CHAPTER-II, SECTION-1

Introduction to small molecule biologically active ligands
2.1.1. Biologically active ligands

Biologically active ligands (Latin ligandum, binding) are molecules capable of binding with biomolecules to form complexes that affect or play vital roles in biological processes. These ligands through highly specific intermolecular forces (ionic bonds, hydrogen bonds, Van der Waals forces), bind to the active site/s of proteins, enzymes etc., thereby lead to activities like triggering of signals, biochemical transformations, mechanical motions, desired or undesired for the living cell. Within the biological systems, binding of ligand with the target molecule, known as docking (association), is usually a reversible reaction. Ligand binding alters the three dimensional shape of the receptor protein, thereby altering its conformational state. These changes in conformational state of a receptor lead to variations in its functional state that prove advantageous or disadvantageous for the organism as a whole. Depending upon the ultimate effect of the docking on the metabolic state of organism, ligands are regarded as inhibitors, activators or neurotransmitters.

The interaction of ligands with their binding sites is characterized in terms of strength/tendency of the receptor sites for their specific ligands, known as binding affinity. In general, high affinity ligand binding results from stronger intermolecular forces between the ligand and its receptor while low affinity ligand binding involves weak intermolecular forces. High affinity binding leads to longer residence time for the ligand at its receptor in comparison to that observed for low affinity binding.

A ligand with affinity to bind to a particular receptor and thereby altering its functions and triggering a physiological response is regarded as an agonist for the said receptor. Agonist binding to a receptor is characterized both in terms of extent of triggered physiological response and in terms of optimal concentration required to produce the physiological response. High-affinity ligand binding implies that ligand has high affinity for receptor sites and hence an optimum physiological response can be triggered at a relatively low concentration of the ligand. Low-affinity binding implies that a relatively high concentration of the ligand is required for maximal occupation of binding sites so as to achieve a maximum physiological response.

2.1.2. Small molecules as biologically active ligands

Therapeutically active ligands validate a target and provide lead structures for downstream drug development. Although most of the bioactive ligands are
macromolecules, there are examples of small molecules that have been found to act as extremely powerful tools for exploring and influencing biological processes. Such molecules referred to as small molecule biologically active ligands are low molecular weight organic compounds that bind with high affinity to a biopolymer such as protein, nucleic acid, or a polysaccharide and thereby alter its activity or function. Small molecules have a variety of biological functions, serving as cell signaling molecules, as tools in molecular biology, as drugs in medicine, as pesticides in farming, etc. These compounds can be natural or artificial and may show a beneficial effect against a disease or may prove detrimental to the organism.

A survey into published reports related to small molecule bioactive ligands indicate that a huge number of simple molecule formulations can be made use of to explore and control many life processes in living organisms. In this regard, tyrosinase inhibition and suppression of fungal growth are two examples which have been explored to investigate the impact of some small molecule bioactive ligands on life processes.

2.1.3. Biological role of tyrosinase and need for tyrosinase inhibitors

Tyrosinase, also known as polyphenol oxidase (PPO), is a glycosylated multifunctional copper-containing enzyme, widely distributed in nature. Its functions include monophenolase (cresolase) and diphenolase (catecholase) activity, besides it plays a key role in the biosynthesis of melanin. In fact, it is considered as rate limiting enzyme for melanin synthesis that catalyses the ortho-hydroxylation of tyrosine (monophenol) 1 to 3,4-dihydroxyphenylalanine or DOPA (o-diphenol) 2 and oxidation of DOPA to dopaquinone 3, through a mechanism that depends on the presence of copper atoms at its active site. Dopaquinone produced by tyrosinase is nonenzymatically converted to dopachrome 4, which is then acted upon by an isomerase producing dihydroxyindoles DHI 6, 7. Finally the oxidation and polymerization of these indoles through a series of enzymatic and non-enzymatic reactions leads to the production of melanin pigments (Figure 1). Nevertheless, it is the oxidation of tyrosine and DOPA that limits the overall rate of melanogenesis and hence the quantity of melanin synthesized in a cell is always proportional to the extent
of tyrosinase activity present in the cell. In view of the above mentioned functions, tyrosinase plays an important role in biological processes responsible for the racial skin pigmentation in living organisms. Besides above mentioned functions, tyrosinase has been reported to participate in several important reactions of host defence, wound healing, and sclerotization in insects and other arthropods. Encapsulation and melanisation of invading foreign organisms in insects are associated with an enzyme
cascade wherein tyrosinase acts as the terminal component. Melanin deposition at the damaged sites prevents the blood loss through wounds in insects. Tyrosinase in association with other enzymes generates reactive intermediates leading to cross-linking of structural proteins and chitin to form hardened cuticle, a process vital for the survival of insects and arthropods. All these reports establish that tyrosinase is one of the most important enzymes in the process of molting in insect and hence tyrosinase inhibitors can be used as alternative insect control agents.

