Validation of a rapid microtiter plate assay to conduct cytochrome P450 2D6 enzyme inhibition studies

Jairam R. Palamanda, Leonard Favreau, Chin-chung Lin and Amin A. Nomeir

Cytochrome P450 (CYP) inhibition studies are often performed on new chemical entities during the drug discovery stage. However, advances in combinatorial chemistry mean that an unprecedented number of compounds now require such evaluation. Recently, a microtiter plate assay that utilizes individually expressed CYP enzymes (Supersomes™) has been developed to facilitate this process. The authors discuss some of their experiences with this technology and its potential impact for higher throughput CYP2D6 enzyme inhibition studies.

The selection of new chemical entities (NCEs) as drug development candidates is a very complex process. An important consideration for certain compounds is the determination of whether or not and to what extent the NCE inhibits a group of heme thiolate proteins, namely the cytochrome P450s (CYP). Oxidative metabolism is catalyzed mainly by CYP to convert a lipophilic molecule into more polar molecule(s) for elimination and/or conjugation[1]. Notably, human liver CYP2D6 and CYP3A4 participate in the oxidative biotransformation of ~80% of commercially available drugs[2]. Thus, inhibition of CYP2D6 may lead to clinically significant drug–drug interactions[2–5].

High-throughput approaches for drug discovery in drug metabolism

Breakthroughs in combinatorial chemistry are generating a vast number of NCEs. Robotics and common end-point assays (e.g. receptor displacement assays) now make it possible to screen hundreds of thousands of compounds relatively quickly for efficacy in *in vitro* pharmacology models, but there is an urgent need to accelerate drug discovery studies related to drug metabolism by using innovative approaches and/or new technologies. So far, some high- (or 'higher') throughput approaches in drug metabolism laboratories have focused on combinatorial pharmacokinetics, stability in liver microsomes and permeability across the human colon carcinoma cell line Caco-2. Combinatorial pharmacokinetics involves dosing laboratory animals with multiple NCEs and assaying the mixture of NCEs in plasma or serum by LC-MS/MS, taking advantage of the superior analytical selectivity attainable in tandem MS (Refs 6,7). These studies are sometimes referred to as 'cassette dosing' or '*n*-in-one dosing'[6].

The stability of NCEs in liver microsomes of animal or human origin is evaluated *in vitro*. NCEs that are metabolized rapidly are discarded, and relatively stable NCEs are progressed further[8]. Higher-throughput microsomal stability-

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studies have been implemented by coupling robotic manipulation of microsomal incubations with LC-MS/MS measurements of NCEs and their metabolites.

Screening for permeability across the human Caco-2 monolayer is gaining popularity in the pharmaceutical industry for the evaluation of NCEs for potential oral absorption. This system can be adapted to higher-throughput screening either by evaluating a mixture of compounds in the same experiment or by pooling samples containing many NCEs and analyzing each NCE selectively by LC-MS/MS.

Higher-throughput CYP enzyme inhibition

A current topic of pivotal importance is the measurement of CYP marker enzyme activities in the presence of selected concentrations of NCEs. Conventional CYP enzyme inhibition studies utilize analyses that rely on chromatographic separation techniques, but these are time-consuming and compromise throughput considerably. Thus, in order to keep pace with the increasing number of NCEs that are being generated by combinatorial chemistry, innovative and rapid throughput approaches for performing

