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A New Human Hepatoma Cell Line to Study Repeated Cell Toxicity

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Summary — Early toxicity screening of new drugs is performed to select candidates for development. Many cell models are used to assess basic cytotoxicity and to show a good correlation with acute toxicity. However, their correlation with chronic *in vivo* exposure is inadequate. The new hepatoma cell line (HBG BC2) possesses the capacities of being reversibly differentiated *in vitro,* and of maintaining a relatively higher metabolic rate when in the differentiated phase (3 weeks) as compared to Hep G2 cells. MTT reduction was used to evaluate the toxicity of propranolol, perhexiline, aspirin and paracetamol, after both single and repeated treatments (three times a week for 2 weeks). Under conditions of repeated treatment, cytotoxicity was observed at lower doses when compared with single administration. Moreover, the first non-toxic doses were in the same range as plasma concentrations measured in humans during therapeutic use. Our results suggest that the new human hepatoma HBG BC2 cell line may be of interest for the evaluation of cell toxicity under repeated treatment conditions.

Key words: *cytotoxicity, HBG BC2 cells, MTT, repeated treatment.*

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Introduction

The development of a new drug involves the performance of many different assays (the majority of which are in animals), in order to identify potential adverse effects, their possible concentration-dependent relationship, their extrapolation (and prediction) for humans, and finally, the calculation of safety margins based upon the expected use of the compound. The majority of studies conducted in animals involve repeated administration. Under these treatment conditions, the amount of compound required for testing is relatively important, and timelines to obtain results are relatively long. Such types of study are implicated in overall drug development activities: improvement of chemical synthetic pathways, safety at work when producing large amounts of a chemical, cost of intermediates, environmental considerations when stocking intermediates, and so forth. The above-mentioned are all justifiable reasons to facilitate the development of the inappropriately named "alternative methods" for toxicity studies. These *in vitro* models are now indispensable in order to be able to evaluate the large numbers of compounds reaching potential development in today's competitive pharmaceutical industry.

The liver, in general, and hepatocytes, in particular, are continually exposed to the parent drug and/or its (reactive) metabolites, and the hepatocyte model is the most suitable for *in vitro* screening purposes (1). In order to develop new experimental approaches (Figure 1) to overcome the known limitations (2) in the conduct of long-term *in vitro* treatments, the new human hepatoma cell line HBG BC2, with its capacity for being reversibly differentiated and its ability to be maintained in culture for more than 3 weeks, has been evaluated and briefly compared with Hep G2 cells.

Materials and Methods

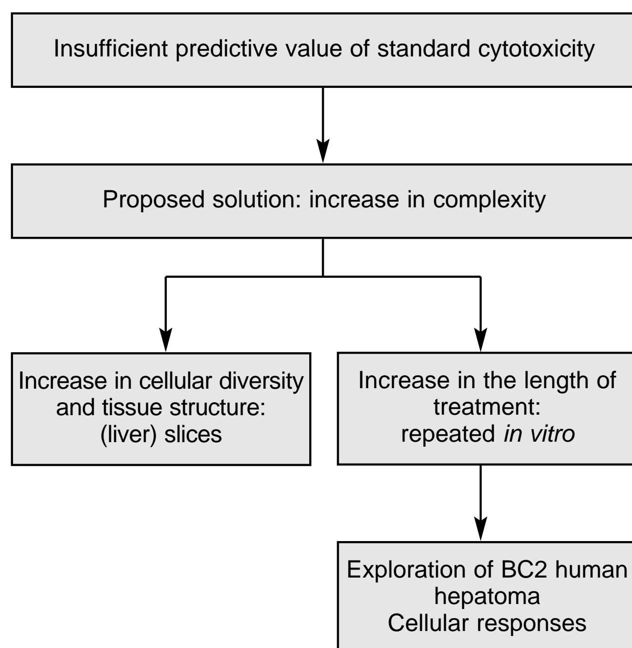
Materials

Acetaminophen, perhexiline, propranolol and acetylsalicylic acid were purchased from Sigma (USA). All culture media and cofactors were purchased from Invitrogen (UK).

Culture conditions

HBG BC2 cells were supplied by Dr C. Guguen-Guillouzo (INSERM 522, Rennes, France). HBG BC2 cell cultures were divided once a week. The culture medium, as previously described (3), was changed three times a week. Cells were cultured in 96-well plates (Costar, USA) for cytotoxic assays and in 25cm^2 flasks for molecular investigations. Once at confluence, cells were maintained under these culture conditions for 3 weeks to allow differentiation to occur.

Figure 1: How it is possible to overcome limitations of repeated *in vitro* tests



Hep G2 cells, supplied by ECCAC (Salisbury, UK), were cultured in MEM containing fetal calf serum (FCS; 10% final concentration), a penicillin (100IU/ml)/streptomycin (100µg/ml) mixture, gentamicin (10µg/ml) and non-essential amino acids (1 X). Cells were cultured in 25cm^2 flasks (Corning, USA) and separated once a week. The culture medium was changed three times a week.

