Chapter 6: CYP Inhibition Study

6.1. Introduction:

Cytochrome (CYP450) constitutes the major drug metabolizing system both at hepatic as well as at intestinal level. Inhibition of CYP450 by concurrent administration of two or more xenobiotics can result in profound alteration in the pharmacokinetics of drugs sometimes even resulting in fatalities. Herbal supplements nowadays are been prescribed along with many medications. It is well documented that principle constituents of such herbal supplements can result in altered pharmacokinetics of many drugs affecting efficacy as well as safety of these drugs. Inhibition of CYP mediated biotransformation of drugs is one of the major reasons of altered pharmacokinetics caused by these herbal supplements. The purpose of present study was to assess the CYP inhibition potential of flavanoids, kaemeferol (KMF), biochanin A (BCA) and formononetin (FMN) which are principle components of many marketed herbal/dietary supplements. These selected flavonoids belong to a special class of flavonoids known as phytoestrogens.

6.2. Materials and Methods:

6.2.1. Chemicals and authentic metabolic standards

Testosterone, nifedipine, diclofenac, mephenytoin, dextromethorphan, phenacetin, chlorzoxazone, 6β-hydroxy testosterone, 4'-hydroxymephenytoin, 4'-hydroxydiclofenac, dextrophan, paracetamol, 6-hydroxy chlorzoxazone, α-naphthoflavone, sulfaphenazole, ticlopidine, quinidine, 4-methyl pyrazole, ketoconazole and NADPH were purchased from Sigma Aldrich Ltd (St Louis, USA). Milli-Q pure water was obtained from a Millipore Elix water purification system purchased from Millipore India Pvt. Ltd. (New Delhi, India). All other chemicals and solvents were of high-performance liquid chromatography grade (HPLC).

6.2.2. Preparation of microsomes

Healthy male Sprague-Dawley rats, weighing 200-220g were procured from the National Laboratory Animal Centre, CDRI (Lucknow, India). Rats were housed in well ventilated cages at room temperature (24±2°C) and 40-60 % relative humidity while on a regular 12 h light-dark cycle. The animals were acclimatized for a minimum period of one week prior to the experiment. The rats were fasted overnight (14-16 h) except prior to the experiment but given free access to water. The livers were perfused and cleaned with KCl (1.15%) to remove
residual blood. Cleaned livers were then weighed and homogenised with homogenization buffer (4 mL/g of liver). The homogenates were submitted to several differential centrifugations, as previously described [1]. The prepared microsomes pellets were then suspended in homogenisation buffer and protein concentration was determined by Folin-Lowry Method using Bovine Serum Albumin (BSA) as standard. The prepared microsomes were stored in aliquots of 20 mg/mL at -80°C until they were used.

6.2.3. Preparation of stocks of specific metabolic substrates and test compounds

Taking into consideration the inhibitory effect of organic solvents on enzymatic activity of CYPs in microsomes, the stocks of substrates, metabolites for different CYPs and test compounds were prepared in organic solvents preferably acetonitrile with least inhibitory effect on CYP activity. The final organic content in the assay medium was maintained ≤1%.

6.2.4. General incubation conditions used for the CYP inhibition study

The inhibitory effect of phytoestrogens, KMF, BCA and FMN on the activity of six different CYPs isoforms mainly involved in the biotransformation of the drugs were studied using rat liver microsomes (RLMs). The activity of these isoforms were analysed utilizing following isoform specific probe reactions: Phenacetin O-deethylation (CYP1A2), Diclofenac 4'-hydroxylation (CYP2C9), S-mephenytoin 4'-hydroxylation (CYP2C19), Dextromethorphan O-demethylation (CYP2D6), Chlorzoxazone 6-Hydroxylation (CYP2E1), Nifedipine 4'-Hydroxylation (CYP3A4) and Testosterone 6β-hydroxylation (CYP3A4). These substrates were selected because their use as isoform selective probe substrate was well documented and also validated in our laboratory. Also, these substrates are approved under the category of accepted and preferred substrates for in vitro drug interaction studies in FDA guidance for industry [2]. Preliminary experiments were conducted so as to obtain incubation conditions specific for each isoform that are within the linear range for the velocity of the reaction (incubation time, substrate concentration and protein concentration). The compounds α-naphthoflavone (25μM), sulfaphenazole (60μM), ticlopidine (10μM), quinidine (5μM), 4-methyl pyrazole (200μM) and ketoconazole (1μM) were used as selective inhibitors of CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A activity, respectively which served as positive control.
The incubation mixture was consisted of phosphate buffer (pH 7.4), RLMs, substrate with or without test compound was pre-warmed for 10 minutes at 37°C. Control samples contained equivalent amount of organic content as that of test compounds (1%). The reaction was started by addition of NADPH (2.4 mM). The reaction was conducted in shaking water bath at 37°C under aerobic conditions and terminated using ice cold organic solvent after predetermined incubation period. The reaction mixture was then vortexed for 5 minutes followed by centrifugation at 3,000xg for 15 minutes resulting in removal of denatured proteins. An aliquot of 200μL of supernatant was transferred to vial and analysed by high performance liquid chromatography (HPLC) using photo diode array (PDA) as detector.

