Chapter 6
Reaction Phenotyping
6.1 Introduction

CYP reaction phenotyping is performed to identify the cytochrome P450s involved in the metabolism of drugs. It involves an integration of three approaches - (1) use of specific chemical and/or antibody inhibitors, (2) use of heterologously expressed CYP isoforms, and correlation and regression analyses [1].

6.1.1 Chemical inhibitors/Inhibitory antibodies

CYP reaction phenotyping using recommended inhibitors is usually performed with liver microsomes. Human liver microsomes are prepared from multiple donors (pooled microsomes) or from individual donor with the intent that all P450 activities are representative of an average in the population [2]. The inhibitors used in the assay are selective for CYPs and are used at potent concentrations. Alternatively, inhibitory antibodies can also be used for phenotyping.

6.1.2 Recombinant enzymes (heterologously expressed CYP isoforms)

With the advent of Recombinant DNA Technology, the application of recombinant CYPs has increased in reaction phenotyping of new chemical entities in drug discovery and development. Human drug metabolizing enzymes have been cloned and expressed in different cell lines, with coexpression of NADPH cytochrome P450 oxidoreductase and, in some cases, cytochrome b5. These enzymes are artificial and may differ in the activity [2].

6.1.3 Correlation and regression analyses

The correlation analysis approach involves the measurement of the reaction velocity of the biotransformation pathway in liver microsomes prepared from a panel of individual donors. Velocities are subjected to multivariate correlation analysis with P450 isoform-specific marker activities. At least ten different liver samples are recommended with minimal covariance of the enzymes of interest [2].

In previous chapters it has been discussed that CYPs may be involved in the biotransformation of CDRI 99/411. Moreover, it was also observed that CYPs
contributed to the formation of regioisomers M1 and M2 in both rat and human. Hence, reaction phenotyping was delimited to CYPs.

CYP reaction phenotyping was performed using substrate depletion and product monitoring approach. However, investigations were restricted with chemical inhibitors.
6.2 Experimental

6.2.1 Materials

HPLC-grade acetonitrile (ACN), tris (hydroxymethyl)aminomethane (tris base), KCl, MgCl$_2$·6H$_2$O, and a reduced form of β-nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sisco Research Laboratory (Mumbai, Maharashtra, India). Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) was obtained from USB Corporation (Cleveland, Oh., USA). Ammonium formate was purchased from Loba Chemie (Mumbai, Maharashtra, India). Male human liver microsomes, α-naphthaflavone, 8-methoxypsoralen, ticlopidine, sulfaphenazole, omeprazole, diethyldithiocarbamic acid, quinidine, troleandomycin and ketoconazole were procured from Sigma (St Louis, MO, USA). 1-Aminobenzotriazole (1-ABT) was procured from Qualigens (Mumbai).

6.2.2 Phenotyping using substrate depletion approach

CDRI 99/411 was phenotyped using specific and potent CYP specific inhibitors. Analyte was incubated at a concentration below EC$_{50}$ with 1 μM of ketoconazole [1], 1000 μM of troleandomycin [1], 1 μM of ticlopidine [3] and 10 μM of quinidine [1] and 10 μM of sulfaphenazole [1]. For mechanism based inhibition the reaction mixture was pre-incubated at ambient temperature 37 ± 1°C for 15 min prior to commencement of the reaction with the substrate. Positive controls (absence of inhibitors) were simultaneously incubated with the test samples. The assay conditions have been described in Table 1 of section 5.2.2. Results were expressed as percentage inhibition compared to positive control.

6.2.3 Phenotyping using product monitoring approach

CYPs involved in the formation of metabolites (M1 and M2 with rat liver microsomes and M2 with human liver microsomes) were identified by incubating CDRI 99/411 with known specific inhibitors at potent concentration (Table 1). The assay conditions have been described in Table 2 of section 5.2.3. For mechanism based
inhibition the reaction mixture was pre-incubated for 15 min prior to commencement of the reaction with the substrate.