Tyrosinase activity causes undesired enzymatic browning of farm products, such as bruised or cut fruits and vegetables, resulting in significant loss in their nutritional and market values. Therefore, designing novel tyrosinase inhibitors is also receiving a considerable attention from food technologists and processors.

In animals, tyrosinase through its impact on synthesis of melanin pigments produced by melanocytes and their deposition in the epidermis plays a key role in determining the skin colour. Disturbances in the amount and distribution of melanin pigments in some cases provides clues about diseases. For example, albinism, a genetic abnormality caused by deficiency in melanin biosynthesis, manifests as hypopigmentation of the skin, hair and eyes. Hypopigmentation in the skin is associated with sensitivity to UV radiation and predisposition to skin cancer. Ultraviolet radiation, chronic inflammation, and release of abnormal α-melanocyte stimulating hormone (α-MSH) are well known triggering factors for hyperpigmentation and inflammatory pigmentation including melasma, freckles, age spots, actinic damage and senile lentigines. Tyrosinase inhibitors therefore can be very useful for the treatment of some dermatological disorders associated with melanin hyperpigmentation. All these reports make the studies aimed at the design and development of novel tyrosinase inhibitors and exploration of their mode of action quite demanding. Such studies seem very informative for understanding the regulation of pigmentation patterns in mammals and are presaged to be very helpful in search for a proper cure to many dermatological disorders in mammals. Such studies can also provide promising ways for design of alternative insect control agents and other products useful for food technology and food processing.
Routes of tyrosinase inhibition

Experimentally it has been proved that presently known inhibitors follow different routes for expressing their tyrosinase inhibitory activity. Some common routes in this regard are:

a) Reducing agents causing chemical reduction of dopaquinone such as ascorbic acid, which is used as a melanogenesis inhibitor because of its capacity to reduce back o-dopaquinone to DOPA, thus avoiding dopachrome and melanin formations.

b) o-Dopaquinone scavenger such as most thio-containing compounds, which are well-known melanogenesis inhibitors, react with dopaquinone to form colorless products. The melanogenetic process is therefore slowed until all the scavenger is consumed, and then it goes at its original rate.

c) Alternative enzyme substrates such as some phenolic compounds, whose quinoid reaction products absorb in a spectral range different from that of dopachrome. When these phenolics show a good affinity for the enzyme, dopachrome formation is prevented, and they could be mistakenly classified as inhibitors.

d) Nonspecific enzyme inactivators such as acids or bases, which nonspecifically denature the enzyme, thus inhibiting its activity.

e) Specific tyrosinase inactivators such as mechanism-based inhibitors, which are also called suicide substrates. These inhibitors can be catalyzed by tyrosinase and form covalent bond with the enzyme, thus irreversibly inactivating the enzyme during catalytic reaction. They inhibit tyrosinase activity by inducing the enzyme catalyzing “suicide reaction.”

f) Specific tyrosinase inhibitors which bind reversibly to tyrosinase and reduce its catalytic capacity.

Working on these leads, researchers so far have been able to discover and design a huge library of small molecule bioactive ligands as tyrosinase inhibitors.

