Metabolism-Induced Toxicity of Selegiline and Carbamazepine Studied with an *In Vitro* Method

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Abstract: Carbamazepine and selegiline, although neuroprotective themselves, are presumed to have toxic metabolites. The aim of this study was to investigate the possible metabolism-induced toxicity of selegiline and carbamazepine with a novel *in vitro* method: The drugs were incubated with target cells (neuroblastoma SH-SY5Y) with or without a pre-incubation with mouse or human hepatocytes. The viability of SH-SY5Y cells was then measured by using total cellular ATP as an indicator of the cell viability. For the pre-incubation with hepatocytes two different methods were used: Hepatocytes were grown either in multiwell plates (Model 1) or in filter inserts (Model 2).

Selegiline itself increased SH-SY5Y viability, but the pre-incubation with both mouse and human hepatocytes made selegiline slightly toxic to SH-SY5Y cells. The biotransformation of carbamazepine seemed to be more complex and showed variation in different hepatocyte models. In general, human hepatocytes increased carbamazepine toxicity to SH-SY5Y cells, whereas mouse hepatocytes had no such effect. The methodology used (especially Model 1) could form a basis in developing a test system for a qualitative detection of metabolism-induced (neuro)toxicity in the early phase of drug discovery. In this respect, the present study might be promising for further evaluation by means of a larger number of independent experiments and different types of compounds.

Key Words: Metabolism-induced drug toxicity, *in vitro*, selegiline, carbamazepine.

INTRODUCTION

Systemically administered drugs are usually extensively metabolized in the liver *in vivo*. Though the principal purpose of the enzymatic biotransformation is the elimination of a drug from the body, hepatic metabolism can sometimes produce toxic metabolites. Metabolism is indeed responsible for a number of adverse drug effects [1-3]. Therefore, when testing drug toxicity *in vitro*, not only the effects of the parent compound, but also those of its possible metabolites should be considered.

Several hepatic *in vitro* models have been used to study xenobiotic metabolism: liver slices, liver spheroids, freshly isolated cells in suspension, primary cells in culture, cryopreserved hepatocytes, continuous cell lines, liver homogenates, subcellular fractions [4-7], and co-cultures of hepatocytes with other non-parenchymal liver-derived or non-liver-derived cells [8, 9]. Due to their strong resemblance to liver *in vivo*, primary hepatocytes are at present a superior model to study drug toxicity and metabolism *in vitro*. However, the drawback of primary hepatocytes is that they lose their metabolic activity in culture and are therefore best suited for testing acute toxicity.

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For studying metabolism-dependent toxicity *in vitro*, several co-culture methods involving a metabolizing system with indicator (target) cells have been presented. These include e.g. microcarrier-attached rat liver cells with BALB/c 3T3 cells [10], a roller chamber co-culture of chick hepatocytes with human fibroblasts [11], and a co-culture of rabbit microsomes with human lymphocytes [12]. However, none of these methods have been validated and accepted into general use, and simplified protocols suitable to high throughput screenings are still missing.

In the present study we evaluated the metabolism-induced toxicity of two neuroactive drugs, selegiline and carbamazepine, with an *in vitro* method using a combination of hepatocytes and neuroblastoma cells. Carbamazepine is a neuroprotective drug used e.g. in the treatment of epilepsy, with some additional side effects reported [13, 14]. It is extensively metabolized in the liver, and the adverse effects are believed to be related to the carbamazepine-10,11-epoxide metabolite [15]. Selegiline is a MAO-B inhibitor used in the treatment of Parkinson's disease. Selegiline is clinically well tolerated with minor adverse effects [16]. However, there are doubts about its safety on account of its detrimental metabolites l-metamphetamine and l-amphetamine [17]. Previously we showed that selegiline and carbamazepine are toxic to mouse primary hepatocytes, but not to neural cells in the concentrations and conditions that we used earlier [18]. According to this we suggested that selegiline and carbama-

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zepine may cause metabolism-dependent toxicity. However, in the previous study the drugs were dosed separately on different cell cultures, and it was not shown whether hepatocytes enhance the toxicity of drugs to target cells.

Our aim was now to evolve a combination of hepatocytes and neuroblastoma cell cultures in order to study the previously observed metabolism-dependent toxicity of those two neuroactive drugs. For this purpose we employed a method with the following characteristics. 1) The test compound was first pre-incubated with hepatocytes, i.e. freshly isolated mouse or human hepatocytes, or cryopreserved human hepatocytes, and then the conditioned medium was dosed to the target neural cells (SH-SY5Y). SH-SY5Y cells were also exposed to test compound without hepatocyte pre-incubation. 2) The viability of SH-SY5Y cells was measured by using total cellular ATP level to estimate cell viability. For pre-incubation, two different exposure methods were used: In Model 1 hepatocytes were grown in multi-well plates, and the hepatocyte-conditioned medium was collected from the top of the hepatocytes. In Model 2 hepatocytes were grown in filter-inserts of multi-well plates and the hepatocyte-conditioned medium was collected below the filter cup. Model 1 is simple and straightforward but requires clearing of the hepatocytes from the medium before dosing it to the target cells, while Model 2 provides a more three-dimensional substratum for the hepatocytes to grow, but it is more complex and less suitable for automation than Model 1.

Neuroblastoma SH-SY5Y cells were used as target cells because neural tissues are the target tissues of the neuroactive drugs studied here. Moreover, neural tissues are especially sensitive to foreign compounds (assuming that they pass the blood-brain barrier), and thus need special focus in safety evaluations. It should be noted, however, that even if showing some properties of neuronal cells, neuroblastoma cells are cancer cells and thus do not maintain all the functions of normal neurons.

Besides of all, an intriguing goal was here to evaluate whether metabolism-induced toxicity of drugs can be qualitatively detected with the method described here, and whether further development of this method would be reasonable.

MATERIALS AND METHODS

Materials

Minimum essential medium (MEM) with Earle's salts, nutrient mixture Ham's F-12K, HepatoZyme-SFM, Dulbecco's MEM/F12, foetal bovine serum, antibiotic-antimycotic solution, L-glutamine and non-essential amino acid solution were obtained from Gibco (Paisley, UK). Human neuroblastoma cell line SH-SY5Y and human hepatoblastoma cell line HepG2 were from ATCC (Rockville, VA/USA). Clonetics® cryopreserved hepatocytes (one donor) (Cryo1D) were from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). Cryopreserved human hepatocytes, 10-donor pooled, (Cryo10D) and *In Vitro*-GRO™HT Medium for thawing were from *In Vitro* Technologies GmbH (Leipzig, Germany). Cryopreserved hepatocytes were metabolically active as verified by the manufacturer. Selegiline hydrochloride was a generous gift from Orion Pharma (Espoo, Finland). Carbamazepine was from Sigma Chemicals Co. (St. Louis, MO/USA). Cell culture plastics and 0.4 μm polycarbonate membrane tissue culture inserts were from Nunc (Roskilde, Denmark). Dulbecco's phosphate-buffered saline (D-PBS) and Hank's balanced salt solution (HBSS) were prepared in our own laboratory. ATP Kit SL was from BioThema (Haninge, Sweden). TRIzol Reagent was from Invitrogen Life Technologies (Paisley, UK). The primers were from Oligomer (Helsinki, Finland). High-Capacity cDNA Archive Kit and SYBR®Green PCR Master Mix were from Applied Biosystems (Foster City, CA/USA).

Cell Cultures

Cell Lines

The SH-SY5Y and HepG2 cells were grown under standard culture conditions in an incubator containing a humidified atmosphere with 5 % CO_2 at 37ºC. The medium used for SH-SY5Y cells was MEM with Earle's salts and Ham's F-12K (1:1) supplemented with 2 mM L-glutamine, 1 % non-essential amino acids and 10 % foetal bovine serum. For the experiments, SH-SY5Y cells were seeded into 96-well plates at a density of 40 000 cells/well and grown for 24 hours prior to drug exposure. HepG2 cells were grown in MEM supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, and 10 % FBS.

Mouse Hepatocytes

The isolation of hepatocytes was based on previous studies [18-20] with some modifications. Mouse (adult NMRI) liver tissue was obtained from other researchers in connection with other studies having the ethical acceptance of the Faculty of Medicine, University of Tampere. The mice were killed by decapitation. Immediately after that the liver was removed and placed in ice-cold D-PBS (without Ca^{2+} and Mg^{2+}) on Petri dish. It was gently crushed with a plastic knife. The connective tissue was removed. The suspension was then transferred to baker glass and washed three times by decanting with ice-cold D-PBS. To remove Ca^{2+}, the suspension was washed another three times with ice-cold D-PBS containing 0.5 mM EGTA. The last washing buffer was carefully removed by suction and replaced by enzyme solution containing 0.05 % collagenase, 0.1 % hyaluronidase and 5 mM $CaCl_2$ in HBSS (15 ml/liver). The suspension was poured into 50 ml Erlenmeyer flasks, 15 ml cell suspension/flask. The tissue suspensions were incubated for 45 minutes in a rotating water bath (37ºC, 85 oscillations/min). The suspension was filtered through six-fold gauze and washed gently 3-5 times by centrifugation (50g 5 min) with DMEM/F12 supplemented with 15 mM HEPES and 1 % antibiotic-antimycotic at room temperature to remove cell debris and contaminating cells. The cell pellet was loosened swirling gently the centrifuge tube. Pipetting was avoided. The cell suspension was transferred to Erlenmayer glass and allowed to recover for 30 min in DMEM/F12 supplemented with 15 mM HEPES, 1 % antibiotic-antimycotic and 10 % FBS in the incubator. During the recovery, the glass was carefully agitated to prevent the attachment of the cells. After recovery, the cells were washed once with serum-free DMEM/F12 and suspended in HepatoZyme medium supplemented with 2 mM L-glutamine. Cell viability was assessed with trypan blue staining and was >80 %. For each

cell preparation, hepatocytes from the livers of 3-4 mice were pooled together.

Human Freshly Isolated Hepatocytes

Human (75 years old female donor) liver sample (1 cm^3) was obtained from Tampere University Hospital in a liver resection. The sample was taken from the "surplus" healthy tissue with the permission of the ethical committee of Pirkanmaa Hospital District. The tissue sample was placed in ice-cold D-PBS buffer with 1 % antibiotics, and transported to the laboratory, where the hepatocyte isolation was performed within 30 minutes after removal of the tissue sample. The hepatocytes were isolated from the tissue samples as explained above for mice livers, and finally suspended in HepatoZyme medium supplemented with 2 mM L-glutamine for the experiments. Cell viability was assessed with trypan blue staining and was >80 %.

Human Cryopreserved Hepatocytes

Human cryopreserved hepatocytes were thawed according to the manufacturers' instructions and allowed to recover for 2.5 hours. Then, the cells were washed twice (5 min 50g) to remove cell debris, and suspended in HepatoZyme media supplemented with 2 mM L-glutamine for the experiments. The viabilities were assessed after the recovery period and were >80%.

The Effect of Hepatic Biotransformation on Drug Toxicity

To investigate the effect of hepatic biotransformation on the drug toxicity, the drugs were first dosed to the hepatocytes and then the pre-incubated (hepatocyte-conditioned) drug exposure medium was collected and dosed to the SH-SY5Y cells. Two different exposure methods were used: In Model 1 (Fig. **1A**), the hepatocytes were seeded into 24-well plates at a density of 100 000 cells/well in 0.5 ml of HepatoZyme medium containing 2 mM L-glutamine, and allowed to settle for 90 min in an incubator containing a humidified atmosphere with 5 % CO_2 at 37ºC. The drug solutions were prepared in HepatoZyme medium containing 2 mM L-glutamine, and were added to the hepatocytes in 0.5 ml volume per well to obtain a total volume of 1 ml and the final concentrations 0, 4.5 and 45 μM for selegiline and 0, 0.1 and 1 mM for carbamazepine. Selegiline was dissolved in the medium, and carbamazepine in DMSO. The final concentration of DMSO in the wells was 0.5 %. The control wells were incubated with equal volumes of drug solvents to avoid changes that could be due to the solvent. After 3-hour incubation, the medium was harvested and cleared from cells by centrifugation (16 000 g 5 min), and 100 μl aliquots of the cell-free supernatant were transferred to SH-SY5Y cells grown in 96-well plates. The exposure time was 24 hours, after which SH-SY5Y viability was measured.