Table 1. Human CYP isoforms and their specific inhibitors at potent concentrations

<table>
<thead>
<tr>
<th>Human CYP isoform</th>
<th>Inhibitor</th>
<th>Conc. (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IA2</td>
<td>α-naphthaflavone</td>
<td>0.5</td>
</tr>
<tr>
<td>2A6</td>
<td>8-methoxypsoralen</td>
<td>2.5</td>
</tr>
<tr>
<td>2B6</td>
<td>Ticlopidine</td>
<td>1</td>
</tr>
<tr>
<td>2C9</td>
<td>Sulfaphenazole</td>
<td>20</td>
</tr>
<tr>
<td>2C19</td>
<td>Omeprazole</td>
<td>10</td>
</tr>
<tr>
<td>2E1</td>
<td>Diethylthiocarbamic acid</td>
<td>50</td>
</tr>
<tr>
<td>2D6</td>
<td>Quinidine</td>
<td>20</td>
</tr>
<tr>
<td>3A4</td>
<td>Ketoconazole, Troleandomycin</td>
<td>5, 100</td>
</tr>
<tr>
<td></td>
<td>l-aminobenzotriazole (suicidal)</td>
<td>1000</td>
</tr>
</tbody>
</table>

6.3 Docking of CDRI 99/411 with CYP 3A4

6.3.1 Material and Methods

Isomers of CDRI 99/411 (A and B) and their metabolites M1 (A and B) and M2 (A and B) (Figure 1) were constructed by full energy minimization using Insight II software [Insight II; Accelrys (2000.1)]. 3-D molecular model of CYP3A4 (PDB ID: 1WOG _A) available in Protein Data bank was used for docking. All water molecules and heteroatom were removed from the enzyme. The binding-site pocket of the
CYP3A4 is large and is defined with heme group present at the floor of the pocket. Binding pocket is bordered by the residues Pro107, Phe108, Ser119, Ile120, Leu210, Leu211, Asp214, Ile301, Phe304, Ala305, Thr309, Ile369, Ala370, Leu373, Ser478 and Leu479 [4].

Docking calculations of isomers of CDRI 99/411 and their metabolites were performed using AUTODOCK 3.0.5. Kollman charges were added and charges on residues were neutralized. Ligands were prepared for calculations by adding gasteiger charges. The binding site of isoform was represented by energy grids using a cubic box of 40 Å × 40 Å × 40 Å with spacing of 0.375 Å, which covered the entire substrate binding cavity of CYP 3A4. The rest of the parameters were set at their default values. A maximum of 20 poses were evaluated for each compound using the genetic algorithm, with the medium number of energy evaluations (250000). The most stable configuration of the protein-ligand complex was then selected for further analysis.

Figure 1: Chemical structures of isomers (A and B) of (a) CDRI 99/411, (b) M1 and (c) M2.
6.4 Results & discussions

6.4.1 Identification of CDRI 99/411 metabolizing CYPs

Prediction and identification of CYP in the metabolism of a candidate drug contributes to the understanding of ligand-enzyme structure-activity relationship, expansion of the database for substrates of the polymorphic isoforms, assessment of potential inter-subject variability and prediction of drug-drug interactions. The involvement of a particular CYP in the metabolism of a drug is usually determined by the application of potent and selective inhibitors, inhibitory antibodies and incubation of substrate with recombinants [1]. Inhibitors serve as preliminary investigational tools (cost-effective, easily available and safe) for identification of CYP [5].

Contribution of CYP in the metabolism of CDRI 99/411 was assessed with inhibitors of CYP1A, CYP2A, CYP2B, CYP2C, CYP2E, CYP2D and CYP3A with rat and human liver microsomes. CYP reaction phenotyping of CDRI 99/411 was investigated using two different approaches. Preliminary investigations were performed using substrate depletion approach with rat liver microsomes. The inhibitor which reduced or stopped the metabolism of CDRI 99/411 was assumed to contribute to the metabolism of CDRI 99/411 [6]. The results illustrated in Table 2 suggest a strong contribution of CYP3A2 in rats. Moreover, it was observed that the formation of regioisomers (M1 and M2 in rat and M2 in human) was primarily inhibited by ketoconazole and troleandomycin (Figure 2 and 3). Figure 4 and 5 shows the inhibition in the formation of regioisomeric metabolites (M1 and M2) by 1-ABT, ketoconazole and troleandomycin with rat and human liver microsomes, respectively. Hence, results using substrate depletion approach conform to the product monitoring approach.

