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INTRODUCTION

1.1 Mono-oxygenase enzyme with special reference to CYP BM3

Cytochrome P450 is a very large and diverse superfamily of heme-thiolate containing proteins (Werck-Reichhart and Feyereisen 2000), found in all forms of life, and currently these enzymes are numbering more than 7700 (Nelson 2007). Cytochromes were initially identified in 1884 by MacMunn, as respiratory pigments. After about four decades, in 1920s, Keilin coined the term Cytochrome, means the cellular pigments. Specifically, P450 monooxygenases were first discovered in rat liver microsomes in 1955 (Axelord J 1955, Brodie B et al 1955, Klingeberg 1958). These monooxygenases activate molecular dioxygen and subsequently insert a single atom of molecular oxygen into an organic molecule or substrate (Ortiz de Montellano PR, ed. 1995, Omura T 1999, Raunio 2001, Denisov et al 2005, Bernhardt 2006).

\[
P450 \\
\text{RH} + \text{O}_2 + \text{NAD(P)H} + \text{H}^+ \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{NAD(P)}^+
\]

Cytochrome P450 monooxygenases are essential for steroid biosynthesis, catabolism of drugs, utilization of carbon compounds as an energy source in bacteria, and in production of various macrolide antibiotics (Duport C et al 1998). P450 monooxygenases originally belonged to two major classes on the basis of their protein components, class I and class II (Degtyarenko KN 1995). Mitochondrial and most of bacterial P450 classified under class I (figure 1.1), having three components i.e. an FAD containing flavoprotein, an iron sulfur protein and the P450 while eukaryotic or microsomal P450 systems come under class II (figure 1.2) i.e. contain two components, NADPH: P450 reductase (a flavoprotein containing both FAD and

Although P450 BM-3 is of bacterial origin, the amino acid sequence of the heme domain has a greater sequence similarity to eukaryotic (fatty acid hydroxylase) P450s than to bacterial Class I P450s. CYP450BM3 from *Bacillus megaterium* contains a P450-heme domain of 54 kDa and an FAD/FMN-reductase domain of 64 kDa on a single polypeptide chain (Narhi LO and Fulco AJ 1986, Ruettinger RT, Wen LP, Fulco AJ 1989, Munro AW and Lindsay JG 1996) and it has been classified as a class II P450 enzyme, together with the microsomal eukaryotic cytochrome P4503A4 (Ravichandran KG et al 1993).

Figure 1.1 Class I type associate electron carrier domains of bacterial or mitochondrial P450 (Degtyarenko KN 1995).

Figure 1.2 Class II type associate electron carrier domains of P450 3A4 (microsomal) P450 BM3 (Degtyarenko KN 1995).
CYP450BM3 monooxygenase is of great interest for chemical and pharmaceutical industry, because of high potential as catalyst for the selective incorporation of molecular oxygen at various substrates level in a region or sterio specific manner (Werck-Reichhart et al 2000, Peters MW et al 2003, and Ortiz de Montellano 2005).

1.2 History of CYP450 BM3

First evidence of a water soluble P450 enzyme from a soil bacterium *Bacillus megaterium* was reported by Miura and Fulco (Miura and Fulco 1974, Miura and Fulco 1975). About ten years later Narhi and co-workers were able to isolate the respective P450 enzyme (Narhi and Fulco 1986). It was the third P450 enzyme isolated from *Bacillus megaterium*, and was therefore called P450 BM-3. In the following years it could possible not only to express the holoenzyme in *E.coli* but also the discrete domains (Wen and Fulco 1987, Li Darwish et al 1991). In 1993, (Ravichandran et al 1993) the crystal structure of the monooxygenase domain of CYP450BM3 was solved, which was helpful in modeling of structure of many microsomal P450s. (Ravichandran et al 1993, Lewis et al 1996, Lewis et al 1999) In the following years wild type enzyme and several mutants of CYP450BM3 were crystallized with and without substrate (Haines and Tomchick et al 2001, Ost et al. 2001, Joyce et al 2004 Li and Poulos 2004). It was a big step forward in understanding the associate molecules responsible for electron transfer system in CYP450BM3 (Sevrioukova et al 1999, Guengerich FP 2005).

