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TIAMULIN INHIBITS HUMAN CYP3A4 ACTIVITY IN AN NIH/3T3 CELL LINE STABLY EXPRESSING CYP3A4 cDNA

ELS M. DE GROENE,*‡ SANDRA M. NIJMEIJER,† G. J. M. JEAN HORBACH* and RENGER F. WITKAMP†

*Research Institute of Toxicology and †Dept. of Veterinary Pharmacology, Pharmacy and Toxicology, Utrecht University, Yalelaan 2, P.O. Box 80.176, NL-3508 TD Utrecht, The Netherlands

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Abstract—Tiamulin is an antibiotic frequently used in veterinary medicine. The drug has been shown to produce clinically important interactions with other compounds that are administered simultaneously. An NIH/3T3 cell line, stably expressing human cytochrome P450 (EC 1.14.14.1) cDNA (CYP3A4), was used to study the effect of tiamulin on CYP3A4 activity. The 6β-hydroxylation activity of testosterone, which is increased in CYP3A4-expressing cells compared to vector-transfected cells, showed reduced activity after incubation with 1 μM tiamulin and was completely reduced to background level after incubation with 2, 5 and 10 μM tiamulin. The CYP3A4-expressing cell line was used in combination with a shuttle vector containing the bacterial lacZ′ gene to study the effect of tiamulin on CYP3A4-mediated mutagenicity of aflatoxin B_1. The mutation frequency of aflatoxin B_1 could be completely inhibited by tiamulin in CYP3A4-expressing cells, but no effect was observed on the mutation frequency of the direct mutagen ethylmethanesulphonate. Western blotting of homogenates of the CYP3A4-expressing cell line showed stabilization of CYP3A4 protein after incubation with tiamulin, supporting the hypothesis that the mechanism of inhibition is by binding of tiamulin to the cytochrome.

Key words: CYP3A4; tiamulin; 3T3/NIH cell line; stable expression

The P450§ enzyme (EC 1.14.1.1) family is involved in the biotransformation of endogenous compounds and of xenobiotics including drugs, environmental chemicals, and carcinogens [1]. During the last few years much research has been done on the expression of human cytochrome P450 cDNAs in transgenic cell lines. The use of these cell lines has made the study of human xenobiotic metabolism and biotransformation-mediated mutagenicity more possible [2]. Recently, a toxicological test system has been developed in which NIH/3T3 cells, stably expressing human CYP3A4 cDNA, are used in combination with a shuttle vector to study mutagenicity [3]. The bacterial lacZ′ gene is present on the shuttle vector as reporter gene for mutations, thereby making it possible to study the role of biotransformation by an individual cytochrome P450 on mutagenic effects of compounds, using a single-cell system. The CYP3A4-expressing cell line developed in our laboratory was shown to be highly active in 6β-hydroxylation of testosterone and had a markedly increased sensitivity towards cytochrome P450-mediated mutagenicity of aflatoxin B_1 (AFB_1). The role of CYP3A in the bioactivation of aflatoxin B_1 leading to mutagenic products has been well established [4].

Tiamulin is a semi-synthetic derivative of the diterpene antibiotic pleuromutulin. The drug is widely used in veterinary medicine for treatment or prevention of pulmonary and gastro-intestinal infections in pigs and poultry. One of the problems associated with its use is the occurrence of clinically relevant and sometimes even lethal interactions with other drugs [5, 6]. In previous studies with hepatic microsomes of rat and pig and with pig hepatocytes, it was demonstrated that this interaction can be partly explained by an inhibition of CYP3A enzyme activity in these species [7, 8].

The aim of the present study is to investigate whether tiamulin also inhibits human CYP3A4 activity by examining its effect on the 6β-hydroxylation activity of testosterone and on AFB_1-induced mutagenicity. In order to provide further support for the idea that the reduction of AFB_1 mutagenicity by tiamulin is due to inhibition of AFB_1 bioactivation, effects of tiamulin on the mutagenicity of ethylmethanesulphonate, a direct mutagen, are investigated. As it has been suggested that tiamulin acts on P450 via the formation of a metabolic intermediate complex [8], the CYP3A4 stabilizing effect of tiamulin is examined.

MATERIALS AND METHODS

ElectroMAX DH10B cells and cell culture media were purchased from Gibco BRL Life Technologies (Gaithersburg, MD, U.S.A.). Aflatoxin B1 and testosterone were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ultraculture medium was purchased from BioWhittaker (Walkerville, MA, U.S.A.). Fetal calf serum was obtained from Flow (Zwanenburg, The Netherlands). Monoclonal antibody against rat CYP3A1 was kindly donated by Dr. P. Kremers (Université de Liège, Belgium). Tiamulin hydrogen fumarate was a generous gift from Biochemie Gesellschaft mbH (Tiamulin Business group, Kundl, Austria).

Plasmid constructions and cell culture

The shuttle vector pSV.SPORTlacZ′ was constructed by insertion of the lacZ′ fragment from pGEM 7zf(+) (Promega, Madison, WI, U.S.A.) in the SalI site of pSV.SPORT1, as described previously [3]. The CYP3A4-expressing and vector-infected NIH/3T3 cells were maintained in Dulbecco's Modified Eagle Me-

‡ Corresponding author. Tel. 31-30-535400, FAX 33-30-535077.

§ Abbreviations: AFB_1, aflatoxin B_1; EMS, ethylmethanesulphonate; P450, cytochrome P450; TIA, tiamulin.

dium, DMEM (supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM l-glutamine) at 37°C and 5% CO_2.

CYP3A4 enzyme activity

Testosterone 6β-hydroxylation activity was determined as described by Wortelboer *et al.* [9]. Incubations were performed using 4×10^6 cells/75 cm^2 culture flask with 5 mL Ultraculture containing 250 μM testosterone. The incubation period with testosterone was 4 h. The effects of tiamulin (0, 1, 2, 5, and 10 μM) were studied after a preincubation period with the compound for 1.5 h.

Mutation analysis

The CYP3A4-expressing cells (3T3-3A4) and vector-transfected cells (3T3-LNCX) were transfected with pSV.SPORT-lacZ′ in suspension [3]. The day after transfection, the cells were incubated for 6 h with 600 ng/mL AFB_1 or with 250 μg/mL ethylmethanesulphonate (EMS) without medium change as EMS has a short half-life in dilute aqueous solutions. In the inhibition studies the cells were preincubated for 1.5 h with 5 or 10 μM tiamulin. The shuttle vectors were rescued from the cells 48 hours later, using Hirt extraction [10], and purified. Plasmids not replicated in the NIH/3T3 cells were removed by DpnI digestion. The plasmids were transformed into electrocompetent DH10B bacteria, and the mutation frequency determined by the ratio of white colonies and the total number of colonies.

Western blot

3T3-3A4 cells (2×10^6 cells/75 cm^2 flask) were grown until they reached confluency in the presence of 0, 5, 10 μM tiamulin. Cells were washed with PBS, scraped, and collected by centrifugation. The pellet was resuspended in 100 μL PBS and frozen/thawed 3 times, centrifugated, and the supernatant frozen at −70°C until use. Protein contents were measured according to Lowry *et al.* [11]. Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) of homogenates (10 μg/lane) was performed, and the resolved proteins blotted on polyvinylidenedifluoride (PVDF) sheets (Millipore, Etten-Leur, The Netherlands). Antibody against rat CYP3A1 was used for immunochemical staining, as described in detail by Wortelboer *et al.* [12].

Statistical evaluation

Statistical calculations were performed using ANOVA followed by a Student's *t*-test. Values were considered to be significantly different if $p < 0.05$.

Table 1. 6β-Hydroxylation activity of testosterone in 3T3-3A4 and 3T3-LNCX cells

Cell line	Tiamulin (μM)	pmol/10^6 cells/min
3T3-LNCX	0	0.56 ± 0.28
3T3-LNCX	10	0.37 ± 0.02
3T3-3A4	0	4.47 ± 0.30
3T3-3A4	1	1.31 ± 0.21*
3T3-3A4	2	0.82 ± 0.02*
3T3-3A4	5	0.55 ± 0.19*,†
3T3-3A4	10	0.71 ± 0.18*,†

3T3-3A4 and 3T3-LNCX cells were incubated for 4 h with 250 μM testosterone. Inhibition studies were carried out by preincubation of the cells with 1, 2, 5, or 10 μM tiamulin prior to testosterone incubation. Activity values are expressed as means ± *SD* for 3 experiments.

* Value differs significantly ($p < 0.05$) from 3T3-3A4 0 μM tiamulin value.

† Value differs significantly ($p < 0.05$) from 3T3-3A4 1 μM tiamulin value.

Table 2. Mutation frequency induced by aflatoxin B_1 in 3T3-3A4 cells after incubation with tiamulin

Cell line	AFB_1 (ng/mL)	Tiamulin (μM)	Mutation freq. ($\times 10^4$)
3T3-3A4	0	0	1.57 ± 0.81
3T3-3A4	600	0	37.13 ± 9.36*
3T3-3A4	600	5	0.97 ± 0.17†
3T3-3A4	600	10	1.21 ± 0.22†

3T3-3A4 cells were transfected with pSV.SPORT-lacZ′ and incubated with 600 ng/mL aflatoxin B_1 (AFB1) in the presence or absence of 5 and 10 μM tiamulin. Mutation frequency values are expressed as means ± *SD* for 3 experiments.

* Value differs significantly ($p < 0.05$) from 3T3-3A4 0 ng/mL AFB_1 value.

† Value differs significantly ($p < 0.05$) from 3T3-3A4 0 μM tiamulin value.

RESULTS AND DISCUSSION

The 6β-hydroxylation activity of testosterone in the CYP3A4-expressing cells was partially reduced after incubation with 1 μM tiamulin, and completely reduced to background level after preincubation with 2, 5, or 10 μM tiamulin (Table 1). AFB_1 is a promutagen that needs metabolic activation to exert its mutagenic activity, and the involvement of the CYP3A family as one of the major enzymes in this activation has been established [4]. The mutagenicity induced by AFB_1 in 3T3-3A4 cells was completely inhibited by tiamulin (Table 2). Both the reduced AFB_1 mutagenicity and the decrease in testosterone 6β-hydroxylation can be assigned to an inhibition of CYP3A4 enzyme activity. From Table 3 it can be seen that control studies using EMS showed no difference in mutation frequency between the CYP3A4-expressing cells and vector-transfected cells. Incubation with tiamulin did not affect the level of mutation frequency, supporting the hypothesis that the action on AFB_1 mutagenicity occurs by inhibition of biotransformation.

Table 3. Mutation frequency induced by ethylmethanesulphonate in 3T3-3A4, in the presence or absence of tiamulin, and 3T3-LNCX cells

Cell line	Tiamulin (μM)	Mutation frequency ($\times 10^4$)
3T3-LNCX	0	8.15 ± 1.14
3T3-3A4	0	9.36 ± 3.68
3T3-3A4	10	7.49 ± 1.96

3T3-3A4 and 3T3-LNCX cells were transfected with pSV.SPORT-lacZ′ and incubated with 250 μg/mL ethylmethanesulphonate (EMS) in the presence or absence of 10 μM tiamulin. Mutation frequency values are expressed as means ± *SD* for 3 experiments.

The regulation of the CYP3A4 gene in this NIH/3T3 cell line differs from the *in vivo* situation, since the gene is under the control of the cytomegalovirus promoter. This is an indication that inhibition by tiamulin has to be at the post-transcriptional level. It can be seen from the Western blot that the amount of CYP3A4 protein, cross-reacting with the monoclonal antibody against rat CYP3A1, was increased with increasing concentrations of tiamulin (Fig. 1). This is in accordance with previous findings showing that tiamulin complexates P450. Studies in pig microsomes and in isolated hepatocytes showed that tiamulin was able to form a metabolic intermediate complex with cytochrome P450 having an absorption maximum at 456 nm and a loss of enzyme activity. The addition of $K_3Fe(CN)_6$ to hepatic microsomes fully restored enzyme activity [8]. The formation of these types of complexes has been reported for several primary, secondary, and tertiary amines, which after being metabolized by P450, complexate the iron (II) [13, 14]. We were not able to detect the metabolic intermediate complex in the CYP3A4-expressing cells after incubation with tiamulin. This is most likely because these cell lines contain only a small amount of cytochrome P450 protein. This is reflected by the 6β-hydroxylation activity of testosterone. In CYP3A4-expressing cells, this activity is 4.47 pmol/10^6 cells/min, which is approximately 30 pmol/mg protein/min, whereas in pig hepatocytes this activity is approximately 200 pmol/mg protein/min [8].

In conclusion, our results illustrate that transgenic cell lines expressing cytochrome P450 enzymes provide a useful system for the study of enzyme inhibition, the role of individual P450 enzymes in biotransformation, and the effect of specific inhibitors on cytochrome P450-mediated mutagenicity. Our data confirm earlier findings that tiamulin is a potent specific inhibitor of CYP3A. Apart from its clinical relevance, the compound provides an interesting tool for further research.

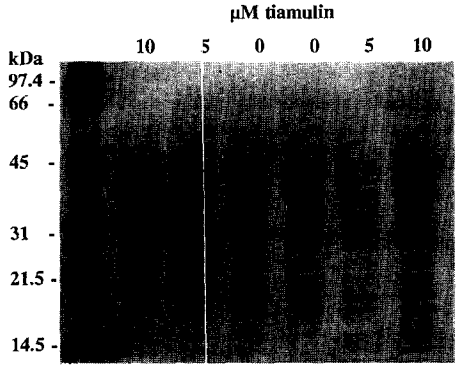

Fig. 1. Western blot of homogenates from 3T3-3A4 cells after incubation with tiamulin. Homogenates of 3T3-3A4 cells were blotted (10 μg of protein/lane) after incubation for 72 h with 0, 5, and 10 μM tiamulin. A monoclonal antibody against rat CYP3A1 was used for immunochemical staining.

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