Acute and repeated cytotoxicity testing

Cells were treated with increasing concentrations of the test compounds (acetaminophen, acetylsalicylic acid, perhexiline and propranolol; 1% DMSO final concentration in the medium), using 21-hour (acute) or the three-times-a-week treatment regimes (Figure 2). For repeated treatment, three plates were used per compound. At the end of each treatment period, one of the plates was processed for cytotoxicity. Thus, cytotoxicity was evaluated following one (acute), three or six treatments. Concentrations overlapped in each series of plates, and lower concentrations were used in the plates treated for longer periods, in order to allow for sufficient survival to determine the degree of toxicity.

The level of cytotoxicity was determined by MTT reduction (4). The concentration that inhibited 50% of the cellular activity (defined as IC50) was calculated, and the NOEL (no effect level) was evaluated as the highest concentration level showing 100% of activity.

Biotransformation-relative basal gene expression

Evaluation of the expression of selected genes was conducted by using reverse transcriptase-polymerase chain reaction (RT-PCR). HBG BC2 (proliferative and 3-week differentiated) and Hep G2 cells were seeded in 25cm^2 flasks. At all sampling times, and after removal of the culture medium, cells were washed with PBS and were immediately frozen at –20°C and stored at –80°C until required for RNA extraction.

Extraction of total RNA was performed using the RNeasy mini kit (Qiagen, Germany). cDNA was synthesised by using the first strand cDNA

Table 1: RT-PCR parameters for gene expression investigations

Gene name	Annealing temperature (°C)	Number of cycles	Primer	
18S	57	30	sense antisense	5´-AACGGCTACCACATCCAAGG-3´ 5´-CCCTCTTAATCATGGCCTCAG-3´
CYP3A4	55	32	sense antisense	5´-GTGTGGGGCTTTTATGATGG-3´ 5´-GGTGGGTGGTGCCTTATTG-3´
CYP2E1	53	30	sense antisense	5´-AGCACAACTCTGATATGG-3´ 5´-ATAGTCACTGTACTTGAACT-3´
GAPDH	55	30	sense antisense	5´-ACCACAGTCCATGCCATCAC-3´ 5´-TCCACCACCCTGTTGCTGTA-3´
GST	57	30	sense antisense	5´-GACCTCACCCAGGTAATGGA-3´ 5´-CCGTGCTCCGACAAATAGTC-3´

Table 2: Acute and repeated cytotoxicity testings

Test compounds	Acute		After 3 treatments		After 6 treatments	
	IC50	NOEL	IC50	NOEL	IC50	NOEL
Acetaminophen	nd	30,000	21,320	10,000	3800	10
Acetylsalicylic acid	47,000	625	7100	500	> 500[a]	500
Perhexiline	50	< 2.5[a]	11	2	4.3	1.5
Propranolol	490	< 250[a]	173	100	43	20

[a]*Highest concentration tested of the experimental concentration range; nd = not determined, outside the concentration range; IC50 and NOEL values are in μmol/l.*

Synthesis kit (Invitrogen), on 5μg of total RNA. The different PCR conditions used are described in Table 1. The amplification products were visualised by use of an ethidium bromide electrophoresis 2% agarose gel in TAE 1X. Readings and semi-quantitation of the spots were performed using the Quantity One software (BioRad, USA). Results were expressed as fluorescence intensity (pixels/surface unit).

Results and Discussion

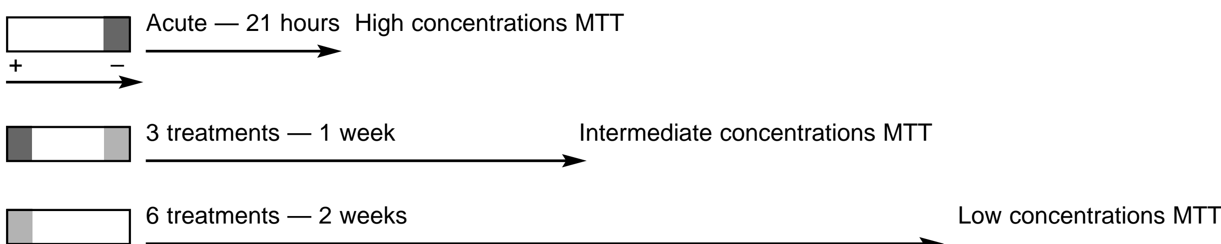
Comparative evaluation of HBG BC2 and Hep G2 cells

The evaluation of the potential of HBG BC2 cells as a new *in vitro* investigative model was performed in comparison with the well-studied Hep G2 cell line. The genes selected were those implicated in biotransformation (cytochrome P450 CYP2E1 and CYP3A4), xenobiotic metabolism (glutathione S-transferase, GST) and housekeeping functions (18S, glyceraldehyde-3-phosphate dehydrogenase [GAPDH]). Basal gene expression using standard RT-PCR, in the proliferating and differentiated HBG BC2 cells and in proliferating Hep G2 cells, was evaluated. The results presented show that the expression of 18S and GAPDH genes was very similar in the three systems that were studied (Figure 3). In this case, no normalisation was necessary. The two biotransformation genes (CYP2E1 and CYP3A4) had a different level of expression and were globally more expressed in differentiated HBG BC2 cells than in Hep G2 cells (from 7 to 30 times higher). This higher expression level was also observed in the HBG BC2 differentiated stage in comparison to the proliferative stage. The expression of the GST gene was similar in both HBG BC2 culture states, and was higher than that in the Hep G2 cells. These results confirmed the greater suitability of differentiated HBG BC2 cells in comparison to proliferative HBG BC2 or Hep G2 cells.

