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SHORT COMMUNICATION

Noninterference of Cytochrome P4501A2 in the Cytotoxicity of Tacrine Using Genetically Engineered V79 Chinese Hamster Cells for Stable Expression of the Human or Rat Isoform and Two Human Hepatocyte Cell Lines

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ABSTRACT. Tacrine (THA) is the only drug currently approved for the treatment of Alzheimer's disease. A common side effect of this drug in humans is major hepatotoxicity. THA-induced toxicity may be related to a metabolic pathway implicating cytochrome P450 1A2 (CYP1A2). The purpose of this study was to clarify the role of the metabolic conversion of THA by CYP1A2 in the cytotoxicity of THA. The cytotoxicity of THA was evaluated in two human hepatocyte cell lines, HepG2 and Chang liver, and on the V79 Chinese hamster cell line, which does not express cytochrome P450 activity, and its variants, genetically engineered for expression of human or rat CYP1A2. Cells expressing human CYP1A2 metabolized THA to form its 1-OH derivative (V_{max} = 9.36 ± 0.57 pmol min^{-1} mg^{-1} total protein), whereas no metabolism was observed with the nonexpressing parental cells. In all cell lines, THA induced a marked decrease in cell viability and a strong inhibition of RNA and protein synthesis. However, these cytotoxic effects did not differ in parental V79 cells and variant cells expressing human or rat CYP1A2. The IC_{50} were tenfold higher for cell viability than for RNA and protein inhibition after 3 hr of incubation but were similar after 24 hr ($P < 0.0001$), indicating that this early inhibition was not a transient effect and could lead to cell death. These results strongly suggest that THA-induced cytotoxicity is not mediated by CYP1A2. *Copyright © 1997 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;3:423–427, 1997.

KEY WORDS. tacrine; cytochrome P450 1A2; toxicity; genetically engineered cells; human hepatocyte cell lines; Chinese hamster cell lines

THA† is a centrally active and reversible cholinesterase inhibitor that improves cognitive function and behavioral deficits in Alzheimer's disease patients [1–3]. The most common adverse effect of this drug is a major but reversible hepatotoxicity, leading to a marked serum alanine aminotransferase elevation [3, 4]. The mechanism of this hepatotoxicity *in vivo* is unclear. *In vitro*, THA can induce a dose-dependent aggregation of ribosomes on endoplasmic reticulum and aggregating nucleic acids in all organelles [5]. THA has been metabolized *in vivo* and *in vitro* by human and rat to hydroxylated metabolites 1-, 2-, 4- or 7-OH THA [6, 7]. The same metabolites and a protein-reactive metabolite that induced an irreversible binding to microsomal proteins have been found *in vitro* [8, 9]. The role of the CYP1A subfamily has been demonstrated by the increased protein binding of [^{14}C]THA with 3-methylcholanthrene-induced rat liver microsomal preparations [9]. Furthermore, experiments with specific inhibitors have demonstrated that the CYP1A2 isoform is implicated in the formation of the protein-reactive metabolite [8, 10]. Formation of a cytotoxic metabolite has also been suggested [10]. However, another study has demonstrated that THA-induced toxicity is similar in hepatocytes from different species despite their different metabolic capacities [11]. The purpose of this study was to clarify the role of the metabolic conversion of THA by CYP1A2 in THA cytotoxicity. We have studied THA-induced cytotoxicity on two human hepatocyte cell lines. We have also used the parental V79 Chinese hamster cell line, spontaneously defective in CYP activity, and V79–derived cell lines, genetically engineered for a stable expression of single forms of human or rat CYP1A2.

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† *Abbreviations:* THA, tacrine, (1,2,3,4,-tetrahydro-9-aminoacridine monohydrochloride monohydrate); CYP, cytochrome P450; CYP1A2, cytochrome P4501A2; MTT, (3-(4,5-dimethylthiazole-2yl)-2,5-diphenyltetrazolium bromide); MEM, minimum essential medium; IC_{50}, inhibitory concentration 50%; PCA, (10% perchloric acid); HPLC, high pressure liquid chromatography.