CYP3A contributes significantly to the metabolism of a wide range of drugs and xenobiotics. It has been reported that a number of CYP3A substrates exhibit sigmoidal kinetics in microsomes [7-9]. The active site of CYP3A is large and can accommodate two molecules (of either the same or different compounds) and exhibit positive cooperativity, homotropic and heterotropic cooperativity [10]. Hence, allosteric behaviour of CYP3A contributed to Hill kinetics (relationship of initial velocity ($v_0$) and
[S] was allosteric with sigmoidal curve) of CDRI 99/411 and it can be presumed to be explained by one substrate - two binding site theory.

Table 2. Determination of CYP in the metabolism of CDRI 99/411

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>% Inhibition compared to control (mean ± S.E.)</th>
<th>p value&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>Ketoconazole</td>
<td>50.41 ± 1.74</td>
<td>0.04</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>41.91 ± 8.27</td>
<td>0.01</td>
</tr>
<tr>
<td>Ticlopidine</td>
<td>0.35 ± 0.21</td>
<td>0.44</td>
</tr>
<tr>
<td>Quinidine</td>
<td>0.64 ± 0.49</td>
<td>0.09</td>
</tr>
<tr>
<td>Sulfaphenazole</td>
<td>0.67 ± 0.75</td>
<td>0.47</td>
</tr>
</tbody>
</table>

<sup>a</sup> Control is samples without inhibitor (n=3, mean ± Standard Error)

<sup>b</sup> Compared with samples without inhibitor (Student t test, p < 0.05)
Figure 2. Identification of CYPs involved in the formation of metabolite M1 (a) and M2 (b) in rat.

(a)

(b)
Figure 3. Identification of CYPs involved in the formation of metabolite M2 in human.

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Figure 4. CYP reaction phenotyping with rat liver microsomes. Typical MRM chromatograms of CDRI 99/411 (m/z 412.5–m/z 185) and metabolites (m/z 428.5–m/z 201) in (a) Control without inhibitor, (b) inhibition with 1-Aminobenzotriazole (1-ABT), (c) inhibition with ketoconazole and (d) inhibition with troleandomycin.
Figure 5. CYP reaction phenotyping with human liver microsomes. Typical MRM chromatograms of CDRI 99/411 (m/z 412.5 –m/z 185) and metabolites (m/z 428.5-m/z 201) in (a) Control without inhibitor, (b) Inhibition with 1-Aminobenzotriazole (1-ABT), (c) inhibition with ketoconazole and (d) inhibition with troleandomycin
(c)

(d)
6.4.2 Docking of CDRI 99/411 with CYP 3A4

Docking study utilizes three-dimensional structures of metabolizing enzymes to calculate the lowest energy conformation and orientations of ligands in the defined active sites. Standard testosterone docking studies were performed with putative active site of CYP 3A4 as described by Toshima et.al. [4]. Docking experiment revealed two different conformations of testosterone. Figure 6 shows one molecule of testosterone bound proximal to heme in an orientation compatible with 6-β hydroxylation whereas the second molecule was bound distal to heme. However, no polar interactions were observed. Whereas, hydrophobic interactions with residues 53 TYROSINE, 57 PHENYLALANINE, 76 ASPARGINE, 106 ARGININE, 108 PHENYLALANINE, 137 PHENYLALANINE, 184 Isoleucine, 215 PHENYLALANINE, 216 LEUCINE, 220 PHENYLALANINE, 223 Isoleucine, 224 THREONINE, 301 Isoleucine, 302 PHENYLALANINE, 305 ALANINE, 309 THREONINE, 313 VALINE, 364 LEUCINE, 368 PROLINE, 369 Isoleucine, 370 ALANINE, 371 METHIONINE, 372 ARGININE, 373 LEUCINE, 374 GLUTAMATE, 434 PROLINE, 435 PHENYLALANINE, 442 CYSTINE, 443 Isoleucine, 447 PHENYLALANINE, 448 ALANINE and 452 METHIONINE were seen.
Figure 6: Binding mode of testosterone in the active site of CYP 3A4. Heme, proximal testosterone, distal testosterone and active site residues are shown in magenta, yellow, cyan, and green, respectively.