1.3 Why CYP BM3

At present, CYP4503A4 enzyme is mainly isolated from microsomes, hepatic cells or rejected blood from donor, and from recombinant bacterial cells. Recently, FDA has put restriction in obtaining CYP450s from human and animal sources due to the possibility of contamination of unwanted proteins. Because of its animal and human origin the cost of isolation, extraction and purification is very expensive, and as a result it is not affordable for common people. Fortunately, CYP450BM3 found in a soil bacterium, *Bacillus megaterium* (Miura and Fulco 1975, Narhi and Fulco 1986, and Ruttinger et al 1989) is homologous to CYP4503A4, which is of great medical and pharmaceutical interest (Narhi and Fulco 1986, Boddupalli et al 1990, Munro et al 1995, Munro and Lindsay 1996, Munro et al 2000, Lewis and Hlavica 2000). So, CYP450BM3 enzyme is used as a substitute of CYP4503A4 for treatment of patients.
suffering from various life threatening diseases (Vugt Lussenberg BM et al 2007). CYP4503A4 enzyme is mainly used in drug detoxifications that are used to cure the patient suffering from drug resistant, hypertension, anxiety, and arrhythmia (Otey CR et al 2006). At present, only few global players are in international market and selling four type of major P450s i.e. 3A4, 1A2, 2D6, 2C19 in different brand name (Table 1.1). These P450s metabolize 90% of total drugs and among these 55% of drugs are metabolized alone by CYP3A4 (Guengerich FP 1990).
<table>
<thead>
<tr>
<th>Name of company</th>
<th>Nature of product</th>
<th>Brand</th>
<th>Patent</th>
<th>Price of different P450 (per vial) in Rs.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphioxus Cell Technologies, Houston, Texas</td>
<td>Human Cytochrome P450 like CYP1A2, CYP3A4, P-Glycoprotein</td>
<td>Active Tox</td>
<td></td>
<td>16,280, 16,120</td>
</tr>
<tr>
<td>Astex Technology Ltd., Astex Therapeutics, Cambridge, UK.</td>
<td>Making of Human Cytochrome P450 3A4, P450 2C9,</td>
<td>Astex</td>
<td>GB2395718</td>
<td>16,500, 20,130</td>
</tr>
<tr>
<td>Astellas Pharma Inc. Japanese (formed after merger of Yamanouchi pharmaceutical Co.Ltd. and Fujisawa pharmaceutical Co. Ltd.)</td>
<td>P450 3A4, 2C9</td>
<td>Astex</td>
<td>WS727713</td>
<td>24,750, 16,120</td>
</tr>
<tr>
<td>Codexis Biotechnology Ltd (created in 2002 on the basis of enzyme optimization technology from Maxygem) Redwood city, CA.</td>
<td>Manufacturer of biocatalysts, also linked to biofuels area, human cytochrome biocatalysts (HCB) screening kit, Micro CYP screening plates (collection of bacterial enzymes, variants of Bacillus megaterium P450s)</td>
<td>Codex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pfizer Pharmaceuticals Ltd. Prague, Czech Republic.</td>
<td>Covering use of crystal structure of human CYP450 3A4</td>
<td></td>
<td>GB2395718</td>
<td>16,770</td>
</tr>
<tr>
<td>Roche Diagnostics, Basel, Switzerland.</td>
<td>Roche Amplichip Cytochrome P450, Cytochrome p450 2D6, 2C19, Medicines for cancer, that are FDA approved.</td>
<td>Roche Amplichip</td>
<td></td>
<td>20,570, 20,130</td>
</tr>
<tr>
<td>Sanofi-Aventis Pharma Ltd. Bridgewater, NJ.</td>
<td>Human CYP 450 3A4, 2D6 working on cardiovascular diseases, oncology, thrombosis, products including Ambien, Taxotere, Eloxatin</td>
<td>Sanofi-Aventis</td>
<td>GB2395718</td>
<td>17,230, 20,540</td>
</tr>
<tr>
<td>Affymetrix Ltd. Santa Clara, California, US.</td>
<td>CYP450 gene chip, role of 2D6 and 2C19 in drug metabolism, manufacturer of DNA microarrays</td>
<td>Affymetrix</td>
<td></td>
<td>20,570</td>
</tr>
<tr>
<td>TNO Pharma Ltd. Netherlands.</td>
<td>Working on human hepatocytes as a model for drug testing, CYP mediated drug metabolism, characterization of CYP450 enzymes by using pig hepatocytes.</td>
<td>TNO Pharma</td>
<td>CA2475230</td>
<td></td>
</tr>
<tr>
<td>AstraZeneca (Anglo-Swedish pharmaceutical company), London, England</td>
<td>CYP450 human crystal structures used to optimize some chemical compounds like certain drugs, working in area of drug development</td>
<td>AstraZeneca</td>
<td></td>
<td>13,890</td>
</tr>
</tbody>
</table>

