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DP7, a novel dihydropyridine multidrug resistance reverter, shows only weak inhibitory activity on human CYP3A enzyme(s)

Paolo D'Elia [a], Francesco De Matteis [b], Stefania Dragoni [a], Anamik Shah [c], Giampietro Sgaragli [a], Massimo Valoti [a,*]

[a] Department of Neurosciences, University of Siena, via A. Moro 2, 53100, Siena, Italy
[b] Medical Research Council Bioanalytical Group, School of Biological and Chemical Sciences, Birkbeck College, University of London, Malet Street, London WC1 7HX, London, UK
[c] Department of Chemistry, Saurashtra University, Rajkot 360 005, India

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ABSTRACT

The aim of this study was to investigate the effects of 3,5-dibenzoyl-4-(3-phenoxy-phenyl)-1,4-dihydro-2,6-dimethylpyridine (DP7), a novel multidrug resistance (MDR) reverter, on cytochrome P450 (CYP)-activities by human and rat liver microsomes. Effects of DP7 were assessed with use of selective substrates, markers of CYP activities. With rat microsomes, ethoxyresorufin (ETR) was used as substrate for CYP1A1, penthoxyresorufin (PTR) for 2B, benzyloxyresorufin (BZR) for 1A1/2, 2B, 2C, 3A. CYP3A enzyme activities of rat (3A2) and human (3A4) liver microsomes, were assessed fluorimetrically using either 7-benzyloxy-quinoline (BQ) or [3-[3(3,4-difluorobenzyl)oxy]-5,5-dimethyl-4-[4-(methylsulfonyl)-phenyl]furan-2-(5H)-one] (DFB). When rat microsomes were incubated with DP7, concentration–inhibition curves were obtained. DP7 inhibitions gave IC_{50} values of 3.8 μM for PTR, 3.8 μM for ETR and 10.4 μM for BZR and were not competitive in nature; moreover, they were reversible. When BQ was used as substrate of rat microsomes, DP7 inhibited its oxidation with an IC_{50} value of 4.17 μM, while this oxidation was inhibited by only 25% at the highest DP7 concentration used (75 μM) with human microsomes. On the contrary, when DFB was used as substrate, DP7 showed identical IC_{50} values (34.67 μM) with microsomal preparations from either species. The moderate inhibition of CYP isoforms of rat liver microsomes and the weak inhibition of human CYP3A4 enzyme activity operated by DP7, suggest that DP7 in man should not give rise to important, unpredictable pharmacokinetic interactions. This conclusion supports the role of this compound as a lead for the development of novel MDR reverterting dihydropyridines of therapeutic interest.

1. Introduction

The membrane efflux protein P-glycoprotein, a member of the ATP binding cassette (ABC) family, recognizes and transports structurally, chemically, and pharmacologically diverse hydrophobic compounds, giving rise to a phenomenon known as multidrug resistance (MDR). MDR is one of the major reasons of the failure of chemotherapy in cancer (Ambudkar et al., 2003), as many cytotoxic drugs used in the cancer chemotherapy are substrates of P-glycoprotein. Because of the clinical importance of P-glycoprotein, various compounds have been tested for their effectiveness to block P-glycoprotein function in vitro. P-glycoprotein inhibitors belong to a number of chemical classes including Ca^{2+} channel blockers, calmodulin inhibitors, coronary vasodilators, indole alkaloids, quinolines, hormones, cyclosporines, surfactants, and antibodies (McDevitt and Callaghan, 2007; Ford and Hait, 1993). These compounds are known as MDR reverters.

Many of the cytotoxic agents are substrates/inhibitors both for P-glycoprotein and the cytochrome P-450 (CYP) isoenzyme 3A4. CYP's enzymes are the principal enzymes involved in the metabolism of drugs and xenobiotics. Modulation of the activity of CYPs by a given drug is a crucial issue for the assessment of safety and efficacy of that drug. In particular the inhibition of CYP enzymes can increase systemic exposure, thereby causing severe toxic effects of the drug or of another concomitantly given medication that is metabolized by the same CYP(s) (Ludwig et al., 1999). The competition between cytotoxic agents and these P-glycoprotein modulators for CYP3A4 activity has resulted in unpredictable pharmacokinetic interactions. The ideal MDR inhibitor should, at a first instance, inhibit P-glycoprotein leaving unaltered CYP-dependent enzymes (Fusi et al., 2006).

Very recently, 3,5-Dibenzoyl-4-(3-phenoxyphenyl)-1,4-dihydro-2,6-dimethylpyridine (DP7) was proposed as a new MDR reverter. It was found that DP7, at a concentration two orders of magnitude higher than its IC_{50} as a P-glycoprotein inhibitor, was devoid of cardiovascular

* Corresponding author. Tel.: +39 0577 234440; fax: +39 0577 234446.
E-mail address: valoti@unisi.it (M. Valoti).

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effects in in vitro rat preparations (Saponara et al., 2004, 2007). These data were not considered sufficient evidence of DP7 safety before this drug could be subjected to clinical investigation. Several 1,4 dihydropyridine calcium antagonists are substrate of CYP enzymes. In particular, CYP3A4 metabolizes dihydropyridines into the corresponding pyridines (Guengerich et al., 1991). The dihydropyridine can also inhibit the activity of CYP3A4. For example, Katoh et al. (2000) reported that nicardipine, benidipine, maidipine and barnidipine strongly inhibited human CYP3A4 activity, while nivaldipine, nifedipine, nitrendepine and amlodipine exhibited a weak inhibition.

