Short Communication

GYKI-47261, A NEW AMPA [2-AMINO-3-(3-HYDROXYMETHYLISOXAZOLE-4-YL)PROPIONIC ACID] ANTAGONIST, IS A CYP2E1 INDUCER

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ABSTRACT:

CYP2E1-inducing capacity of xenobiotics was determined in cultured hepatocytes on the basis of enzyme activities (chloroxzone 6-hydroxylation and 7-ethoxycoumarin O-dealkylation) and protein levels. Hepatocytes in culture showed rapid loss of CYP2E1 enzyme during 72 h. CYP2E1 inducers (ethanol, dimethyl sulfoxide, acetone, isopropanol, pyrazole, and imidazole) were able to prevent the fast decrease of the activities and protein levels of CYP2E1 enzyme. Imidazole was found to be the most effective inducer in rat hepatocytes, and it was selected as a reference in subsequent experiments. The effect of GYKI-47261 [6-(4-aminophenyl)-8-chloro-2-methyl-11H-imidazo[1,2c] [2,3]benzodiazepine], a new AMPA [2-amino-3-(3-hydroxymethylisoxazole-4-yl)propionic acid] antagonist drug-candidate, was also tested in the in vitro system. On the basis of enzyme activities and CYP2E1 protein content of rat hepatocytes, GYKI-47261 was considered as a potent CYP2E1 inducer. Furthermore, it was more effective than imidazole, since 10 μM GYKI-47261 produced the maximal induction, whereas 500 μM imidazole brought about the maximal response. Human hepatocytes were more sensitive to GYKI-47261 than were rat cells. In rat hepatocytes, 10 μM caused maximal increase, whereas 0.01 μM produced the highest induction in human cells. Elevation of CYP2E1 gene transcription as the mechanism of induction caused by GYKI-47261 can be excluded. It seems to act mainly on stabilization of CYP2E1 enzyme protein, whereas the role of stabilization of CYP2E1 mRNA can be considered negligible. Although the imidazole part of GYKI-47261 can explain its CYP2E1-inducing capacity, the other part of the molecule must contribute to the final inducing potency.

CYP2E1 participates in the metabolic activation of several precarcinogens such as nitrosamines (Shu and Hollenberg, 1996) and cytotoxic agents like aceterminophen, or halogenated compounds (Hinson et al., 1995; Hammond and Fry, 1997). It is also suggested that CYP2E1 has a great role in lipid peroxidation and contributes to oxidative stress in cells (Caro and Cederbaum, 2001). CYP2E1 is effectively induced by structurally diverse chemicals, including ethanol, acetone, imidazole, and pyrazole. During the treatment of laboratory animals with these xenobiotics, CYP2E1 protein level was elevated in the absence of an increase of CYP2E1 mRNA as a result of post-translational regulation. The inducer stabilizes the enzyme protein and prevents its phosphorylation and rapid degradation by proteases (Ronis et al., 1996).

The aim of our work was to determine the CYP2E1-inducing effect of xenobiotics in hepatocyte culture. Several reports have demonstrated that increased levels of CYP2E1 protein were detected as a result of xenobiotic treatment of primary hepatocytes cultured mainly on a Matrigel or Vitrogen substrate (Zangar et al., 1995; Wu et al., 1997; Woodcroft and Novak, 1998). The objective of the present study was to investigate CYP2E1 induction caused by well known inducers (ethanol, acetone, dimethyl sulfoxide, isopropanol, pyrazole, and imidazole) in hepatocytes cultured on collagen surface. The effect of these chemicals was detected on the basis of protein levels as well as of enzyme activities (chloroxzone 6-hydroxylation and 7-ethoxycoumarin O-dealkylation) in intact cells. 6-Hydroxylation of chloroxzone is catalyzed primarily by CYP2E1 (Peter et al., 1990; Lucas et al., 1996); therefore, the chemical probe would offer a simple and fast method for measuring CYP2E1 induction in cell culture. Although several enzymes (CYP1A, CYP2B, CYP2E1) are responsible for O-dealkylation of 7-ethoxycoumarin, this probe can provide additional evidence for CYP2E1 induction (Yamazaki et al., 1996). A further aim of our studies was to test the CYP2E1-inducing capacity of a new drug-candidate, GYKI-472611 (Fig. 1; [6-(4-aminophenyl)-8-chloro-2-methyl-11H-imidazo[1,2c][2,3]benzodiazepine]), developed by IVAX Drug Research Institute Ltd. (Budapest, Hungary) for treatment of epilepsy and parkinsonism (Sólyom, 2001).