Table 1.1 Global players involved in manufacturing different CYP450s.
The CYP450BM3 enzyme structure can also be engineered on the basis of its requirement for metabolism of specific drugs (Peters MW et al 2003, Urlacher VB and Eiben S 2006, Di Nardo G et al 2007). The CYP450BM3 has 52% amino acid sequence homology with human CYP4503A4 (Munro AW et al 2002). The domain character of this bacterial cytochrome is very much identical to that of the microsomal cytochromes (Li HV, Darwish K and Poulos TL 1991). The CYP450BM3 is a cytosolic in nature, and is easily separable as compared to membrane bound microsomal cytochromes. One more interesting feature of this enzyme is its water soluble nature (Narhi LO and Fulco AJ 1987), and as a result it can be isolated and purified in much higher amounts. Interestingly, the substrate conversion efficiency of CYP450BM3 is about 1000 times more then human CYPs, till today (Nobel et al 1998, Di Nardo G et al 2007). Quite a good number of literatures are available on drug metabolism by CYP4503A4 (Table-1.2).
<table>
<thead>
<tr>
<th>Name of Drug</th>
<th>Use</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cimetidine</td>
<td>Histamine H2-receptor antagonist</td>
<td>Whitney, Jake 2006.</td>
</tr>
<tr>
<td>Clozapine (also CYP1A2)</td>
<td>Antipsychotic used in treatment of Schizophrenia</td>
<td>Meltzer HY 1997.</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>Antibiotic</td>
<td>Ray WA et al. 2004</td>
</tr>
<tr>
<td>Flutamide</td>
<td>Antoandrogen drug</td>
<td>Chrousos, George P et al 2001</td>
</tr>
<tr>
<td>Imipramine</td>
<td>Antidepressant</td>
<td>Mayo 2001</td>
</tr>
<tr>
<td>Proquanil</td>
<td>Antimalarial</td>
<td>Kevin SG et al 2007</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>Immunosuppresor</td>
<td>Ponner B and Cvach B 2005</td>
</tr>
<tr>
<td>Trimethadone</td>
<td>Anticonvulsant</td>
<td>Teratology and Drug Used During Pregnancy 2007.</td>
</tr>
<tr>
<td>Zonisamide</td>
<td>Anticonvulsant</td>
<td>Dainippon Sumitomo Pharma Co., Ltd. 2005</td>
</tr>
</tbody>
</table>

Table 1.2 Metabolism of different type of drugs by CYP4503A4, mostly derived from hepatic cell culture and liver microsomes of human beings and pigs.
1.4 Spectral properties

Monooxygenases (CYP450) are named on the basis of absorption maximum at 450 nm. (Omura T and Sato R 1964). The heme iron of this molecule binds six ligands, four nitrogens are provided by porphyrin ring which built a plane. The fifth and sixth ligands bind above (distal) and below (proximal) this plane. In cytochrome P450s, the fifth ligand is a thiolate anion, sulphur with a negative charge which is provided by a conserved cysteine. The sixth ligand in the resting state is a water molecule (Denisov et al 2005). This characteristic is unusual because most other heme-proteins do not show a similar spectral band for e.g. the ferrous – CO complex of myoglobin has a maximum absorption at 423nm (Ortiz de Montellano 1997). Because of this spectral property this family of proteins is called Cytochrome P450 (Omura T and Sato R 1964). Cytochrome P450 proteins usually contain approximate five hundred amino acids with heme as a prosthetic group (Estabrook 1999). Iron in the heme uses thiol-group from cysteine of the protein as fifth legand; the other four legands are from porphyrin group of heme (Guengerich 1991, Estabrook 1999). Most other heme-proteins have nitrogen from histidine as a ligand (Ortiz de Montellano 1997, Estabrook 1999). Hence, when carbon monoxide binds to opposite side of thiolate ligand coordinated heme-iron, this Fe$^{2+}$- CO complex of cytochrome P450 shows a distinctive absorbance band near 450nm (Ortiz de Montellano 1997). This unique spectrum was also used for measurement of P450 content (Omura and Sato 1964b, Ortiz de Montellano 1997). However, this thiol-legand is easily destroyed so, the non-functional form (P420) (Omura and Sato 1964a, Omura 1999), whose carbon-monooxide binding compound has an absorption at 420nm, can be detected (figure 1.3). Therefore, the absorption maximum at 450nm is necessary for cytochrome P450 activity (Ortiz de Montellano 1997).
Figure 1.3 Spectral characteristics of CYP102A1 (CYPBM3) (Omura and Sato 1964).