The present study was performed to test the in vitro interactions between DP7 and CYP450 isoforms both in human and rat liver microsomes, in order to elucidate whether the inhibitory activity of DP7 is confined to P-glycoprotein or also affects CYP enzymes.

2. Materials and methods

2.1. Materials

NADP and NADPH, D-glucose-6-phospate, glucose-6-phosphate dehydrogenase, benzyloxyresorufin (BZR), ethoxyresorufin (ETR), pentoxyresorufin (PTR), resorufin, 7-benzyloxy-quinoline (BQ), oxidized glutatione, glutatione reductase, tris[hydroxymethyl]aminomethane (TRIZMA-base) were all purchased from Sigma-Aldrich (Milan, Italy). [3-[(3,4-difluorobenzyl)oxy]-5,5-dimethyl-4-[4-(methylsulfonyl)phenyl]furan-2(5H)-one] (DFB) and [3-hydroxy-5,5-dimethyl-4-[4-(methylsulfonyl)phenyl]furan-2(5H)-one] (DFH) were kindly provided by Merck Frosst Canada L.T.D. (Pointe Claire, Quebec). 3,5-dibenzoyl-4-(3-phenoxy-phenyl)-1,4-dihydro-2,6-dimethylpyridine (DP7) was synthetized as described elsewhere (Kawase et al., 2002).

All other chemicals were of the highest purity available and were purchased from commercial sources.

2.2. Rat and human liver microsomes

The animal protocols used were reviewed and approved by the Animal Care and Ethics Committee of the Università degli Studi di Siena, Italy.

Male Sprague–Dawley rats (weight 250–350 g) were anesthetized with a mixture of 0.3 mg/kg Ketavet® (Gellini, Italy) and 0.08 mg/kg Rampum® (Bayer, Germany) and then sacrificed. Livers were perfused with physiological saline (0.9% NaCl, w/v) and homogenized in 0.154 M KCl using a glass/teflon, Potter–Elvehjem homogenizer. The homogenate was then centrifuged at 10,000 *g* for 30 min and the post-mitochondrial supernatant decanted and centrifuged at 105,000 *g* for 1 h using a Beckman preparative OPTIMA™ LE-70 ultracentrifuge. The pellet, containing the microsomal fraction, was suspended in 2 ml of Tris–HCl buffer (10 mM, pH 7.6) containing 0.154 M KCl and 1 mM EDTA. Aliquots of equal volume of this suspension were stored at −80 °C.

Pooled human liver microsomes were obtained from BD Biosciences (Woburn, MA, USA).

2.3. Tissue protein concentration

The protein content of rat liver microsomes was determined according to Lowry et al. (1951) using serum albumin as a standard. The protein concentration of the pooled human liver microsomal fraction was 20 mg/ml.

2.4. Alkoxyresorufin assay for determination of inhibition of human and rat liver microsomes CYP-activity

Pentoxyresorufin (PTR), ethoxyresorufin (ETR) and benzyloxyresorufin (BZR) are O-dealkylated to the fluorescent compound resorufin, sometimes selectively, by different CYP isoforms, thus becoming selective substrate markers: ETR for CYP1A, PTR for 2B family both in human and rat liver microsomal preparations, and BZR for 1A, 2B, 2C and 3A families in rat while CYP2B6 and partially 3A4 are the isozymes preferentially involved in the BZR metabolism by human preparations (Niwa et al., 2003; Donato et al., 2004; Abass et al., 2007; Chovan et al., 2007).

The incubation mixture (total volume 1300 µl) contained the following components (final concentration): Tris–HCl, pH 7.6 (50 mM), $MgCl_2$ (25 mM), human or rat liver microsomes (0.5 mg protein/ml), NADPH (0.215 mM) and probe substrate [ETR (2 µM), PTR (10 µM) or BZR (5 µM)]. DP7 was added at various concentrations (ranging from 0.5 to 75 µM) dissolved, as the probe substrate, in DMSO. The concentration of DMSO in the incubation mixture never exceeded 1%. At this concentration, DMSO alone had no effect on ETR-O-dealkylase (EROD), PTR-O-dealkylase (PROD) and BZR-O-dealkylase (BROD) activities. Reactions were started by adding NADPH and after 10 min of incubation at 37 °C, the amount of resorufin formed was measured by means of a Shimadzu RF-5000 Spectrofluorophotometer, using wavelengths of 522 nm and 586 nm for excitation and emission, respectively. The amount of fluorescence detected was referred to a standard curve made with pure resorufin (0–0.1 µM) dissolved in Tris–HCl/$MgCl_2$ buffer).

2.5. Determination of BQ-dealkylase activity

Assay of CYP3A4 activity in human and CYP3A2 in rat microsomal preparations with BQ as a substrate was based on a previously published method (Stresser et al., 2002) with minor modifications. Human and rat liver microsomes (final microsomal protein concentration 0.5 mg/ml) were suspended in potassium phosphate buffer 200 mM pH 7.4 containing an NADPH-regenerating system ($NADPH^+$ 1 mM, D-glucose-6-phoshate 4 mM, 4 U/ml D-glucose-6-phospate-dehydrogenase in $MgCl_2$ 48 mM). Various concentrations of DP7 (0.5–75 µM) were added to the reaction mixture together with the substrate BQ (the latter at 40 µM final concentration). The final volume of the incubation mixtures was 800 µl. DP7 and BQ were dissolved in DMSO and acetonitrile, respectively. The final concentration of both solvents was kept below 1% and no effects on BQ-O-dealkylase activity was noted. The samples were incubated for 20 min at 37 °C and the reaction was terminated by adding 500 µl of a mixture of acetonitrile and 0.5 M Tris base (80:20 v/v). Fluorescence was measured at $E_x\lambda$: 410 nm and $E_m\lambda$: 538 nm for emission and excitation, respectively.

2.6. Determination of DFB-dealkylase activity

Assay of CYP3A activity with DFB was based on a previously published method (Chauret et al., 1999) with minor modifications. DFB is O-dealkylated by CYP3A2 (rat) and CYP3A4 (human) isoforms to 3-hydroxy-5,5-dimethyl-4-[4-(methylsulfonyl)phenyl]furan-2(5H)-one (DFH). Human and rat liver microsomes were suspended (at a final protein concentration of 0.2 mg/ml) in 125 mM phosphate buffer, pH 7.4. Various concentrations of DP7 (1–90 µM) and the substrate DFB (40 µM final concentration, from 2.5 mM acetonitrile stock solution) were then added to the samples. The reaction mixture was pre-incubated for 5 min at 37 °C. The reaction was then started by adding NADPH (0.215 mM). The final volume of the samples was 500 µl. After incubation at 37 °C for 15 min, oxidized glutathione (10 µl of a 100 mM water solution) and glutathione reductase (10 µl of a 200 U/ml solution in phosphate buffer) were added and the sample was left in the dark at room temperature for 15 min. Finally, the reaction was quenched with 480 µl of 60% acetonitrile in Tris buffer (0.05 M, pH 10) and the fluorescence was read ($E_x\lambda$: 360 nm, $E_m\lambda$: 460 nm).

The production of DFH was quantified by referring to a standard curve made with pure DFH (0–10 µM in 50% Tris–acetonitrile in phosphate buffer).

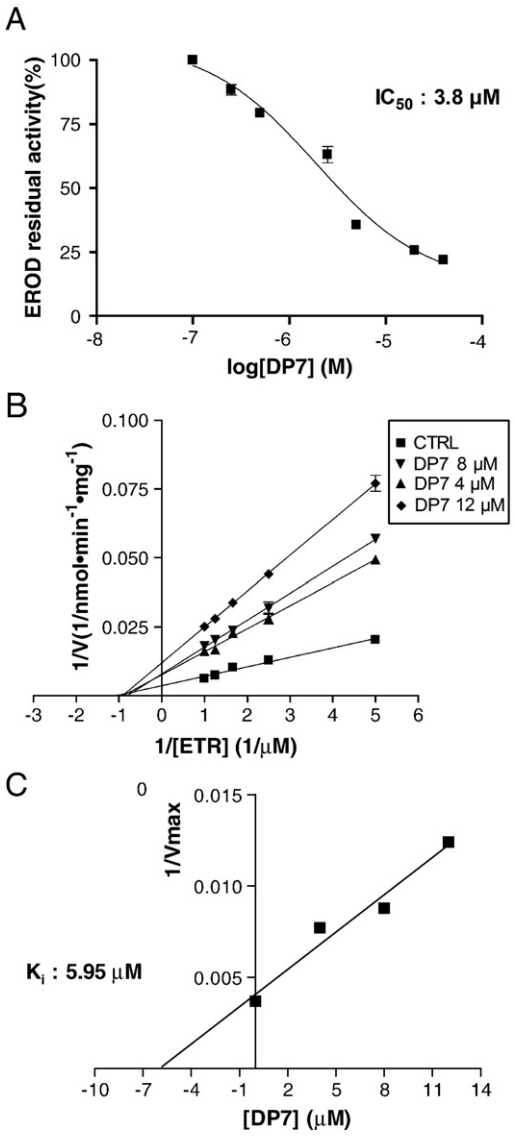

Fig. 1. Effects of DP7 on rat liver EROD activity (CYP1A): A. Inhibition–concentration curve. B. Lineweaver–Burk plot of DP7 inhibition on EROD activity in rat liver microsomes. C. Secondary plot of reciprocals of Vmax, taken from Lineweaver–Burk plot, vs DP7 concentrations. Incubation conditions are described in the Materials and methods section. Each data point represents the mean ± S.E.M. derived from at least 3 different experiments.

2.7. Kinetic analysis of inhibition by DP7 of alkoxyresorufin-O-dealkylase activity

Kinetic analysis was performed using the above described reaction mixture and information on type of inhibition and value of K_i were obtained by varying the concentrations of probe substrates (1–5 µM ETR 0.2–2 µM PTR or 1–2.5 µM BZR) and those of DP7 (0–15 µM). A double-reciprocal (Lineweaver–Burk) plot of activity versus probe substrate concentration was then drawn.

