In vitro Metabolic Stability Study of New Cyclen Based Antimalarial Drug Leads Using RP-HPLC and LC-MS/MS

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Abstract

Metabolic stability of the new antimalarial drug leads is determined using Human Liver Microsome (HLM) and specific cytochrome P450 enzyme (CYP2C8) taking the clinically used antimalarial drug chloroquine as a positive control. Experiment is done using standard methods. All the assays were conducted in 0.5 M phosphate buffer at pH 7.4. In general the metabolic reaction was initiated by adding 1 mM NADPH and 0.5 mg of enzyme. Incubations were done with time frequency of 0 hr, 1 hr, and 2 hrs at 37°C and the reactions were terminated by adding acetonitrile in the equal amounts of the assay mixture taken. The samples were centrifuged for 15 minutes at 10,000×g at 4°C and an aliquot of the supernatant was subjected to analysis using HPLC as well as LC-MS to confirm the masses of the drug and/or metabolite(s), if any. While chloroquine was found to be metabolized in a predictable manner by both HLM and CYP2C8, the drug leads were metabolically stable at similar experimental conditions. This study demonstrated that the new drug leads are worth conducting further preclinical evaluations.

Keywords: Metabolic stability; Preclinical study; Drug lead analysis; Drug discovery

Introduction

With the arrival of new combinational drug synthesis, the need for an expeditious evaluation of drug safety has become a paramount point in drug discovery. The duration and impact of drug discovery revolving around drug absorption, distribution, metabolism and excretion will be evaluated according to their determined metabolic stability. The major organ for drug metabolism is liver that contains major drug metabolizing enzyme called Cytochrome P450 (CYP) system [1]. Drug metabolism refers to the susceptibility of compounds to biotransformation that depends on the presence of groups in the molecule those are open to enzyme catalyzed transformation [2]. Drug metabolism can be divided into two phases, Phase I and phase II. Phase I involves oxidation, reduction and hydrolysis reactions, which are catalyzed by the CYP and flavin containing monooxygenases (FMOs) whereas Phase II involves conjugation reactions catalyzed by metabolic enzymes like UDP-glucuronon transferases (UGTs) and sulfotransferases [3,4].

Recognizing the metabolites of drugs is of paramount importance in drug discovery and development. The identification of drug metabolites in the early stages of the drug discovery is important in the development processes. The analytical tools like Liquid Chromatography-Mass Spectrometry (LC-MS) and HPLC play prominent role in these processes. Through this process of identification, the pharmacokinetic profiles can be assessed that are highly significant in detecting safety processes. Through this process of identification, the pharmacokinetic Spectrometry (LC-MS) and HPLC play prominent role in these drug discovery and development. The identification of drug metabolites [3,4].

The main purpose of the present study was to determine the metabolic stability of a series of newly discovered cyclen bisquino line antimalarial drug leads [8] and related compounds (Figure 1) by RP-HPLC and LC-MS techniques. Compound B was shown to be highly effective antimalarial agent both in vitro and in vivo and was found to work by inhibiting β-hematin formation [8]. This lead structure fulfilled few important criteria for a new drug lead: 1) it is a 4-aminoquinoline derivative that are the most trusted class of antimalarials, 2) active against chloroquine-resistant as well as multidrug resistant isolates of Plasmodium falciparum, 3) simple pharmacophore structure that will afford low cost manufacturing, and 4) it also showed oral efficacy in mice model. All these features warranted further preclinical studies with these leads and related newly synthesized compounds as shown in Figure 1. Compounds A, C, D and E were also shown to have potent in vitro antimalarial activity and also serve as drug leads (manuscript is under preparation). The present study was thus aimed at determining the in vitro metabolic stability and identifying potential metabolites by HPLC and LC-MS techniques using HLM and CYP enzymes. The study was designed based on the known metabolic pathway of the related clinically used drug chloroquine (CQ) [9-12] and thus utilized both pooled human liver microsomes as well as specific isozyme CYP2C8 (Figure 1).

