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Title page

Title:

Mechanism-based inactivation of human cytochrome P450 1A2 and 3A4 isoenzymes by anti-tumour triazoloacridinone C-1305.

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Abstract

1. 5-Dimethylaminopropylamino-8-hydroxytriazoloacridinone, C-1305, is a promising anti-tumour therapeutic agent with high activity against several experimental tumours.
2. It was determined to be a potent and selective inhibitor of liver microsomal and human recombinant cytochrome P450 (CYP) 1A2 and 3A4 isoenzymes. Therefore, C-1305 might modulate the effectiveness of other drugs used in multidrug therapy.
3. The objective of the present study was to investigate the mechanism of the observed C-1305-mediated inactivation of CYP1A2 and CYP3A4.
4. Our findings indicated that C-1305 produced a time- and concentration-dependent decrease in 7-ethoxycoumarin O-deethylation (CYP1A2, K_I = 10.8±2.14 μM) and testosterone 6β-hydroxylation (CYP3A4, K_I = 9.1±2.82 μM). The inactivation required the presence of NADPH, was unaffected by a nucleophilic trapping agent (glutathione) and a reactive oxygen species scavenger (catalase), attenuated by a CYP-specific substrate (7-ethoxycoumarin or testosterone), and was not reversed by potassium ferricyanide. The estimated partition ratios of 1086 and 197 were calculated for the inactivation of CYP1A2 and CYP3A4, respectively.
5. In conclusion, C-1305 inhibited human recombinant CYP1A2 and CYP3A4 isoenzymes by mechanism-based inactivation. The obtained knowledge about specific interactions between C-1305 and/or its metabolites, and CYP isoforms would be useful for predicting the possible drug-drug interactions in potent multidrug therapy.

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Introduction

5-Dimethylaminopropylamino-8-hydroxytriazoloacridinone, C-1305 (Figure 1), is a representative derivative among triazoloacridinones (TAs), anti-tumour compounds developed at the Gdańsk University of Technology (Cholody et al. 1990a). It is a promising therapeutic agent with high anti-tumour activity against several experimental cellular models and tumours in mice, particularly leukemias and colon carcinomas (Kusnierczyk et al. 1994). The clinical potential of the compound is being currently evaluated in extended preclinical trials. Simultaneously, the extensive studies on the biologic and biochemical mechanisms of C-1305 action have been conducted (Augustin et al. 2006; Fedejko-Kap et al. 2011, 2012).

It was postulated that an ability of C-1305 to bind to cellular DNA by intercalation (Lemke at al. 2005; Koba & Konopa 2007) may be crucial for the molecular mechanism of its anti-tumour activity. Apart from the reversible DNA interaction, it has been also reported that C-1305 induced interstrand DNA crosslinking in tumour cells, which occurred only after metabolic activation (Dziegielewski & Konopa 1996; Koba & Konopa 2007). Another unique mode of action of this TA is the inhibition of the catalytic activity of topoisomerase II by stabilizing the formation of a cleavable complex between DNA and topoisomerase II *in vitro* and in living cells (Skladanowski et al. 1996; Dziegielewski et al. 2002). The above-mentioned features of C-1305 confirm that it should be considered as a valuable anti-tumour drug candidate.

The previous suggestions that the metabolic activation by intracellular enzymes might be a prerequisite for biochemical action of C-1305 on the cellular level (Dziegielewski & Konopa 1996; Koba & Konopa 2007) prompted us to study on the role of drug-metabolizing enzymes in metabolic transformation of this compound (Fedejko-Kap et al. 2011, 2012; Pawlowska et al. 2012). Our recent studies revealed that, contrary to the majority of therapeutic agents, C-1305 and its less active 8-methoxy derivative, C-1299 (Figure 1), were not metabolized by the main cytochrome P450 (CYP) families, CYP1, CYP2 and CYP3. Both C-1305 as well as C-1299 were found to be selective inhibitors of human (unpublished results) and rat liver microsomal, and human recombinant CYP1A2 and CYP3A4 isoenzymes. The determined IC_{50} values of C-1305 were 0.95 μM and 3.12 μM, respectively (Fedejko-Kap et al. 2011). The type