6.2.5. Enzyme specific assay conditions used for inhibition study

6.2.5.1. CYP1A2 activity (Phenacetin O-deethylation to acetaminophen) [3]

RLMs (1 mg/mL), phenacetin (25 μM), phosphate buffer (pH 7.4) and NADPH (2.4 mM) were incubated at 37°C for 20 minutes with or without different concentrations of KMF (0.1-100 μM), BCA (0.1-100 μM) and FMN (0.1-50 μM). Reaction was terminated with acetonitrile containing the internal standard. Samples were processed as described earlier and analysed using HPLC.

6.2.5.2. CYP2C9 activity (Diclofenac 4-hydroxylation to 4-hydroxy Diclofenac) [4]

RLMs (0.5 mg/mL), diclofenac (40 μM), phosphate buffer (pH 7.4) and NADPH (2.4 mM) were incubated at 37°C for 30 minutes with or without different concentrations of KMF (0.1-100 μM), BCA (0.1-100 μM) and FMN (0.1-50 μM). The final incubation mixture volume was 500 μL. Ice cold acetonitrile (500 μL) containing the internal standard was used to terminate the enzymatic reaction. Samples were processed as described earlier and analysed using HPLC.

6.2.5.3. CYP2C19 activity (S-mephenytoin 4'-hydroxylation to 4-hydroxy mephenytoin) [5]

RLMs (1 mg/mL), mephenytoin (50 μM), phosphate buffer (pH 7.4) and NADPH (2.4 mM) were incubated at 37°C for 40 minutes with or without different concentrations of KMF (0.1-100 μM), BCA (0.1-100 μM) and FMN (0.1-50 μM). Reaction was terminated with
acetonitrile: acetic acid (94:6) containing the internal standard. Samples were processed as described earlier and analysed using HPLC.

6.2.5.4. CYP2D6 activity (Dextromethorphan O-demethylation to Dextrorphan) [6]

RLMs (1 mg/mL), dextromethorphan (10 µM), phosphate buffer (pH 7.4) and NADPH (2.4 mM) were incubated at 37°C for 30 minutes with or without different concentrations of KMF (0.1-100 µM), BCA (0.1-100 µM) and FMN (0.1-50 µM). Reaction was terminated with acetonitrile containing the internal standard. Samples were processed as described earlier and analysed using HPLC.

6.2.5.5. CYP2E1 activity (Chlorzoxazone 6-Hydroxylation to 6-hydroxy Chlorzoxazone) [2]

RLMs (1 mg/mL), chlorzoxazone (40 µM), phosphate buffer (pH 7.4) and NADPH (2.4 mM) were incubated at 37°C for 40 minutes with or without different concentrations of KMF (0.1-100 µM), BCA (0.1-100 µM) and FMN (0.1-50 µM). Reaction was terminated with acetonitrile containing the internal standard. Samples were processed as described earlier and analysed using high performance liquid chromatography.

6.2.5.6. CYP3A activity

Since CYP3A is involved in the metabolism of more than 50% of the drug substrates and it has broad substrate specificity, two structurally unrelated substrates are necessary to assess the potential for drug interactions using this enzyme system [2, 7]. For this reason, we have chosen two probe substrate; nifedipine and testosterone for characterizing the inhibitory effect of test flavonoids on CYP3A.

6.2.5.6.1. Nifedipine 4'-Hydroxylation to 4-hydroxy nifedipine [7]

RLM (0.5 mg/mL), nifedipine (20 µM), phosphate buffer (pH 7.4) and NADPH (2.4 mM) were incubated at 37°C for 3 minutes with or without different concentrations of KMF (0.1-100 µM), BCA (0.1-100 µM) and FMN (0.1-50 µM). Reaction was terminated with acetonitrile containing the internal standard. Samples were processed as described earlier and analysed using HPLC.

6.2.5.6.2. Testosterone 6β-hydroxylation to 6β-hydroxy testosterone [7]
Chapter 6: CYP Inhibition Study

RLM (0.5 mg/mL), testosterone (25 μM), phosphate buffer (pH 7.4) and NADPH (2.4 mM) were incubated at 37°C for 5 minutes with or without different concentrations of KMF (0.1-100 μM), BCA (0.1-100 μM) and FMN (0.1-50 μM). Reaction was terminated with acetonitrile containing the internal standard. Samples were processed as described earlier and analysed using HPLC.

6.2.6. Analytical Procedure

Quantitative evaluation of metabolites and internal standards were carried out by using HPLC with PDA detection. The liquid chromatographic system consisted of a Shimadzu LC-10 AT VP pump, SIL-10 AD VP autosampler, DGU-14A degasser, SPD- M 10A VP photo diode array detector and CTO-10A-VP column oven (Shimadzu Corporation, Kyoto, Japan). All the parameters of HPLC were controlled by Class VP software. The rate of production of each metabolite from the substrate probes were quantified using the ratio of the peak area of the metabolites to the peak area of internal standard.