2.1.3.1. Tyrosinase inhibitors from natural sources

Several tyrosinase inhibitors such as hydroquinone, ascorbic acid derivatives, azelaic acid, retinoids, arbutin, kojic acid, metallothionein and others have been identified from several organisms, ranging from fungi to higher plants. Among the naturally
existing tyrosinase inhibitors, kojic acid (9) (5-Hydroxy-2-hydroxymethyl-4H-pyran-4-one) is an antibiotic that is produced by many species of *Aspergillus* and *Penicillium*. It is a good chelator of transition metal ions such as Fe$^{3+}$ and Cu$^{2+}$ and is a safe and mild agent for treating hyperpigmentation disorders.\textsuperscript{14} It has been extensively used as a cosmetic agent with skin-whitening effect.\textsuperscript{14} Safety is a primary consideration for tyrosinase inhibitors, especially for those to be used in food and cosmetic products, which may be utilized in unregulated quantities on a regular basis. Hence, inhibitors from natural sources which are considered to be safe and largely free from adverse side effects, have great potential use in the food industry. However, while some inhibitors from natural sources like aromatic aldehydes such as cinnamaldehyde and benzaldehyde are generally recognized as safe, others such as (2E)-alkenals were reported to exhibit potent mutagenic activity.\textsuperscript{13}

### 2.1.3.2. Tyrosinase inhibitors with synthetic origins

A number of tyrosinase inhibitors, \textit{e.g.}, captopril (10), tropolone (11), and hydroxylamines, have been synthesised till date.\textsuperscript{13} Captopril [(2S)-\textit{N}-(3-Mercapto-2-methylpropionyl)-L-proline] is a drug widely used in the treatment of hypertension and heart failure through its inhibitory effect on the angiotensin-converting enzyme. This drug shows irreversible noncompetitive inhibition on the monophenolase activity of mushroom tyrosinase, while exhibiting irreversible competitive inhibition for the diphenolase activity of tyrosinase.\textsuperscript{13} Captopril can also interact with the enzymatically generated \textit{o}-quinone giving rise to a colourless conjugate. Inhibition of both monophenolase and diphenolase activities of tyrosinase by captopril shows positive kinetic co-operativity which arises from the protection of both substrate and \textit{o}-quinone against inhibition by captopril.\textsuperscript{15} Captopril is a known copper chelator; therefore, it is reasonable to think that captopril mainly exerts tyrosinase inhibitory effect by chelating copper ions at the active site of enzyme. In addition, the inhibitory process may involve a disulfide interchange reaction between captopril and cysteine-rich domains at the active site of the enzyme. Among all the inhibitors assayed till date, tropolone (2-Hydroxy-2,4,6-cycloheptatriene) is one of the most potent tyrosinase inhibitors. It is structurally analogous to \textit{o}-diphenolic substrates of tyrosinase, as well as an effective copper chelator. Slow-binding inhibition is characterized by the nonimmediate response of enzymatic reaction to the presence of
inhibitor, which assumes the formation of an enzyme-inhibitor complex that undergoes slow and favorable isomerisation. The equilibria between enzyme, inhibitor and enzyme-inhibitor complexes occur slowly in the steady-state time scale.

Figure 2: Chemical structures of selected tyrosinase inhibitors
In addition to the standard tyrosinase inhibitors, a huge number of new inhibitors including polyphenols, flavonols, flavones, flavanols, flavanones, isoflavones, stilbenes, bibenzyl derivatives, coumarins, chalcones, benzaldehyde and benzoate derivatives, long-chain lipids and steroids (Figure 2).

Among all the above studied small molecule biologically active ligands for tyrosinase inhibition, stilbenes have been found to more potent.