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and the exact mechanism of the observed inhibition was not assessed then. However, these results indicated that, whereas the substitution of a hydroxyl group at position 8 provides C-1305 high anti-tumour activity, other moieties within TA molecule are presumably responsible for the inactivation of CYP1A2 and CYP3A4 isoenzymes. In contrast, CYP2 family isoforms were not inactivated by C-1305 and C-1299. On the other hand, flavin-containing monooxygenases (FMOs) 1 and 3 were demonstrated to be crucial enzymes metabolizing both compounds in rat and human liver microsomes and in human hepatocellular liver carcinoma cell line (HepG2) cells (Fedejko-Kap et al. 2011). In addition, we showed that C-1305 underwent UDP-glucuronosyltransferase (UGT)-mediated metabolism in human liver and intestinal microsomes to 8-O-glucuronide. The observed glucuronidation was catalysed mainly by UGT1A10 of the UGT1A family, whereas UGT2B family isoforms did not participate in C-1305 metabolism (Fedejko-Kap et al. 2012; Pawlowska et al. 2012).

Considering that C-1305 inhibits the catalytic activity of CYP1A2 and CYP3A4 isoforms, the purpose of the present study was to extensively evaluate the specific interactions between C-1305 and CYP1A2 and CYP3A4 isoenzymes. Among 57 human cytochromes P450, the 1A2 and 3A4 isoforms are two of the most abundant and important, because they metabolize the majority of the administered drugs (Guengerich 1995). A remarkable feature of CYP1A2 and CYP3A4 is the extreme diversity in substrate specificity and cooperative substrate binding, so their activity can be modulated by drugs. Consequently, changes in drug metabolism might result in clinically significant drug-drug interactions, leading to undesirable adverse reactions or therapeutic failures. Such interactions involving, *e.g.*, warfarin, antidepressants, anti-epileptic drugs, and statins, often relate to changes in CYPs' activity (Kivisto et al. 1995; Guengerich 1999; Bibi 2008). Knowledge about the ability of drugs to be metabolized by cytochrome P450 isoenzymes and about their influence on the activity of enzymes can help to minimize the possibility of adverse drug reactions and interactions. C-1305, being a potent inhibitor of CYP1A2 and CYP3A4, might modulate the effectiveness of other drugs used in multidrug therapy. Among drugs that cause clinically significant pharmacokinetic drug-drug interactions, mechanism-based inactivators of cytochromes P450 represent several of those

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agents that cause interactions of the greatest magnitude (Venkatakrishnan et al. 2007). It has previously been confirmed for a number of drugs with a triazole moiety (Grimm & Dyroff 1997; Stresser et al. 2004) or with a diaminoalkyl side chain (Murray & Murray 2003; Kalgutkar et al. 2007). Both functional groups are also present in the C-1305 molecule. It was believed that they formed a reactive intermediate that could irreversibly modify critical active site moieties and thus inactivate the enzyme in a process characterized as mechanism-based inactivation, MBI (Kalgutkar et al. 2007). In view of this fact we expected that such interactions between C-1305 and CYPs may also occur.

Therefore, the current study was designed to determine whether C-1305 is a mechanism-based inactivator of CYP1A2 and CYP3A4 isoforms. A kinetic study of CYPs' inactivation by C-1305 was carried out. The inactivation experiments, which involved a 10-fold dilution step, were performed *in vitro* using the human recombinant CYP1A2 and CYP3A4 (Bactosomes). CYP isoenzyme activities were determined using the CYP-specific reactions, 7-ethoxycoumarin O-deethylation (CYP1A2) (Yamazaki et al. 1996) and testosterone 6β-hydroxylation (CYP3A4) (Yuan et al. 2002). The concentrations of CYP-specific substrates and their metabolites formed by CYP isoenzymes were measured by reversed-phase high performance liquid chromatography (RP-HPLC) with UV-Vis and/or diode array and multiple wavelength detection. The elucidation of the mechanism of C-1305-mediated inactivation of CYP1A2 and CYP3A4 may provide new insights into the role of C-1305-CYP interactions in the anti-tumour action of this drug, particularly considering that the metabolism of C-1305 by CYPs was not observed. This knowledge would be applied to predict the possible drug-drug interactions induced by this agent in potent multidrug therapy.