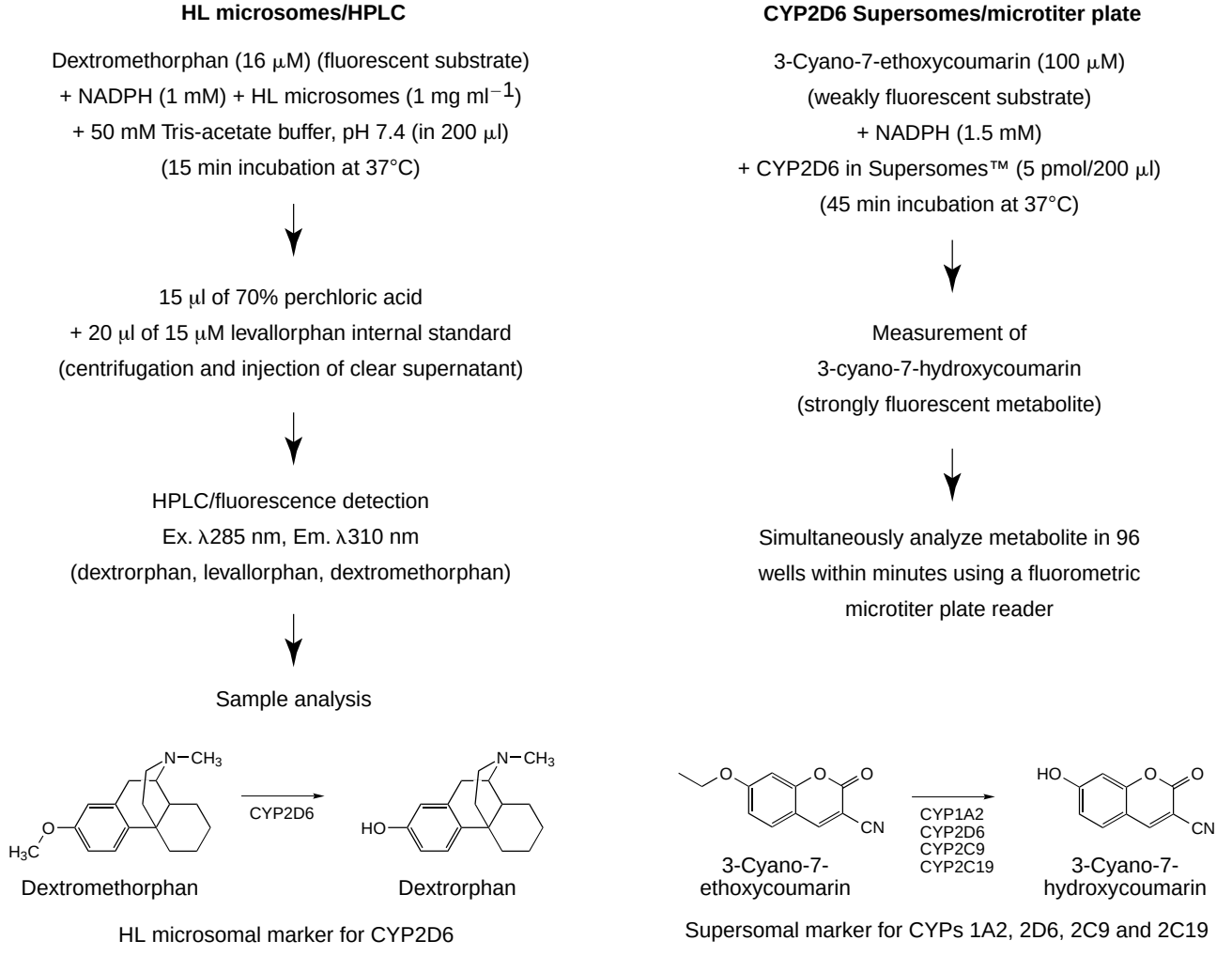

Figure 1. *Comparison of CYP2D6 activity analysis in human liver (HL) microsomes and Supersomes. The formation of dextrorphan was quantified by HPLC using a 3 μm Microsorb MV phenyl column (10 cm × 4.6 mm ID). The mobile phase was a mixture of 30% acetonitrile, 1% acetic acid and 0.05% triethylamine in water, delivered isocratically at 0.75 ml min^{-1}. Dextrorphan (product), levallorphan (internal standard) and dextromethorphan (substrate) were detected fluorometrically at excitation and emission wavelengths of 285 and 310 nm, respectively. The retention times for dextrorphan, levallorphan and dextromethorphan were 4.5, 7.0 and 12.0 min, respectively. Also shown are the marker substrates and reactions used for the determination of CYP2D6 activities in each assay.*

CYP2D6 inhibition studies are required. One such approach has been to incubate [*O*-methyl-^{14}C]dextromethorphan with human liver (HL) microsomes and NADPH (Ref. 9). The basis of the assay is the quantitative measurement of [^{14}C]formaldehyde formed in the incubation buffer after extraction of unmetabolized [*O*-methyl-^{14}C]dextromethorphan with methylene chloride.

