Time-Course Disposition of Ritonavir and Prototypical Hepatic Inducers in Cultures of Primary Human Hepatocytes: Context for Induction & Inhibition Concentration Responses

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Introduction

Primary human hepatocytes are recognized as the "gold standard" model system for the assessment of cytochrome P450 induction potential in humans as they are known to retain the appropriate molecular signaling pathways involved in the control of CYP450 expression levels in human liver. These cultures represent a dynamic, responsive cellular system that has been shown to effectively model drug metabolism, induction, and inhibition in vitro.

To model human liver with complex drug-drug interactions properties (e.g. ritonavir that both inhibits and induces liver metabolic activity as well as being rapidly metabolized in humans) we have utilized this model system to simultaneously assess the effects of several drugs (e.g. inducers, inhibitors, time-dependent inhibitors, dual inhibitor/inducers) on liver function markers via mRNA expression, protein content, and metabolic enzymatic activity determinations. In addition we have assessed the concentrations of these chemicals within culture monolayers to correlate data with known cell-free parameters such as receptor binding affinity and inhibition equilibrium constants (e.g. Ki). This approach demonstrates the utility in using temporal response profiles to identify and model the kinetics of drugs with complex drug interaction potential.

Materials & Methods

Primary cultures of human hepatocytes were prepared from human liver tissue derived from normal remnants of resected liver tissue. Hepatocytes were isolated by a collagenase perfusion method described by LeCluyse, et al. (2005). Culture media (William's E) containing 50 nM dexamethasone, ITS+ and 0.25 mg/ml Matrigel® was changed on a daily basis. Hepatocyte cultures in 24-well format and 60 mm dishes were incubated with positive control inducers, positive control inhibitors, and dual inhibitors/inducers. At time points of 0.25, 0.5, 2, 6, 24, 48, and 72-hours (and 96 hrs for ritonavir only), cultures were harvested by an initial 10 min wash with HBSS, followed by incubations with selective probe substrates for CYP2B6, CYP3A, and CYP1A2. Cell plates were treated with ABI Nucleic Acid Lysis Solution™ and frozen for mRNA analysis by RT-qPCR (TaqMan®). Total RNA was isolated using a 6100 Nucleic Acid Prepstation (ABI). RT reactions were completed with High Capacity cDNA Archive Kit (ABI) and qPCR was performed with a 7900HT detection system (ABI).

Abbreviations: 3-methylcholanthrene (3-MC), phenobarbital (PB), rifampicin (RIF), omeprazole (OMP), chenodeoxycholic acid (CDCA), fenofibric acid (FFA).

Figure 1 – Hepatocyte Monolayer

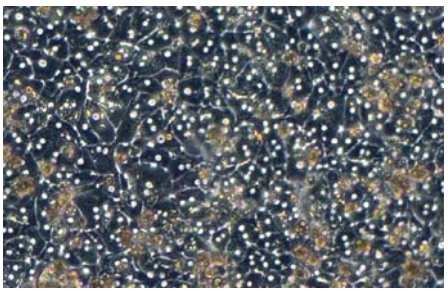

Table 1 – Ritonavir Disposition in Sandwich Cultured Human Hepatocytes

Summary of ritonavir concentrations inside and outside sandwich cultured hepatocyte monolayers at a few notable concentrations as determined culture supernatants and monolayer fractions using LC-MS/MS based detection.

Time	[Ritonavir] Added	Percent in Monolayer	Supernatant Concentration (µM)	Est. Monolayer Concentration (µM)†
15 min	0.01 µM	8.75	0.00665	0.213
	0.1 µM	13.5	0.0738	3.83
	1 µM	23.1	0.532	53.3
	10 µM	16.1	5.67	363
24 hr	0.01 µM	100	0*	0.0262
	0.1 µM	99.4	0.000287	15.1
	1 µM	79.5	0.0791	102
	10 µM	19.2	5.34	424
48 hr	0.01 µM	100	0*	0.209
	0.1 µM	92.5	0.00653	27.0
	1 µM	73.5	0.113	1056
	10 µM	18.9	7.14	554

*below detection limits
†Assumed 4 µL volume per million cells

Figure 1 –Ritonavir Disposition Time Course in Cultures of Primary Human Hepatocytes

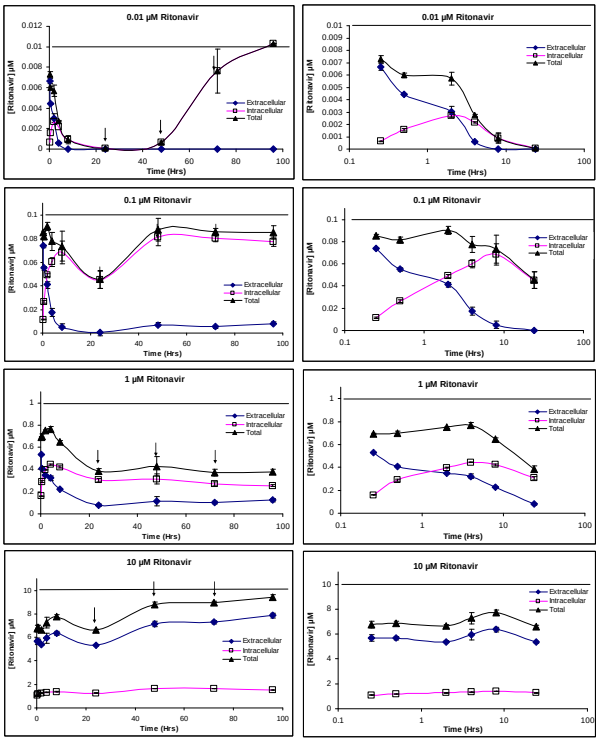

Figure 1. Concentrations of ritonavir detected in the cell monolayer and supernatants (media) after 15 min, 30 min, 2 hr, 6 hr, 24 hr, 48 hr, 72 hr, and 96 hr in culture. Media containing inducers was refreshed daily.

Figure 3 – Effects of Ritonavir on CYP3A4 Activity & mRNA Expression

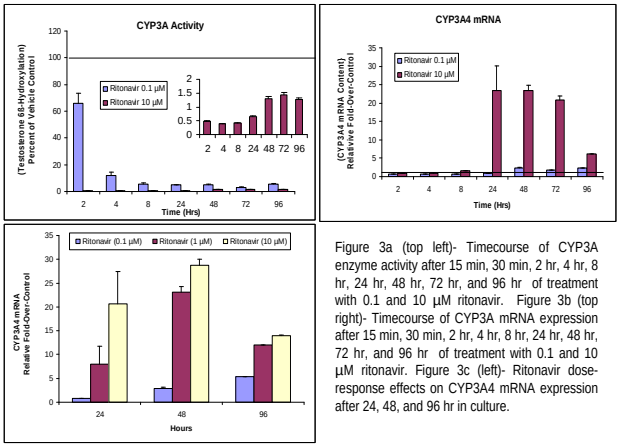

Figure 3a (top left)- Timecourse of CYP3A enzyme activity after 15 min, 30 min, 2 hr, 4 hr, 8 hr, 24 hr, 48 hr, 72 hr, and 96 hr of treatment with 0.1 and 10 µM ritonavir. Figure 3b (top right)- Timecourse of CYP3A mRNA expression after 15 min, 30 min, 2 hr, 4 hr, 8 hr, 24 hr, 48 hr, 72 hr, and 96 hr of treatment with 0.1 and 10 µM ritonavir. Figure 3c (left)- Ritonavir dose-response effects on CYP3A4 mRNA expression after 24, 48, and 96 hr in culture.

Figure 4 – Effects of Rifampicin on Enzyme Activity & mRNA Expression

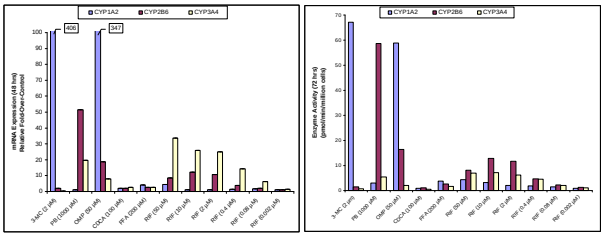

Figure 4 – Effects of prototypical positive control inducer (3-MC, PB, OMP, CDCA, FFA, and RIF) on CYP1A2, CYP2B6, & CYP3A4 mRNA expression at 48 hrs (left) and enzyme activity at 72 hrs (right). Media containing inducers was refreshed daily.

Table 2 – Rifampicin Disposition in Sandwich Cultured Human Hepatocytes

Time	[RIF] Added	Percent in Monolayer	Supernatant Concentration (µM)	Est. Monolayer Concentration (µM)†
15 min	50 µM	6.64	29.5	700
	10 µM	8.69	4.05	129
	2 µM	21.2	0.492	44.2
	0.4 µM	7.38	0.168	4.47
	0.08 µM	8.61	0.0275	0.862
	0.002 µM	8.98	0.00404	0.133
24 hr	50 µM	5.10	20.6	370
	10 µM	7.18	3.08	79.6
	2 µM	12.6	0.355	17.1
	0.4 µM	20.4	0.0382	3.26
	0.08 µM	14.2	0.00970	0.537
	0.002 µM	17.2	0.000896	0.0621
48 hr	50 µM	4.92	29.4	506
	10 µM	6.93	3.52	87.3
	2 µM	13.3	0.451	23.0
	0.4 µM	18.4	0.0547	4.12
	0.08 µM	14.6	0.0113	0.647
	0.002 µM	15.0	0.00277	0.163

†Assumed 4 µL volume per million cells

Table 3 – Other prototypical Inducer Disposition in Sandwich Cultures of Human Hepatocytes

Time	[Inducer] Added	Percent in Monolayer	Supernatant Concentration (µM)	Est. Monolayer Concentration (µM)†
15 min	3-MC 2 µM	5.32	6.91	129
	OMP 50 µM	11.5	80.9	3490
	PB 1000 µM	3.18	923	10100
	CDCA 100 µM	6.55	90.7	2120
	FFA 200 µM	3.27	201	2270
24 hr	3-MC 2 µM	21.2	1.79	161
	OMP 50 µM	9.86	15.7	571
	PB 1000 µM	2.26	836	6450
	CDCA 100 µM	8.84	33.5	1080
	FFA 200 µM	3.47	119	1420
48 hr	3-MC 2 µM	14.6	2.57	146
	OMP 50 µM	7.71	7.51	209
	PB 1000 µM	2.07	836	5900
	CDCA 100 µM	8.90	41.0	1330
	FFA 200 µM	3.63	123	1540

†Assumed 4 µL volume per million cells

Table 4 – Potency Implications: Relevant Parameters

	Ritonavir	Rifampicin
EC_{50} PXR Activation	~ 2 µM	~1 µM
K_D PXR Binding	~ 2 µM	
EC_{50} of CYP3A4 induction in primary human hepatocytes	~ 0.15 µM	0.50 µM
K_i TDI in HLM	0.17 µM	n/a
IC_{50} in HLM	0.14 µM	n/a
~IC_{50} in primary human hepatocytes	~ 0.01 µM	n/a

Results & Conclusions

• Measuring disposition of drugs such as ritonavir and rifampicin in cultures of human hepatocytes provides 'intracellular' concentrations context to induction, inhibition, and other data generated in vitro.

•Addition of low concentrations (e.g. 0.1 µM) of ritonavir in human hepatocyte cultures produced profoundly higher 'intracellular' concentrations (e.g. 100X).

•Potency responses with functional phenotypes (e.g. induction, inhibition responses) and cell-free endpoints (e.g. nuclear receptor potency and mRNA induction EC_{50}s, K_i) are more closely correlated when accounting for 'intracellular' exposures.

• Ritonavir is a potent inhibitor of CYP3A activity but is a highly efficacious inducer of CYP3A4 mRNA content with a net effect of inhibition at higher concentrations due to the high potency of inhibition.

•Ritonavir is rapidly metabolized in human liver microsomes; however in vitro and higher 'intracellular' concentrations (e.g. ~100X concentrations added to cultures) appear to accumulate to produce induction and inhibition that may be reflective of zone-3 type concentrating effects in the liver.

•Ritonavir at low concentrations resides mostly inside the cell monolayer, however at 10 µM the majority remains in the supernatant, while for rifampicin, the relative proportion inside and outside monolayers appears to be maintained at all concentrations examined.

• Prototypical inducers 3-MC, PB, CDCA, and FFA all appear to exhibit intracellular versus supernatant profiles similar to RIF, with relative proportions remaining constant across all concentrations. Metabolism appears to play a significant role in the disposition of OMP, with only 2% of the parent compound (at 50 µM) remaining at 72 hrs.

References

LeCluyse EL, Alexandre E, Hamilton GA, Viollon-Abadie C, Coon DJ, Jolley S, and Richert L (2004). Isolation and culture of primary human hepatocytes. *Methods Mol. Biol.* 290: 207-230.