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List of abbreviations:

CAT, catalase;

CYP, cytochrome P450;

C-1305, 5-dimethylaminopropylamino-8-hydroxytriazoloacridinone;

7EC, 7-ethoxycoumarin;

$t_{1/2inact}$, inactivation half-life;

MBI, mechanism-based inactivation;

NADPH, β-nicotinamide adenine dinucleotide phosphate, tetrasodium salt;

P, partition ratio of inactivation;

RP-HPLC, reversed-phase high performance liquid chromatography;

TT, testosterone;

K_I, the concentration required for half-maximum inactivation;

k_{inact}, the maximum rate of inactivation at saturation;

k_{obs}, the observed inactivation rate constant;

TA, triazoloacridinone;

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Materials and methods

Chemicals and reagents

A triazoloacridinone derivative C-1305 was synthesized as hydrochloride in the Department of Pharmaceutical Technology and Biochemistry, Gdańsk University of Technology (Gdańsk, Poland), as described earlier (Cholody et al. 1990a). 7-Ethoxycoumarin (7EC), formic acid, L-glutathione reduced (GSH), potassium ferricyanide, and testosterone (TT) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Methanol (gradient grade for liquid chromatography) and β-nicotinamide adenine dinucleotide phosphate, tetrasodium salt (NADPH) were obtained from Merck KGaA (Darmstadt, Germany). Ammonium formate was from Fisher Scientific (Loughborough, UK). All other commercially available chemicals and solvents were of the highest purity available. Redistilled water (R > 18 M*cm) was used to prepare all the solutions.

Stock solutions of C-1305 (2 and 4 mM) and NADPH (20 mM) were freshly prepared in filter sterilized 0.1 M potassium phosphate (pH 7.4 buffer solution). 7EC (20 mM) and TT (20 mM) were prepared in DMSO, and then diluted 10-fold with water for each experiment. GSH (10 mM) and potassium ferricyanide (20 mM) were freshly prepared in water. To obtain 0.1 M potassium phosphate (pH 7.4 buffer solution) 0.1 M monobasic potassium phosphate and 0.1 M dibasic potassium phosphate were mixed, and the correct pH was adjusted by adding 1 N potassium hydroxide.

Enzymes

The human recombinant cytochrome P450 (CYP) isoenzymes: 1A2 (10.5 μM stock solution in Bactosome storage buffer, 50 mM Tris acetate pH 7.6, 250 mM sucrose, 0.25 mM EDTA) and 3A4 (7.2 μM stock solution in Bactosome storage buffer), co-expressed with human NADPH-cytochrome P450 oxidoreductase (POR) in *Escherichia coli* cells (Bactosomes), were purchased from Tebu-bio (Le Perray-En-Yvelines, France). Catalase from bovine liver (20000 U/mL stock solution in water) was obtained from Sigma-Aldrich Co. (St. Louis, MO).

Instrumentation (HPLC analysis)

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The concentrations of CYP-specific substrates (7EC or TT) and their metabolites, formed in Bactosomes 1A2 or 3A4, were measured by RP-HPLC with UV-Vis detection and/or diode array and multiple wavelength detection. The supernatants, obtained according to the procedures described below, were analysed with a reversed-phase 5-μm Suplex pKb-100 analytical column (0.46 cm x 25 cm, C18) (Supelco Inc., Bellefonte, PA) with Waters Associates HPLC systems (Waters Co., Milford, MA). One Waters HPLC system was equipped with a model 600E system controller, a model 7725i Rheodyne injector, and a model 2996 photodiode array detector (DAD) controlled with Millennium software. The second Waters HPLC system contained: a model 1525 binary HPLC pump, a model 7725i Rheodyne injector, a model 2487 dual λ absorbance detector, and a model 717 plus autosampler controlled with Breeze software. The HPLC analyses were carried out at a flow rate of 1 mL/min with the following mobile phase system: a linear gradient from 15% to 80% methanol in ammonium formate (0.05 M, pH 3.4) for 25 min, followed by a linear gradient from 80% to 100% methanol in ammonium formate for 3 min. The eluates were monitored at 324 nm and 245 nm for 7ET and TT, respectively.

Enzyme inactivation assays

The activities of CYP isoenzymes were determined by measuring the rate of the CYP-specific reactions, 7-ethoxycoumarin O-deethylation (CYP1A2) and testosterone 6β-hydroxylation (CYP3A4) (Yamazaki et al. 1996; Yuan et al. 2002).