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MATERIALS AND METHODS

Cell Line Culture

Media and culture reagents were obtained from Gibco BRL (Cergy Pontoise, France). Fetal calf serum was furnished by

Boehringer Mannheim (Meylan, France). Human hepatoma HepG2 (ECACC 85011430) and nonmalignant hepatocyte Chang liver cell lines (ECACC 88021102) were used. These cell lines were cultured in monolayer, at 37°C in 95% air/5% CO_2, in complete medium consisting of MEM supplemented with 10% fetal calf serum, 1% nonessential amino acids, HEPES 30 mM and 100 UI/mL of penicillin. The V79 Chinese hamster fibroblast cell line is spontaneously defective in CYP activity, and V79-derived sublines are cells genetically engineered for a stable expression of human (V79MZh1A2) [12, 13] and rat CYP1A2 (V79MZr1A2) [14]. The cell lines were cultured in monolayer at 37°C in 95% air/5% CO_2, in complete medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1 mM of L-glutamine and 100 UI/mL of penicillin. Genetically engineered cells were maintained by adding G418 (400 μg/mL, Gibco BRL). However, before the experiments, the V79-derived cell lines were grown for a minimum of 72 hr in a G418-free medium to avoid interference.

Toxicity Studies

THA-mediated toxicity was evaluated by the MTT assay as previously described [15]. Cells were seeded in 24-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ, USA) at a density of 1.10^4 cells/cm^2 and cultured for 24 hr. Cytotoxicity was assessed after a 3- or 24-hr incubation period in the corresponding complete medium containing 50–3000 μM of THA (Aldrich, L'Isle d'Abeau Chesnes, France) or without THA (control). The results were expressed as the percentage of viability vs. control and IC_{50} (mean ± SD of three independent experiments, each performed in duplicate).

The inhibition of RNA and protein synthesis by THA was assessed by incorporation of radioactive precursors for 3 hr (0.4 μCi/mL of L-[4,5-^{3}H]leucine or [5,6-^{3}H]uridine; THA: 50–400 μM). Cells were seeded in 6-well plates at a density of 2.10^4 cells/cm^2. Radioactivity incorporation into the acido-insoluble pellet was assessed by liquid scintillation. The results were expressed as the percentage of inhibition of protein or RNA synthesis vs. that of control (no drug) and by the respective IC_{50} (mean ± SD of three independent experiments, each performed in duplicate).

Metabolic Study

The parental V79 cell line, its derived sublines and HepG2 cells (1.10^5 cells/cm^2) were incubated in 6-well plates for 24 hr at 37°C in 1 mL of complete medium containing THA at concentrations of 0.25–50 μM. An aliquot of the incubation medium was then treated with an equal volume of cold methanol. The amount of 1-OH THA formed was determined in the supernatant (10,000*g*, 10 min) by HPLC as previously described [6]. The respective rates of formation were expressed in pmol min^{-1} mg^{-1} total protein. V_{max} was obtained by using the Michaelis–Menten equation.

Analysis of Data

Dose–response curves were fitted to a sigmoidal model using nonlinear regression (Kaleidagraph, Synergy Software, Reading, PA, USA). Data were analyzed for significance ($P < 0.05$) by using a single-factor analysis of variance and the Student–Newman–Keuls multiple comparison test. The Kruskal–Wallis nonparametric test was also used when appropriate.

RESULTS AND DISCUSSION

The cytotoxicity of THA was determined on two human hepatocyte cell lines, HepG2 and Chang liver, and on the CYP-defective V79 Chinese hamster cell line and its derived cell lines genetically engineered for expression of human and rat CYP1A2. The toxic effects of THA, as measured by cell viability and inhibition of RNA and protein synthesis, did not differ in parental V79 cells, defective in CYP activity, and V79-derived cells expressing CYP1A2 activity. The dose–response curves for each test were quite superimposable (Fig. 1). Hence, THA-mediated cytotoxicity on V79 cell lines, as quantified by the IC_{50}, was not significantly affected by the expression of human or rat CYP1A2 (Table 1). These IC_{50}s were about tenfold higher for cell viability than for RNA and protein inhibition after

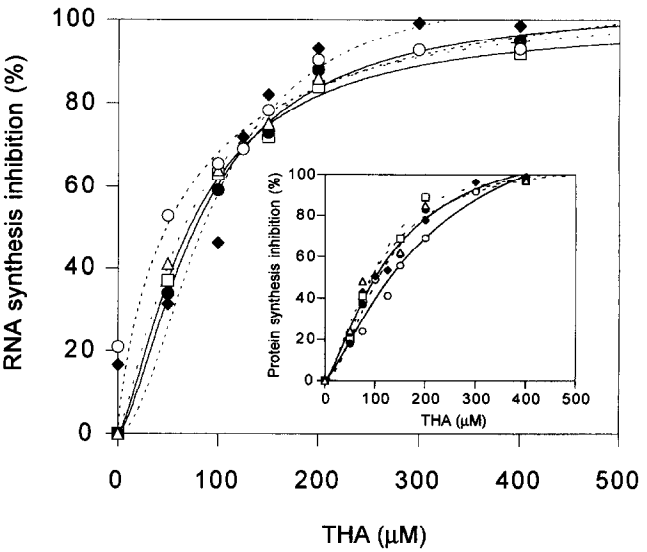

FIG. 1. Effect of THA on RNA and protein synthesis (inset) in HepG2 (♦) and Chang liver (○) hepatocyte cell lines and in V79 cell lines, both non-expressing (□) and expressing the human (●) or rat (△) CYP1A2. Cells were exposed to THA for 3 hr with 0.4 μCi/mL of [5,6-^{3}H]uridine or L-[4,5-^{3}H]leucine. Precursor incorporation was measured in the acido-insoluble pellet. Effect was expressed as the percentage of inhibition vs. that of the control (no drug). Dose–response curves were obtained using a sigmoidal model. Each curve represents the mean of three experiments performed in duplicate. Error bars were omitted for clarity (coefficient of variation is <10% for all points). No difference was observed in all cell lines regardless of CYP1A2 expression.

3 hr of incubation but were similar after 24 hr of incubation ($P < 0.0001$). Moreover, the IC_{50}s for RNA and protein synthesis inhibition were not different after 3 and 24 hr of incubation. V79MZh1A2 cells, expressing the human isoform, were able to metabolize THA as demonstrated by the release into the incubation medium of its 1-OH derivative (1.90 μM of 1-OH THA for 25 μM of THA; V_{max} = 9.29 ± 0.30 pmol min^{-1} mg^{-1} total protein) and of an unidentified metabolite (X). No THA metabolism was obtained with the parental V79 cell line, defective in CYP activity (Fig. 2). Only trace amounts of 1-OH THA were obtained with V79MZr1A2 cells, expressing the rat isoform. HepG2 cells also expressed a low but significant ability to metabolize THA (V_{max} = 2.42 ± 0.27 pmol min^{-1} mg^{-1} total protein).

Similar toxicity profiles were obtained on two human hepatocyte cell lines (HepG2 and Chang liver). Cell viabilities of these cell lines as estimated by the IC_{50} were not different, nor were inhibitions of RNA and protein synthesis, as shown by similar dose–response curves (Table 1, Fig. 1) and by the IC_{50}. As for the V79 cells, the IC_{50}s were higher for the viability test than for protein and RNA synthesis inhibition (Table 1, $P < 0.002$).

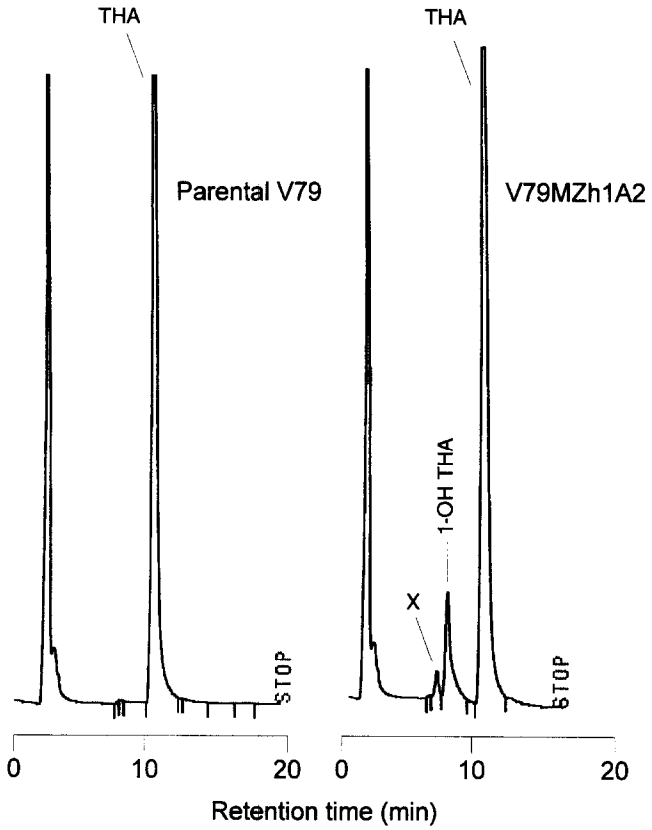

FIG. 2. Influence of the expression of CYP1A2 on the metabolism of THA. Parenteral V79 cells (deficients in CYP) and CYP1A2-expressing parental V79 cells and CYP1A2-expressing V79MZh1A2 cells (1.10^6/mL) were incubated for 24 hr with 25 μM THA. After removal of proteins by the addition of an equal volume of methanol, the incubation medium was analyzed by HPLC. No metabolite was obtained with the parental V79 cells. The 1-OH metabolite (1.36 μM; V_{max} = 9.29 ± 0.30 pmol min^{-1} mg^{-1} total protein) and an unidentified metabolite (X) were obtained with the V79MZh1A2 cells expressing human CYP1A2.

The toxicity profiles showed only a partial similarity between the V79 and hepatocyte cell lines. The THA-mediated cytotoxic effect on cell viability was higher on the HepG2 and Chang liver cells than on the V79 cells, where or not expressing human or rat CYP1A2 (Chang liver: $P < 0.01$, HepG2: $P < 0.002$). However, the IC_{50}s for RNA and protein synthesis did not differ from one cell line to the next (Table 1). For all cell lines, a complete inhibition of RNA and protein synthesis was observed for THA concentrations higher than 400 μM (Fig. 2). Except for HepG2 cells, the IC_{50}s for RNA synthesis were lower than for protein synthesis ($P < 0.01$) after 3 and 24 hr of incubation.

Our results strongly suggest that the bioactivation pathway using CYP1A2 is not implicated in the expression of THA-induced cytotoxicity. This THA-induced cytotoxicity as estimated by cell survival and inhibition of protein and RNA synthesis was identical in the CYP-defective V79 cell line and in the V79-derived cells genetically engineered for stable expression of the human or rat CYP1A2 isoform.

A marked hydroxylase and O-deethylation activity has been reported for these V79-derived cells [12]. We have shown that the V79MZh1A2 subline, expressing human CYP1A2, was also able to metabolize THA *in vitro*, as demonstrated by the formation of its 1-OH derivative, the major metabolite of THA obtained *in vivo* [6]. The rate of 1-OH formation has been studied by Spaldin *et al.* in 16 human liver microsomal samples [7]. This rate varied from 19.2 to 101 pmol min^{-1} mg^{-1}. Considering that our metabolic studies were performed on whole cells, our value is in good accordance with this range. This result suggests that the CYP1A2 activity in V79MZh1A2 cells and human hepatocytes could be similar. This metabolism was low in cells expressing the rat isoform, according to the species difference of the THA metabolism previously described [9, 10]. As expected, CYP-defective V79 cells were unable to metabolize THA. HepG2 cells also led to the formation of the 1-OH derivative but to a lesser extent than did V79MZh1A2 cells. However, the dose–response curves and the IC_{50}s for RNA and protein synthesis were very similar in all V79 and human hepatocyte cell lines, suggesting a comparable toxic effect regardless of species, cell origin difference, CYP-expressing status and metabolizing capacities. This finding is in agreement with the results of a previous study on HepG2 and rat hepatocyte cells, which showed a similar toxic response to THA exposition [11].

In all cell lines, THA led to a rapid and complete dose-dependent inhibition of protein synthesis, as previously reported [5]. Moreover, our results demonstrate that THA is also able to lead to a complete RNA synthesis inhibition in a dose-dependent manner. Except for HepG2 cells, the IC_{50}s for RNA synthesis were significantly lower than for the protein synthesis in other cell lines, suggesting an ear-

TABLE 1. Cytotoxicity of THA on the HepG2 and Chang liver hepatocyte cell lines and on the non-CYP-expressing V79 cell line and CYP1A2-expressing V79-derived cells

	IC_{50} for cell lines (μM)				
Test	**Parental V79**	**V79MZh1A2**	**V79MZr1A2**	**HepG2**	**Chang liver**
Cell viability					
3 h	1275 ± 132	1262 ± 179	1255 ± 55	854 ± 78	1089 ± 81
24 h	121 ± 14*	126 ± 20*	ND	ND	
RNA synthesis					
3 h	82 ± 7	84 ± 6	78 ± 2	94 ± 11	73 ± 9
24 h	77 ± 4	81 ± 2	82 ± 5	ND	ND
Protein synthesis					
3 h	110 ± 6	116 ± 7	106 ± 16	102 ± 9	121 ± 20
24 h	120 ± 25†	122 ± 20†	113 ± 9†	ND	ND

Cell viability was assessed by the MTT test after 3 and 24 hr of incubation with THA. RNA and protein synthesis inhibition were estimated by radioactive precursor incorporation after 3 and 24 hr of incubation with THA. Values are expressed as the IC_{50} ± SD (μM) of three experiments each performed in duplicate.

* $P < 0.0001$ for cell viability vs. 3 and 24 hr of incubation; ND, not determined.

† $P < 0.05$ for protein inhibition vs. RNA inhibition both for 3 and 24 hr of incubation.

lier toxic effect. Moreover, the IC_{50}s were higher for cell viability than for RNA and protein synthesis inhibition after 3 hr of incubation but were similar for all tests after 24 hr. The IC_{50}s for RNA and protein synthesis inhibition were not different after 3 and 24 hr of incubation, strongly suggesting that effects on RNA and protein were not transient and could be responsible for cell death. This inhibition of RNA synthesis has not been previously reported, although THA has been shown to reduce cellular RNA content in rat hepatocyte suspensions [5]. This previous study demonstrated that THA induced a dose-dependent aggregation of ribosomes on endoplasmic reticulum and the aggregation of nucleic acids in nucleus and mitochondria. This aggregation could result from intercalation of THA with overlapping RNA single strand to form double strands [5]. Thus, our results could provide additional support for the hypothesis that THA hepatotoxicity is primarily caused by a drug-induced ribosomal dysfunction [5].

Previous *in vitro* studies using incubation of [^{14}C]THA with human or rat liver microsomes and specific CYP inhibitors have shown that the CYP1A subfamily, particularly the CYP1A2 isoform, is strongly implicated in THA metabolism, as demonstrated by the formation of several stable hydroxylated derivatives and a reactive-protein metabolite [8–10]. The reactive metabolite has not been identified, but a putative quinone methide(s) intermediate has been postulated in the irreversible protein binding of THA [8, 9]. However, the role of this metabolite in the toxicity of THA on whole cells has not been clearly demonstrated. A species difference in THA-derived irreversible protein binding has also been demonstrated in the same model. The extent of this irreversible binding differed markedly in incubation experiments using human and rat liver microsomal preparations [8–10]. However, this species difference was not found in our study for THA-induced cytotoxicity, despite the use of V79 cell sublines expressing the human or rat isoform. Furthermore, this difference has only been demonstrated for the bioactivation and irreversible protein binding of THA and not for its cytotoxic effect on whole cells.

THA metabolism by rat liver microsomal preparations added in the incubation medium leads to the formation of a cytotoxic metabolite(s) on a lymphoblastic cell line [10]. This effect was attenuated by the addition of reduced glutathione, suggesting that a reactive metabolite was responsible for the observed cytotoxicity. In this model, no intracellular bioactivation was observed. However, because no evidence for penetration of this cytotoxic metabolite(s) inside cells was presented, this effect could also be interpreted in terms of a membrane-directed toxic mechanism. Thus, this cytotoxicity cannot be definitively ascribed to an intracellular activation of THA.

In summary, if several studies have shown that CYP1A2 is clearly implicated in the metabolism of THA, the relationship between this process and the THA cytotoxicity has not been definitely established. On the contrary, our model of similar cell lines, either CYP-defective or expressing human or rat CYP1A2, strongly suggests that this isoform is not implicated in the cytotoxic effects of THA that include a marked decrease in cell viability and a strong inhibition of RNA and protein synthesis. Furthermore, this finding provides additional support for the hypothesis that the effect of THA on ribosomal function could be responsible for THA-mediated hepatotoxicity.

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