In the case of CDRI 99/411, both isomers were bound proximal to heme with opposite orientation. The isomers were in a pose close to ferric atom with a distance of approximately 4Å (Figure 7). Binding energy was calculated using the following equation.

\[ \Delta G_{\text{binding, solution}} = \Delta G_{\text{binding, vacuo}} + \Delta G_{\text{solvation}} - \Delta G_{\text{solvation(E + I)}} \]

Where, E is the enzyme, I is the inhibitor and \( \Delta G \) is the Gibbs free energy change.

The binding energy of CDRI 99/411(A) is slightly higher than that of CDRI 99/411(B) (Table 3), which may be due to the structural differences at the chiral centre in the molecule. The strong hydrogen bond interactions with the residues 105 ARGinine, 143 ISOleucine, 309 THreonine, 442 CYStine, 443 ISOleucine and 444 GLYCine were reported. Hydrophobic interactions with some side chains lining the binding cavity were also seen. These include 118 ISOleucine, 137 PHENylalanine, 301 ISOleucine, 302 PHENylalanine, 305 ALANine, 309
THREONINE, 313 VALINE, 364 LEUCINE, 369 ISOLEUCINE, 370 ALANINE, 373 LEUCINE, 434 PROLINE, 435 PHENYLALANINE, 440 ARGinine, 443 ISOLEUCINE, 448 ALANINE and 452 METHIONINE residues.

Figure 7: Binding mode of CDRI 99/411 in the active site of CYP 3A4. Heme, CDRI 99/411 A, CDRI 99/411 B and active site residues are shown in pale green, magenta, cyan and green, respectively.

Similarly, both isomers of M1 and M2 were bound proximal to heme iron with a distance of approximately 4Å. Binding energy of both isomers of M1 and M2 occurs with a difference of 1.7 and 2.9 kcal/mol, respectively. Polar interactions with 105 ARGinine, 119 SERine, 301 ISOLEUCINE, 309 THREONINE, 370 ALANINE, 435 PHENYLALANINE, 440 ARGinine, 441 ASPARAGINE, 442 CYSTINE, 443 ISOLEUCINE and 444 GLYCINE were observed. Non polar hydrophobic interactions with some side chains lining the binding cavity can be seen. These include 94 LEUCINE, 108 PHENYLALANINE, 118 ISOLEUCINE, 120 ISOLEUCINE, 137 PHENYLALANINE, 271 PHENYLALANINE, 301 ISOLEUCINE, 302 PHENYLALANINE, 304 PHENYLALANINE, 305 ALANINE, 309 THREONINE, 313
Table 3. Binding energies of isomers of CDRI 99/411 and their metabolites

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Binding energies (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDRI 99/411</td>
<td>A -11.1</td>
</tr>
<tr>
<td>M1</td>
<td>-11.0</td>
</tr>
<tr>
<td>M2</td>
<td>-11.1</td>
</tr>
</tbody>
</table>

6.5 Conclusion

Reaction phenotyping of CDRI 99/411 was performed using substrate depletion and product monitoring approach. It was found that 1-ABT inhibited the metabolism of CDRI 99/411 with human and rat liver microsomes. Thus, it was concluded that CYPs are involved in the metabolism of CDRI 99/411. Furthermore, it was found that CYP 3A is the principal enzyme involved in the Phase 1 metabolism of CDRI 99/411 in rat and human. Moreover, it was also concluded that CYP 3A contributed to Hill kinetics of CDRI 99/411.

In silico docking studies were performed to understand the interaction of CDRI 99/411 and its metabolites with CYP 3A4 isoenzyme. It was found that testosterone and CDRI 99/411 were binding at the same site and in a similar fashion. Thus, they exhibit Hill kinetics. Moreover, M1 and M2 were also binding to the active site of CYP 3A4. Hence, M1 and M2 are potential substrates for CYP 3A isoform. It was also observed that 105 ARGinine, 309 threonine, 442 cystine, 443 isoleucine, 444 cystine, 447 phenylalanine, 448 alanine and 452 methionine residues.
GLYCINE were within hydrogen bonding for all the compounds. Thus, these residues may be important for substrate binding.
Reference