The method used for the inactivation experiments involved two steps: a primary (preincubation) reaction with the inhibitor and a secondary (incubation) reaction with the substrate, with these two steps conducted in separation reaction tubes, all presented in Figure 2. Primary reaction mixture containing potassium phosphate buffer (0.1 M, pH 7.4), CYP1A2 or CYP3A4 (0.5 μM), and increasing concentrations of C-1305 (0, 5, 10, 25, 50, and 100 μM) was pre-warmed for 3 min at 37°C in a shaking water bath. Enzymatic reaction was initiated by adding NADPH (0.5 mM), which was excluded in negative control incubations, and preincubated for varying lengths of time (as specified in the figure legends). Subsequently, an aliquot (10 μL) of the primary reaction mixture was periodically transferred to 90 μL of pre-

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warmed (for 3 min at 37°C) secondary incubation mixture (total volume of 100 µL) containing potassium phosphate buffer, 7EC or TT (20 µM), and NADPH (0.5 mM). CYP-specific substrates were added from 2 mM stock solutions in 10% DMSO (the final concentration of DMSO in the incubation mixture was 0.1%). The enzymatic reaction in the secondary incubation mixture was incubated for 30 min at 37°C and terminated by adding of ice-cold methanol (1:1, v/v). The incubation mixture was placed in ice for 10 min and then centrifuged for 5 min at 12 000*g*. An aliquot of the supernatant (100 µL) was then analyzed directly by RP-HPLC to establish remaining enzyme activity.

The 10-fold dilution of the primary reaction mixture allowed differentiation of covalent (irreversible) inactivation from competitive inhibition by C-1305. Results representative of at least three independent experiments were considered. The calculation of kinetic constants, which describe mechanism-based enzyme inactivators, was performed as described previously (Kitz & Wilson 1962; Hollenberg et al. 2008). Briefly, the relative inhibition of 7-hydroxycoumarin or 6β-hydroxytestosterone formation were determined by comparing peak area ratios of time-matched samples with C-1305 versus control samples without C-1305. These natural logarithm of relative inhibition values were plotted versus preincubation time for each concentration of C-1305 used, and the slopes were obtained by linear regression (GraphPad Prism 6.05; GraphPad Software, Inc., San Diego, CA). These slopes represent the observed inactivation rate constants (k_{obs}), which were used to calculate the half-life of the inactivation reaction. A Kitz-Wilson plot was then constructed using the calculated half-life values (*y*-axis) and the reciprocal of the associated C-1305 concentration (*x*-axis). The apparent K_I (the concentration required for half-maximum inactivation) and k_{inact} (the maximum rate of inactivation at saturation) values were determined from the reciprocal of the *y*-axis intercept and the negative reciprocal of the *x*-axis intercept of the Kitz-Wilson plot, respectively. The time required for half of the enzyme molecules to be inactivated ($t_{1/2}$) was determined by the equation:

$t_{1/2} = \ln 2/k_{inact}$.

Effects of a competing CYP-specific substrate

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The inactivation of CYP1A2 or CYP3A4 (0.5 μM) by C-1305 (20 μM) in the absence and presence of a CYP-specific substrate was investigated by adding a 2- and 4-fold molar excess of 7-ethoxycoumarin or testosterone, respectively, over C-1305 to the primary reaction mixture. At the end of the incubation time, aliquots (10 μL) of the primary reaction mixtures in triplicate were periodically removed and assayed for remaining enzyme activity in a secondary incubation mixture as described above.

Effects of exogenous nucleophilic trapping agent (GSH) and scavenger of reactive oxygen species (catalase)

GSH (1, 2, and 5 mM) was included in the primary reaction mixture with CYP1A2 or CYP3A4 (0.5 μM), C-1305 (20 μM), and NADPH (0.5 mM). Aliquots (10 μL) of the primary reaction mixtures in triplicate were periodically removed to estimate the remaining enzyme activity in a secondary incubation mixture as described above. In control samples, potassium phosphate buffer was added in place of GSH. In a separate study, CYP1A2 or CYP3A4 was incubated with C-1305 and NADPH in the absence and presence of catalase (1000 and 2000 U/mL).