1.5 Location of CYPs

Most of the CYP450s in human beings are found in liver (Paine 1981; Guengerich FP 1990; Foidart A et al 1994) (figure 1.4). Some amount is present in small intestine also (McKinnon RA et al 1995). Very low levels of CYPs are found in extra hepatic tissues such as lungs, kidney, skin and gastrointestinal tract (Macica C et al 1993, Jugert FK et al 1994, Gonzalez and Lee 1996). These are also found in mitochondria and endoplasmic reticulum. CYPBM3 is found in cytosol of soil bacterium, *Bacillus megaterium*, it is not membrane bound like microsomal cytochromes (Miura Y and Fulco AJ 1974). Liver is the main organ for drug metabolism and detoxification (figure 1.5), and about 55% of drugs are metabolized by liver CYP4503A4 and remaining by extrahepatic P450s. (Guengerich FP 1995, Tanaka E Hisawa L 1999).
Figure 1.4 Distribution of CYPs in human body (Compiled from Shimda et al 1994, Imaka et al 1996).
Figure 1.5 Metabolic contribution of various P450s (Data compiled from Shimda et al 1994, Imaka et al 1996).
1.6 Structure and function of cytochrome P450BM3

Core region of protein CYP450BM3 is having the highest structural conservation around heme. Conserved core is formed by a bundle of four – helices that are D, E, I and L, these helices are again surrounded by J and K helices, two sets of β-sheets and a coil like structure called meander. The prosthetic protoporphyrin IX (heme) is placed between distal helix I and the proximal helix L. In proximal helix conserved cysteinate ligand of the heme iron is situated in the cysteine pocket. Distal helix I is also a part of defined substrate access channel. This helix also contains a conserved amino acid sequence including the absolutely conserved threonine. Beside these conserved regions there are also some extremely variable regions, which constitute the substrate binding site for a variety of substrates, mainly include the B-C and F-G loops. These loops are located along the substrate access channel therefore, situated distal of the protoporphyrin system. There are total six substrate recognition and binding sites that include the B’ helix (SRS1), parts of helix F (SRS2), G (SRS3), I (SRS4), the β4-hairpin (SRS5) and the β2-loop (SRS6). Mutations in these regions, marked red, have a high impact on substrate specificity. Substrate binding region of CYP450BM3 is very flexible and susceptible to structural reorganization upon substrate binding encouraging an induced fit model (Li and Poulos 2004). This accounts for the broad substrate spectra of many P450 monooxygenases especially the microsomal ones. CYP450BM3 can hydroxylate carboxylic acids, alcohols and amides of a chain length from ~C12 to C22 at sub terminal positions. Their region-specificity and activity depends on chain length of the fatty acid. A cysteine residue housed by the bulge, is responsible for holding the iron center of the heme active site in place (figure 1.6). This heme active site (figure 1.7) is a porphyrin ring system that contains a redox active iron ion and is identical to the cofactor found in hemoglobin, myoglobin and many other biologically important redox enzymes. Several angstroms from the porphyrin ring system lay key residues that determine what particular substrate will be allowed entry to the active site of the enzyme. Also these amino acid residues mediate electron transfer within the enzyme (Poulos T 1995)
Figure 1.6 Tertiary structure of CYP BM3 (With courtesy from Ravichandaran et al 1993).
Figure 1.7 Active site structure of P450BM3 (Poulos T 1995).
1.7 Catalytic reaction of cytochrome P450