Recently, a microtiter plate method that utilizes Supersomes™, or individually expressed recombinant CYPs, has become available[10]. This allows the simultaneous measurement of 96 samples in ~10 min, which is much faster than conventional HPLC methods using HL microsomes. As part of the validation of this approach, the present study was initiated to compare CYP2D6 inhibition by 62 NCEs in both Supersomes and HL microsomes. The NCEs can be broadly classified into imidazole- and non-imidazole-containing compounds. Most of the NCEs (79%) contained one imidazole ring, and 5% contained two imidazole rings. The remaining 16% contained either pyrrolidine or piperidine or cycloheptane 1-aza ring structures.

Approaches for conducting CYP2D6 inhibition studies

Conventional approach using HL microsomes and HPLC analysis

Figure 1 illustrates the procedure that was used for the measurement of HL microsomal CYP2D6 activity. The analysis was performed by HPLC as previously described[11] (see Fig. 1 for details).

When the fluorescent marker substrate dextromethorphan is incubated at low micromolar concentrations with HL microsomes and NADPH, CYP2D6 almost exclusively *O*-demethylates dextromethorphan to its fluorescent metabolite, dextrorphan[11]. Because dextromethorphan and dextrorphan are both fluorescent at the same excitation and emission wavelengths, it is not possible to use a microtiter plate reader to measure dextrorphan formation alone. Dextrorphan is therefore separated from dextromethorphan by HPLC and analyzed fluorimetrically. Sample analysis by HPLC is a time-consuming process that requires inspection, sorting and processing of innumerable chromatograms.

Microtiter plate assay for CYP2D6

The microtiter plate assay utilizes the method of Crespi *et al.*[10] As shown in Fig. 1, the microtiter plate assay for CYP2D6 is performed using the weakly fluorescent substrate 3-cyano-7-ethoxycoumarin, which is metabolized to the strongly fluorescent metabolite 3-cyano-7-hydroxy-

Table 1. NCEs with matching supersomal and human liver (HL) microsomal IC_{50} values (≤ fivefold difference)

Compound number	IC_{50} HL microsomes (μM)	IC_{50} Supersomes™ (μM)	Fold difference[a]
1	1.40	0.48	−2.9
2	0.73	0.67	−1.1
3	1.30	0.35	−3.7
4	0.08	0.08	No difference
5	0.09	0.05	−1.8
6	0.54	0.86	+1.6
7	0.72	0.87	+1.2
8	0.46	1.03	+2.2
9	2.70	1.61	−1.7
10	~40.00	22.80	−4.4
11	0.14	0.10	−1.4
12	No inhibition	No inhibition	No difference
13	0.68	1.21	+1.8
14	0.09	0.28	+3.1
15	No inhibition	No inhibition	No difference
16	0.43	0.20	−2.2
17	0.08	0.06	−1.3
18	2.00	1.00	−2.0
19	0.11	0.04	−2.8
20	0.20	0.25	+1.3
21	1.50	1.25	−1.3
22	0.50	0.33	−1.5
23	1.00	2.00	+2.0
24	0.90	1.00	+1.1
25	~40.00	10.00	−4.0
26	0.08	0.12	+1.5
27	0.80	1.50	+1.9
28	8.00	8.00	No difference
29	0.70	1.80	+2.6
30	0.20	0.45	+2.3
31	0.07	0.05	+1.2
32	15.00	25.00	+1.7
33	0.30	0.15	−2.0
34	0.13	0.40	+3.1
35	~40.00	10.00	−4.0
36	1.00	0.24	−4.2
37	0.10	0.022	−4.5
38	No inhibition	No inhibition	No difference
39	11.00	3.30	−3.3
40	0.80	0.36	−2.3
41	9.00	5.20	−1.7
42	0.57	0.29	−2.0
43	0.08	0.37	+4.6
44	0.43	0.56	+1.3
45	0.17	0.48	+2.8
46	0.14	0.05	−2.8
47	1.56	3.80	−2.4
48	0.08	0.37	+4.6
49	15.30	5.20	−2.9
50	0.77	0.16	−4.8
51	30.00	30.00	No difference
52	14.00	16.80	+1.0
53	0.05	0.13	+2.6

[a]+, indicates higher IC_{50} values in Supersomes™; −, indicates higher IC_{50} values in HL microsomes.

coumarin. In addition to CYP2D6, the reaction is catalyzed by CYP1A2, CYP2C9 and CYP2C19, and it has been used to determine the activity of each of these isoforms by using individually expressed recombinant enzymes[10]. Because the substrate 3-cyano-7-ethoxycoumarin is weakly fluorescent and the metabolite 3-cyano-7-hydroxycoumarin is strongly fluorescent, the formation of the metabolite can easily be measured using a fluorescent microtiter plate reader after subtracting the background fluorescence. We used a CytoFluor Series 4000 Multi-well Plate Reader (PerSeptive Biosystems, Framingham, MA, USA) set at an excitation wavelength of 420 nm with a band width of 50 nm and an emission wavelength of 490 nm with a band width of 40 nm to quantify 3-cyano-7-hydroxycoumarin. The microtiter plate reader allows the rapid measurement of 96 samples within 10 min. Relative CYP2D6 activity is calculated from the fluorescence data using CytoCalc Data Analysis Software (PerSeptive Biosystems). The IC_{50} values were determined by semi-log plotting over three orders of magnitude of inhibitor concentration (0.03–30 μM) on the *x*-axis versus percentage of remaining activity on the *y*-axis.

Potential of higher-throughput screening to predict CYP2D6 inhibition

Initially, we determined the IC_{50} for quinidine, a prototype CYP2D6 inhibitor, and compared it to literature values. The IC_{50} for quinidine in the microtiter plate assay was 9.5 nM, which is consistent with that reported earlier in the same assay[10] (8.9 nM) and in agreement with the reported K_i (27 nM) in HL microsomes for CYP2D6 (Ref. 12).

Application of the rapid microtiter plate assay for estimating CYP2D6 IC_{50} values showed that, for 53 of the 62 NCEs evaluated, supersomal IC_{50} values agreed within a fivefold difference with HL microsomal IC_{50} data (Table 1). A difference of fivefold or less in the IC_{50} values is considered acceptable because data from a single CYP isoform (Supersomes) are being compared with data from a mixture of CYP enzymes (HL microsomes) and the substrates are different. Also, literature IC_{50} values for quinidine inhibition of CYP2D6 using dextromethorphan[13] and bufuralol[14] as substrates are 0.22 and 0.04 μM, respectively – a difference slightly over fivefold. Overall, supersomal IC_{50} curves could be superimposed on HL microsomal IC_{50} curves over a wide range of inhibition, from very potent (IC_{50} <0.5 μM) to intermediate (IC_{50} >0.5–5 μM) and weak (IC_{50} >5 μM) inhibitors (Fig. 2a–2c, respectively).

Deviation of supersomal IC_{50} values from HL microsomal IC_{50} values

For nine of the 62 NCEs, supersomal and HL microsomal IC_{50} values differed by more than fivefold (Table 2). Compared to HL microsomal IC_{50}s, supersomal IC_{50} values