Effects of potassium ferricyanide (irreversibility of inactivation)

The experiment was divided into three parts: the inactivation assay (preincubation), the restoration of activity assay, and the remaining activity assay. Primary reaction mixture containing potassium phosphate buffer, CYP1A2 or CYP3A4 (0.5 μM), C-1305 (20 μM), and NADPH (0.5 mM) was preincubated for 30 min at 37°C in a shaking water bath. Negative control incubations lacked NADPH. An aliquot (10 μL) of the primary reaction mixture in triplicate was periodically removed and added to the restoration of activity plate, which had 50 μL of the potassium ferricyanide (2 mM; potassium phosphate buffer for the controls). After a further 15-min incubation, another 10-μL aliquot was taken and assayed for the determination of the remaining CYP1A2 and CYP3A4 activity in a secondary incubation mixture as described above.

Partition ratio determination

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Primary reaction and secondary reaction mixtures were prepared similarly to those reported in the section on enzyme inactivation assays. In this experiment, incubations were conducted at various concentrations of C-1305 (0-2.5 mM) for 30 min to ensure complete inactivation. Negative control incubations lacked NADPH. Aliquots (10 µL) of the primary reaction mixtures were periodically removed and assayed for remaining enzyme activity in a secondary incubation mixture as described above. The percentage remaining enzyme activity was plotted versus the ratio [C-1305]/[CYP]. As previously described (Silverman 1996; Kent et al. 1998), extrapolating the intercept of the linear regression of lower C-1305 concentrations and the horizontal line from saturating C-1305 concentrations yielded the partition ratio of inactivation (P).

Statistical analysis

All of the data reported represent the mean ± standard deviation (SD). Statistical evaluation was performed using GraphPad Prism 6.05 (GraphPad Software, Inc., San Diego, CA) and employed an unpaired *t*-test or a two-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test, and $p<0.05$ was accepted to be statistically significant.

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Results

Kinetics of the inactivation of human recombinant CYP1A2 and CYP3A4 by C-1305

Time-dependent enzyme inhibition is the primary criterion to determine irreversible or mechanism-based inhibitors (Kalgutkar et al. 2007). To characterize in detail the kinetics of CYP1A2 and CYP3A4 inactivation, varying concentrations of C-1305 were preincubated with human recombinant CYP isoform and NADPH at 37°C for varying lengths of time. As shown in Figure 3, both 7-ethoxycoumarin O-deethylation as well as testosterone 6β-hydroxylation declined in a time- and concentration-dependent manner when CYP isoform was preincubated with C-1305 in the presence of NADPH for up to 60 min. Enzyme kinetic analyses of CYPs' inactivation by C-1305 were conducted by the Kitz-Wilson plot (Figure 3, the insets). With this method, we estimated the maximum rates of inactivation at saturation (k_{inact}) of 0.016 min^{-1} (CYP1A2) and 0.022 min^{-1} (CYP3A4). The concentrations of C-1305 required to produce half-maximum CYP inactivation (K_I) were 10.8±2.14 μM (CYP1A2) and 9.1±2.82 μM (CYP3A4). The times required for half of the enzymes to be inactivated ($t_{1/2inact}$) were 43.3 min (CYP1A2) and 31.5 min (CYP3A4). Hence, the ratio of k_{inact} to K_I, which is used to assess the efficiency of enzyme inactivation (Kalgutkar et al. 2007), was 0.0015 $min^{-1}\mu M^{-1}$ (CYP1A2) and 0.0024 $min^{-1}\mu M^{-1}$ (CYP3A4). The values of all kinetic parameters are presented in Table 1.

NADPH-dependent inactivation of human recombinant CYP1A2 and CYP3A4 by C-1305

To assess whether the inactivation of CYP1A2 and CYP3A4 by C-1305 required NADPH, we performed experiments in which varying concentrations of C-1305 were preincubated with human recombinant CYP isoform at 37°C for 60 min in the presence or absence of NADPH. The remaining CYP1A2 activity in samples co-incubated with C-1305 and 1 mM NADPH was 23±2.8% and 20±1.3% lower at C-1305 concentrations of 5 and 100 μM, respectively, compared to NADPH-free incubations (Figure 4A). The remaining CYP3A4 activity in samples that had C-1305 co-incubated with 1 mM NADPH decreased 26±1.9% and 19±2.4% at C-1305 concentrations of 5 and 100 μM, respectively, compared to samples that lacked NADPH (Figure 4B). Incubations under identical conditions but in the absence of C-1305 did not lead to a significant loss of CYP activities. These results suggested that NADPH-dependent

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metabolic activation of C-1305 to reactive intermediate by CYP1A2 or CYP3A4 was required for enzyme inactivation, though in the absence of NADPH a low level of inhibition was still observed.

Attenuation of C-1305-mediated inactivation of human recombinant CYP1A2 and CYP3A4 by a CYP-specific substrate

In order to determine whether CYP1A2 or CYP3A4 inactivation by C-1305 was protected by the presence of a competing CYP substrate, 7-ethoxycoumarin and testosterone, respectively, varying concentrations of a CYP-specific substrate were preincubated with C-1305, human recombinant CYP isoform, and NADPH at 37°C for varying lengths of time. 7EC or TT at concentrations of 20, 40, and 80 μM corresponded to a molar ratio of 7EC (TT) to C-1305 of 1, 2 and 4, respectively. Our results indicated that both substrates reduced the ability of C-1305 to inactivate CYP1A2 or CYP3A4, what is illustrated in Figure 5. The remaining CYP1A2 activity in samples where C-1305 was co-incubated with 7EC at a molar ratio of 1:4 increased 48±2.8% in comparison to 7EC-free incubations after 60 min of preincubation (Figure 5A). In turn, C-1305 decreased the activity of CYP3A4 to 28±0.9% in TT-free incubations after 60 min of preincubation, and increasing concentrations of TT attenuated the extent of inactivation (Figure 5B). The obtained results indicated that 7EC and TT compete with C-1305 for metabolism by CYP1A2 and CYP3A4 isoforms, respectively, and thereby protect against inactivation. They also suggested that the inactivation of CYP1A2 or CYP3A4 proceeded via the attachment of C-1305 reactive intermediate to the active site of the enzyme.

Lack of GSH and catalase effects on inactivation of human recombinant CYP1A2 and CYP3A4 by C-1305

To evaluate whether CYP1A2 and CYP3A4 inactivation by C-1305 was confined to the active site, the drug was preincubated with human recombinant CYP isoform and NADPH at 37°C for varying lengths of time in the presence or absence of a nucleophilic trapping agent (glutathione) or a scavenger of reactive oxygen species (catalase). As shown in Figure 6A, C-1305 alone decreased 7-ethoxycoumarin O-deethylation to 36±0.4% and testosterone 6β-

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hydroxylation to 36±3.9% after 60 min of preincubation, but the addition of GSH (1, 2, and 5 mM) did not attenuate the inactivation of CYP1A2 by C-1305 and had only a very little (~6%) protective effect against CYP3A4 inactivation by the TA. Similarly, catalase (1000 and 2000 U/mL) did not protect CYP1A2 and CYP3A4 against the inactivation by C-1305 (Figure 6B).

Irreversibility of human recombinant CYP1A2 and CYP3A4 inactivation by C-1305

To examine whether the inactivation of CYP1A2 and CYP3A4 by C-1305 was irreversible, the studied drug was preincubated with human recombinant CYP isoform and NADPH at 37°C for 30 min before an aliquot was subjected to potassium ferricyanide for 15 min. These samples were also compared with that lacked potassium ferricyanide. Figure 7 shows that, as expected, the addition of potassium ferricyanide to the inactivation mixture did not affect the magnitude of the enzymes' inactivation significantly.

Partition ratio for the inactivation of human recombinant CYP1A2 and CYP3A4 by C-1305

Given that C-1305 inactivated human recombinant CYP1A2 and CYP3A4 isoforms, we determined, in the next step, the partition ratio for the C-1305-mediated CYP1A2 and CYP3A4 inactivation. The partition ratio P, is defined as the number of inactivator molecules metabolized per molecule of enzyme inactivated (Hollenberg et al. 2008). It was estimated graphically using the method described previously (Silverman 1996; Kent et al. 1998). CYP isoform was incubated with various concentrations of C-1305, and the inactivation was allowed to progress for 60 min, until it was essentially complete. The percentage of remaining activity was plotted as a function of the molar ratio of C-1305 to CYP. The turnover number (P+1) was estimated from the intercept of the linear regression line obtained from lower ratios of C-1305 to CYP and with the straight line derived from the higher ratios of C-1305 to CYP (Figure 8). With this method, we estimated the turnover numbers of ~1087 (CYP1A2) and ~198 (CYP3A4), and, consequently, the Ps were ~1086 and ~197, respectively.