To examine whether DP7 inhibited CYPs in a time-dependent manner, rat liver microsomes were preincubated alone or in presence of NADPH generating system with DP7 (at a concentration close to the IC_{50} value) for various periods of time (0, 10, 15 and 20 min). The reaction was then started by the addition of the appropriate substrate.

In order to assess the reversibility of the inhibition rat microsomes (5 mg protein/ml) were incubated in presence of DP7 (at a concentration two times the IC_{50} value for the enzyme activity under assay) and NADPH. After 15 min samples were diluted ten folds with buffer and the enzyme activity was assayed by adding the substrate. The decrease of the concentration of a reversible inhibitor was expected to give rise to a decrease of inhibition, whereas no restoration of activity will occur for an irreversible inhibitor (Tipton, 2001).

2.8. Data analysis

Data are reported as means ± S.E.M. of the number of different experiments indicated in parentheses. Analysis of data was accomplished using GraphPad Prism version 4.03 (GraphPad Software, U.S.A.). Statistical analyses and significance as measured by ANOVA (followed by Dunnet's post test) or by One-sample *T* test, were made by means of GraphPad InStat version 3.06 (GraphPad Software, U.S.A.) as appropriate. In all comparisons, $P<0.05$ was considered significant.

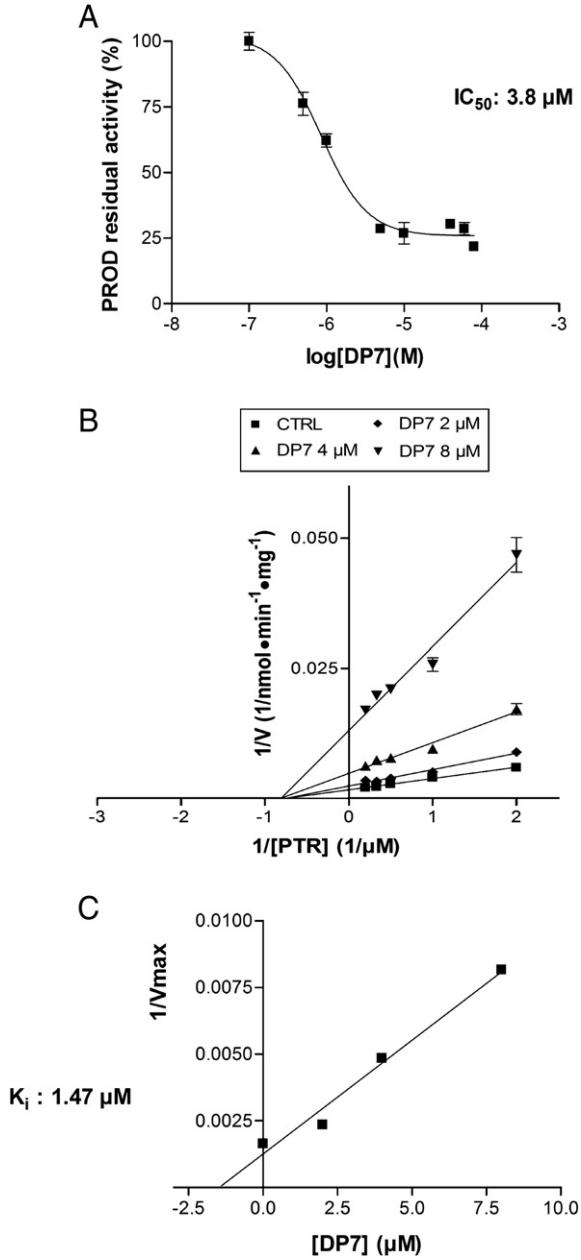

Fig. 2. Effects of DP7 on rat liver PROD activity (CYP2B): A. Inhibition–concentration curve. B. Lineweaver–Burk plot of DP7 inhibition on PROD activity in rat liver microsomes. C. Secondary plot of reciprocals of Vmax, taken from Lineweaver–Burk plot, vs DP7 concentrations. Incubation conditions are described in the Materials and methods section. Each data point represents the mean ± S.E.M. derived from at least 3 different experiments.

3. Results

The analysis of DP7 inhibition was first performed on rat liver microsomes using marker substrates (alkoxyresorufins) of various CYP isoforms. DP7 inhibited metabolism of each alkoxyresorufin investigated. A kinetic analysis was also performed to determine the type of inhibition mechanism and to calculate the corresponding K_i.

As shown in Fig. 1A, DP7 inhibited rat liver EROD activity in a concentration-dependent manner with an IC_{50} value of 3.8 μM. A Lineweaver–Burk plot (Fig. 1B) suggested the inhibition to be of a noncompetitive type, with a K_i value of 5.95 μM, calculated by plotting the reciprocals of the Vmax values, obtained from the Lineweaver–Burk plot, versus DP7 concentrations (Fig. 1C).