Materials and Methods

Chemicals. GYKI-47261 [6-(4-aminophenyl)-8-chloro-2-methyl-11H-imidazo[1,2c][2,3]benzodiazepine] was provided by IVAX Drug Research Institute Ltd. (Budapest, Hungary). Chloroxzone, 7-ethoxycoumarin, imidazole, pyrazole and dimethyl sulfoxide were the products of Sigma Chemie (Deisenhofen, Germany). Ethanol, dichloromethane, isopropanol, acetonitrile, and β-glucuronidase/arylsulfatase were purchased from Merck (Darmstadt, Germany). All other chemicals were obtained from Reanal Finechemical Co. (Budapest, Hungary).

Isolation and Culture of Hepatocytes. Experiments were carried out by using hepatocytes prepared from male Wistar rats (weighing 200 g) (ToxiCoop Safety Toxicological Study Center, Budapest, Hungary). Human livers were

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1 Abbreviations used are: GYKI-47261, 6-(4-aminophenyl)-8-chloro-2-methyl-11H-imidazo[1,2c][2,3]benzodiazepine; P450, cytochrome P450; AMPA, 2-amino-3-(3-hydroxymethylisoxazole-4-yl)propionic acid.
obtained from kidney transplant donors at the Transplantation and Surgery Clinic, Semmelweis University (Budapest, Hungary). Permission of the Local Research Ethics Committee was obtained to use human tissues. The liver cells were isolated by the method of Bayliss and Skett (1996). The hepatocytes with better than 90% viability, determined by trypan blue exclusion (Berry et al., 1991), were used in experiments. The cells were plated at a density of 4 × 10^6 cells/dish onto 60-mm plastic dishes precoated with collagen in medium described by Ferrini et al. (1998). After a 4-h attachment, the medium was changed and renewed every 24 h thereafter in the absence of serum. Ethanol, dimethyl sulfoxide, acetone, and isopropanol were directly added to the medium; pyrazole and imidazole were prepared as a stock solution in sterile water. Although GYKI-47261 was dissolved in dimethyl sulfoxide, the final concentration of the vehicle (0.1 mM) did not have an effect on the CYP2E1 enzyme. The cytotoxic effect of GYKI-47261 was determined on the basis of trypan blue exclusion and glutathione levels (Anderson, 1985).

**Enzyme Assays.** Catalytic activity of CYP2E1 was evaluated by measuring 6-hydroxylation of chlorozoxazone. The medium over the cells was replaced with Hanks' balanced salt solution containing 400 μM chlorozoxazone. After a 45-min incubation, the medium was removed. Phenacetin (17.2 μM) as an internal standard and phosphoric acid (final concentration of 0.34 M) were added to the medium. 6-Hydroxy-chlorzoxazone was extracted three times with 5 ml of dichloromethane. The organic phase was evaporated to dryness and the residue was dissolved in 40% acetonitrile. The amount of 6-hydroxy-chlorzoxazone was determined by high-performance liquid chromatography [column: 125 × 4 mm LiChrospher RP18, 5 μm; mobile phase: acetonitrile, 0.2% phosphoric acid, 25:75 (v/v); flow rate: 1 ml/min; wavelength: 290 nm]. O-Dealkylation of 7-ethoxycoumarin was determined as previously described (Monostory and Vereczkey, 1996).