In Model 2 (Fig. **1B**), primary hepatocytes were seeded into polycarbonate filter inserts at the density of 150 000 cells/filter insert in 0.5 ml of HepatoZyme medium containing 2 mM L-glutamine. The filter inserts were placed into 24-well plates containing 0.5 ml culture medium. After 90 min, the medium of the filter insert was replaced with 0.5 ml exposure medium. The drug solutions were as explained above. After 3-hour incubation, 100 μl aliquots were collected under the filter inserts and transferred into the SH-SY5Y grown in 96-well plates. The exposure time was 24 hours, after which SH-SY5Y viability was measured.

To study the effects of the parent drugs themselves on SH-SY5Y cells, i.e. in a direct exposure, SH-SY5Y cells were exposed to the same drug solutions as explained above but without pre-incubation with hepatocytes. For Model 1, SY-SY5Y cells were exposed to the exposure medium as such. For Model 2, the exposure medium was dosed into the filter cups, and three hours later the exposure medium was collected under the filter cups and dosed on SH-SY5Y cells. The exposure time for SH-SY5Y cells was always 24 hours.

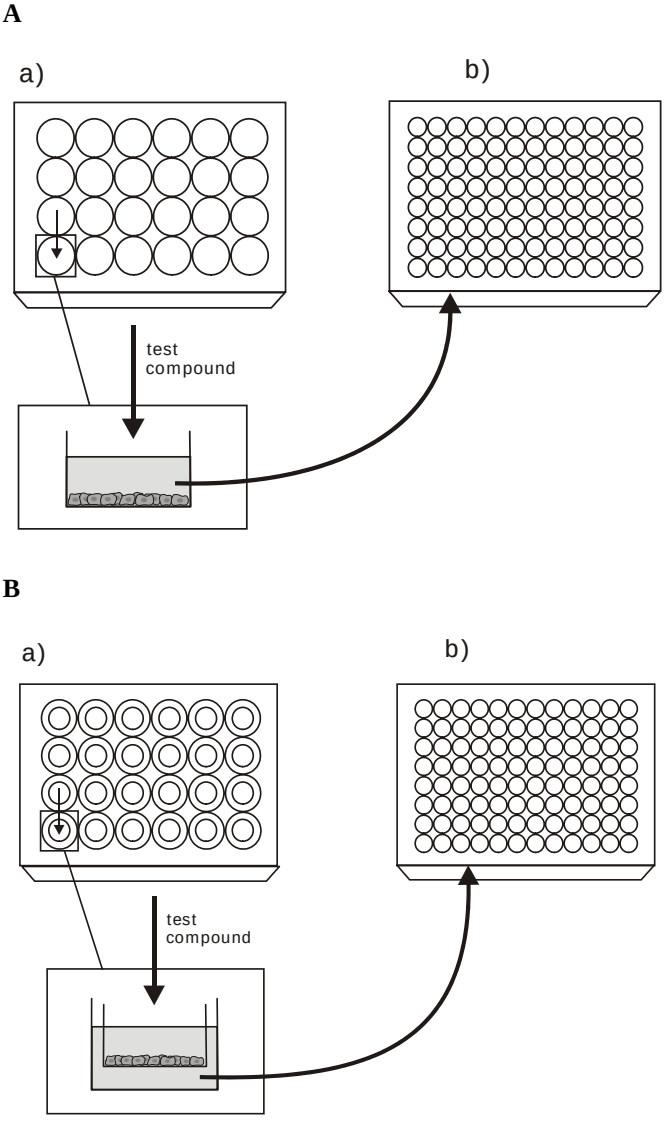

Fig. (1). The drug exposure models presented schematically. In Model 1 the test compound is dosed on the hepatocytes grown in 24-well plates and exposed for 3 hours (**A, a**). The "conditioned medium" is harvested from the top of the hepatocytes and dosed on target SH-SY5Y cells grown in 96-well plates (**A, b**). The viability of SH-SY5Y is determined after 24-hour exposure.

In Model 2 the test compound is dosed on the hepatocytes grown in polycarbonate filter cups and exposed for 3 hours (**B, a**) The "conditioned medium" is harvested under the filter cup and dosed on SH-SY5Y cells grown in 96-well plates (**B, b**). The viability of SH-SY5Y cells is determined after 24-hour exposure.

The effect of HepatoZyme medium itself on SH-SY5Y cells was tested by running experiments where the drug toxicity was tested parallelly using MEM and HepatoZyme medium, but it was found to be negligible. Moreover, within each experiment the effect of the drug solutions on the viability of hepatocytes was also tested after the 3-hour drug exposure.

ATP Measurement

The cell viabilities were determined by luminescence-based ATP measurement (ATP Kit SL, BioThema). ATP measurement is an index of mitochondrial function and energy metabolism, and thus ATP content is proportional to cell viability. After the drug exposure, 5 µl 10 % TCA was added to 96-well plates/well and 50 µl to 24-well plates/well. After the application of TCA, the plates were agitated and frozen in –75 °C overnight. The next day the plates were thawed and agitated. 25 µl of cell suspension was transferred to black 96-well plate (Black Cliniplate). 100 µl ATP monitoring reagent (containing a mixture of luciferase and D-luciferin) and Tris acetate buffer (1:5) were added to each well, followed by immediate measurement of luminesence with Luminoscan luminometer (Labsystems, Turku, Finland). Cell viability in treated wells was expressed as a percentage of absorbance of control wells.

CYP1A2 and CYP3A4 Gene Expression in Hepatocyte Cultures

To get an idea of the metabolic capacity of the hepatocyte cultures, the gene expression levels of two cytochrome P450 enzymes, CYP1A2 and CYP3A4 (representing phase I enzyme activity) were studied and compared to that of HepG2 cells, which are known for their low metabolic activity [21]. CYP expression levels were measured by quantitative real time PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression.

First, total RNA was extracted from the hepatocytes using TRIzol reagent for homogenization, followed by chloroform-isopropylalcohol-treatment. RNA was quantified by measuring the optical density of samples at a wavelength of 260 nm. RNA was converted to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems) according to manufacturer's instructions. The reverse transcription reaction was performed for 10 min at 25ºC and for 120 min at 37ºC (Gene Amp PCR System 2400, Perkin Elmer).

The pairs of primers were designed using Primer Express 2.0 software (Applied Biosystems). The nucleotides were as follows (the GenBank accession numbers are given in the parenthesis):

1. Mouse GAPDH (XM_132897)
   forward primer 5´ -CATGGCCTTCCGTGTTCCTA-3´,
   reverse primer 5´- GCGGCACGTCAGATCCA-3´
2. Mouse CYP1A2 (NM_009993)
   forward primer 5´-CAAAGACAATGGCGGTCTCA
   reverse primer 5´-CCAAAGATGTCATTGACAATG TTG
3. Mouse CYP3A4 (AB033414)
   forward primer 5´-TGGCCAGAGCCTGAAGAATT-3´
   reverse primer 5´- GATCAATGCTGCCCTTGTTCTC-3´
4. Human GAPDH (BC013310)
   forward primer 5´- ATGGAAATCCCATCACCATCT T-3´
   reverse primer 5´- CGCCCCACTTGATTTTGG-3´
5. Human CYP1A2 (NM_000761)
   forward primer 5´-GCCCGGCCCACAATTAA-3´
   reverse primer 5´-GCTAATGGGTGCAGGGTTTC-3´
6. Human CYP3A4 (NM_017460)
   forward primer 5´- GCTGTGCAGGGCAGGAAA-3´
   reverse primer 5´- TTTCAGCTCTGTGTTGCTCTTT G-3´

Real-time PCR was performed using an ABI PRISM 7700 Sequence Detection System and SYBR® Green PCR master mix (Applied Biosystems) according to manufactorer's instructions. The amplification program consisted of an initial denaturation step at 95ºC for 10 min. This was followed by cycles of 15 s at 95ºC and 1 min at 60ºC. The cycle number was 45. In each experiment, water was used as a negative control for contamination. Standards were performed in duplicates and the samples in triplicates. The results were calculated using a standard curve method (Applied Biosystems, User Bulletin #2, 2001).

Statistics

Comparisons between drug treatments and corresponding controls were made by using one-way ANOVA with Tukey's post test (GraphPad Prism 3.0). Comparisons between different drug exposures (direct or *via* hepatocytes) at each concentration were made by Student's t-test (SigmaPlot 7.0). The numbers of independent experiments, *N*, are indicated in the figure legends. Due to the limited availability of freshly isolated human hepatocytes, they were used in one experimental set-up only, i.e. in carbamazepine exposure using Model 1.

RESULTS

Metabolism-Dependent Toxicity of Selegiline

Selegiline itself (direct exposure) increased the ATP level of SH-SY5Y cells by ~18 % compared to corresponding control (w/o selegiline) (Fig. **2A**). When Model 2 was used and selegiline was dosed through empty filter cup, this increase was not statistically significant (Fig. **2B**). After pre-incubation with mouse and human Cryo1D and Cryo10D hepatocytes, 45 µM selegiline decreased SH-SY5Y viability (compared to corresponding control) up to 26.6±5.7%, 7.7±4.5, and 14.5±3.8%, respectively, in Model 1 (Fig. **2A**). In Model 2, after pre-incubation with mouse hepatocytes, 4.5 µM selegiline caused a 43.2±24.3% reduction in SH-SY5Y viability, whereas pre-incubation with human Cryo1D and Cryo10D hepatocytes had no significant effect (Fig. **2B**). Since the data in the figures are given as a relation to corresponding control, which is set to 100 %, the impact caused by different drug exposure systems cannot be directly compared. To overcome this problem we summarized the percent changes in the total cellular ATP content of SH-SY5Y cells between direct drug exposure and hepatocyte-condi-

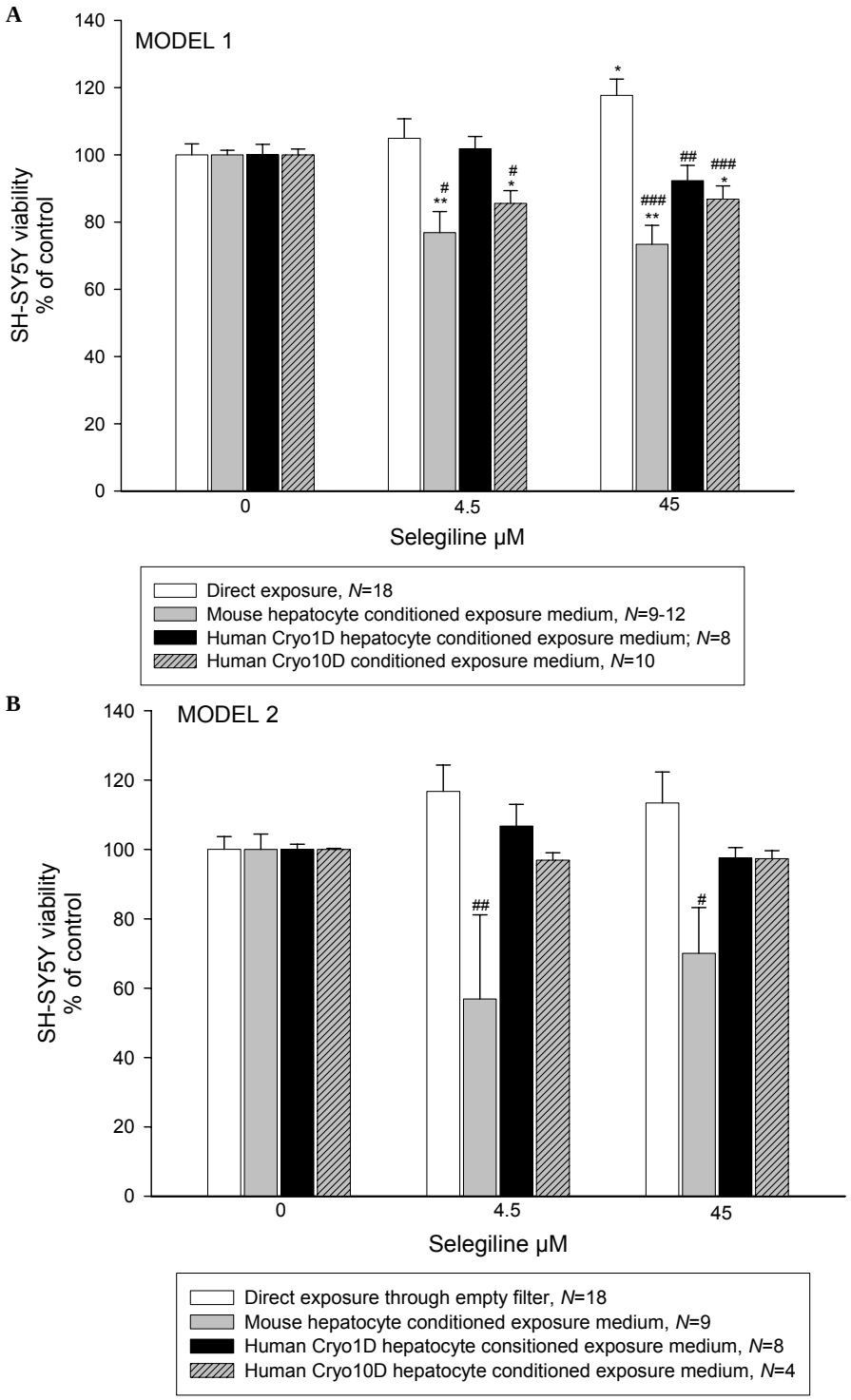

Fig. (2). The effect of selegiline (24 h exposure) on the viability (ATP content) of SH-SY5Y cells as dosed directly or after its pre-incubation with different hepatocyte cultures. The bars (means±SEM) in A present the results obtained by using Model 1, and in B those obtained by using Model 2. The statistically significant differences between treated cells and corresponding controls are indicated as *P<0.05 and **P<0.01. Significant differences between direct drug exposure and exposure with hepatocyte-conditioned drug are indicated as # P<0.05, ## P<0.01 and ### P<0.001.

tioned drug exposure at each concentration level (in both models) in Table **1**.

Metabolism-Dependent Toxicity of Carbamazepine

Carbamazepine itself was toxic to SH-SY5Ycells. The highest concentration 1 mM decreased SH-SY5Y viability by 52.3±3.8% when dosed on the cells directly (Fig. **3A**), and by 44.8±2.3% when dosed through empty filter cup (Fig. **3B**). When 1 mM carbamazepine was first pre-incubated with human freshly isolated, Cryo1D and Cryo10D hepatocytes according to Model 1, its toxicity increased causing 86.7±0.9, 69.2±3.7 and 73.8±3.9% reductions in SH-SY5Y

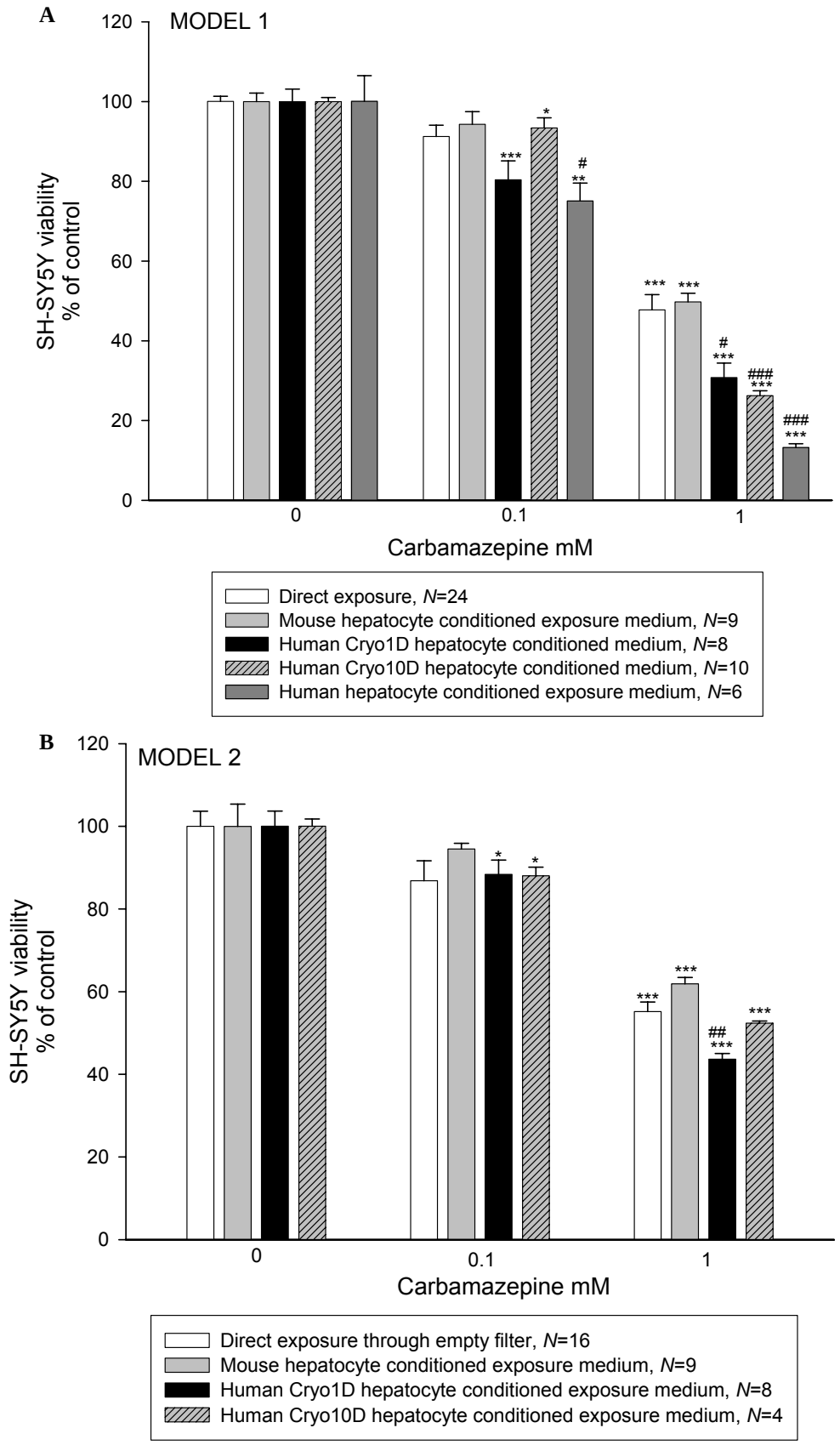

Fig. (3). The effect of carbamazepine (24 h exposure) on the viability (ATP content) of SH-SY5Y cells as dosed directly or after its pre-incubation with different hepatocyte cultures. The bars (means±SEM) in A present the results obtained by using Model 1, and in B those obtained by using Model 2. The statistically significant differences between treated cells and corresponding controls are indicated as *P<0.05, **P<0.01 and ***P<0.001. Significant differences between direct drug exposure and exposure with hepatocyte-conditioned drug are indicated as # P<0.05 and ### P<0.001.

Table 1. The Changes [Decrease (-) or Increase (+)] in Total Cellular ATP Contents of SH-SY5Y Cells Caused by Hepatocyte-Conditioned Exposure Medium, Compared to Direct Drug Exposure. The Values are Percentages, means±SEM

	MODEL 1				MODEL 2			
	Selegiline µM		Carbamazepine mM		Selegiline µM		Carbamazepine mM	
	4.5	45	0.1	1	4.5	45	0.1	1
Mouse hepatocytes	-23.0±4.5	-34.6±2.8	+4.9.±2.7	+11.6±5.4	-48.5±4.7	-32.4±6.0	+13.8±6.3	+13.3±3.7
Human Cryo1D[1]	-2.0±5.7	-19.7±2.8	-10.5±2.6	-20.0±4.9	-3.8±5.1	-7.0±7.0	+5.8±5.3	-20.0±2.4
Human Cryo10D[2]	-14.6±4.5	-24.6±2.6	+3.9±2.6	-41.3±2.8	-12.3±4.9	-4.2±8.1	+6.27±6.2	-3.7±3.3
Human hepatocytes[3]	-	-	-16.3±2.4	-70.0±1.6	-	-	-	-

[1]Cryopreserved human hepatocytes, 1 donor.
[2]Cryopreserved human hepatocytes, 10-donor pooled cell suspension.
[3]Freshly isolated human hepatocytes.

viability, respectively (Fig. **3A**). Pre-incubation with mouse hepatocytes did not alter carbamazepine toxicity (Fig. **3A**). In Model 2, only human Cryo1D hepatocytes increased carbamazepine toxicity; the viability of SH-SY5Y decreased by 56.4±1.4% (Fig. **3B**). Freshly isolated human hepatocytes were not used in these experiments.

Hepatocyte Viability

Selegiline (4.5 and 45 µM) and carbamazepine (0.1 and 1 mM) had minor effects on the viability of mouse and human cryopreserved hepatocytes during the 3-hour exposure (Table **2**). The only statistically significant effect was the 17.9±1.7% reduction of mouse hepatocyte viability caused by 1 mM carbamazepine. No statistical evaluations were possible with freshly isolated human hepatocytes, due to the limited number of independent experiments (*N*=2).

The Drug-Metabolizing Capacity of Hepatocytes

The mRNA expressions of CYP1A2 and CYP3A4 are shown in Fig. (**4**). Human and mouse freshly isolated and human cryopreserved hepatocytes showed high expression of CYP1A2 and CYP3A4 compared to HepG2 cells, where the expression were hardly detectable. Different mouse hepatocyte batches gave similarly repeatable results. Among human hepatocytes, the hepatocytes isolated in our laboratory showed highest CYP activities, followed by the cryopreserved hepatocytes.

DISCUSSION

Compared to several other liver-based *in v itro* models, such as microsomal preparations, hepatocytes are a superior model for studying drug toxicity and metabolism *in v itro*, because they encompass complete metabolic routes, i.e., phase I and II activity, as well as intact plasma membrane transport of drugs and their metabolites, i.e., phase III activity [3, 22]. In the present study, the CYP1A2 and CYP3A4 expressions of the freshly isolated mouse and human hepatocyte preparations were several-fold higher compared to metabolically less active HepG2 cells. This suggest a good phase I enzyme activity of the freshly isolated cells. Since enzyme function does not necessarily correlate with gene expression, we have previously verified the functionality of similarly isolated mouse hepatocytes by showing the disap-

Table 2. The Effect of Selegiline and Carbamazepine on the Viability of the Hepatocytes Assayed with ATP Measurement. Exposure Time 3 hours. The Results are Given as Percentages of Viable Cells Compared to Controls. Values are MEAN±SEM, *N*. The Significant Differences between Drug Treatments and Corresponding Controls were Tested, *** P<0.001

	Selegiline µM			Carbamazepine mM		
	0	4.5	45	0	0.1	1
Mouse hepatocytes	100.0±3.2 (*N*=6)	95.2±4.0 (*N*=6)	95.7±5.9 (*N*=6)	100.1±2.2 (*N*=6)	99.0±3.3 (*N*=6)	82.1±1.7*** (*N*=6)
Human Cryo1D[1]	100.0±3.8 (*N*=3)	101.1±6.7 (*N*=3)	90.1±2.4 (*N*=3)	100.0±1.0 (*N*=3)	102.9±6.3 (*N*=3)	65.3±17.3 (*N*=3)
Human Cryo10D[2]	100.0±3.2 (*N*=3)	91.3±9.2 (*N*=3)	87.6±25.8 (*N*=3)	100.0±28.1 (*N*=3)	107.5±32.6 (*N*=3)	77.1±5.4 (*N*=3)
Human hepatocytes[3]	-	-	-	100.0 (*N*=2)	78.8 (*N*=2)	96.6 (*N*=2)

[1]Cryopreserved human hepatocytes, 1 donor.
[2]Cryopreserved human hepatocytes, 10-donor pooled cell suspension.
[3]Freshly isolated human hepatocytes.

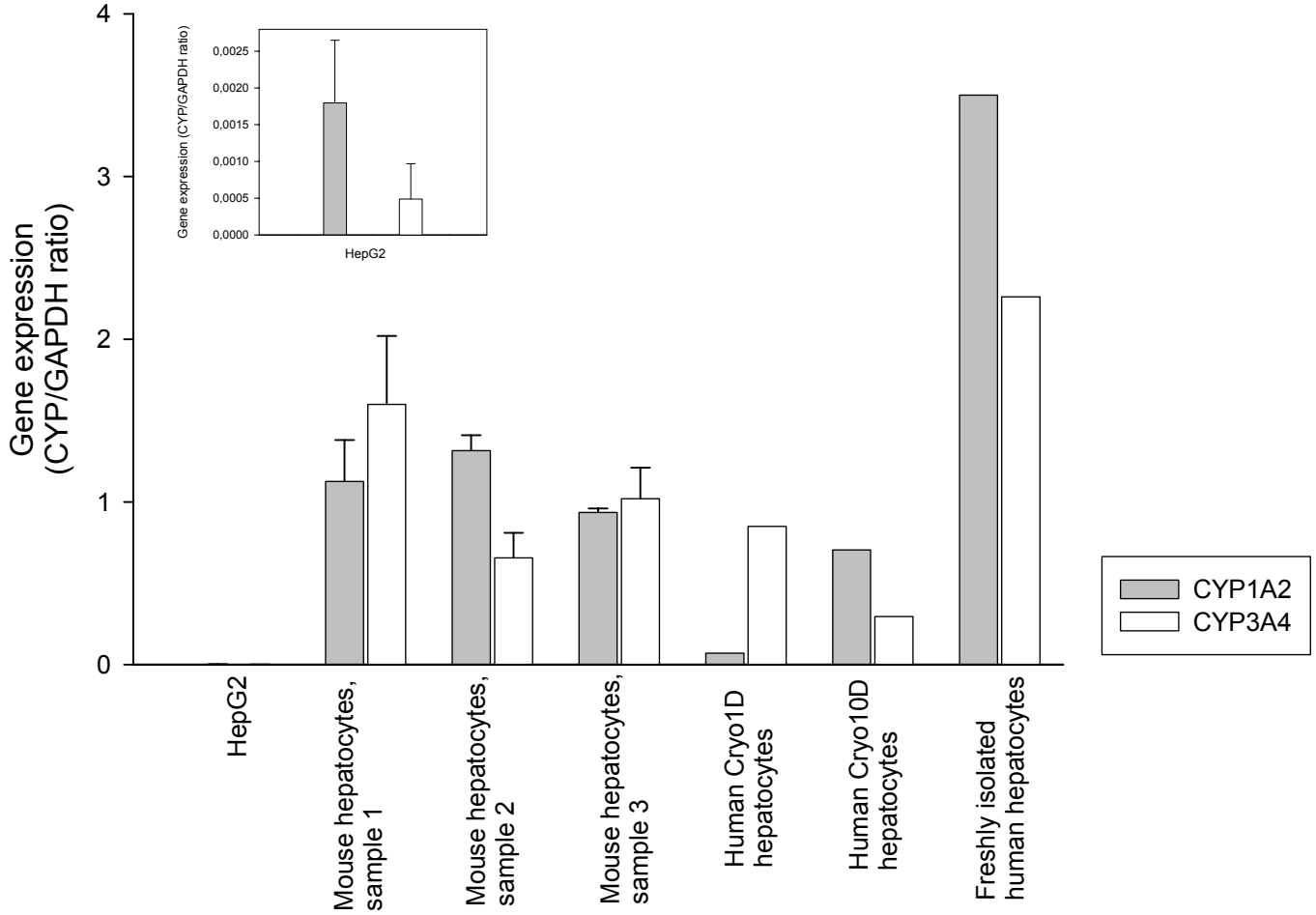

Fig. (4). Expression of CYP1A2 and CYP3A4 in hepatocyte cultures. The bars present CYP expressions in relation to respective GAPDH expression. The mouse hepatocyte CYP expressions were investigated in three different cell preparations (samples 1-3). Two PCR runs were carried out with mouse hepatocyte and HepG2 samples, and one PCR run with human hepatocyte samples.

pearance of the parent drug in the exposure media during 24-hour hepatocyte treatment [18]. Since the availability of human tissue for generating primary cultures is limited, commercially available cryopreserved human hepatocytes were also used in this study. Their use is well supported by the fact that they show fair metabolic activity and are well suited for short-term studies [4, 23]. In the present study, all the cryopreserved hepatocytes expressed CYP1A2 and CYP3A4, though not as much as freshly isolated human hepatocytes.

As it is shown in this and our previous study [18], selegiline itself increased SH-SY5Y viability. When selegiline exposure medium was pre-conditioned with hepatocytes, it became slightly toxic to SH-SY5Y cells, which may reflect a metabolism-induced toxicity of selegiline. Although there is controversy about the toxic potential of the metabolites of selegiline [24], our earlier *in vi tro* findings with a method using mouse hepatocytes as the metabolizing component also support the toxic nature of selegiline metabolites [18]. Selegiline is known to be metabolized by CYP1A2 and CYP3A4 [25]. The changes in selegiline toxicity caused by different hepatocyte cultures correlated with the gene expression data: Mouse hepatocytes, which showed the biggest CYP1A2 expression (except for freshly isolated human hepatocytes, which were not used in this test), increased selegiline toxicity to the highest extent. In turn, human Cryo1D, where CYP1A2 expression was severalfold smaller than in other hepatocytes (except for HepG2), increased selegiline toxicity least.

Carbamazepine itself, at 1 mM concentration, was toxic to SH-SY5Y cells, causing a 45% (Model 1) or 53% (Model 2) reduction in the cell viability. Pre-incubating carbamazepine with human freshly isolated and cryopreserved hepatocytes according to Model 1 further increased its toxicity. This was assumed to be due to the formation of metabolites that were more toxic than carbamazepine itself. There is also earlier *in vi tro* evidence that carbamazepine may have metabolism-dependent toxicity [12, 18, 26].

In freshly isolated human hepatocytes, carbamazepine metabolism seemed to be particularly active (compared to the other hepatocytes), since exposure to carbamazepine and its (possible) metabolites resulted in almost 90% loss of SH-SY5Y viability. This is consistent with the CYP expression data: CYP3A4 expression was severalfold higher in the freshly isolated human hepatocytes compared to other hepatocytes studied. Carbamazepine is metabolized by CYP3A4 both in human [27] and mouse [28] hepatocytes. It is noteworthy, however, that freshly isolated human hepatocytes represented one donor only.

Unlike human hepatocytes, mouse hepatocytes did not alter the toxicity of carbamazepine, possibly due to species-specific differences in the drug metabolism. The opinion that animal models do not reliably predict human metabolism has been frequently presented [1, 21, 22, 29]. Moreover, due to the polymorphism of cytochrome P450 enzymes, the metabolism of drugs can vary considerably between individuals [21, 30]. This can explain the slightly different results obtained with human Cryo1D and Cryo10D hepatocytes. The use of hepatocyte pools of several donors contains samples of different types of metabolizers, but may, on the other hand, mask exceptional metabolic characteristics.

Filter inserts are believed to maintain better hepatocyte morphology and function than simple monolayers [31]. Despite of this, the results obtained by using Model 2 were not as clear and uniform as those obtained by using Model 1. In Model 2 the variation in the data was somewhat greater lacking statistically significant effects, especially in the case of selegiline. Carbamazepine was generally less toxic in Model 2 than in Model 1. This is somewhat surprising, especially since 1.5 times more hepatocytes were used in Model 2. Thus greater toxic effects would be expected with Model 2. On the other hand the filter material itself might bind drugs and their metabolites and thus could interfere with the results. This could be one explanation to the lack of toxicity of selegiline, treated by human cryopreserved hepatocytes using Model 2. Moreover, there might be some incompatibility of membrane materials with different types of hepatocytes.

The plating densities of the hepatocytes were a result of several pilot experiments. Our target was to get a confluent layer of hepatocytes in the filter inserts in order to maximize the contact of drugs with the hepatocytes and to avoid the passage of drugs through the filter insert without a contact with the hepatocytes. In the pilot studies it was apparent, that relatively more hepatocytes were required to obtain a confluent cell layer in the filter inserts than in the wells. This may be due to the uneven surface of the filter insert and/or different morphologies of the hepatocytes in the filter inserts and the wells.

The drug incubation times, 3 hours for hepatocytes and 24 hours for target SH-SY5Y cells, were chosen for three main reasons: 1) *In vivo* orally administered drugs *pass* the liver, where they are suspected for liver biotransformation, and thereafter distribute and *accumulate* into target tissues. Thus, the target tissue might be enforced to sustain the drugs and/or their metabolites for longer periods (and maybe higher concentrations) than the liver itself. 2) 24-hour incubation time is generally used to study acute toxicity, and was consequently chosen to investigate the drug effects on SH-SY5Y cells. 3) There is evidence, with compounds other than selegiline and carbamazepine, that within three hours, human and rat hepatocytes produce the same metabolites that are found *in vivo* [29].

Selegiline is quickly and extensively metabolized, and its metabolites reach their maximal plasma concentrations within a couple of hours after its dosage as shown in previous clinical studies [32]. Carbamazepine, in turn, is a slow-clearance drug, but at least carbamazepine-10,11-epoxide is produced within 3 hours after exposure in different liver-derived systems (hepatocytes, microsomes and liver slices) [27].

A longer incubation time than 3 hours with hepatocytes might have resulted in reduced viability of hepatocytes and confusion with the possible cytotoxic effects of compounds liberated from lysing hepatocytes. During the 3-hour exposure time, 45 μM selegiline and 0.1 mM carbamazepine did not affect the viability of most of the hepatocytes. Only the greatest carbamazepine concentration (1 mM) resulted in an 18 % decrease in mouse hepatocyte viability. Due to small sample size with human freshly isolated hepatocytes (N=2), the data obtained cannot exclude the possible hepatotoxic effects of selegiline and carbamazepine on human freshly isolated hepatocytes themselves and its possible effects on the results. A three-hour incubation may be too short to allow the generation of some (less than 3%) phase II metabolites [7]. On the other hand, during 3-hour incubation, some short-lived metabolites may be lost, since the target cells are exposed to conditioned medium instead of the use of a co-culture system [3]. Further studies are needed to evaluate the importance of the time factor.

It is to be noted that the concentrations of selegiline and carbamazepine where metabolism-induced toxicity was found were higher than their therapeutic blood levels. Carbamazepine concentrations in this study were 0-240 μg/ml, while the therapeutic blood level is 1.4-12 μg/ml [33]. Selegiline concentrations were 0-10 μg/ml, the therapeutic blood level being 0.009-0.019 μg/ml [33]. These relatively high drug concentrations were used to ensure that the concentrations of metabolites produced would be high enough to cause detectable effects on the target cells. Moreover, neuroactive drugs may accumulate in brain tissue, causing markedly higher concentrations compared to those in blood [34].

In conclusion, the method combining metabolically competent hepatocytes and (neural) target cells might be promising in detecting metabolism-induced (neuro)toxicity of drugs. However, the experimental procedure should be standardized (e.g. in terms of sample sizes, number of hepatocyte and target cell wells etc.), tested with different types of test compounds, including negative and positive controls, and finally validated. After these improvements, the method introduced here will be efficient to predict qualitatively metabolism-induced toxicity of different compounds in the early phase of drug development and safety evaluations.

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