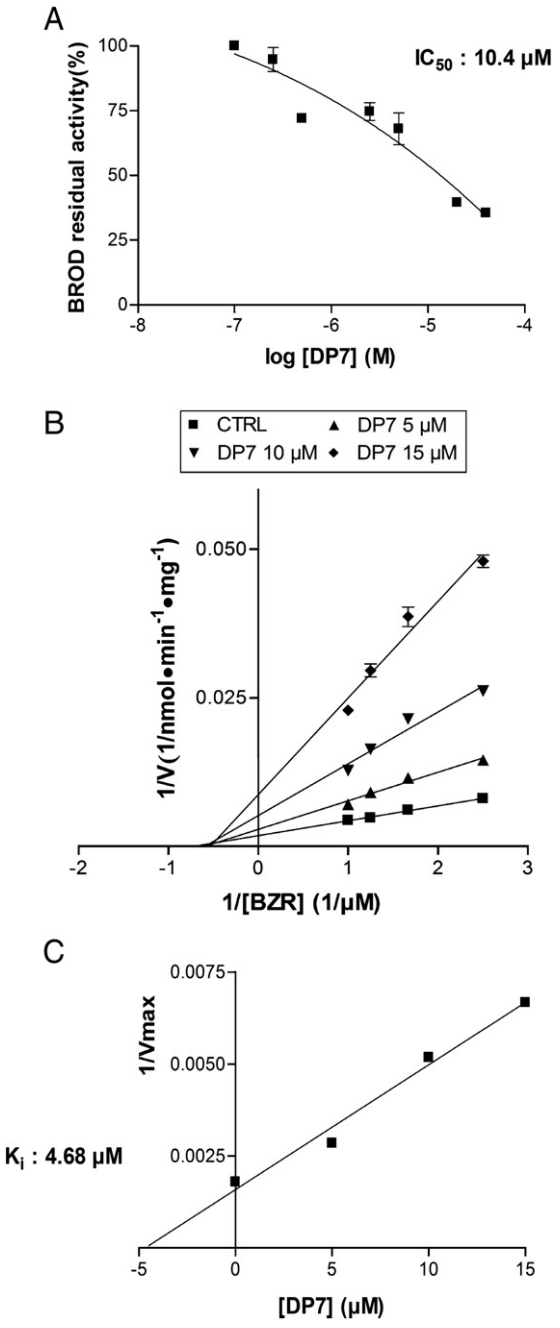

Fig. 3. Effects of DP7 on rat liver BROD activity (CYP1A-2B-2C-3A): A. Inhibition–concentration curve. B. Lineweaver–Burk plot of DP7 inhibition on BROD activity in rat liver microsomes. C. Secondary plot of reciprocals of Vmax, taken from Lineweaver–Burk plot, vs DP7 concentrations. Incubation conditions are described in the Materials and methods section. Each data point represents the mean ± S.E.M. derived from at least 3 different experiments.

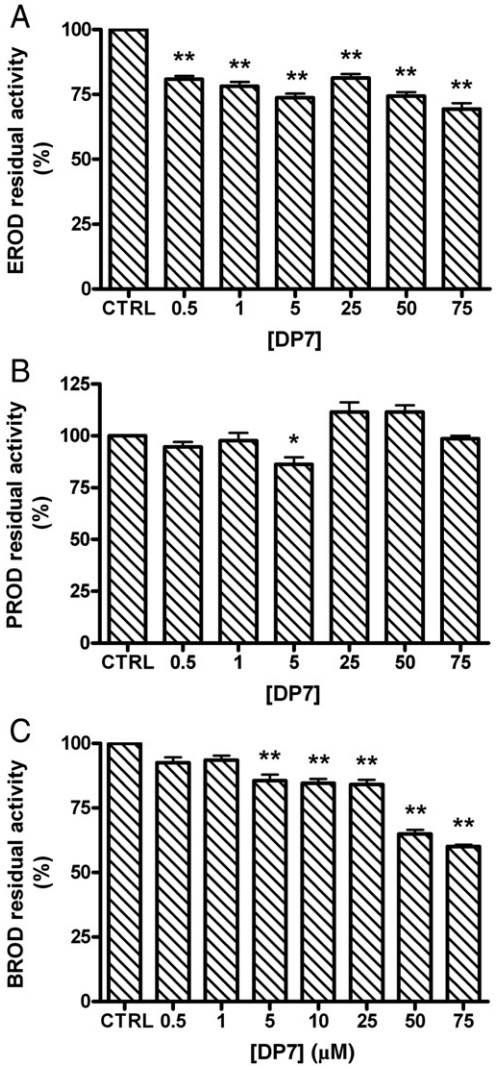

Fig. 4. Effects of DP7 on EROD (A), PROD (B) and BROD (C) activities in human live microsomal preparations. Incubation conditions are described in the Materials and methods section. Each data point represents the mean ± S.E.M. derived from at least 3 different experiments. Statistical analysis was performed by using One-sample *t*-test $^{*}P<0.05$, $^{**}P<0.01$ vs CTRL.

As shown in Fig. 2B, DP7 inhibited rat liver PROD activity in a concentration-dependent manner. The concentration needed for 50% inhibition was 3.8 μM (Fig. 2A). The Lineweaver–Burk plot (Fig. 2B) suggested a non competitive type of inhibition. The apparent K_i value was 1.47 μM (Fig. 2C).