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Materials and Methods

Materials

Chloroquine diphosphate purchased from Pfaltz & Bauer. NADPH, anhydrous with assay 93–100% (HPLC), was purchased from Sigma Aldrich. 0.5 M Potassium phosphate buffer pH 7.4 was purchased from BD Gentest. Human Liver Microsome (HLM) was purchased from BD Bioscience and Corning. Human cDNA expressing CYP 450 and 2C8 (BD Gentest. Human Liver Microsome (HLM) was purchased from BD Bioscience and Corning. Human cDNA expressing CYP 450 and 2C8, 3A4, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4, 4A11, FMO, UGT1A1, UGT1A4, and UGT1A9 as shown in Table 1 [9]. The flow rate was maintained at 1.0 ml/min, the column oven temperature at 40°C and the effluent was monitored at 325 nm (based on the λ). The peak purity data were obtained using Photodiode Array (PDA) detector in the sample chromatograms. The method was found linear over the concentration range of 3.5–200.1 μg/ml (R²=0.99) for drug lead B.

General procedure

The method used to determine the metabolism was a modified method obtained from BD Bioscience, which is summarized here. Metabolic stability study of a drug can be determined by treating it with the liver enzyme that correlates with the in vivo conditions. The assay mixture is prepared by the combination of substrate (0.01 mM), enzyme (0.5 mg), buffer (0.5 M), and 1 mM NADPH cofactor. The order of addition of the assay component also plays a major role in the stability study. Metabolism can be initiated by pre-warming or incubating the substrate, buffer, and the cofactor to 37°C and then adding liver microsomes or CYP2C8 to the mixture. It is customary that the assay mixture should be thoroughly mixed. After the preparation of the Total Assay Mixture (TAM), required volume of the sample is collected at different time intervals. The reaction is then terminated using acetonitrile in equal volumes as that of the sample solution collected. The samples are then transferred into the microcentrifuge tubes, vortexed for 2 minutes and then centrifuged for 10,000 xg for 15 minutes. The purpose of centrifugation is to remove the protein. The sample solution is then separated from the protein pellet by the supernatants. These supernatant solutions are transferred into the HPLC auto sampler vials and are analyzed according to the analytical method.

Metabolic stability in HLM using HPLC Method 1

The HLM used was pooled and prepared from freshly frozen human tissues which were tested negative for pathogens using PCR. The HLM used in this study was comprised of 330 pmoles/mg of total P450 and 0.5 ml and the protein content was 20 mg/ml in 250 mM sucrose. All the incubations were performed in duplicates. Therefore, all together there were 6 samples for each drug, one blank and 6 samples of the positive control. The total experiment with 5 drugs has about 65 samples. All the components used in the TAM are shown in Table 1.
Metabolic stability in CYP2C8 using HPLC method 1

The CYP enzyme is comprised of a human CYP2C8 and human CYP-reductase co-expressed in *Escherichia coli*. The concentration of the CYP P450 is 1.0 nmol/ml and its protein concentration is 10.0 mg/ml. All the assays were conducted in 0.2 M phosphate buffer at pH 7.4. One blank for each sample A, B, C, D, and E was prepared by adding 180 µl of the sample in 1620 µl of the buffer. The total assay mixture contained 25 pmoles/ml of CYP2C8 and 0.5 mM of sample as shown in the table 3.

<table>
<thead>
<tr>
<th>Time (Min)/solvent</th>
<th>0.04% formic acid in Water</th>
<th>Acetonitrile</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>96</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>64</td>
<td>30</td>
<td>6</td>
</tr>
<tr>
<td>12</td>
<td>4</td>
<td>90</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 1: Mobile phase for metabolic stability study: Gradient elution mode.

180 µl of Sample, 1275 µl buffer, and 300 µl of 1 mM NADPH was taken into a reaction flask and was pre-incubated for 2 minutes. The reaction was initiated by adding 45 µl enzyme. Incubations were done with increasing time (t=0 hr, 1 hr, 2 hrs) at 37°C. Immediately at time=0 hr, 300 µl of the assay mixture was pipetted into a centrifuge tube and 300 µl of acetonitrile was added to it to stop the reaction. All the samples were done in triplicates. Therefore, all together there were 9 samples for each drug, one blank and 9 samples of the positive control. The total experiment with 5 drugs had about 95 samples.

