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Differential drug-induced mRNA expression of human CYP3A4 compared to CYP3A5, CYP3A7 and CYP3A43

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Abstract

Drug-mediated regulation of mRNA expression of all members of the cytochrome P450 3A (CYP3A) subfamily has been measured by reverse transcription–polymerase chain reaction (RT-PCR) in the human hepatocellular carcinoma cell line, HepG2. Transcriptional regulation was proved by inhibition of induction with actinomycin D. Besides the positive control dexamethasone, the H^+/K^+-ATPase inhibitors omeprazole, lansoprazole, pantoprazole, and rabeprazole, and the herbal antidepressant St. John's wort (Hypericum extract) were studied. All CYP3A mRNAs were induced by dexamethasone. CYP3A4 was the only CYP3A isoform that was induced by all of the four benzimidazole derivatives, while CYP3A5, CYP3A7, and CYP3A43 were unaffected or even slightly downregulated by these drugs. St. John's wort also increased CYP3A4 mRNA exclusively, leaving CYP3A5 and CYP3A43 unaffected, whereas CYP3A7 was decreased. Depending on the inducer, expression of CYP3A4 is differently regulated from CYP3A5, CYP3A7, and CYP3A43.

Keywords: CYP3A (Cytochrome P450 3A); Omeprazole; Pantoprazole; St. John's wort; Induction of transcription; RT-PCR (reverse transcription–polymerase chain reaction)

1. Introduction

Cytochrome P450 3A (CYP3A) enzymes are involved in the metabolism of about 50% of all drugs, which makes them an intrinsic molecular target for drug–drug interactions. The three known isoforms of this subfamily, CYP3A4, CYP3A5, and CYP3A7, are expressed at different levels in liver, kidney, and gastrointestinal tract (Thummel and Wilkinson, 1998). CYP3A7 is the predominant variant occurring prenatally (Schuetz et al., 1994); CYP3A4 is the most abundant enzyme in adult liver, whereas CYP3A5 is mainly expressed in kidney (Haehner et al., 1996). Recently, a new CYP3A related gene has been identified, named CYP3A43 (Domanski et al., 2001; Gellner et al., 2001; Westlind et al., 2001). Low amounts of CYP3A43 mRNA have been detected in liver, kidney, pancreas, and prostate (Domanski et al., 2001).

Until recently, metabolic activity of hepatic CYP3A4 has not been distinguished from whole CYP3A activity, because CYP3A4 is the main liver cytochrome P450 in most individuals. However, new data published by Kuehl et al. (2001) implicate a more relevant participation of CYP3A5 in CYP3A-mediated drug metabolism. These authors found high expression of this enzyme in liver linked to a polymorphism in the CYP3A5 gene. Depending on that polymorphism, CYP3A5 ranges from undetectable to up to more than 50% of whole CYP3A liver enzyme in single persons (Kuehl et al., 2001).

Most of the substrates of CYP3A4 can also be metabolized by the other CYP3A enzymes, but comparative studies with recombinant enzymes revealed different metabolic capacities of CYP3A4, CYP3A5, and CYP3A7 to transform several substrates, concerning metabolites and substrate binding (Wrighton et al., 1990; Ohmori et al., 1998; Gibbs et al., 1999; Williams et al., 2002). For most but not all substrates, metabolic capability of CYP3A4 seems to be higher than of CYP3A5 and CYP3A7 (Williams et al., 2002).

Though 5′ flanking regions of CYP3A4 and CYP3A7 are 90% identical up to −8.8 kb (Bertilsson et al., 2001), constitutive expression of these genes varies during onto-

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genesis. Recently, a NFκB like element specific for the CYP3A7 promoter has been reported to convey constitutive expression of this gene in HepG2 cells (Saito et al., 2001). Binding sites of different transcription factors are involved in regulation of CYP3A5 expression (Iwano et al., 2001).

In contrast to constitutive expression, different inducibility of these genes has not yet been elucidated in detail. Induction of expression of CYP3A is dependent on activation of the pregnane X receptor. Specific receptor binding sites have been identified in the 5′ region of CYP3A4, CYP3A7, and CY3A5 genes (Goodwin et al., 1999; Pascussi et al., 1999; Schuetz et al., 1996). A great variety of structurally different substances have been shown to activate the pregnane X receptor and to induce expression of CYP3A mRNA and protein in cell culture (for review, see LeCluyse, 2001). However, due to high homology at the protein as well as DNA level, most studies do not discriminate between the CYP3A isoforms. Therefore, it is not known whether the four human CYP3A enzymes are co-induced by drugs or whether induction is restricted to CYP3A4.