6.2.7. Data Analysis

IC50 values of the test compounds were determined graphically by non-linear regression analysis of the plot of logarithm of the inhibitor concentration and percent of remaining activity using GraPadPrism 5 (GraphPad Co. Ltd, USA). The enzyme activity in the presence of inhibitors was compared with the enzyme activity in control incubations (incubation containing solvent but not inhibitor).

6.3. Results and Discussion:

The inhibitory effect of test compounds was determined in the concentration range in which they do not precipitate in the incubation mixture. The concentration range selected for KMF and BCA was 0.1-100 μM whereas, for FMN, the concentration range of 0.1-50 μM was selected. Since the concentration range we selected was governed by solubility issues, IC50 values of our test flavanoids mainly FMN and BCA cannot be determined for most of the CYP isoforms. However, for KMF it had been possible to demonstrate its inhibitory potential on various CYPs in the concentration range examined. The results clearly demonstrate that KMF is the moderate inhibitor of CYP2C9, CYP2C19, CYP2E1 and CYP3A4 (testosterone as substrate) with IC50 value of 17.75 μM, 26.34 μM, 13.32 μM and 12.72 μM, respectively.
Chapter 6: CYP Inhibition Study

(Figure 6.1.1. and Table 6.1.1.). The KMF was found to be the weak inhibitor of CYP1A2 with IC50 value of 48.82 μM. KMF does not exhibit significant inhibitory effect on CYP2D6 with IC50 value exceeding 100 μM (Figure 6.1.1. and Table 6.1.1.).

FMN and BCA appear to be relatively safe with IC50 values for most of the CYPs greater than 50 and 100 μM, respectively, except CYP3A4 where IC50 value of BCA is 66.82 μM and 52.18 μM using nifedipine and testosterone as substrate, respectively (Figure 6.1.2 & 6.1.3. and Table 6.1.1.).

These flavanoids are less likely to cause hepatic CYP-mediated drug-drug interaction since it is less likely to attain such a high concentration of these flavanoids in blood because of low bioavailability of these flavonoids. However, since hepatic and intestinal CYPs cDNA are identical, the proteins expressed in these organs are probably the same. Therefore, the results of study conducted here with rat liver microsomes can also apply to intestinal CYPs where it is more likely to attain high concentrations of flavanoids.

Table 6.1.1. IC50 values of Kaempferol, Formononetin and Biochanin A on CYP-450 specific probe activity

<table>
<thead>
<tr>
<th>P-450 Isoform</th>
<th>Reaction</th>
<th>IC50 (μM) in rat liver microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Kaempferol</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>Phenacetin O-deethylation</td>
<td>48.82</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac 4-hydroxylation</td>
<td>17.75</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>S-mephenytoin 4'-hydroxylation</td>
<td>26.34</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan O-demethylation</td>
<td>&gt;100</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Chlorzoxazone 6-Hydroxylation</td>
<td>13.32</td>
</tr>
<tr>
<td>CYP3A</td>
<td>Nifedipine 4'-Hydroxylation</td>
<td>&gt;100</td>
</tr>
<tr>
<td></td>
<td>Testosterone 6β-hydroxylation</td>
<td>12.72</td>
</tr>
</tbody>
</table>

*NE – No effect
Chapter 6: CYP Inhibition Study

**Figure. 6.1.1.** Inhibition of CYP isoforms by kaempferol (KMF) in rat liver microsomes (RLMs). Each point represents the average of duplicate incubations.

**Figure. 6.1.2.** Inhibition of CYP isoforms by biochanin A (BCA) in rat liver microsomes (RLMs). Each point represents the average of duplicate incubations.
Chapter 6: CYP Inhibition Study

Figure. 6.1.3. Inhibition of CYP isoforms by formononetin (FMN) in rat liver microsomes (RLMs). Each point represents the average of duplicate incubations.

6.4. Conclusion:

FMN has not shown significant inhibitory effect on any of the CYP isoforms examined and appears to be relatively safe while co-administering with drugs. It exhibits least potential to alter pharmacokinetics of other concurrently administered medications.

BCA also has not shown significant inhibitory effect on most of the CYP isoforms except CYP3A with IC50 value of 66.82 μM and 52.18 μM for nifedipine and testosterone as probe substrate, respectively.

KMF appears to be moderate inhibitor for CYP2C9, CYP2C19, CYP2E1 and CYP3A4, and weak inhibitor of CYP1A2. However, it has not shown significant inhibitory effect on CYP2D6. Inhibitory effect of KMF on major CYPs isoforms arise major safety concerns regarding its co-administration with drugs metabolised by same CYP isoforms which may result in potential drug-drug interaction and alteration of pharmacokinetics of many medications.
Chapter 6: CYP Inhibition Study

6.5. References:


