Evaluation of dilution, dialysis and ultracentrifugation methods to assess the reversibility of metabolism-dependent inhibitors (MDIs) of cytochrome P450 (CYP) enzymes

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INTRODUCTION

Metabolism-dependent inhibition (MDI) of P450 enzymes is a well-recognized cause of clinically significant drug-drug interactions (DDI). For this reason, the US Food & Drug Administration (FDA) and the European Medicines Agency (EMA) have both published draft guidance documents on DDI that require an *in vitro* assessment of the ability of drug candidates to cause MDI of the major drug-metabolizing P450 enzymes (2006, 2010). The most recent PhRMA publication and EMA draft guidance discuss *in vitro* experiments, to not only identify the potential for a drug candidate to cause MDI (e.g. IC_{50} shift experiments), but also to evaluate whether MDI involves reversible or irreversible inhibition (Grimm *et al.*, 2009).

To assess whether the mechanism of MDI involves reversible or irreversible inhibition, researchers often employ a dilution, dialysis or ultracentrifugation procedure (Grimm *et al.*, 2009). The dilution method necessitates the use of relatively high concentrations of human liver microsomes (HLM) (≥ 1 mg/mL) in a pre-incubation step, and this feature is frequently used with the dialysis method.

Dialysis procedures can be problematic when assessing the reversibility of MDIs. Ma et al (2000) reported that CYP3A4 inhibition by three well-established quasi-irreversible inhibitors, namely troleandomycin, verapamil, and diltiazem, was reversed by dialysis alone.

In the present study, we evaluated an ultracentrifugation method (also referred to as microsomal re-isolation or washing method) to determine the mechanism of MDI (i.e., to distinguish reversible from [quasi]-irreversible inhibitors) by 19 well-established MDIs. The present study underscores the potential for the dilution and dialysis methods to misidentify the mechanism of MDI and supports the use of the ultracentrifugation method to evaluate the reversibility of MDIs.

MATERIALS & METHODS

Chemicals and Reagents:
Gemfibrozil glucuronide, tienilic acid and azamulin were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada); Cypex (Dundee, Scotland, UK); and BD Biosciences (Bedford, MA), respectively. Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Methods:

Assessment of MDI reversibility:

A: Dilution
30-min pre-incubation → 25x dilution → activity assay

B: Dialysis
30-min pre-incubation → 24h dialysis at 4°C, mwco 20K dalton → activity assay + BCA

C: Ultracentrifugation
30-min pre-incubation → 10 min +/- 2 mM potassium ferricyanide → centrifuge 30000 rpm, 15 min at 4°C → activity assay + BCA

Assessment	Pre-incubation (PI)		Activity assay		
	Inhibitor (μM)	PI protein (mg/mL)	Substrate (μM)	Protein (mg/mL)	Incubation time (min)
A	Mibefradil (0.1)	1.25	Midazolam (40)	0.05	5
B	Mibefradil (2)	1	Testosterone (250)	0.2	5
	Troleandomycin (50)	1	Testosterone (250)	0.2	5
C	Various see Table 2	0.0125 - 1 mg/mL	Various (~10x Km)	0.0125 - 0.2	5

RESULTS

Figure 1 shows the effects of the dilution method on the evaluation of CYP3A4 MDI reversibility by mibefradil (Posicor®). **Figure 1A** demonstrates that a low concentration of mibefradil (0.1 μM) inhibits CYP3A4 activity in a time-dependent manner when the 30 minute pre-incubation step is conducted with a low concentration of HLM (0.05 mg/mL) and no dilution. **Figure 1B** shows that the same low concentration of mibefradil (0.1 μM) causes little to no inhibition of CYP3A4 when it is pre-incubated with a 25-fold higher concentration of HLM (1.25 mg/mL) followed by a 25-fold dilution prior to measurement of residual CYP3A4 activity. **Figure 1C** demonstrates that, for the dilution method, CYP3A4 inhibition is apparent after increasing the concentration of mibefradil in the same ratio as conducted with the HLM concentration (*i.e.* 25-fold to 2.5 μM).

Figure 1

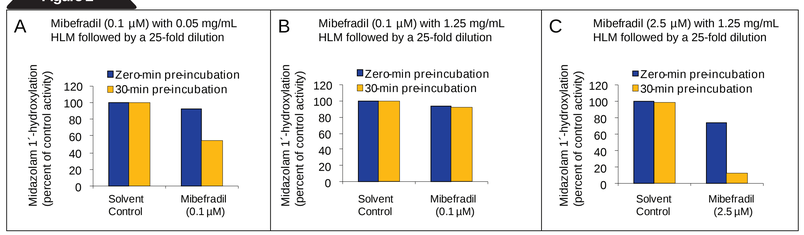

Figure 2 shows the effects of dialysis and microsomal re-isolation/washing on the reversibility of three MDIs that irreversibly inhibit CYP3A4 activity by either MIC formation (troleandomycin and verapamil) or covalent binding (mibefradil). Following an overnight dialysis step, the MDI of CYP3A4 was still observed with mibefradil; however, the inhibition of CYP3A4 activity by troleandomycin and verapamil was reversed following the dialysis procedure, in contrast to re-isolation, for which inhibition of CYP3A4 activity was still observed. Inhibition of CYP3A4 activity by troleandomycin and verapamil was reversed by treatment with potassium ferricyanide. The inhibition of CYP3A4 by mibefradil, an irreversible inhibitor that covalently modifies the enzyme, was not reversed by re-isolation or treatment with potassium ferricyanide. In the absence of NADPH, none of the MDIs caused a marked inhibition of CYP3A4 (data not shown).

Figure 2

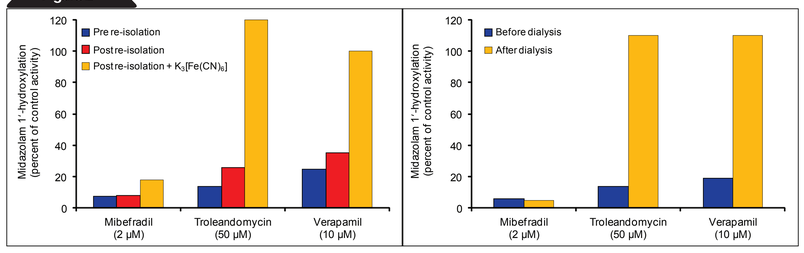

Table 1 summarizes an evaluation of the microsomal re-isolation method to assess the reversibility of 19 MDIs of various CYP enzymes. In the case of MIC formation, treatment with potassium ferricyanide reversed those nitrogenous-based MI complexes; whereas it did not reverse non-nitrogenous MI complexes.

Table 1

P450 Enzyme	Inhibitor	[Inhibitor] (μM)	Percent Residual Activity				Mechanism of MDI		
			Solvent Control	Without Re-isolation	With Re-isolation	$K_3[Fe(CN)_6]$/ Re-isolation	Experimentally Determined	Reported in literature (with Reference)	
CYP1A2	Furafylline	1	100	36	62	76	Irreversible	Irreversible	Kunze and Trager, 1993
CYP2A6	8-MOPs	0.05	100	23	31	57	Irreversible	Irreversible	Koenigs et al., 1997
CYP2B6	Phencyclidine	30	100	24	30	32	Irreversible	Irreversible	Jushchyshyn et al., 2003
CYP2C8	Gemfibrozil glucuronide	25	100	7.0	8.5	11	Irreversible	Irreversible	Ogilvie et al., 2006
CYP2C9	Tienilic acid	0.5	100	15	15	19	Irreversible	Irreversible	Lopez-Garcia et al., 1994
CYP2C19	Fluoxetine (racemic)	2	100	22	73	110	Reversible	MIC[b]	Murray and Murray, 2003
CYP2C19	*R*-Fluoxetine	10	100	16	76	93	Reversible	MIC	Vandenbrink et al., 2010
CYP2C19	*S*-Fluoxetine	10	100	7.2	13	96	MIC	MIC	Hansen et al., 2010
CYP2C19	Ticlopidine	0.75	100	21	17	13	Irreversible	Irreversible	Ha-Duong et al., 2001
CYP2D6	MDMA	10	100	43	45	52	Irreversible[c]	MIC[c]	Delaforge et al., 1999
CYP2D6	Paroxetine	0.3	100	52	44	48	Irreversible[c]	MIC[c]	Bertelsen et al., 2003
CYP3A4	Azamulin	0.5	100	4.2	5.4	7.4	Irreversible	Irreversible	Stresser et al., 2004
CYP3A4	Clarithromycin	25	100	62	64	96	MIC	MIC	Mayhew et al., 2000
CYP3A4	Diltiazem	50	100	30	43	130	MIC	MIC	Ma et al., 2000
CYP3A4	Hydrastine	10	100	43	49	47	Irreversible[c]	MIC[c]	Chatterjee and Franklin, 2003
CYP3A4	Mibefradil[a]	0.1	100	29	37	42	Irreversible	Irreversible	Prueksaritanont et al., 1999 and Foti et al., 2011
CYP3A4	Mibefradil	2	100	3.7	3.7	7.8	Irreversible	Irreversible	Prueksaritanont et al., 1999 and Foti et al., 2011
CYP3A4	Mifepristone	5	100	3.0	2.5	5.0	Irreversible	Irreversible	He et al., 1999
CYP3A4	Troleandomycin[a]	5	100	21	32	110	MIC	MIC	Pessayre et al., 1981
CYP3A4	Troleandomycin	25	100	16	19	82	MIC	MIC	Pessayre et al., 1981
CYP3A4	Verapamil	10	100	22	26	100	MIC	MIC	Ma et al., 2000

[a] Marker substrate reactions for CYP3A4 activity were conducted with midazolam, whereas all others were conducted with testosterone.
[b] Fluoxetine (racemic mixture) formed a MIC with rat CYP2C11.
[c] These inhibitors contain a methylenedioxyphenyl moiety and were reported to form a non-nitrogenous MIC. Restoration of enzyme activity was reported to be resistant to potassium ferricyanide treatment with this type of MIC (Franklin 1977).
All values were rounded to two significant figures.

CONCLUSIONS

- These data demonstrate the potential for irreversible MDIs of CYP enzymes to be incorrectly identified as reversible when the assessment of reversibility is conducted with a dilution method.
- Under these experimental conditions, dialysis has the potential to reverse some nitrogen-based MICs for quasi-irreversible inhibitors such as troleandomycin and verapamil, in contrast to the clinical outcome.
- These data support the use of ultracentrifugation procedures and microsomal re-isolation coupled with potassium ferricyanide treatment to evaluate MDI reversibility and identification of nitrogen-based MICs.

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