In this study, we used HepG2 cells to compare inducibility of all human CYP3A forms, CYP3A4, CYP3A5, CYP3A7, and CYP3A43, by different groups of drugs, which have been reported to increase expression of CYP3A in primary hepatocytes. Drug-mediated induction of endogenous mRNA expression was measured by semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR).

2. Materials and methods

2.1. Cell culture and drug treatment

HepG2 cells (human hepatocellular carcinoma, German Collection of Microorganisms and Cell Cultures, DSMZ) were grown in Dulbecco's Modified Eagle Medium supplemented with 5% fetal bovine serum at 5% CO_2 and 37 °C. Cells were seeded in 6-well dishes and grown to confluence.

Solutions of the inducers omeprazole (Antra™, Astra), lansoprazole (Takeda), pantoprazole (Pantozol™, Byk Gulden), rabeprazole (Eisai), and dexamethasone (Sigma-Aldrich) in dimethylsulfoxide (DMSO, Sigma-Aldrich) were added to the culture medium at the concentrations indicated. Concentration of DMSO was 0.1% in all experiments. Two formulations of St. John's wort were used, Ariston™ (Steiner) and Esbericum™ (Schaper and Bruemmer). Content of 1 Esbericum capsule was extracted with 1 ml ethanol and cells were treated with a final concentration of 0.1% (v/v). Dexamethasone was used as positive control for induction of CYP3A mRNA (100%). Values are given relative to this control.

Several concentrations of each substance were applied to the cells in order to avoid interaction of specific CYP induction with unspecific stress-mediated effects on gene expression that usually precede toxicity. We observed that a slight rounding of the cells precedes decrease of growth rate, which is accompanied by a decrease of RNA content of the well that can be quantified photometrically. The highest concentration of each substance that did not result in morphological changes of the cell shape within 48 h was selected for the subsequent experiments. Expression of β-actin used as an internal control was not affected by drug treatment.

2.2. Harvest of cells and RNA preparation

Cells were washed twice with phosphate buffered saline, scraped from culture dish in lysis buffer (Qiagen) and homogenized by centrifugation through a Qiashredder (Qiagen). RNA preparation was performed using a Qiagen RNeasy Mini Kit according to the manufacturer's instructions. RNA concentration was determined photometrically (Uvicon photometer, Kontron), measuring absorbance at 260 nm (concentration), 280 nm (A260/A280 ratio), and 320 nm (background correction). RNA was immediately used for cDNA synthesis or stored at −80 °C.

2.3. Reverse transcription–PCR

Reverse transcription was performed using Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Life Technologies) and 2 μg of RNA in a 15 μl assay. After establishing suitable cycle numbers for each pair of primers to confirm linearity of the reaction, cDNA equivalents of

Table 1
Gene specific primers

Gene	Accession	Position	Sequence	Product (bp)
CYP3A4	M14096	121	GCC TGG TGC TCC TCT ATC TA	187
		307	GGC TGT TGA CCA TCA TAA AAG	
CYP3A5	J04813	290	ATG GAA AAA TGT GGG GAA CG	216
		505	CGC TGG TGA AGG TTG GAG AC	
CYP3A7	D00408	212	AAG TCT GGG GTA TTT ATG ACT	210
		421	CGC TGG TGA ATG TTG GAG AC	
CYP3A43	AF319634	31	ACA TGG GTT CTT GTG GCT AC	243
		273	TTT GAT CAT GTC GGG ATC CA	
β-actin	X00351	486	ACT GGC ATC GTG ATG GAC TC	425
		910	CGG ATG TCC ACG TCA CAC TT	

12.5, 25, and 50 ng RNA were submitted to PCR. After initial denaturation at 96 °C for 1 min, PCR was performed as follows: 94 °C/40 s, 50–54 °C/40 s (depending on the primers), 72 °C/50 s in a 20 μl assay, cycles as indicated, followed by an elongation reaction of 10 min/72 °C. PCR products were separated on 2% agarose gels. Gels were treated with ethidium bromide and quantified by UV fluorescence (BioDoc Analyze Biometra, software TINA (Raytest)). Values were normalized to β-actin, which was used as an internal control for the amounts of mRNA.

Because of high homology between CYP3A4, CYP3A7, CYP3A5, and also CYP3A43, specificity of primers and PCR products was analyzed carefully by restriction analysis and sequencing. The primers used in this study discriminate between CYP3A4, CYP3A5, CYP3A7, and CYP3A43 (Table 1).

3. Results

3.1. Induction of CYP3A mRNAs in HepG2 cells

Fig. 1 summarizes the effects of four benzimidazole derivatives, two hypericum extracts, and of dexamethasone as positive control (set 100% in all experiments), on mRNA levels of all four known CYP3A isoforms. As expected, all CYP3A mRNAs were significantly induced by dexamethasone. This increase was about 5 times for CYP3A4, 3 times for CYP3A43, 2 times for CYP3A7, and 1.5 times for CYP3A5.

CYP3A4 was induced by all benzimidazoles and by both hypericum extracts ($P<0.01$). CYP3A4 induction by omeprazole, lansoprazole, and pantoprazole reached about 50% of the dexamethasone level and was significantly lower than dexamethasone-mediated induction ($P<0.01$). After treatment of cells with rabeprazole and hypericum extracts, mRNA amounts did not differ from positive control (dexamethasone).

CYP3A5, CYP3A7, and CYP3A43 were not induced by benzimidazoles or hypericum extracts. While St. John's wort did not change mRNA levels of CYP3A5 and CYP3A43 as compared to the vehicle (ethanol), CYP3A7 was even downregulated. The mRNA amounts of CYP3A5, CYP3A7, and CYP3A43 were slightly lower in cells incubated with benzimidazoles rather than DMSO or untreated. This decrease was significant for CYP3A5 in cells treated with omeprazole ($P<0.05$), lansoprazole ($P<0.01$), or pantoprazole ($P<0.01$), and for CYP3A7 in cells treated with lansoprazole or pantoprazole ($P<0.05$).

3.2. Inhibition of CYP3A4 induction by actinomycin D

To confirm induction by de novo synthesis of mRNA, actinomycin D (10 μg/ml) was added to the medium 10 min prior to the inducers omeprazole, pantoprazole, or dexame-

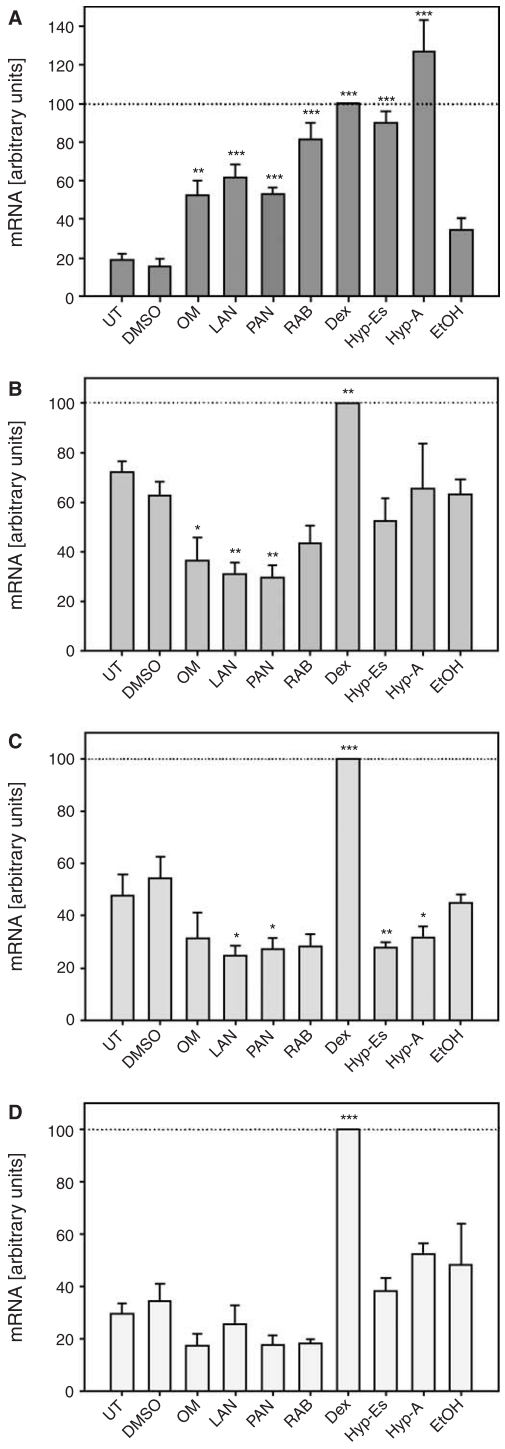

Fig. 1. Induction of CYP3A isoforms in HepG2 cells. HepG2 cells were untreated (UT), treated with vehicle (DMSO or ethanol (EtOH) for hypericum extracts), 100 μM omeprazole (OM), 50 μM lansoprazole (LAN), 200 μM pantoprazole (PAN), 25 μM rabeprazole (RAB), 20 μM dexamethasone (Dex, set 100%), or hypericum extracts (Hyp-Es: Esbericum, Hyp-A: Ariston) for 24 h. PCR conditions were: CYP3A4, 35 cycles; CYP3A5, 28 cycles; CYP3A7, 28 cycles; CYP3A43, 35 cycles; β-actin, 15 cycles. Results represent means and S.E.M. of four or five experiments. Marked columns are significantly different from vehicle control, determined by one-way ANOVA, $*P<0.05$, $**P<0.01$, $***P<0.001$. (A) CYP3A4, (B) CYP3A5, (C) CYP3A7, (D) CYP3A43.

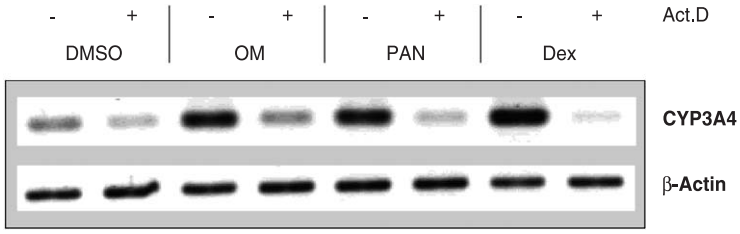

Fig. 2. Inhibition of CYP3A4 induction by actinomycin D. Pretreatment of HepG2 cells with 10 μg/ml actinomycin D or vehicle (DMSO) for 10 min was followed by addition of 100 μM omeprazole (OM), 200 μM pantoprazole (PAN), 20 μM dexamethasone (Dex), or vehicle (DMSO) and subsequent incubation of cells for 6 h. The 25 ng of total RNA was submitted to RT-PCR (CYP3A4, 35 cycles; actin, 17 cycles).

thasone. In all cases, induction of CYP3A4 mRNA synthesis was completely prevented by actinomycin D pretreatment (Fig. 2).

4. Discussion

CYP3A4 is the predominant CYP3A enzyme in human liver. However, as in other permanent cell lines, CYP3A4 is decreased in the hepatoma cell line HepG2, whereas the fetal CYP3A7 is increased in these cells. As a result of these changes in cell culture, the dominant CYP3A form in non-induced HepG2 cells is CYP3A7, followed by CYP3A5 and CYP3A4. Low amounts of CYP3A43 mRNA were detected in HepG2 cells (Fig. 1D).

Rifampicin and dexamethasone have been frequently used as typical inducers of CYP3A. Both inducers are dependent on pregnane X receptor (Lehmann et al., 1998). Nevertheless, the time course of rifampicin-induced CYP3A mRNA accumulation is different from what would be expected for direct pregnane X receptor-mediated transcription, because increase of CYP3A mRNA occurs only after 48 h (Sumida et al., 1999b). There had been some conflicting reports about the mechanism of CYP3A induction by dexamethasone, particularly about a possible involvement of the glucocorticoid receptor. Recently, Pascussi et al. (2001) provided evidence that the glucocorticoid receptor is involved only in induction mediated by low dexamethasone concentrations (nM). Concentrations of dexamethasone higher than 10 μM induce transcription of CYP3A4 directly by activating the pregnane X receptor. Therefore, we decided to include 20 μM dexamethasone as a positive control.

We observed a different regulation of CYP3A expression by benzimidazoles and dexamethasone. Levels of all four CYP3A mRNAs were upregulated by dexamethasone, although to a different extent, whereas only CYP3A4 was induced by all four benzimidazoles tested. Unexpectedly, pantoprazole, which is different from the other benzimidazoles in that it failed to induce CYP1 (Krusekopf et al., 1997, in press), corresponded to omeprazole, lansoprazole, and rabeprazole concerning induction of CYP3A4. CYP3A5, CYP3A7, and CYP3A43 were even slightly (not always significantly) downregulated by the benzimidazole derivatives.

St. John's wort has been reported to induce CYP3A (probably CYP3A4) in primary human hepatocytes (Moore et al., 2000). This is in agreement with our results obtained for HepG2 cells. Both of the St. John's wort extracts induced CYP3A4 mRNA only. CYP3A5 and CYP3A43 remained unchanged, while CYP3A7 mRNA was significantly downregulated. Regulation of the CYP3A mRNAs by the hypericum extracts was similar to benzimidazoles and different from dexamethasone.

Induction of CYP3A4 mRNA by omeprazole, pantoprazole and dexamethasone was completely inhibited by actinomycin D. This result confirms de novo synthesis of mRNA as the mechanism of induction, rather than post-transcriptional mechanisms like RNA stabilization. Transcriptional regulation of CYP3A4 has been supported by reporter gene assays (Ogg et al., 1999) and also by comparison of mRNA content and enzyme activity (testosterone 6β-hydroxylation) of hepatic CYP3A4 (Sumida et al., 1999a; Rodriguez-Antona et al., 2001; Sy et al., 2002). Therefore, measurement of mRNA has proved a suitable way of determining induction of this enzyme.

Dexamethasone-mediated induction of CYP3A in HepG2 cells had been reported previously (Sumida et al., 1999b). But, contrary to results obtained in primary human hepatocytes (Curi-Pedrosa et al., 1994), Sumida et al. (1999b) did not detect induction of CYP3A by omeprazole, having used primers not discriminating between CYP3A isoforms. The data presented here provide an explanation for this apparently different regulation in primary and permanent hepatocytes. Since CYP3A4 is the only CYP3A enzyme induced by omeprazole (Fig. 1), experimental procedures not discriminating between the different CYP3A isoforms, e.g. RT-PCR with unspecific primers, Northern blots with probes from the coding region, Western blots with non isoenzyme-specific antibodies or activity tests, will have a different outcome in primary and permanent hepatocytes, due to the different amounts of the single CYP3A isoforms. While induction of the CYP3A subfamily by omeprazole occurs in most primary hepatocytes because of the high proportion of CYP3A4 in these cells, this effect is not observed in HepG2 cells, because the increase of CYP3A4 in these cells is concealed by downregulation of the other more abundant CYP3A isoforms.

There are considerable individual differences concerning the level of expression of the distinct CYP3A isoforms in human liver. Especially CYP3A5 ranges from undetectable

to up to more than 50% of whole CYP3A liver enzyme in single persons (Kuehl et al., 2001). The different regulation of expression of CYP3A4 and CYP3A5 by benzimidazoles (Fig. 1A and 1B) provides a possible explanation for the failure of benzimidazole derivatives to induce CYP3A in single subjects or primary hepatocytes, although CYP3A could be induced by other substances (rifampicin or dexamethasone) in these subjects or cells (Masubuchi and Okazaki, 1998). The data presented here propose a contrary influence of benzimidazoles and hypericum extracts on expression of CYP3A4 on the one hand and of CYP3A5, CYP3A7, and CYP3A43 on the other. As a consequence, the influence of benzimidazoles and possibly other drugs on the level of total liver CYP3A in a single person could depend on the ratio of expression of CYP3A4, CYP3A5, CYP3A7, and CYP3A43 in that person.

To our knowledge, this is the first study showing in detail different transcriptional regulation of the four human CYP3A isoforms by two groups of drugs, benzimidazole derivatives and hypericum extracts. Treatment of HepG2 cells with these drugs increased only CYP3A4 mRNA, leaving CYP3A5, CYP3A7, and CYP3A43 unchanged or slightly downregulated. This distinct diversity between regulation of CYP3A4 and the other CYP3A mRNAs did not occur following treatment with dexamethasone.

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