2.1.3.3. Stilbenes as small molecule tyrosinase inhibitors

Stilbenes are C6 (aromatic)-C2-C6 (aromatic) compounds that are found in nature as monomers and oligomers. A number of naturally occurring stilbenes with polyoxyoxygenation have been reported to possess tyrosinase inhibitory activity. They are classified depending upon the degree of oxygenation as di-, tri- and tetra-oxyoxygenated stilbenes. So far, only one dioxygenated stilbene, i.e., pinosylvin (17) has been studied for its tyrosinase inhibitory potential. It has been found to show weak activity, probably due to the lack of 4-alkyl resorcinol structure. In the group of tri-oxyoxygenated stilbenes, resveratrol (3,5,4'-Trihydroxy-trans-stilbene) (18) represents the simplest structure, with free phenolic groups at positions 4', 3 and 5. It has shown stronger DOPA oxidase inhibitory activity than kojic acid. A variety of trans-resveratrol derivatives are known to be abundantly distributed in the plants belonging to Dipterocarpaceae, Vitaceae, Gnetaceae, Leguminosae and Cyperaceae. Some of their derivatives have also been reported to possess various biological and pharmacological activities such as anti-carcinogenic and anti-inflammatory effects. Therefore, stilbene derivatives composed of resveratrol are considered to be useful resources for pharmacological agents. Among tetra-oxyoxygenated stilbenes, oxyresveratrol (2,4,3',5'-Tetrahydroxy-trans-stilbene) (19), in view of its IC$_{50}$ value is claimed to display a nine-fold stronger inhibitory effect on mushroom tyrosinase than resveratrol. Kim et al., have demonstrated that oxyresveratrol from Morus alba Linne show a potent inhibitory effect on tyrosinase activity as a non-competitive inhibitor. Following oxyresveratrol, another three hydroxystilbenes have been purified and identified as potent tyrosinase inhibitors. Thus while chloroporin (4-Geranyl-3,5,2',4'-tetrahydroxy-trans-stilbene) (20) isolated and purified from the heartwood of Chlorophora excels displayed 14.8-fold inhibitory activity than that of kojic acid against diphenolase of mushroom tyrosinase, gnetol (2,3',5',6-Tetrahydroxy-trans-...
stilbene) \((21)\), a naturally occurring compound isolated from the roots of \textit{Gnetum gnemon}, exhibited 30-fold more diphenolase inhibitory activity for murine tyrosinase than that shown by kojic acid.\textsuperscript{16} Recently, piceatannol \((3,4,3',5'-\text{Tetrahydroxy-trans-stilbene}) \((22)\), isolated from grapes and red wine, has been found to exhibit 32.7 times stronger antimonophenolase activity than kojic acid towards mushroom tyrosinase.\textsuperscript{16} Besides so much of studies like these, the detailed mechanism for tyrosinase inhibition by stilbenoid moieties remains far from being clearly understood. Recently it has been found that the phenolic hydroxy groups and \textit{trans}-olefin structure of the parent stilbene skeleton contribute to the inhibitory potency of hydroxystilbene for tyrosinase inhibitory activity.\textsuperscript{7} Though all the naturally existing hydroxystilbenes possessing potent antityrosinase activity are \textit{trans}-isomers, recently, Song \textit{et al.}, from their studies\textsuperscript{18} concluded that the diphenolase inhibitory capacity on mushroom tyrosinase by \textit{cis}-3,5-dihydroxystilbene was stronger than that by its corresponding \textit{trans}-isomer. The justifications proposed for the explanation of these results seem unsatisfactory. Similarly, in one of the studies Ohguchi \textit{et al.},\textsuperscript{7} have demonstrated that dihydrognetol, a gnetol analog, which loses the double bond between the two aromatic rings, exerts a greatly lowered inhibitory effect on diphenolase activity of murine tyrosinase, thus highlighting the critical role of double bond in the stilbene skeleton for tyrosinase inhibition. Attempts to prepare tyrosinase inhibitors with other types of stilbene-related structures have been made. Recently, hydroxy-2-phenylnaphthalenes \((23)\), which are isosteric with resveratrol and oxyresveratrol respectively, were synthesized and found to be potent tyrosinase inhibitors.\textsuperscript{16}

2.1.4. Fungi and small molecule ligands as antifungal agents

As the sister group of animals and part of the eukaryotic crown group that radiated about a billion years ago, the fungi constitute an independent group equal in rank to that of plants and animals. The organisms of the fungal lineage include mushrooms, rusts, smuts, puffballs, truffles, morels, molds and yeasts, as well as many less well-known organisms. While presence of 1.5 million species of fungi is estimated, more than 70,000 species of fungi have been described so far.\textsuperscript{19} Within the fungal group a large diversity in habitat preference is observed. While some fungal species feed on dead and decaying organisms (saprophytic fungi), others are found inside or on a living host (biotrophic). Fungi have fundamental functions in terrestrial ecosystems, in
degradation of organic matter and in nutrient uptake of plants through mycorrhizal interactions. The interaction between the fungus and the environment can be very diverse, ranging from beneficial (symbiotic) to harmful (pathogen). Therefore, from a human perspective, there are both good and bad fungi. Among the good ones, some are delicious and edible, some are used in production of food like soy sauce, tempeh and bread and some serve as a source of important drugs like penicillins, cholesterol-lowering lovastatin and cyclosporins, drugs which counteract the rejection of transplanted organs. On the downside, fungi cause enormous economic loss in agriculture and food industry by destroying crops and plants in the field and during storage.

Although number of different kinds of fungi is vast, only a small subset is capable of infecting humans. The following is a very general breakdown of types of fungal infections that occur based on site of infection: Cutaneous (Athlete’s foot’, Ringworm, and Tinea cruris), mucocutaneous (Candida albicans), pulmonary/systemic (Invasive Aspergillus, cryptococcal meningitis, pulmonary histoplasmosis; also, systemic candidiasis).

Fungal infection can occur in two ways. Some fungi, such as Candida, are usually found in the bodies of healthy people and cause little or no harm. However when the immune system is weak, these fungi begin to grow and cause infection. Other mode of fungal infection is when the fungus is either inhaled into the lungs or comes into contact with an operated wound. Fungi, such as Aspergillus and Cryptococcus, are found in the air and can get inhaled into lungs or come in contact with blood supply at place of wounds where blood is exposed to environment. Thus an exposure to A. fumigatus in immunocompromised individuals can lead to aspergillosis, a pulmonary infection.

**Antifungal agents**

Since fungal infections are caused by eukaryotic organisms, these infections generally present more difficult therapeutic problems than bacterial infections do. In many cases fungal infections prove difficult to be treated (often referred to as ‘stubborn’), even when the offending organism is identified and appropriate therapy is applied. It is because of these reasons that compared to the repertoire of antibiotics used in the management of bacterial diseases; far fewer drugs are currently available for
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treatment of fungal infections.\textsuperscript{20} A survey into the published reports indicates that despite recent progresses in the management of invasive fungal infections, the incidence of fungal infections is increasing and response rates to first-line therapy remains insufficient.\textsuperscript{21} In absence of suitable drugs and problems associated with therapeutic treatment, fungal infections (mycoses) though not as frequent as bacterial or viral infections, are emerging as a major cause of morbidity and often mortality in immunocompromised and debilitated patients\textsuperscript{22}. Though sound agriculture and silviculture practices \textit{e.g.}, maximum use of suppressive soils, crop rotation and removal of potential inocula, can minimize the spread of fungal pathogens to some extent, the overall impact of such practices proves to be very limited. Hence to combat fungal pathogens, a wide array of antifungal agents that include inorganic and synthetic organic compounds, antifungal antibiotics and compounds based on natural products have been tested and some of them clinically approved. The antifungals are classified by structure or mechanism and not by site of action, as some of them may be used, for example, either topically or systemically depending on the type of infection. The major classes of antifungals currently in use are:

\textbf{Polyenes}

It has been shown that few polyenes bind to ergosterol present within the fungal cell wall membrane and thereby disrupting its permeability by forming oligodendromes functioning as pores that lead to subsequent efflux of potassium and intracellular molecules causing fungal death. Amphotericin B (AMB) (\textsuperscript{29}), the “gold standard” in the antifungal armamentarium and nystatin (\textsuperscript{30}) are the currently available polyenes (\textbf{Figure 3}).\textsuperscript{23} Before the development of alternative agents, AMB was the recommended first-line agent for invasive candidal infections. Prolonged use of AMB in AIDS patients induces drug resistance in fungal strains, a common cause of death in these immunosuppressed patients. It has also been reported that prolonged use of AMB can induce disseminated candidiasis in humans. Moreover it may produce adverse effects, such as fever, nausea, hypokalemia, nephrotoxicity, hepatotoxicity, leukocytopenia, thrombocytopenia, anaemia and chills.