The response of HBG BC2 cells to toxicants

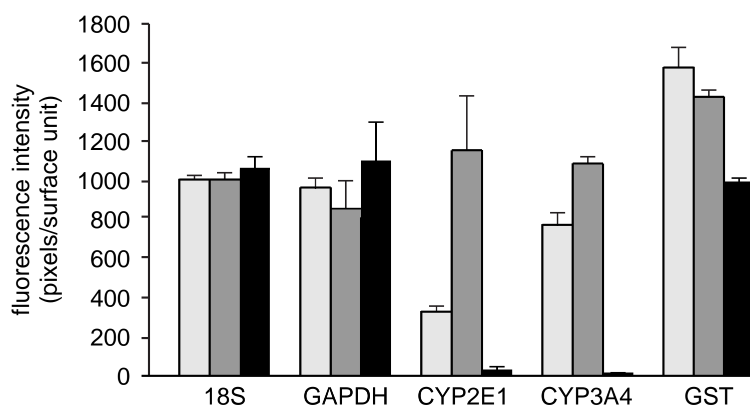
The new human hepatoma cell line, HBG BC2, has the property of maintaining a stable hepatic-like phenotype over a sufficiently long period of time to allow repeated treatment *in vitro*. It was then decided to explore the response of the model to four widely used drugs (acetaminophen, acetylsalicylic acid, propranolol and perhexiline), with the use of MTT reduction as a parameter to evaluate cell toxicity. MTT reduction was selected from the differ-

Figure 2: Experimental design of acute and repeated cytotoxicity assays



Tinted boxes show the concentration overlap in each plate.

Figure 3: RT-PCR results for some selected biotransformation genes



□ = *HBG BC2 proliferating;* ▨ = *HBG BC2 differentiated;* ■ = *Hep G2.*

ent parameters available for cell toxicity in our laboratory, since it is widely used and covers a wide range of cellular toxic phenomena and can be easily automated (4). The results (Table 2) confirmed that cytotoxicity was more important after repeated treatment compared to acute conditions.

The IC50 values for the four compounds were reduced more than ten-fold following 3–6 treatments. Furthermore, the traditionally used in *in vivo* studies non-toxic concentration or no observable effect level (NOEL) decreased by more than 50-fold under conditions of repeated treatment, reaching a 3000-fold reduction in the case of acetaminophen.

The data from the literature (5) show that the toxic and non-toxic concentrations determined in the HBG BC2 cell model were of the same order as those seen in humans. In the case of acetaminophen and acetylsalicylic acid, the NOEL was in the same range as the therapeutic concentrations in humans.

The higher metabolic gene expression level of HBG BC2 may explain the increased sensitivity in metabolism-related toxicity of the four selected compounds. The HBG BC2 cell line presents a more appropriate metabolic profile than the widely used Hep G2 hepatoma cells, and thus, concords more closely with the toxic response generated in *in vivo* studies.

Conclusions and Perspectives

The results presented here show that the HBG BC2 cell model can be a new investigative tool for toxicology *in vitro*. In addition, this new cell line opens a broad range of possibilities for exploring the toxic response of human hepatic cells in culture under conditions of treatment more relevant to human therapy. More compounds need to be studied to fully establish the place of this model in the development of new compounds of therapeutic interest.

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References

1. Paillard, F., Finot, F., Mouche, I., Prenez, A. & Vericat, J.A. (1999). Use of primary cultures of rat hepatocytes to predict toxicity in the early development of new chemical entities. *Toxicology in Vitro* **13**, 693–700.
2. Eisenbrand, G., Pool-Zobel, B., Baker, V., Balls, M., Blaauboer, B.J., Boobis, A., Carer, A., Kevekordes, S., Lhuguenot, J.C., Pieters, R. & Kleiner, J. (2002). Methods of *in vitro* toxicology. *Food and Chemical Toxicology* **40**, 193–236.
3. Glaise, D., Ilyin, G.P., Loyer, P., Cariou, S., Bilodeau, M., Lucas, J., Puisieux, A., Ozturk, M. & Guguen-Guillouzo, C. (1998). Cell cycle gene regulation in reversibly differentiated new human hepatoma cell lines. *Cell Growth and Differentiation* **9**, 165–176.
4. Edmondson, J.M., Armstrong, L.S. & Martinez, A.O. (1988). A rapid and simple MTT-based spectrophotometric assay for determining drug sensitivity in monolayer cultures. *Journal of Tissue Culture Methods* **11**, 15–17.
5. Meyer, F.P. (1994). Indicative therapeutic and toxic drug concentrations in plasma: a tabulation. *International Journal of Clinical Pharmacology and Therapeutics* **32**, 71–81.