A scheme for the catalytic cycle of cytochrome P450 has been proposed (Guengerich et al 1991, Guengerich and Johnson 1997, Ortiz de Montellano 1997, Guengerich et al 1998, Mansuy 1998). There are nine steps in the reaction cycle as shown in Figure 1.8. Substrate binding is the first step. The P450-substrate complex then receives the first electron from cytochrome P450 reductase. The Fe$^{3+}$ in the heme is reduced. The reduced P450-Fe$^{2+}$ intermediate binds to O$_2$, and then receives the second electron from cytochrome b5. The substrate is oxidized by P450 iron-oxo intermediate. One oxygen atom is transferred to the substrate; another oxygen atom forms H$_2$O molecule. The oxidized substrate is released from cytochrome P450 and the iron in P450 returns to Fe$^{3+}$ form. Although this catalytic cycle is generally accepted, details of some steps are still poorly understood because some intermediates have a lifespan too short to be detected (Mansuy 1998). Substrate binding is well studied among these steps. The spin state of iron in the heme is different in the absence and presence of substrate (Jefcoate 1978, Ortiz de Montellano 1997). Without substrate, the ferric in a heme group has six ligands, nitrogen atoms of porphyrin provide four ligands, cysteine from cytochrome P450 is the fifth ligand and water molecule binds to iron as the sixth ligand (Ortiz de Montellano 1997). This state is called low spin state. After substrate binding to cytochrome P450, the substrate replaces water and as a result, the ferric state changes from hexacoordination to pentacoordination (Ortiz de Montellano 1997). This state is called high spin state.
Figure 1.8 A model for catalytic cycle of P450BM3 monooxygenase showing the process of substrate metabolism (Guengerich 1991).
1.8 Function of CYP450s

The function of P450 has been intensely studied. The typical function of P450 is monooxygenation activity but beside that these are involve in various catalytic reactions such as dehydration, isomerization and dehydrogenation have been found (Mansuy 1998).

Main functions performed by CYP450s:

i) Metabolism of exogenous compounds

Cytochromes P450 can introduce a polar group, such as hydroxyl group, into hydrophobic drugs, which makes these drugs more hydrophilic and easier to be excreted or metabolized by enzymes (Smith et al 1998). In human beings, three CYP families including CYP1, CYP2 and CYP3 are principally involved in the metabolism of various drugs (Smith et al 1998). These CYPs play an important role in the metabolism of drugs (Parikh et al 1997, Smith et al 1998, Murray 1999). CYP 3A4, alone can metabolise a wide range of clinical drugs (Hasler 1999, van der Weide and Steijns 1999). Therefore, the activity, induction and inhibition of these CYPs controls the elimination rate of drugs and as a result, the therapeutic response, side effects and drug-drug interaction are often related to these CYPs (Farrell 1999, van der Weide and Steijns 1999). Therefore, studying these CYPs may change our diagnosis methods (Boitier and Beaune 1999, Gelboin et al 1999, van der Weide and Steijns 1999). Human CYPs are also associated with producing carcinogens. The polycyclic aromatic hydrocarbons (PAHs), which are strong carcinogens, can be bioactivated by these CYPs (Guengerich 1991, Kaminsky and Spivack 1999, Omura 1999). Therefore, these CYPs may provide a novel target enzyme against cancer.

ii) Biosynthesis and biotransformation of endogenous chemicals

These CYPs have an important role in steroid hormone biosynthesis (Omura 1999, Pikuleva and Waterman 1999), in the synthesis of cholesterol (Pikuleva and Waterman 1999). In insects and plants, CYPs are involved in the biosynthesis of certain hormones (Stegeman and Livingstone 1998) and phyto-hormones, development of fruit ripening as well as fragrance, flavor and color development in plants (Berg et al 1988, Feyereisen 1999, Morant et al 2003, Schuler and Werck-Reichhart 2003) (figure 1.9).
iii) Their role in xenobiotic metabolism in mammals/humans is very crucial, especially for activation/clearance of many life saving drugs used by common people that are lipophilic in nature and can not be easily removed. The metabolism of these compounds takes place in two phases, Phase I and Phase II. In Phase I reactions there occur addition of a functional group to the substrate (drug) that can be a hydroxyl group is used to attach a conjugate. The conjugate makes the modified compound more water soluble and easily excreted in the urine (Meyer 1996). The hydroxyl group then serves as the site for further modifications in Phase II drug metabolism (Goldstein and Faletto 1993; Ioannides and Lewis 2004).