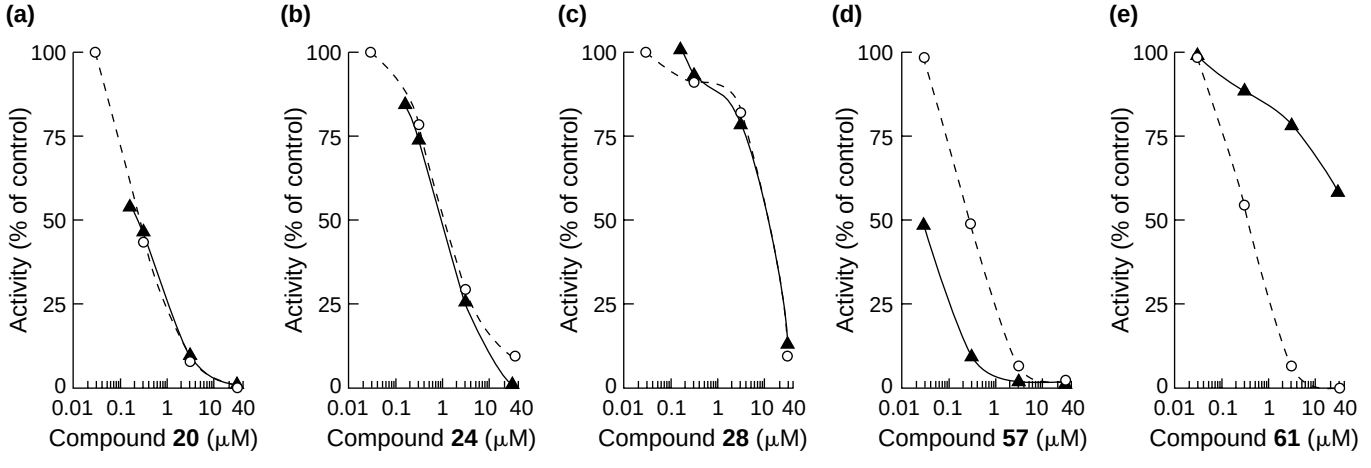

Figure 2. *Human liver microsomal and supersomal IC_{50} curves for (a) highly potent, (b) intermediate and (c) weak CYP2D6 inhibitors. Parts (d) shows underestimated and (e) shows overestimated human liver microsomal CYP2D6 inhibition by new chemical entities. Circles represent Supersomes, triangles represent HL microsomes.*

Table 2. NCEs with non-matching supersomal and human liver (HL) microsomal IC_{50} values (> fivefold difference)

Compound number	IC_{50} HL microsomes (μM)	IC_{50} Supersomes™ (μM)	Fold difference[a]
54	0.08	0.76	+9.5
55	0.05	0.70	+14.0
56	0.12	1.30	+11.0
57	0.03	0.27	+9.0
58	0.06	0.74	+12.0
59	0.05	1.40	+28.0
60	>30.00	3.10	>−9.7
61	~40.00	0.43	−93.0
62	0.61	0.10	−6.1

[a]+, indicates higher IC_{50} values in Supersomes™ −, indicates higher IC_{50} values in HL microsomes.

were lower for three NCEs and higher for six other NCEs (Table 2). These deviations resulted in supersomal IC_{50} curves that either underestimated (Fig. 2d) or overestimated (Fig. 2e) HL microsomal IC_{50}.

Speculation about the deviation

The reasons for the greater than fivefold differences between supersomal and HL microsomal IC_{50} values that were observed for ~14% of the NCEs (9/62) are not fully understood. However, we speculate that, if the NCE is rapidly metabolized by CYP isoforms other than CYP2D6 that are present only in HL microsomes, the concentration of the NCE in the HL microsomal incubation is decreasing continuously. Therefore, a lower concentration of the NCE is present at a given incubation time in HL microsomal incubations compared with supersomal incubations. Hence, lower apparent IC_{50} values (three of the nine deviant NCEs) are obtained using Supersomes.

For NCEs that are rapidly metabolized by CYP2D6, the greater amount of CYP2D6 present in Supersomes compared with HL microsomes (5 pmol versus an estimated 1 pmol in our HL microsomal incubation) coupled with the longer incubation time of 45 min will result in rapid metabolism of the NCE in the supersomal incubations and thus a lower concentration of the NCE in the supersomal incubation mixture after prolonged incubation. Consequently, higher IC_{50} values/curves (six of nine deviant NCEs) are obtained using Supersomes. Indeed, if confirmed, such a comparison may actually be used to predict potential CYP2D6 substrates. Nevertheless, the basis for these apparent deviations needs further investigation.

Impact of the higher-throughput approach

The utility of the microtiter plate assay for conducting CYP2D6 enzyme inhibition studies using Supersomes is proving to be invaluable in discovery programs that require early assessment of the potential of NCEs to inhibit CYP2D6. The data presented in this review are from such a discovery program. The finding that six of the 62 NCEs (9.7%) are more potent inhibitors of HL microsomal CYP2D6 than of supersomal CYP2D6 is not of major concern (false positives) because these NCEs would be eliminated by further tests (i.e. the HL microsomal screen); but the finding that three of the 62 NCEs (4.8%) are more potent inhibitors of supersomal CYP2D6 than of HL microsomal CYP2D6 is of concern because of the possibility that these three NCEs might be discarded as false negatives. However, overall, the use of Supersomes coupled with the microtiter plate technology has greatly improved the selection of appropriate NCEs quickly by speeding up the analytical throughput and data throughput by tenfold. There is a 'balancing act' between discarding a few false negatives and enhanced throughput, but the overall benefit derived by accelerating our discovery program outweighs the loss of ~5% of NCEs during the first supersomal screen.

Conclusions

The results of the present study demonstrate that Supersomes could be used for conducting higher-throughput CYP2D6 inhibition studies. However, human liver microsomes should subsequently be used to confirm the supersomal results on the few selected NCEs that are likely to be recommended for further development.

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