The remaining TAM was incubated for 1 hr and 2 hrs, same procedure of sampling was followed as mentioned in the HLM section. Then the samples were centrifuged for 15 minutes at 10,000 x g at 4°C. An aliquot about 500 µl of the supernatant fraction were collected and subjected to analysis using the above HPLC conditions as shown in Table 1. The retention times were noted and the amounts of drugs metabolized were calculated according to their percentage peak area using the equation: % peak area of the metabolite/(% peak area of the drug+% peak area of the metabolite). After running HPLC the samples were frozen and then lyophilized to remove the solvent. The dried samples were then dissolved in methanol and water (50:50 v/v) and tested for their mass. The mass of the drug and the metabolite were determined by using LC-MS.

Metabolic stability in HLM and CYP2C8 using HPLC method 2

To verify the metabolic stability results using HPLC method 1, HPLC method 2 was carried out using drug lead B, and CQ as a positive control. For that we prepared sample, standard and blank for our drug lead B and CQ in duplicates as mentioned in the following Table 4. All the ingredients in the TAM were taken in the order of their serial number (Table 4).

Sample stock solutions of Chloroquine diphosphate and Cylen Bisquinoline Hydrochloride Salt were prepared by dissolving in water. 0.5 M potassium phosphate pH 7.4 (BD Biosciences Cat No. 451201) was used as a buffer. Sample solutions were prepared by adding 20 µl of substrate, 400 µl of the buffer solution, 200 µl of the NADPH solution and 1355 µl of water. Standard solutions were prepared by adding 20 µl of Substrate, 400 µl of the buffer solution and 1580 µl of water. Blank solutions were prepared by adding 400 µl of the buffer solution, 200 µl of the NADPH solution and 1375 µl of water.

All the above solutions were then pre-incubated for 5 minutes in a shaking water bath at 37°C. Reaction was initiated by adding 25 µl of the enzyme (from Corning HLM-452161 and CYP2C8-456252). These TAMS were mixed thoroughly by inverting a couple of times. Immediately, at time t=0 hr, 300 µl of the TAM was taken into a reaction flask and was pre-incubated for 2 minutes. The reaction was terminated by adding 30 µl of 1 mM NADPH and 300 µl of 0.5 M K phosphate solution and 1500 µl of water.

The remaining TAM was warmed in a shaking water bath at 37°C for 2 hrs and the same procedure was followed for the samples at time=1 hr and t=2 hr. The incubation mixtures were then centrifuged for 15 minutes at 10,000 x g at 4°C. About 500 µl aliquots of the supernatant were collected and were subjected to analysis using the HPLC conditions as shown in Table 1. After running HPLC the samples were frozen and then lyophilized to remove the solvent. The dried samples were then dissolved in methanol and water (50:50 v/v) for LC-MS analyses.

Results and Discussion

HPLC Method 1

Results using HLM: Under the provided experimental conditions
and 3) (Table 5).

peaks (m/z) were similar to those of their molecular weights (Figures 2 the drug leads when they were analyzed by LC-MS. The molecular ion were stable. There was no evidence of any metabolite mass of any of At time t=1 hr CQ showed some metabolism whereas the drug leads and CQ obtained at time t =1 hr and 2 hr are shown in the Table 5. At time t=0 min there was no metabolism with any of the drug samples. 3. The retention times and the peak areas of the drug leads A, B, C, D, E and CQ obtained at time t =1 hr and 2 hrs are shown in the Table 5. At

<table>
<thead>
<tr>
<th>Time t=1 hr</th>
<th>Time t=2 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drugs</td>
<td>RT</td>
</tr>
<tr>
<td>A</td>
<td>8.136</td>
</tr>
<tr>
<td>B</td>
<td>5.737</td>
</tr>
<tr>
<td>C</td>
<td>4.869</td>
</tr>
<tr>
<td>D</td>
<td>5.818</td>
</tr>
<tr>
<td>E</td>
<td>5.822</td>
</tr>
<tr>
<td>CQ/DCQ</td>
<td>6.801/6.485</td>
</tr>
</tbody>
</table>

*Percentage of the metabolite formation was calculated according to the obtained percentage peak areas of the metabolite and the drug. The percent of CQ metabolized using HLM at time t=1 hr and 2 hrs was 5.12% and 6.146%, respectively.

Table 5: Data obtained from HPLC and MS for the drugs using HLM.

it was observed that CQ was metabolized in a predictable manner by HLM into the metabolite DCQ; contrarily all our experimental drug leads were metabolically stable. Not only was the metabolite for CQ identified in HPLC (Figure 2) but also in MS (Figure 7) reconfirming our results. Both drug (320 m/z) and metabolite masses (292 m/z) were observed in the LC-MS analysis of the samples. According to the area under curve ratios, CQ was converted to about 4.29% of its N-deethylated metabolite after 1

Figure 2: HPLC chromatogram of chloroquine at time A) 0 hour and B) 2 hour using HLM.

Figure 3: HPLC chromatogram of drug lead B at time A) 0 hour and B) 2 hour using HLM.


2 hours of incubation, whereas the drug lead B was stable as shown in Figure 5. The drug leads A (523 m/z), B (495 m/z), C (561 m/z), D (885 m/z), and E (619 m/z) were metabolically stable at similar experimental conditions. The retention times of the drugs A, B, C, D, E and CQ obtained at time t=0 were 8.136, 5.971, 4.869, 5822, 5.817 and 6.998, respectively, and no metabolite peak was evident. The retention times of the drug leads and the CQ at time t=1 hr and t=2 hr is shown in the Table 6. All the samples were estimated in triplicates and the results shown are the average of the three trials (Figures 4 and 5) (Table 6).

With these results, it was confirmed that CYP2C8 has an important role in the metabolism of chloroquine and the drug leads were metabolically stable. The experiment was extended up to 24 hours for the drug leads and consequently no metabolism was observed. The drug masses were confirmed by MS (Figures 6-9).

HPLC Method 2

Results using HLM: Under provided experimental conditions using HPLC method 2, similar results were obtained, CQ was metabolized by HLM into the metabolite DCQ and contrarily the drug lead B was metabolically stable. The metabolite was identified by HPLC and also by LC-MS reconfirming the results shown in method 1. Both drug (320 m/z) and metabolite (292 m/z) masses were observed in the LC-MS analysis of the samples. According to the area under curve ratios, CQ was converted to about 4.29% of its N-deethylated metabolite after 1

Figure 4: HPLC chromatogram of chloroquine at time A) 0 hour and B) 2 hour using CYP2C8.

Figure 5: HPLC chromatogram of drug B at time A) 0 min and B) 2 hour using CYP2C8.

it was observed that CQ was metabolized in a predictable manner by HLM into the metabolite DCQ; contrarily all our experimental drug leads were metabolically stable. Not only was the metabolite for CQ identified in HPLC (Figure 2) but also in MS (Figure 7) reconfirming our results. Both drug (320 m/z) and metabolite masses (292 m/z) were observed in the LC-MS analysis of the samples. According to the area under curve ratios, CQ was converted to about 4.29% of its N-deethylated metabolite after 1

Table 6: Data obtained from HPLC and MS for the drugs using CYP2C8.
The Table 8. All the samples were estimated in duplicates and the results of the drug lead B and the CQ at time t=1 hr and t=2 hr are shown in at similar experimental conditions (Figure 11D). The retention times of incubation. The drug lead B (495 m/z) was metabolically stable was transformed into DCQ after 1 hour of incubation and 2.89% after 2 hours of incubation. On the other hand, the lead drugs B (495 m/z) was found to be metabolically stable in both HPLC and LC-MS analyses. A sample chromatogram of CQ and compound B are shown in Figure 10C and Figure 11C, respectively. The compound B are shown in Figure 11D. The retention times of the drug lead B and the CQ at time t=1 hr and t=2 hr are shown in the Table 8. All the samples were estimated in duplicates and the results shown are the average of the two trials (Figures 10 and 11) (Table 8).

According to the results obtained from HPLC method I and II, it was confirmed that both HLM and CYP2C8 has an important role in the metabolism of chloroquine whereas, all drug leads were metabolically stable to these enzyme.

**Discussion**

The *in vitro* metabolism of the new drug leads compared to the positive control chloroquine in the presence of HLM and cDNA expressing CYP2C8 have been demonstrated in this study. In the pharmacokinetic studies of CQ, Frisk-Holmberg [6,7] reported the single dose kinetics of CQ and its major metabolite DCQ in healthy subjects and identified that CQ is dealkylated into two main metabolites, N-desethylchloroquine (DCQ) and N-bis-desethylchloroquine (BDCQ). Identification of metabolites is done by LC-MS. Mass spectrometers help in separating the compounds from each other using the difference in mass-to-charge ratio (m/z) of ionized compounds.

In blood and plasma, the concentration of DCQ was detected up to 20 to 50% of those of the parent compound CQ, whereas the conversion to BDCQ was <10% [6,7]. It is known that CYPs are major drug metabolizing enzyme system. Ofori-Adjei and Ericsson [13] estimated that 30 to 50% of an administered dose of chloroquine is metabolized by the liver via cytochrome P450 enzymes. Identification of the major enzymes, associated with drug's metabolism therefore started by evaluating and identifying the role of CYP isoforms. Xue-Qing Li et al. [14] identified human CYPs that metabolize anti-parasitic drugs using three approaches, (a) Relative Activity Factor (RAF), (b) correlation analysis of their activity, and (c) inhibitors diagnostics. They reported that the major CYPs involved in the metabolism are CYP isoforms 2C8, 3A4, and 2D6. The RAF approach is used significantly to bridge the gap between liver microsomes and recombinant systems. It also estimates the individual P450 contributions to drug metabolism [15,16]. Using the same approach, Projean et al. [9] also reported that CYPs 2C8, 3A4, and 2D6 are the main isoforms catalyzing DCQ formation. This was the first investigation of CQ metabolism in HLM and they identified that CYP2C8 and CYP3A4 contributes collectively

<table>
<thead>
<tr>
<th>Time t=1 hr</th>
<th>Time t=2 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drugs</td>
<td>RT</td>
</tr>
<tr>
<td>B</td>
<td>13.135</td>
</tr>
<tr>
<td>CQ/DCQ</td>
<td>5.46/04.480</td>
</tr>
</tbody>
</table>

*The percent of CQ metabolized using HLM at time t=1 hr and 2 hrs was 4.29% and 6.61%, respectively.

**Table 7:** Data obtained from HPLC and MS for the drug using HLM.
Table 8: Data obtained from HPLC and MS for the drugs using CYP2C8.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>RT (m/z)</th>
<th>Metabolism</th>
<th>MS- (m/z)</th>
<th>RT (m/z)</th>
<th>Metabolism</th>
<th>MS- (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>13.145</td>
<td>NO</td>
<td>495</td>
<td>13.129</td>
<td>NO</td>
<td>495</td>
</tr>
<tr>
<td>CQ/DCQ</td>
<td>5.486/4.533</td>
<td>YES</td>
<td>320,292*</td>
<td>5.432/4.517</td>
<td>YES</td>
<td>320, 292*</td>
</tr>
</tbody>
</table>

*The percent of CQ metabolized using CYP2C8 at time t=1 hr and 2 hrs was 12.26% and 10.33%, respectively.

In the Figure 10, the HPLC chromatograms clearly showed that CQ was metabolized and there was an additional peak before the parent peak which was absent in blank and control sample, partially confirms the metabolism of chloroquine. Moreover, when the samples were tested with LC-MS (Figure 7), positive control chloroquine sample at time t=2 hrs gave two ion peaks, one at 320 m/z and another at 292 m/z, which confirms the metabolism of the drug.

In the Figure 11, the HPLC chromatograms clearly showed that drug lead B was not metabolized but the appearance of an additional peak before the parent peak in blank and control sample confirms the presence of impurity in drug lead B.
from LC-MS showed the ion peak of the drug lead B at time $t=2$ hrs as 495 m/z, which confirms its molecular weight. Henceforth there is no metabolism of the drug lead B.

As discussed earlier, CYP enzymes provide information regarding the metabolites. Nebert and Russell [21] mentioned that there are 270 different CYP gene families, and 18 of those gene families were recorded in mammals that encrypt 57 CYP genes in humans. Out of all CYP enzymes, CYP2C8, 3A4, and 2D6 are involved in Chloroquine metabolism. This was proven in our experiment where CYP2C8 contributed to more than 10% of the total CQ $N$-desethylation over a 100 µM concentration [22]. It has low affinity and high capacity in metabolizing the chloroquine. Further investigations would be conducted utilizing more CYP isozymes at different concentration ranges and time intervals to identify any possible metabolic pathways for these drug leads in near future as a part of our continuous preclinical analysis. This study demonstrated a convenient method development and also a metabolic stability of the drug leads against most important CYP isozyme that is involved in the metabolism of standard drug chloroquine.

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References


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