Comparison of Primary vs. Proliferative Human Hepatocytes in Long-Term Culture: Metabolic Capability and Usefulness for Clearance Prediction

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1. Abstract

The purpose of this study was to evaluate upcyte® human hepatocytes (UHH) as an alternative *in vitro* system for studying hepatic drug metabolism and for clearance prediction of metabolically stable compounds. Derived from primary human hepatocytes (PHH) by lentiviral transduction of proliferation stimulating genes, UHH provide a virtually unlimited source of non-transformed primary hepatocytes. Differentiated UHH express adult hepatic markers and are responsive to prototypical inducers of cytochrome P450 enzymes[1,2]. Compared to immortalized cell lines, confluent UHH retain expression and functionality of nuclear receptors and drug metabolizing enzymes with regard to major CYP, UGT and SULT at donor-specific levels[3,4].

We assessed the metabolic capability of UHH in sandwich culture for up to 21 days, focusing on functional *in situ* enzyme activity and relative mRNA expression of selected phase I and phase II enzymes. Absolute enzyme protein expression was determined by LC-MS/MS quantification. *In vivo* hepatic clearance (CL_H) was predicted for a set of slowly and intermediate cleared reference drugs by scaling from *in vitro* intrinsic clearance (CL_{int}). The *in vitro* metabolite pattern was semi-quantitatively analyzed exemplarily for alprazolam and meloxicam. Data from the above experiments were compared to those obtained from sandwich cultures of cryopreserved PHH.

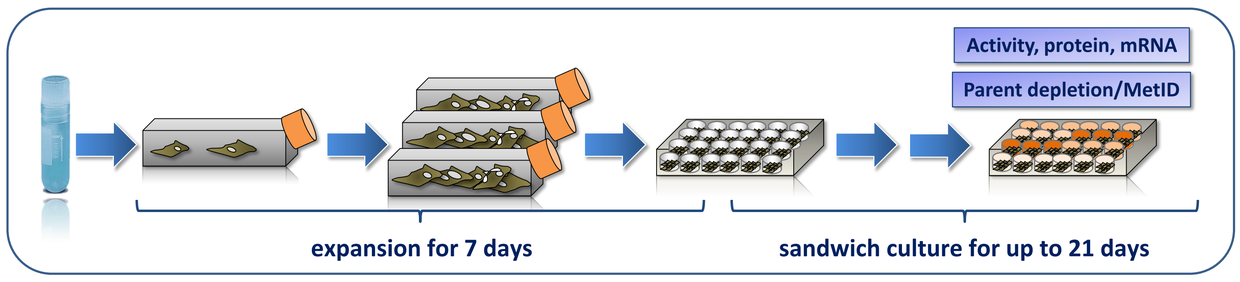
2. Objectives

Focus of the investigations was on the assessment of upcyte® human hepatocytes and comparison with standard primary human hepatocyte cultures regarding:

- **Activity-time profile for key P450s, UGTs, and SULT in sandwich culture**
- **Utility for CL_H prediction of slowly metabolized compounds**
- **Metabolite pattern for selected reference drugs**

3. Methods

Experimental workflow for expansion and culturing of Upcyte® human hepatocytes (UHH)



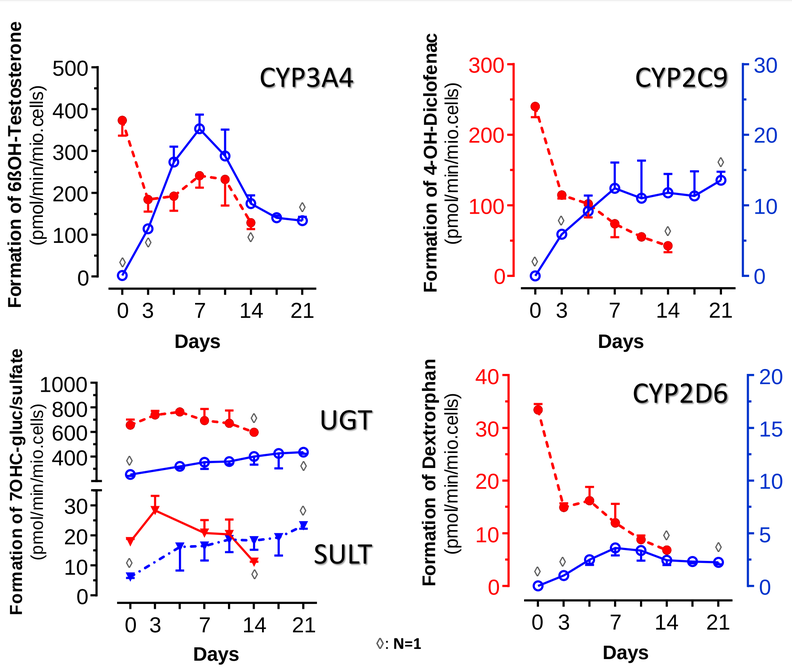
Analysis of enzyme activity, mRNA & protein expression, and metabolite pattern

Activities of cytochrome P450, UGT and SULT enzymes were determined *in situ* via metabolite formation (LC-MS/MS) after incubation with probe substrates[3]. Relative mRNA levels were determined by Taqman RT-PCR[3]. Protein quantification was performed from microsomal preparations by LC-MS/MS quantification of specific tryptic fragments[5]. Metabolites for reference drugs were identified from supernatants by exact mass (LTQ Orbitrap) according to literature[6,7] and relative abundance was determined semi-quantitatively by extracted-ion count (XIC).

In vivo CL_H prediction

Reference drugs with low to intermediate *in vivo* $CL_{nonrenal}$ and metabolism as primary elimination pathway were incubated in 24-well format for up to 120 h. *In vivo* CL_H was scaled from *in vitro* CL_{int} applying the physiologically based *in vitro-in vivo* direct scaling approach and the well-stirred model[3].

4. Results



4.1 Metabolic activity over time in sandwich culture

Enzyme activities (exemplarily shown for CYP3A4, 2C9, 2D6, UGT, and SULT) were determined by *in situ* metabolite formation in 24-well sandwich culture for up to 21 days. Data represent mean ± SD of N=2-5 experiments. UHH donor 151-03 (blue) showed relatively lower levels at start of culture compared to PHH donor Hu1601 (red), with increasing and maintained activity over the study period. Activities in PHH decreased readily from start of sandwich culture until day 14 as study end.

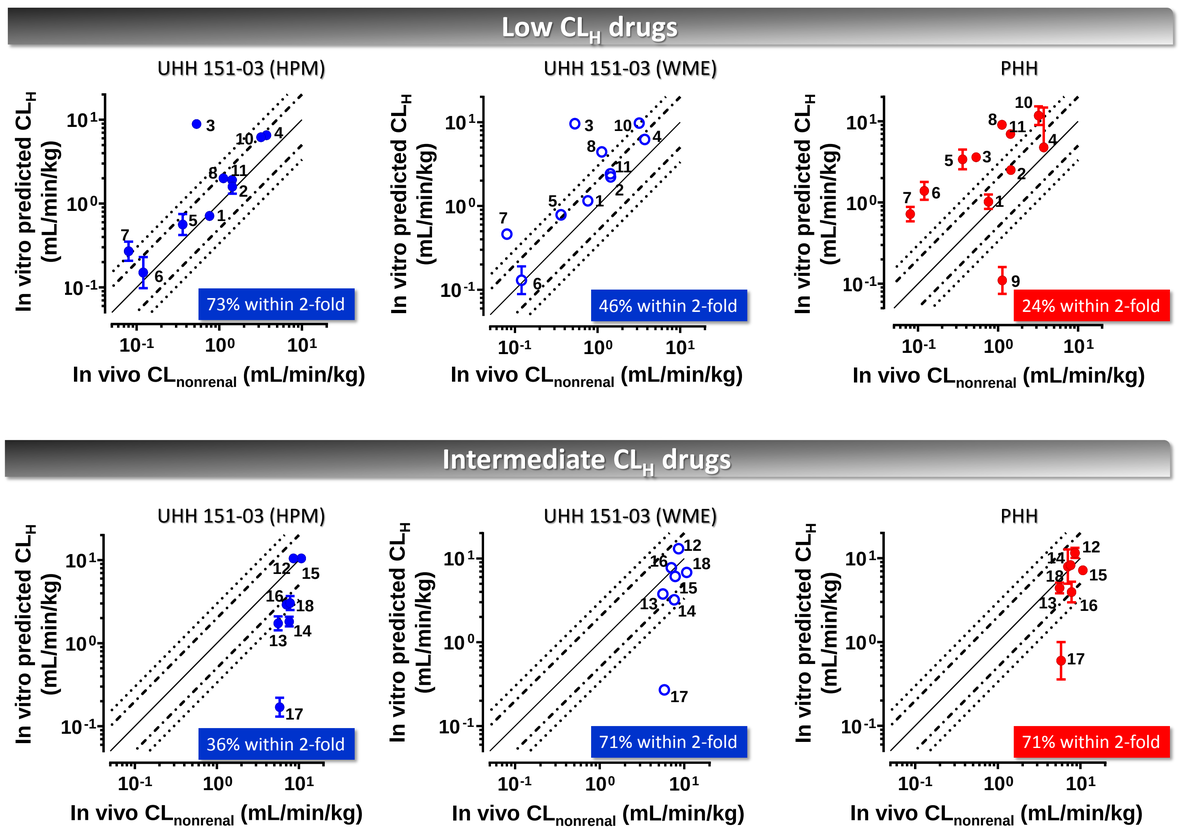
	PHH (Hu1601)			UHH (151-03)		
	Activity	mRNA	Protein	Activity	mRNA	Protein
CYP1A2	67.4 ± 50.3	115 ± 10	56.7 ± 2.0	3.80 ± 0.78	0.11 ± 0.10	<1.0
CYP2B6	3.60 ± 2.11	9.02 ± 0.89	1.66 ± 0.17	227 ± 92	919 ± 265	11.9 ± 1.9
CYP2C8	21.1 ± 1.1	25.2 ± 3.5	3.15 ± 1.72	11.2 ± 6.0	49.5 ± 19.1	3.13 ± 1.05
CYP2C9	73.9 ± 18.9	65.3 ± 8.5	182 ± 51	12.0 ± 4.6	111 ± 45	8.80 ± 2.08
CYP2C19	6.84 ± 1.42	4.58 ± 0.94	2.71 ± 0.35	17.7 ± 4.4	17.1 ± 8.4	4.88 ± 0.11
CYP2D6	12.0 ± 3.6	62.6 ± 8.43	7.88 ± 0.70	3.18 ± 0.56	6.87 ± 4.95	1.26 ± 0.29
CYP3A4	241 ± 98	102 ± 15	50.8 ± 10.0	339 ± 37	329 ± 141	45.1 ± 8.2
UGT1A1	65.0 ± 0.1	347 ± 60	42.5 ± 4.3	111 ± 17	508 ± 106	96.2 ± 2.7
UGT1A9	768 ± 93	36.3 ± 7.8	1.99 ± 0.86	426 ± 18	8.06 ± 1.93	1.73 ± 0.15
UGT2B7		221 ± 33	7.04 ± 0.15		39.4 ± 2.8	1.86 ± 0.30
UGT2B15		51.3 ± 12.0	15.9 ± 2.0		4.05 ± 1.00	2.19 ± 0.15
SULT	20.6 ± 0.7	n.a.	n.a.	15.4 ± 0.4	n.a.	n.a.
Na/K-ATPase			11.9 ± 0.3			8.11 ± 0.43

4.2 Enzyme activity, protein and mRNA expression

PHH and UHH were assessed at culture day 7 in 24-well sandwich culture. Enzyme activities are depicted as pmol/min/mio. cells, mRNA levels as 10^3-fold of endogenous control β-actin, and protein as fmol/µg of microsomal protein. Na/K-ATPase was used as endogenous control for protein quantification. Activity values represent mean ± SD (N=2-5 individual experiments), mRNA and protein data mean ± SEM (N=1). Non-specific UGT/SULT activity data was determined by 7-OH-coumarin glucuronidation and sulfatation, respectively. Overall, CYP1A2 and CYP2B6 data showed most pronounced differences between PHH and UHH.

4.3 CL prediction performance of UHH and PHH sandwich cultures

Correlation of *in vitro* predicted CL_H and *in vivo* $CL_{nonrenal}$ for 18 reference drugs in sandwich cultured UHH (blue) and PHH (red). Incubations were performed for 96-120 h. High Performance Medium (HPM, ●) or Williams´ Medium E (WME, ○) was used for UHH, WME only for PHH. Data represent mean ± SD of individual experiments for UHH (151-03), and mean ± SD for three PHH donors (BD371, HC3-31, Hu1601) with each N=1 experiment, respectively. Correlation plots depict subsets of low (upper panels) and intermediate (lower panels) clearance drugs. The solid line represents conformity, dotted lines 2- and 3-fold error range, respectively. Theophylline (**9**) was excluded from the UHH plots as depletion could not be reliably determined. Good prediction performance was seen with UHH 151-03 in HPM for the set of low CL_H drugs (*in vivo* $CL_{nonrenal}$ ≤5mL/min/kg), for which PHH generally overpredicted $CL_{nonrenal}$. In contrast, UHH in HPM showed a clear trend towards underprediction of the intermediate cleared reference drugs (5-15 mL/min/kg). Pre-culture and incubation of UHH in WME resulted in prediction performance comparable to PHH.



1: alprazolam, **2**: prednisolone, **3**: diazepam, **4**: voriconazole, **5**: tolbutamide, **6**: meloxicam, **7**: warfarin, **8**: glimepiride, **9**: theophylline, **10**: riluzole, **11**: oxazepam, **12**: midazolam, **13**: atazanavir, **14**: diclofenac, **15**: lidocaine, **16**: risperidone, **17**: flecainide, **18**: atomoxetine

Data for UHH represent Mean ± SD of N=3 experiments for HPM and Mean ± SD for triplicate determinations of N=1 experiment for WME

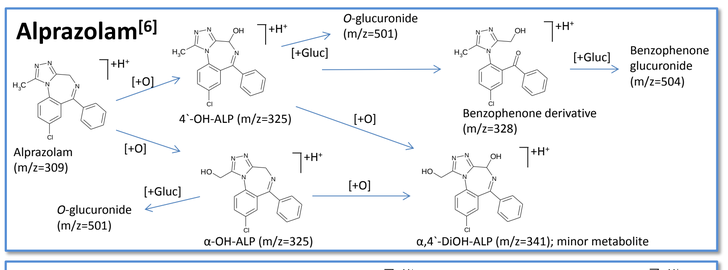
Alprazolam (ALP; m/z = 309)			PHH			UHH (151-03)	
			Hu1601	HC3-31	BD371	WME	HPM
% parent remaining after 120 h			36.7 ± 4.4	39.3 ± 0.2	22.7 ± 1.0	34.0 ± 3.5	59.8 ± 2.1
In vitro predicted CL_H (mL/min/kg)			0.96 ± 0.07	0.83 ± 0.01	1.27 ± 0.06	1.15 ± 0.07	0.48 ± 0.06
Initial CYP3A4 activity (pmol/min/mio. cells)			184 ± 8	341 ± 23	488 ± 52	722 ± 16	362 ± 1
Biotransformation		**m/z**	**Relative metabolite abundance after 120 h**				
Hydroxylation [+O]		325(1)/(2)/(3)	+/+/(+)	+/+/(+)	(+)/(+)/(+)	(+)/+/(+)	+/+/+
Dihydroxylation [+2O]		341	-	-	-	-	-
Benzophenone formation [-N, +H, +2O]		328	(+)	(+)	+	+	+
Phase II	[+Gluc]	485(1)/(2)	+++/(+)	+/(+)	+	+	+
Phase II	[+O, +Gluc]	501(1)/(2)/(3)	+++/++/++	++/+/++	++/+/++	+/+/+	++/+/+
Phase II	[-N, +H, +2O, +Gluc]	504	+++	+++	+++	+++	+++

(+): <1%, **+**: <10%, **++**: >10%, **+++**: >25% of total metabolites analyzed, **-** : metabolite not detected

Meloxicam (ME; m/z = 352)		PHH			UHH (151-03)	
		Hu1601	HC3-31	BD371	WME	HPM
% parent remaining after 120 h		14.4 ± 4.2	19.2 ± 2.0	32.4 ± 3.2	87.2 ± 0.1	99.2 ± 0.01
In vitro predicted CL_H (mL/min/kg)		1.79 ± 0.11	1.40 ± 0.04	0.98 ± 0.10	0.13 ± 0.06	n.d.
Initial CYP2C9 activity (pmol/min/mio. cells)		102 ± 12	42.1 ± 2.6	55.3 ± 5.0	9.85 ± 0.50	8.44 ± 0.54
Biotransformation	**m/z**	**Relative metabolite abundance after 120 h**				
Hydroxylation [+O]	368	+	+	+	+++	+++
Dihydroxylation, dehydrogenation [+2O, -2H]	382	+++	+++	+++	++	++
Phase II [+O, +Gluc]	544	+	+	+	(+)	-
Phase II [+2O, -2H, +Gluc]	558	++	+ +	+	-	-

4.4 Metabolite pattern

Comparison of relative metabolite amounts for ALP (predominantly metabolized by CYP3A4) and ME (CYP2C9) present after 120 h of incubation (n=3) in supernatants of PHH cultures from three donors in WME and of UHH 151-03 cultured in either WME or HPM. Metabolites analyzed were selected based on major phase I and II biotransformation pathways reported in literature[6,7]. Relative abundance was ranked according to fraction of total metabolites analyzed by XIC. Overall, similar metabolite patterns were observed for ALP in the PHH and UHH cultures, however, detailed structural elucidation of metabolites is needed for confirmation. Turnover of meloxicam was altogether lower in UHH cultures, and consequently no significant levels of *O*- or acyl glucuronides were identified. Structures of reported metabolites are depicted below:



5. Conclusions

- → **UHH in sandwich culture showed donor- and enzyme specific expression/activity profiles; levels were found initially lower compared to PHH, however increased over time and were maintained up to 21 days**
- → **UHH incubations in HPM most accurately predicted *in vivo* $CL_{nonrenal}$ for a subset of 11 slowly metabolized reference drugs; cultured PHH provided better predictions for the 7 intermediate CL compounds tested**
- → **Prediction performance appeared affected by enzyme activity levels, as indicated by switch of culture medium from HPM to WME for UHH**
- → **Metabolite pattern for the reference drugs tested were essentially comparable among PHH and UHH sandwich cultures, suggesting similar metabolic degradation pathways**

6. References

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