\textbf{Triazoles}

A thorough literature survey reveals that triazoles exert their effects within the fungal cell membrane.\textsuperscript{23} The inhibition of cytochrome P450 (CYP)-dependent 14a-
demethylase prevents the conversion of lanosterol to ergosterol. This mechanism results in the accumulation of toxic methylsterols and resultant inhibition of fungal cell growth and replication. Cross-inhibition of several human CYP-dependent enzymes (3A4, 2C9, and 2C19) is responsible for most of the clinical side effects and drug interaction profiles that have been associated with this class. In addition, these compounds are found to be embryotoxic and teratogenic. Some of the prescribed drugs from this class are:

i) Fluconazole (31) remains one of the most frequently prescribed triazoles because of its excellent bioavailability, tolerability, and side effect profile. Fluconazole is active against most Candida species with the exception of C. krusei and C. glabrata isolates.

ii) Itraconazole (32) is currently available as capsules and as an oral solution suspended in hydroxypropyl-β-cyclodextrin (HPCD). The most frequent of its side effects include: nausea and vomiting (<10%), hypertriglyceridemia (9%), hypokalemia (6%), liver enzyme elevations (5%), etc.

iii) Voriconazole (33) exhibits a broad spectrum of activity against molds with the exception of the Zygomycetes.23 Voriconazole has become the drug of choice for most cases of invasive aspergillosis. Well-known side effects of voriconazole therapy include skin rash and transaminase elevation.

Echinocandins

Echinocandins (caspofungin, micafungin, anidulafungin) are synthetic compounds that inhibit the synthesis of β-1,3-glucan, by inhibiting the activity of glucan synthase. This mechanism impairs cell wall integrity and leads to osmotic lysis.23 Their clinical use is primarily limited to Candida species and Aspergillus species. Caspofungin was the first available agent of this class.

Oximes

Since last few decades oximes and their derivatives have been attracting a considerable attention on account of their chemotherapeutic value. A large number of oximes and their derivatives (34, 35) with lesser side effects have been developed as anti-microbial agents.24
Other antifungal agents currently in use include: imidazoles, antimetabolites, allylamines, thiocarbamates, fluoropyrimidines etc.\textsuperscript{25}

Though trials with natural product based antifungal have demonstrated remarkable success in combating fungal pathogens, synthetic efforts still remain the mainstream in antifungal drug discovery, as evidenced by the fact that 18 out of 23 antifungal drugs approved from 1980 to 2002 are synthetic (83% belong to the single azole class). The number of agents available to treat fungal infections has increased by 30% since the year 2000, yet still only 15 agents are currently approved for clinical use.\textsuperscript{23}

Though many of the antifungal agents can keep fungal infections at an acceptable level, there are always some concerns regarding their therapeutic use. Many of the currently available drugs are toxic, produce recurrence because they are fungi static and not fungicidal or lead to the development of resistance among fungal strains.
especially after the prolonged periods of administration of the available antifungal
drugs. The usage of most antimicrobial agents is limited, not only by the rapidly
developing drug resistance, but also by the unsatisfactory status of present treatment
of bacterial and fungal infections and drug side effects. Antimicrobial
chemotherapy, including the treatment of infections associated with AIDS, cancer,
and organ transplantation, places humans at risk for acquiring opportunistic fungal
infections. Although a new generation of triazoles, polyenes, echinocandins or the
combination therapy have been introduced as alternatives in the last ten years, fungal
infections still remain difficult to eradicate. These above mentioned limitations
associated with currently available antifungal drugs, increased incidence of systemic
fungal infections and rapid development of drug resistance have set the need for the
discovery of new antifungal agents. Like bacteria, fungi have unique characteristics,
distinct from their mammalian hosts; such features provide important clues for the
design of highly selective therapeutic drugs. In this regard the fungal cell wall whose
chitin structure differentiates it from human cells, can serve as a prime target site for
the newly designed selectively toxic antifungal agents.

2.1.5. Concluding remarks
Inspired by the literature reports related to tyrosinase inhibitors and antifungal agents,
we aimed to design small molecule bioactive ligands with potential to act as tyrosinase
inhibitors or antifungal agents. In the current section of thesis we present a detailed
account of our studies related to design of small molecule stilbene derivatives and
oxime derivatives and biological investigations related to their use as tyrosinase
inhibitors and antifungal agents respectively.
2.1.6. References


