SUPPORTING INFORMATION

Effects of pharmaceuticals on the expression of genes involved in detoxification in a carp primary hepatocyte model.

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Supplemental experimental section

Hepatocyte isolation

The isolation of hepatocytes was performed using a two-step collagenase perfusion technique, carried out at room temperature *in situ*, using a protocol described previously [1] and in accordance with the UK Animals (Scientific Procedures) Act, 1986.

The liver was perfused via the *aerteria coeliaca* with pre-perfusion HEPES-buffered Hank's solution, containing EDTA, at a flow rate of 15 ml min^{-1}. All solutions used in the isolation procedure based on the HEPES-buffered Hank's solution, were adjusted to pH7.5 and sterile filtered. The perfusion solution, containing 0.02% collagenase D [w/v] (Roche Diagnostics, Switzerland) was then supplied at a rate of 10 ml min^{-1}, until the liver became soft and malleable. At this stage, the solution was changed to a post-perfusion solution, again containing EDTA, and the liver was perfused at a flow rate of 15 ml min^{-1}. The liver was then dissected from the body cavity, placed into a Ca^{2+} - and Mg^{2+} -free HEPES-buffered Hanks salt solution, finely minced, and the cell suspension flushed through nylon screens (250, 100 and 55 µm). Hepatocytes were separated by low speed centrifugation (55 x g, 4 °C for 5 minutes), and the cell pellet obtained was washed twice and suspended in M199 culture medium. Initial cell viability was assessed using the trypan blue exclusion assay and only those isolates showing >90% viability were used in the subsequent analyses.

Primary hepatocyte culture

Hepatocytes were counted using a haemocytometer and seeded into 24-well BD Falcon Primaria cell culture plates at a density of 1.5 x 10^6 cells mL^{-1} (400 µl or 6 x 10^5 cells $well^{-1}$) in M199 culture medium (supplemented with 3.5 mM HEPES, 4.1 mM $NaHCO_3$, 3.4 mM $CaCl_2$, 10% foetal bovine serum, 2mM L-glutamine, 10 U mL^{-1} penicillin and 10 µg mL^{-1} streptomycin). Cells were cultured in a humidified atmosphere at 20°C, and allowed to attach to

the culture plates for 24 h without exposure to chemicals. Hepatocytes were then exposed to IBU, CFA, CTZ, PRO or RIF for 72 h, at concentrations of 0.01, 1 and 100 µM. 72 h was chosen as the culture period for study of the treated cells to allow for a sufficient period of time for any modulation of gene expression to occur in the treatment regimes, whilst also ensuring high cell viability. In a time course study hepatocytes were exposed to either RIF [10 µM] or M199 only (controls) and sampled at dosing periods of 6, 12, 24, 36, 48, 60 and 72 h. At 24 h, and each subsequent 24h interval thereafter, 50% of the culture medium was removed and replaced with fresh medium, without FBS, containing the appropriate concentration of test chemical dissolved in DMSO (final solvent concentration 0.01%). For the inhibition studies, where cells were exposed to RIF and KET simultaneously, the appropriate concentrations, in DMSO, were added to the medium, with a final solvent concentration of 0.02%. The hepatocyte cultures in this work were prepared from 13 fish, yielding between 3 and 9 culture plates per fish. Each treatment was replicated for at least 4 separate cultures, with minimally duplicate wells for each culture treatment.

Primer design

Where partial mRNA sequences were available for common carp (*gstα, gstπ, mdr1 and mrp2),* primers were designed using BeaconDesigner 3.0 software (Premier Biosoft International, Palo Alto, USA). Gene sequence data for common carp *cyp2k* and *cyp3a* were not available, and so partial sequences were first established for these genes. Briefly, the known sequence for the target gene from at least two related cyprinid species were aligned using Clustal W [2], degenerate primers designed using Primer3 software [3] and PCR performed. The PCR products were purified, sequenced (Eurofins MWG Operon) and the sequences obtained were analysed using BioEdit software [4]; carp specific primers were then designed from these sequences.