iv) Mutations in cytochrome P450 genes, expression levels or deficiencies of the enzymes are related with several human diseases (Liu et al 2004, Villeneuve and Pichette 2004). Induction of some P450s is a risk factor in several cancers since these enzymes can convert procarcinogens to carcinogens (Agundez 2004, Tsigelny et al 2004).

v) From industrial point of view P450 enzymes are interesting targets for the production of macrolide- and peptide- antibiotics as well as fine chemicals (Hezari and Croteau 1997, Betlach et al 1998, Zerbe et al 2004). For the pharmaceutical industry it is necessary to know how a putative drug will be processed in the human liver to prevent health risks during the longsome and expensive trial phase or to avoid recourse expanses.
Figure 1.9 Mechanisms of metabolic transformations of different substrates to its water soluble form by CYP450 (Meinhold P et al 2005).
1.9 Characterization of P450BM3 and its homologue enzyme

P450BM3 belongs to family CYP102A. This CYP102A family represents a unique group of bacterial self-sufficient cytochrome P450 monooxygenases. These P450s consist of a heme domain fused to a diflavin reductase. Up to now eight members have been identified within the CYP102A subfamily. According to the sequence homology of their monooxygenase domains the enzymes can be divided into four subgroups represented by the phylogenetic tree of the monooxygenase domains (Figure 1.10). Group 1 and 2 are consisting just of one enzyme, CYP102A1 (CYP450BM3) from *Bacillus megaterium* and CYP102A6 from *Bradyrhizobium japonicum*. Group 3 includes CYP102A3 from *Bacillus subtilis* and CYP102A7 from *Bacillus licheniformis*. The biggest group 4 contains four enzymes. Three of them CYP102A4 from *Bacillus anthracis*, CYP102A5 from *Bacillus cereus* and YP_037304 from *Bacillus thuringiensis* demonstrating more than 90 % homology have never been characterized. The last member of this group is CYP102A2 from *Bacillus subtilis* exhibiting at least 80 % homology to the former three enzymes.
Figure 1.10 Phylogenetic tree of the CYP450BM3 monooxygenase domains

Identities were obtained by alignment using ClustalW and the Blosom matrix, the tree was generated by treev32 (Schmid RD 2007).
1.10 Future prospective

Use of life threatening drugs has been increasing dramatically for the last two decades, and as a result excess load of drug in the body, in particular in the liver cause drug-drug interaction leading to adverse drug reactions. CYPs used to prevent drug-drug interaction and adverse drug reactions are very costly. Variety of CYP450s are presently available in the market (table 1.1) to give relief to the patient suffering from adverse drug reactions, and problems related to liver functions. Most of the CYP450 available in the market are from cultured human liver cells and rejected blood samples. Big players like Amphioxus Cell Technologies market about eleven CYP450s manufactured from human hepatocytes. Out of these 11 CYPs mainly 5 i.e. CYP3A4, CYP2D6, CYP1A2, CYP2C9 and CYP2E1 are dominating in the market. The other competitors for CYP450s are Astex, Invitrogen, Roche and Invitrogen. There are only few companies like TNO Pharma, Netherland manufacturing CYP450 from animal (pig) hepatocytes. Recently, FDA has put ban on manufacturing CYP from animal and human sources.

Fortunately, bacterial CYP450BM3 was noticed to be 52% homologues to CYP4503A4, and having 1000 times more catalytic efficiency to substrate metabolism than the CYP450s of human origin (Nobel et al 1999).

At present, hepatic disorder cases in developed countries is about 1.42 million per year. This is mainly due to the reason of increasing cases of alcoholism, smoking and adverse drug reactions. In India, deaths due to adverse drug reactions and other hepatic disorders are about 35000 per year. So, there is an immediate demand of CYP450 as pharmacophore under economical budget.
1.11 REFERENCES:


