

Time-dependent NSAIDs bind the COX active site first in a loose interaction and then in a productive tight complex. The rate-limiting step in drug binding is the formation of the tight binding conformation of the NSAID within the COX channel. Second step in NSAID binding is the constriction point created by the hydrogen bonding network of Arg 120, Tyr 355, and Glu524 and the proposed difficulty for some NSAIDs to traverse it. The interactions results in tight binding of many NSAIDs at the constriction point of the channel, where they totally block entry of AA.

2.5.4.4 Selective inhibitors of COX-2 (SC-558 and Celecoxib)

The discovery of COX-2, which is expressed in inflammatory cells and central nervous system, but not in the gastric mucosa, offers the impetus to develop anti inflammatory and analgesic agents that is devoid of gastrointestinal toxicity where they spare mucosal prostaglandin synthesis. Celecoxib and rofecoxib were marketed in 1999 as the first NSAIDs developed as selective COX-2 inhibitors. Other NSAIDs including meloxicam, nimesulide, and etodolac, which were marketed earlier as safer NSAIDs, were found after the discovery of COX-2 to be
preferential inhibitors of this enzyme. Currently, second generation COX-2 inhibitors, such as valdecoxib (Smith and Baird, 2003) and etoricoxib (Hunt et al., 2003) are other selective COX-2 agents in use.

2.5.5 Cyclooxygenase isoenzymes in human diseases

Several COX products like PGE2, PGI2 have been found in the synovial fluid from knee joints of arthritic patients (Bombardier et al., 1981) and in inflammatory conditions in animal models (Portanova et al., 1996; Zhang et al., 1997; Rossi et al., 2000). COX-2 is primarily involved in the fever response to LPS (Zhang et al., 2003). Large amounts of PGs production mediated by COX-2 by certain tumour cells (Bennet et al., 1997) which induce a generalized state of immune deficiency (Plescia et al., 1975). COX-2 induction by tumour cells contributes to tumour angiogenesis and ultimately the growth of the tumours (Williams et al., 2000). COX-2 can be induced in neurons, microglia and astrocytes by a variety of neurotoxic stimuli including hypoxia and excitotoxins (Adams et al., 1996; Tocco et al., 1997; Tomimoto et al., 2000). Elevated COX-2 is found in neurogenerative diseases such as sporadic amyotrophic lateral sclerosis (Almer et al., 2001). A number of excellent reviews have explored the complex and yet unclear roles of COX enzymes in alzheimer’s disease (O’Banion, 1999; McGeer, 2000; Pasinetti, 2001).

2.5.5.1 COX-2 in inflammation

NSAIDs are currently used as first line therapeutics in the treatment of osteoarthritis (OA), rheumatoid arthritis (RA), systemic lupus erythematosis (SLS) and other inflammatory syndromes. Treatment of inflammation with NSAID is palliative than disease modifying. They reduce inflammation and pain and hence
COX-2 inhibitors are used as pain relievers. In 1980s, anti inflammatory drugs with less gastric injury were developed. Nimesulide, ecodolac and meloxicam emerged from pre clinical studies as less toxic drugs and later proved to be selective inhibitor of COX-2. Selectivity for the inducible isoform was established by comparing the inhibitory potency against COX-1 measured as IC$_{50}$ with inhibition of COX-2 in isolated enzymes, cultured cells or in the whole blood assay.

Nimesulide did not have any effect on gastric PG levels and did not cause bleeding in gastric mucosa. The therapeutic efficacy of nimesulide has been demonstrated in clinical trials for inflammation and pain (Huskinson et al., 1999; Bennett, 2001). However it was withdrawn in 2002 due to hepatic toxicity. Etodolac has a pyranocarboxylic acid structure showed anti inflammatory effect without gastric injury in a range of preclinical trials (Jones, 2001). The renal toxicity of etodolac was also demonstrated to be minimal (Shand et al., 1986). Meloxicam also emerged as anti inflammatory drug with low GI toxicity. Its chemical structure is that of an enolcarboxamide and has been reported as selective COX-2 inhibitor.

2.5.5.2 COX-2 in cancer and angiogenesis

Many human malignancies results in production of increased amounts of prostaglandins than in the normal tissues, a consequence of enhanced COX-2 expression (Eberhart et al., 1994; Sano et al., 1995). PGs are important in pathogenesis of cancer due to the effects on cell proliferation, angiogenesis, immune surveillance and apoptosis (Tsujii et al., 1998; Williams et al., 2000). Shiotani et al., 2001 and Tiano et al., 2002 reported that pharmacologic inhibition of COX-2 reduces the formation of various tumour in animal models, suggesting selective COX-2 inhibitors might be useful for prevention of cancer.
The role of COX products in the formation of new blood vessels, a process commonly referred to as angiogenesis (Hla et al., 1993) has received attention in recent years. The induction of angiogenesis by the COX derived PGE2 may be potentially involved in colon cancer (Tsujii et al., 1998; Hansen-Petrik et al., 2002) and the angiogenic factors like VEGF was modulated by COX-2 over expression. Hence use of COX-2 inhibitors may exert anti angiogenic effect.

2.5.5.3 COX-2 in Alzheimer’s disease

Epidemiologic evidence indicates that NSAID use is associated with a lower incidence of Alzheimer’s disease (McGeer et al., 1996; Hendrie, 1997). Proinflammatory cytokines, acute phase proteins, prostaglandins and other mediators of inflammation are elevated in and around the senile plaques present in Alzheimer’s disease brains (Oka and Takashima, 1997; Kitamura et al., 1999) at the same time it is important to note that COX-2 is also normally expressed in neurons of neocortex and hippocampus (Kemmann et al., 1996; Yasojima et al., 1999; Ho et al., 2001). However the normal function of COX-2 in brain neurons is not known. The current debate is whether COX-2 induction after neuronal insult serves to protect against cell death or promote apoptosis. Findings using animal models and in vitro systems support both protective (Kunz and Oliw, 2001) and pro apoptotic roles (Iadecola et al., 2001). Findings of Baik et al., (1999) suggests that inhibition of COX-2 induced by excitotoxins may be neuroprotective, but inhibition of constitutive COX-2 expression may lead to be deleterious. Many question remained to be answered regarding the use of selective COX-2 inhibitors in neurodegenerative diseases. The motivation for research in this area is, of course, to develop more selective anti inflammatory drugs, but recent research also points to possible applications for selective COX-2 inhibitors as drugs for cancer and Alzheimer’s disease (Vane and Botting, 1998).
Two major approaches are currently in use for discovery of lead molecules: one uses the chemical diversity of nature and the other applies combinatorial chemistry. Knowledge of the structural features that explain the differential inhibitory profile can be useful for the design of new selective COX-2 inhibitors. Nimesulide is one of the first NSAID marketed with a preferential COX-2 inhibition (Cullen et al., 1998). Analysis of the crystal structures of COX inhibitors (Picot et al., 1994; Kurumbail et al., 1996) reveals a network of hydrogen bonds involving Arg 120, Tyr 355, His 513 and Glu 524 that are thought to act as a gate for ligand entrance to the COX active site.

2.5.6 p38 MAPK structure and function

p38 have typical kinase fold with a small N-terminal domain dominated by β-strands, a larger C-terminal domain consisting of α-helices and an ATP binding site in the interface between the two domains. The ATP binding site is formed by the flexible glycine rich loop, that connects the N-terminal domain with the large α-helical domain and the catalytic loop. The kinase domains of p38 have a substrate binding groove which is at the interface of the N and C terminal domains. p38 has an additional docking groove for activating kinases, substrate kinases and inactivating phosphatases. The docking groove contains glutamate-aspartate and common docking regions and is conserved among MAP kinases. The p38 activation loop contain two phosphorylating sites (Thr 180 and Tyr 182) which when modified stabilize its activated conformation and open the substrate binding groove. p38 have a docking groove for binding with their activating kinases, inactivating phosphatases and substrates. This docking groove is different from the substrate binding groove at which the phosphorylation takes place. The p38 docking groove contains CD region is part of a shallow groove formed by the acidic residues Asp313, Asp316,Glu81 and the aromatic residues Phe 129 and Tyr 311. The phosphorylation lip in p38
consists of 13 residues; Gly 170 to Thr 185. p38 is activated by phosphorylation on Thr and Tyr within a Thr-X-Tyr motif where X is Gly or p38 and pro and Gly for ERK and JNK respectively (Wilson et al., 1996).

2.5.7 Role of p38 MAP kinase in diseases

2.5.7.1 Inflammatory disease

p38 kinase is involved in regulation of inflammatory cytokines and enzymes responsible for inflammatory mediators like COX-2, iNOS and MMP (Dean et al., 1999; Underwood et al., 2000). Many MAPK inhibitors have progressed to testing in clinical trials, however some of them failed due to safety and other reasons. Many of the p38 inhibitor work through inhibition of TNF-α production which in turn inhibits proinflammatory mediators such as COX-2 and iNOS (Lee et al., 1994; Foey et al., 1998).

2.5.7.2 Pulmonary disease

Inflammatory cytokines play an important role in airways inflammation. Cytokines such as TNF-α, IFN-γ, IL-4, IL-5 and chemokines such as IL-8 and RANTES have been shown to regulate or support chronic airway inflammation (Barnes et al., 1998). The production and action of these potential mediators have been shown to be dependent upon the p38 MAPK cascade. The major pulmonary diseases such as chronic obstructive pulmonary disease (COPD) and asthma may result from chronic hypoxia which is mediated by p38 MAPK pathway (Scott et al., 1998).
2.5.7.3 Neurodegenerative disease

ATF 2, a substrate for p38 MAP kinase is highly expressed in neurons (Martini-villalba et al., 1998), which play a vital role in neuronal development and survival. The expression and phosphorylation of ATF-2 increased with various neurodegeneration insults TNF-α and iNOS gene expression in LPS stimulated microglia and astrocyte correlated with activation of p38 (Bhat et al., 1998).

2.5.7.4 p38 and cancer

p38 MAPK is not only involved in regulation of cytokine expression but also plays important role in apoptosis, cell cycle regulation, proliferation, development and differentiation of immune cells (Aouadi et al., 2006; Khiem et al., 2008). Multiple myeloma is a B-cell malignancy where overproduction of IL-6, TNF-α and IL-1 can be observed. Nikas and Drogog, (2004) reported that SC10-469, a potential anticancer agent inhibited p38 MAPK activation and cytokine production in multiple myeloma cells. SC10-469 in combination with proteasome inhibitor enhanced the reduction in multiple myeloma cell proliferation and increased protease inhibition induced apoptosis. Pre clinical studies with p38 inhibitors have demonstrated significant efficacy in many diseases but most of them failed due to side effects. Hence there is a need for identification of newer class of compounds with specificity and less toxicity.