**Immunoblot Analyses.** Hepatocytes were harvested in phosphate-buffered saline [pH 7.4], and microsomes were prepared according to the method of van der Hoeven and Coon (1974). The amount of CYP2E1 in hepatocytes was determined by Western blot analysis using anti-rat CYP2E1 as primary antibody (Towbin et al., 1979).

**Preparation and Analysis of Total RNA.** Total RNA was isolated using TRizol reagent (Invitrogen, Carlsbad, CA) from 10^5 cultured hepatocytes or 0.5 g of liver tissues according to the manufacturer’s instructions. The RNA was subjected to electrophoresis on a 1.2% agarose/formaldehyde denaturing gel (15 μg/lane), transferred onto a nylon membrane (Sartorius AG, Goettingen, Germany), and hybridized with radiolabeled cDNA probes specific for CYP2E1. The probes were obtained by reverse transcription-polymerase chain reaction amplification of rat and human liver RNA, respectively, and labeled with [α-32P]dCTP using the Rad Prime DNA labeling system (Invitrogen) as previously described (Nedelcheva et al., 1996; Simi and Ingelman-Sundberg, 1999). The filters were washed and exposed to autoradiography as described (Simi and Ingelman-Sundberg, 1999) and scanned using LAS-1000 (Fujifilm, Dusseldorf, Germany).

**In Vivo Induction Studies.** Male Wistar rats (150 g) were treated i.p. with 20 mg/kg GYKI-47261 (in corn oil) and with 300 mg/kg imidazole (pH 7.4 in water) for 4 consecutive days. Microsomes and total mRNA were prepared from the livers. Chlorozoxazone 6-hydroxylation activity (Peter et al., 1990) and CYP2E1 protein content of the microsomes as well as CYP2E1 mRNA were determined.

**Results and Discussion**

**CYP2E1 Induction in Rat.** Preliminary experiments involved adaptation of chlorozoxazone 6-hydroxylation probe developed as a microsomal assay (Peter et al., 1990) for intact hepatocytes. Comparison of the efficacy of the extraction from the medium and from the medium plus cells showed that only 3 to 4% of the metabolite remained inside the hepatocytes after removing the medium; therefore, the formation of 6-hydroxy-chlorozoxazone was determined from the medium.

Hepatocytes were prepared from untreated rats and maintained in culture for 72 h. Chlorozoxazone 6-hydroxylation activity of CYP2E1 decreased rapidly in the cell culture since only 20% of the initial activity remained after 72 h (Fig. 2A). Although the same amount of CYP2E1 apoprotein was detected in hepatocytes at 24 h and freshly isolated cells, rapid decrease occurred at 48 and 72 h (Fig. 2B). Fast loss of CYP2E1 mRNA was also demonstrated in untreated hepatocytes (Fig. 3A), which is in accordance with the previous findings (Eliasson et al., 1988; Woodcroft and Novak, 1998). CYP2E1 activity was also measured in the cells treated with well known inducers: ethanol (10 and 100 mM), dimethyl sulfoxide (5 and 50 mM), isopropanol (5 and 50 mM), acetone (5 and 50 mM), pyrazole (50 and 500 μM), and imidazole (50 and 500 μM) (Fig. 4A). Although all inducers were able to prevent the decrease of chlorozoxazone 6-hydroxylation activity of the cells cultured for 48 h, none of them elevated CYP2E1 activity above the initial level (the activity of freshly isolated hepatocytes). Ethanol (100 mM) and acetone (5 mM) treatment resulted in about 25 to 26% increase compared with untreated cells. The effect of dimethyl sulfoxide, isopropanol, and pyrazole was more pronounced mainly at the higher concentrations. The most effective chemical in preservation of chlorozoxazone 6-hydroxylation activity was imidazole. The hepatocytes treated with 500 μM imidazole displayed about 4-fold higher activity than did the untreated...
GYKI-47261 for 4 consecutive days (C). UT, untreated.