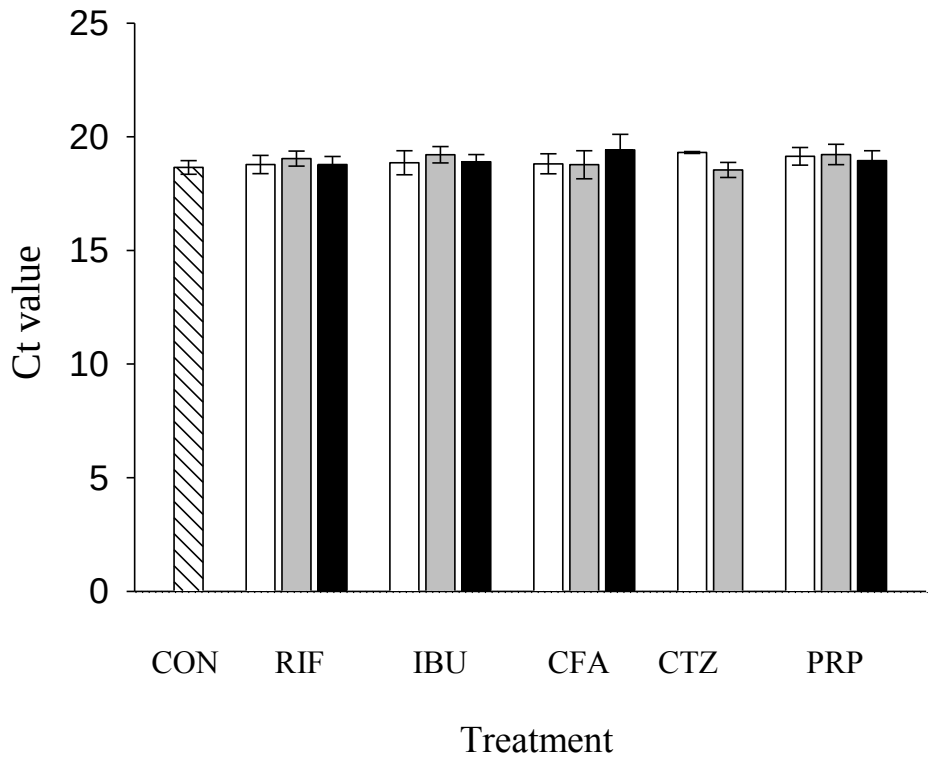
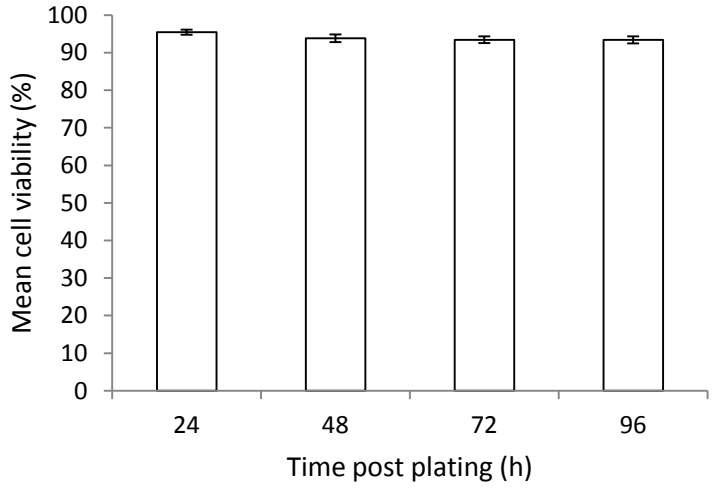


Figure S1: Rpl8 expression expressed as Ct value (threshold cycle) in hepatocytes exposed to pharmaceuticals (rifampicin (RIF), ibuprofen (IBU), clofibric acid (CFA), clotrimazole (CTZ) and propranolol (PRP)) at three different concentrations [0.01 µM (white bars), 1 µM (grey bars), 100 µM (black bars)] and controls (striped bar) for 72 h. There was no significant difference in Ct value between treatment groups or between cultures (p>0.05 in all cases). N = 5 cultures. Error bars represent standard error.

(a)



(b)