Rat liver BROD activity was inhibited by DP7 in a concentration-dependent manner with an IC_{50} value of 10.4 μM (Fig. 3A). Lineweaver–Burk plot (Fig. 3B) suggested a noncompetitive type of inhibition, and K_i was 4.68 μM (Fig. 3C.).

In a second set of experiments, rat liver microsomes were pre-incubated with DP7, at IC_{50} concentrations, in presence or absence of NADPH at different times up to 20 min; after this pre-incubation, alkoxyresorufin substrate was added and activity measured up to 10 min. In all experiments, DP7 inhibition of PROD, EROD and BROD activity did not result to be dependent on pre-incubation time (data not shown).

DP7 reversible inhibition of resorufin substrates metabolism was assessed by dilution experiments. Reaction mixtures (containing microsomes, DP7, NADPH and various substrates) were pre-incubated for

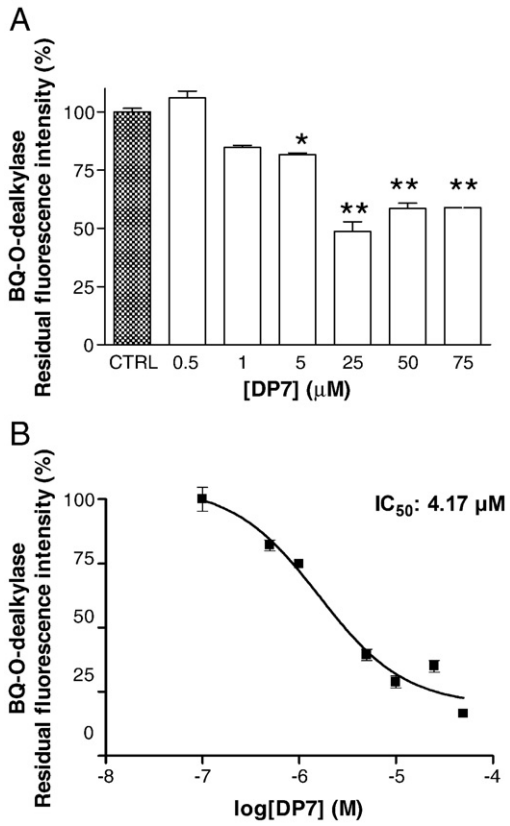

Fig. 5. Effects of DP7 on BQ-O-dealkylase activity (CYP3A) in human (A) and rat (B) liver microsomes. Incubation conditions are described in the Materials and methods sections. Each data point represents the mean ± S.E.M. derived from at least 3 different experiments. Statistical analysis was performed by using ANOVA (followed by Dunnet's post test): *$P<0.05$, **$P<0.01$ vs CTRL.

15 min and than diluted 10 folds. The residual resorufin-oxydase activities observed exhibited values close to those of control conditions, no DP7 added (Fig. 7), suggesting that all inhibitions were reversible.

To evaluate potential inhibition of DP7 on human CYP isoforms, alkoxyresorufin-O-dealkylase activities were also evaluated in human liver microsomal preparations. As shown in Fig. 4, at the highest DP7 concentration used the inhibition of EROD was about 30%, while no inhibition of PROD activity was recorded and the BZR metabolism was inhibited of about 40%.

CYP 3A is a very important enzyme in clinical practice as it is thought to be responsible for the metabolic inactivation of more than half of the drugs currently employed in human therapy (Zhou, 2008). Even though BROD is a marker of CYP3A-dependent metabolism, other CYP enzymes are implicated. In order to assess the potential inhibitory activity of DP7 on CYP3A, two additional substrates were employed which are considered to be selective for this subfamily.

BQ-O-dealkylase activity is marker of human (CYP3A4) and rat (CYP3A2) liver activity. In Fig. 5A the effect of DP7 on BQ-O-dealkylase activity in human liver microsomes is shown. At the highest DP7 concentration used (75 μM), this activity was inhibited only by 25%. When BQ was used as marker substrate in rat liver microsomes, DP7 inhibited its oxidation with an IC_{50} value of 4.17 μM (Fig. 5B). This inhibition was not time- or NADPH-dependent, thus suggesting that DP7 is not a mechanism-based inhibitor of either rat or human liver CYP3A isoform (data not shown).

As shown in Fig. 6A and B, DFB-O-dealkylase activity, another marker of the liver CYP3A family, was inhibited by DP7 in a concentration-dependent manner with IC_{50} values (34.67 μM) which were comparable for either microsomal preparations.

4. Discussion

Drug–drug interactions are a major clinical problem. The ability of drugs to act as inducers, inhibitors, or substrates for CYP3A is predictive of whether concurrent administration of these compounds with a CYP3A substrate might lead to altered drug efficacy or toxicity.

The activity of CYP group of enzymes or even of a single CYP can determine a patient's response to drug therapy. Therefore, modulation of the activity of CYPs by a given drug is a critical issue for the assessment of safety and efficacy of a drug. In particular, inhibition of CYP can increase systemic exposure, thereby causing severe toxic effects of the drug or another concomitantly given medicine that is metabolized by the same CYP(s), thus undergoing inhibition of its own metabolism (Jurima-Romet et al., 1994; Wandel et al., 1998).

P-glycoprotein inhibitors—especially those belonging to the so-called second generation of MDR reverters—have usually also effects on CYP enzymes and co-administration with cytotoxic drugs can induce drug–drug interactions and may result in serious clinical consequences.

DP7, a 1,4 dihydropyridine derivative, is a novel potent MDR inhibitor (Saponara et al., 2004). However, as dihydropyridine derivative, this compound was expected to be active also on the cardiovascular system. Previous results have, however, shown that DP7 was without cardiovascular effects at concentrations at which it was active as a P-glycoprotein inhibitor (10^{-7} M) (Saponara et al., 2004, 2007). Before proceeding to a clinical investigation of DP7, it was also necessary to exclude a strong inhibitory activity of this compound on the CYP drug-metabolizing system. The present study has investigated the effects of DP7 on rat and human liver CYP isoforms. Using microsomal preparations the inhibitory effect of DP7 on CYP enzymes has been tested in vitro. We have used fluorescent probe substrates and measured CYP inhibition over a range of DP7 concentrations. In rat liver microsomal preparations DP7 inhibited the metabolism of O-alkylated resorufins in a concentration-dependent manner, with IC_{50} values of 3.8 μM for EROD, 3.8 μM for PROD and 10.4 μM for BROD, respectively.

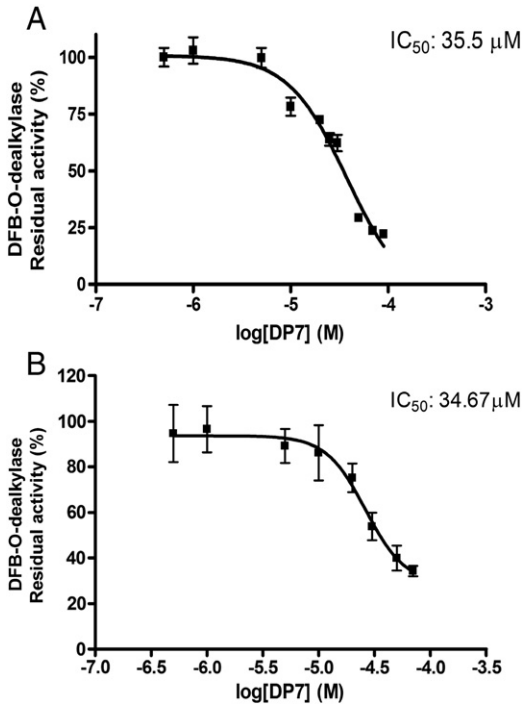

Fig. 6. Inhibitory effect of DP7 on DFB-O-dealkylase activity in human (A) and rat (B) liver microsomes. Incubation conditions are described in the Materials and methods section. Each data point represents the mean ± S.E.M. derived from at least 3 different experiments.

Lineweaver–Burk plots of double reciprocals of activity vs. concentration of substrate showed a non competitive type of inhibition for all three substrates, with K_i values of 5.95 μM for EROD, 1.47 μM for PROD and 4.58 μM for BROD, thus posing DP7 in the group of CYP inhibitors with intermediate potency. On the contrary the alkoxyresorufin-O-dealkylase activities were slightly inhibited by DP7 in human liver microsomal preparations. This difference is explained by the different distribution in the CYP isoenzymes and CYP affinity towards these substrates by the two mammalian species, as observed in several studies (Bogaards et al., 2000; Stresser et al., 2000; Chovan et al., 2007).

A large number of xenobiotic compounds have been shown to undergo metabolic activation by the cytochrome P450 enzymes to form biologically reactive intermediates that may, in turn, target the P450 for inactivation. These compounds were defined as suicide or mechanism-based inhibitors (Massey et al., 1970; Rando, 1984). Mechanism-based inhibitors can be broadly classified as substrates for the P450 that, in the process of metabolism by the enzyme, are converted to reactive intermediates, which then irreversibly inactivate the enzyme without leaving the active site (Silverman, 1995). Previous work has shown some dihydropyridines to inhibit CYP enzymes by a suicidal mechanism, whereby the enzyme activity is irreversibly lost, either because a fragment of the dihydropyridine becomes attached to one of the pyrrole nitrogen atoms of the haem prosthetic group, or through alkylation of the CYP protein moiety (De Matteis et al., 1982; McCluskey et al., 1986, 1992; Ortiz de Montellano, 1995). A mechanism of this type would lead to an irreversible inhibition characterized both by NADPH- and time-dependence. In contrast we have found the inhibition caused by DP7 on the rat CYP system to be reversible and independent of preincubation time and of NADPH addition, as summarized in Table 1.

The interaction between DP7 and CYP3A isoforms was then examined, both in human (CYP3A4) and rat (CYP3A2) liver microsomal preparations. The CYP3A substrates used were BQ and DFB.

When the effect of DP7 on DFB-O-dealkylase activity was measured, a concentration-dependent inhibition was found both in human and rat preparations. The two values of IC_{50} were similar (35 μM).

When BQ was used as substrate, DP7 inhibited rat CYP3A2 isoform in a concentration-dependent manner with a value of IC_{50} of 4.17 μM. In contrast, with the human CYP3A4 isoform, a very slight inhibition was only found at the highest concentration tested (75 μM), and it was not possible to calculate the IC_{50} value.

We conclude that DP7 inhibits different human and rat CYP isoforms, but the inhibition is reversible and not of a mechanism-based type. By using selective substrates of CYP 3A enzymes, an inhibition of CYP enzymes of this subfamily could also be documented both in rat and human preparations, but the inhibitory effect was found to be much less marked on human CYPs than on rat CYP3A2, as shown—in the case of the former enzyme—by the higher IC_{50} value or by a slight inhibition only seen at very high DP7 concentrations.

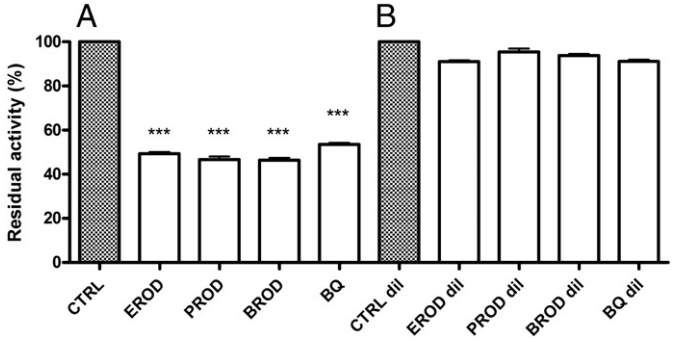

Fig. 7. Reversibility of DP7 inhibition of dealkylase activities on rat liver microsomes. A: Residual activity values in concentrated microsomes (DP7 was used at 2 times IC_{50} concentration) B: Residual activity values in 10 times diluted microsomes after preincubation in presence of DP7 and NADPH. Incubation conditions are described in the Materials and methods section. Each data point represents the mean ± S.E.M. derived from at least 3 different experiments. Statistical analysis was performed by using One-sample *t*-test: ***$P<0.001$ vs the corresponding CTRL.

Table 1
Inhibition parameters of DP7 on rat alkoxyresorufin-O-dealkylase activities.

Activity	IC_{50} (μM)	K_i (μM)	Inhibition type	Time-dependence	NADPH-dependence	Reversibility
EROD	3.8	5.95	Non competitive	No	No	Yes
PROD	3.8	1.47	Non competitive	No	No	Yes
BROD	10.4	4.58	Non competitive	No	No	Yes

Katoh et al. (2000) showed that 1,4 dihydropyridine calcium antagonists have different inhibitory potencies and selectivities on human CYP activities. Particularly, nifedipine, benidipine and barnidipine exhibited strong inhibition towards many CYP activities. The interactions of nicardipine and other dihydropyridines that are metabolised by CYP3A4 in humans should be considered to have important clinical implications. These dihydropyridines strongly inhibit human CYP3A4: the K_i values of nicardipine, benidipine and barnidipine are 0.06, 0.88 and 0.20 μM, respectively. The inhibition by DP7 of CYP3A4 is very weak ($>10^{-5}$ M) as compared to that of the previous reported 1,4 dihydropyridines.

In a previous study (Fusi et al., 2006) where we described the strong inhibition by DP7 of some CYP-dependent O-dealkylase activities, particularly BROD activity, the conclusion was drawn that DP7 behaved like a typical member of the second generation MDR reverters, not very promising with respect to its clinical application. In the present study, the IC_{50} values of inhibition of CYP enzymes (in particular CYP3A isoforms) were over two orders of magnitude higher (10^{-5} M) than that as a P-glycoprotein inhibitor (10^{-7} M). These results are at variance with those reported in the previous study with use of a different experimental procedure. In the previous one, in fact, male rat liver BROD activity was measured in presence of dicumarol; this compound is a specific activator of this CYP-dependent activity in rat liver preparations (Ninci and DeMatteis, 2005). BZR, at variance with other CYP3A substrates, seems very sensitive towards the enzyme activation promoted by a second compound (Stresser et al., 2002) and influences also the extent of CYP inhibition. The lowest IC_{50} value was observed only in male rat microsomes in the presence of dicumarol and BZR. The CYP-dependent metabolism of other 3A substrates such as BQ and DFB was not activated by dicumarol and DP7 showed the same IC_{50} value either in presence or absence of dicumarol both in rat and human microsomal preparations (D'Elia personal communication).

It is not excluded that DP7 could interact with nuclear receptors, such as PXR and/or CAR, to promote the induction of the metabolism of xenobiotics thus causing pharmacokinetic interactions. In literature studies are present that describe the induction at various extents of CYP isozymes by some dihydropyridine compounds. These effects, however, are less potent and promoted at higher concentrations (10–100 aM) as compared to the classic inducers like rifampicin (Drocourt et al., 2001; Zhang et al., 2006). It is worth to underline that these concentrations are two orders of magnitude higher than the IC50 value of DP7 as MDR reverter. However, experiments are in progress to assess the interaction of DP7 with nuclear receptors *in vivo* in experimental animals.

Inhibition by DP7 of CYPs is not as strong as that of many previously studied P-glycoprotein inhibitors. Since this was one of the main reasons why P-glycoprotein inhibitors of the second generation have been unsuccessful in clinical applications, we can conclude that DP7 is a good lead compound for the synthesis of new dihydropyridine derivatives which are safe and more potent and selective P-glycoprotein inhibitors.

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