Hepatocytes (A and B) were exposed to 500 μM imidazole and different concentrations (1, 0.1 mM; 2, 1 mM; 3, 10 mM; 4, 100 mM; 5, 1 μM; 6, 10 μM; 7, 50 μM) of GYKI-47261 for 48 h. Rats were treated i.p. with 300 mg/kg imidazole M) of GYKI-47261 for 48 h. Rats were treated i.p. with 300 mg/kg imidazole M) or GYKI-47261.

Cell population. The findings determined on the basis of chlorzoxazone 6-hydroxylation activity were confirmed by the results of CYP2E1 content of the hepatocytes (Fig. 4B). On the basis of these results, imidazole at a concentration of 500 μM was selected as a reference inducer in subsequent experiments.

Based on preliminary experiments, the effect of GYKI-47261 (at a concentration range of 0.01–50 μM) on chlorzoxazone 6-hydroxylase activity was investigated and the results were compared with the effect of 500 μM imidazole (Fig. 5A). Significant increase of CYP2E1 activity was detected in the cells treated with GYKI-47261 at concentrations as low as 0.01 μM, and the maximal response was observed at a concentration of 10 μM. However, the activity determined in 50 μM GYKI-47261-treated cells did not differ from that of the control cells. In comparison, 500 μM imidazole treatment caused about a 2- to 3-fold increase. Similar results were obtained from the determination of 7-ethoxyresorufin O-dealkylation of the hepatocytes (Fig. 5B). Maximal induction could be reached at a concentration of 10 μM GYKI-47261, whereas the cells treated with 50 μM showed lower activity than those given 10 μM. It should be noted that 50 μM GYKI-47261 had a cytotoxic effect on primary hepatocytes, which can be considered as the reason for decreasing activities. The cells exposed to 50 μM GYKI-47261 did not form a perfect monolayer; some cells detached from the collagen surface. The viability of the hepatocytes decreased by 20% as a result of the treatment for 3 h. Reduced glutathione level of the cells also decreased by 20%.

Elevated CYP2E1 protein levels were also detected in the microsomes of the hepatocytes treated with GYKI-47261 or imidazole (Fig. 5C). A good correlation was observed between CYP2E1 activities and immunoreactive protein levels. During the 72-h exposure, GYKI-47261 was able to prevent or slow down the loss of CYP2E1 activity; however, the cells treated with GYKI-47261 for 72 h showed somewhat lower chlorzoxazone 6-hydroxylase activity than did freshly isolated hepatocytes (Fig. 2A). Although CYP2E1 mRNA of the cultured cells rapidly decreased, a small amount (about 6% of the initial level) was detected in GYKI-47261-treated (10 and 50 μM) cells at 48 h. CYP2E1 mRNA totally disappeared from the cells maintained for 72 h (Fig. 3A). These facts provided the evidence that GYKI-47261 did not elevate the transcription of CYP2E1 gene, but caused slight stabilization of CYP2E1 mRNA and markedly decreased the loss of CYP2E1 activities and enzyme protein.

From in vitro results it can be concluded that GYKI-47261 is an inducer of CYP2E1 and is more efficacious than imidazole. A 500 μM concentration of imidazole was able to bring about the maximal induction of CYP2E1, whereas 10 μM GYKI-47261 produced the same or even higher response. It was also evaluated whether GYKI-47261 had CYP2E1-inducing capacity in vivo. In vivo treatment of male rats (n = 3/group) with GYKI-47261 caused significant increase of chlorzoxazone 6-hydroxylation activity (Fig. 6A) and CYP2E1 protein level (Fig. 6B). Slight, but significant increase of CYP2E1 mRNA (by 35%, compared with controls) was also observed (Fig. 3C), which confirmed in vitro findings of a weak mRNA-stabilizing capacity of CYP2E1. Imidazole, the reference inducer, also elevated the activity and the protein level of CYP2E1 (Fig. 6, A and B) without any increase in CYP2E1 mRNA levels (Fig. 3C). It should be noted that the difference in efficacy between GYKI-47261 and imidazole could also be observed in the in vivo experiments, since a 20 mg/kg dose of GYKI-47261 produced induction similar to that of a 300 mg/kg dose of imidazole.

It should be mentioned that the activities of other cytochrome P450 (P450) enzymes investigated in our in vitro or in vivo experiments (CYP1A, ethoxyresorufin O-dealkylation; CYP2B, pentoxyresorufin O-dealkylation; CYP3A, ethylmorphine and aminopyrine N-demethylation) did not increase in the presence of GYKI-47261. Total P450 content as well as the activities of cytochrome P450 and NADPH-P450 reductase were also unchanged.

CYP2E1 Induction in Human. Hepatocyte culture offers a simple tool for the extrapolation of data from laboratory animals to humans. CYP2E1 induction studies were also carried out in human hepatocytes prepared from four donors (HH-042, HH-043, HH-044, and HH-050). The basal CYP2E1 activities (measured in control cells) were differ-

![Fig. 3. CYP2E1 mRNA levels in rat (A) and human (HH-050) hepatocytes (B) and rat livers (C) treated with imidazole (IM) or GYKI-47261.](image-url)
ent; HH-043 displayed the lowest chlorzoxazone 6-hydroxylation and 7-ethoxycoumarin O-dealkylation activities (Fig. 7, A and B). Imidazole (500 μM) produced marked increase of both CYP2E1 activities in human hepatocytes. The effect of GYKI-47261 in human cells was somewhat different from what was observed in rat cells. The hepatocytes of HH-043, HH-044, and HH-050 were treated at low concentrations of 0.01 and 0.1 μM. Similar or even higher induction of CYP2E1 activities was observed in the cells treated with 0.01 μM GYKI-47261 than in those treated with 500 μM imidazole. However, higher concentrations were not able to cause further induction. On the other hand, 50 μM GYKI-47261 decreased CYP2E1 activities. A cytotoxic effect of GYKI-47261 may serve as the explanation since it was found in the case of rat hepatocytes. A 4- to 5-fold elevation of CYP2E1 protein content resulted as an effect of GYKI-47261 treatment (Fig. 7C). CYP2E1 mRNA content of untreated human hepatocytes decreased in culture; however, the decrease of mRNA level was lower than that in rat cells (Fig. 3B). CYP2E1 mRNA was present in trace in human cells cultured for even 72 h, whereas it failed to be detected in rat hepatocytes at 48 h. GYKI-47261 (10 μM) seemed to cause slight elevation of CYP2E1 mRNA at 24 h, but the mRNA levels at 48 or 72 h did not differ from those of the control or imidazole-treated cell population. The results obtained in human hepatocytes also demonstrated that GYKI-47261 was able to induce CYP2E1 mainly via enzyme protein stabilization and not at the level of gene transcription.

In summary, CYP2E1 induction caused by xenobiotics was investigated in rat and human primary hepatocytes. In rat hepatocytes, imidazole was found to be the most effective inducer on the basis of enzyme activities (chlorzoxazone 6-hydroxylation and 7-ethoxycoumarin O-dealkylation). The results were also confirmed by the determination of CYP2E1 protein levels. GYKI-47261, a new AMPA
Human hepatocytes from four donors (HH-042, HH-043, HH-044, and HH-050) were treated with different concentrations (1, 0.1 nM; 2, 1 nM; 3, 10 nM; 4, 100 nM; 5, 1 µM; 6, 10 µM; 7, 50 µM) of GYKI-47261 or with imidazole (IM; 500 µM) as a reference inducer, and the results were compared with those of untreated (UT) hepatocytes. Activities were determined in triplicate.

antagonist drug-candidate, was also tested in this in vitro system. The results indicated that GYKI-47261 was a potent inducer of CYP2E1 and was more effective than imidazole. In rat hepatocytes, 10 µM caused maximal increase, whereas 0.01 nM/H9262 hepatocytes. Activities were determined in triplicate.

Hammond AH and Fry JR (1997) Involvement of cytochrome P450SE1 in the toxicity of dichloropropanol to rat hepatocyte cultures. Toxicology 118:171–179.