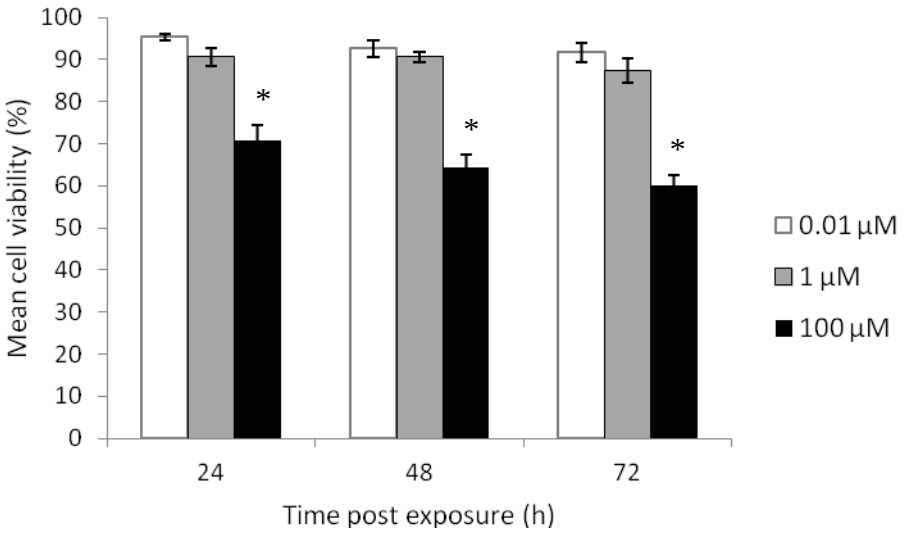


Figure S2: cell viability over culture duration, as measured by LDH leakage into the medium in (a) control cells and (b) cells exposed to CTZ. Cell viability is expressed as percentage viability based on maximum LDH leakage being 0% viability, and no LDH leakage being 100% viability. Error bars represent standard error. $n = 7$ for control cultures, and $n = 4$ for each CTZ treatment. An asterisk denotes a significant difference from control ($p<0.05$).

Gene		Application	NCBI accession number or reference	Sense primer (5'-3')	Antisense primer (5'-3')	Ta (°C)	PCR efficiency	Product size (base pairs)
Cytochrome P450 2K	*cyp2k*	RT-PCR to obtain partial carp-specific sequences	N/A*	F1:TTGAMMGAGTGATCGGTGGA F2:TTGGAGAGAGTTTGGCCAG F3:CCTCATATACAGGATCGAGTTCA	R1:CTGGCCAAACTCTCTCCAA R2:TKGTGTGGCKWTGGATTYA R3:TGGTAATCCATACGCCAAA	F1/R1 53 F1/R2 50 F2/R2 50 F3/R3 50	N/A	F1/R1 342 F1/R2 472 F2/R2 150 F3/R3 690
Cytochrome P450 3A	*cyp3a*		N/A*	F1:TGCGTACAGTATGGATGTGG F2:AAGAARACRGTGGASATCAA	R1:TTGATSTCCACYGTYTTCTT R2:GGRGMCAGTAGRCCAYTGA	F1/R1 50 F1/R2 55 F2/R2 50	N/A	F1/R1 639 F1/R2 962 F2/R2 323
Cytochrome P450 2K	*cyp2k*	RT-qPCR	GU19996	GCTCTTCCTGTTCTTC	TGTGACTTCTACTACTC	60.0	2.07	103
Cytochrome P450 3A	*cyp3a*		GU19997	CCAAGGACCACAAGAAGAAG	AGCCGCCGAAGATGAAG	60.0	1.921	159
Glutathione-S-transferase α	*gstα*		DQ411310	TACAATACTTTCACGCTTTCCC	GGCTCAACACCTCCTTCAC	61.5	1.979	149
Glutathione-S-transferase π	*gstπ*		DQ411313	GTCCTTTGCTCTGCCTCTCTG	TTACTGCTTGCCATTGCCATTG	60.5	2.103	141
P-glycoprotein	*mdr1 (abcb1)*		AY999964	TTGCGGCTGTGGGAAGAG	GTGGATGTTCAGTTGCTTTGTG	58.5	2.104	109
Multidrug resistant protein 2	*mrp2 (abcc2)*		AY679169	TTCGGCTCTAATCTGGATG	CTCACCCGCTGTTTCTG	58.5	2.08	149
Ribosomal protein 8	*rpl8*		See[1]	CTCCGTCTTCAAAGCCCATGT	TCCTTCACGATCCCCTTGATG	60.0	2.14	N/A

Table 1: Details of primers used with PCR and RT-qPCR; Ta is annealing temperature; PCR efficiency represents the 'E' value. * Degenerate primers were designed by aligning sequences available in other cyprinid species; letters denote where the sequence may be more than one specific base as follows: M (A or C), R (A or G), S (G or C), K (G or T), W (A or T), Y (C or T). Multiple primers were designed to gain overlapping sequence segments for *cyp2k* and *cyp3a* genes, designated F1, F2, etc. (sense) and R1, R2, etc. (antisense), from which a consensus sequence was established, and carp-specific rt-qPCR primers designed.

References

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(2) Thompson, J.D., Higgins, D.G., Gibson, T.J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994, 22, 4673-4680

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