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Discussion

The results of the previous studies on the biologic and biochemical mechanisms of C-1305 action makes the compound very attractive as a potent anti-tumour agent for clinical development. In a separate study, we demonstrated that, contrary to the majority of therapeutic agents, C-1305 was not metabolized by the main CYP families, CYP1, CYP2 and CYP3. In contrast, C-1305 was determined to be a selective inhibitor of human (unpublished results) and rat liver microsomal, and human recombinant CYP1A2 and CYP3A4 isoenzymes (Fedejko-Kap et al. 2011), but the actual mechanism of the inhibition was not assessed. Thus, we undertook here to examine this issue, because C-1305, being a potent inhibitor of CYP1A2 and CYP3A4, might modulate the effectiveness of other drugs used in multidrug therapy.

The obtained results revealed that C-1305 is a mechanism-based inactivator of human recombinant CYP1A2 and CYP3A4 isoenzymes. It produced a time- and concentration-dependent inactivation of both enzymes. To more clearly define the inhibition potency of C-1305 against CYP1A2 and CYP3A4 the values of K_I and k_{inact} were determined (Figure 3, Table 1). The values of K_I (10.8 and 9.1 μM) indicated that this compound is a very potent inactivator of the studied CYP isoforms, although we observed the slightly different potential of C-1305 to inhibit both CYPs. The potency of CYP1A2 and CYP3A4 inactivation (K_I) by C-1305 was similar to that for other inactivators, *e.g.*, delavirdine (Voorman et al. 1998), raloxifene (Chen et al. 2002), and *trans*-resveratrol (Chang et al. 2001). The values of k_{inact} (0.016 and 0.022 min^{-1}) were lesser than other drugs, *e.g.*, irinotecan (Hanioka et al. 2002), tamoxifen (Zhao et al. 2002), and dihydralazine (Masubuchi & Horie 1999). We also showed that a high level of inactivation was observed if NADPH was present in the incubation system (Figure 4). It strongly suggests that C-1305 itself is not an inactivator of CYP1A2 and CYP3A4, and that the loss of the catalytic activity of the enzymes requires metabolic activation of C-1305. A low level of inhibition was also observed in the absence of NADPH in a primary mixture. We suppose that NADPH added with the CYP-specific substrate in a secondary mixture could initiate the additional interactions between C-1305 and CYPs studied, and interactions of C-1305 with its metabolites. They were independent of the mechanism-based inactivation and could influence

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the observed inhibition effect. The protective effect of 7-ethoxycoumarin on CYP1A2 and testosterone on CYP3A4 inactivation observed in a competing CYP-specific substrate experiments (Figure 5), indicates that C-1305 competed with 7-ethoxycoumarin or testosterone for the active site of the enzyme. As a result the generation of its reactive metabolites was diminished. These critical findings of substrate protection provide an evidence that bioactivation of C-1305 occurs in the active site of the enzyme.

Further evidence for the involvement of the CYP active site in the metabolic activation of C-1305 was provided by the observation that CYP1A2 and CYP3A4 inactivation was not protected by a nucleophilic trapping agent such as glutathione (exogenous nucleophile) (Figure 6A). This implies in part that CYP1A2 and CYP3A4 are covalently modified by electrophilic metabolites of C-1305 before escaping from the active site. Additionally, catalase, a scavenger of reactive oxygen species, did not prevent CYP1A2 and CYP3A4 inactivation induced by C-1305, suggesting that reactive oxygen species (*i.e.*, hydrogen peroxide, superoxide anion) were not involved in the inactivation event (Figure 6B). We also considered that mechanism-based enzyme inactivators are often irreversible inhibitors. Irreversibility of inactivation was evaluated here by oxidation with potassium ferricyanide. Activities of both CYP isoforms could not be recovered by potassium ferricyanide, which indicated the irreversible nature of inhibition (Figure 7).

In sum, C-1305 fulfilled the criteria for a mechanism-based inhibitor (Silverman 1996; Kalgutkar et al. 2007; Hollenberg et al. 2008). The results obtained in the present study gave a good support for earlier suggestions about the existence of a catalysis-dependent inactivation of CYPs upon oxidation of C-1305 to a reactive intermediate. The mechanism-based inactivation (MBI) of CYPs has been reported earlier for several therapeutic agents, including anti-tumour drugs such as thioTEPA (Richter et al. 2005), tamoxifen (Sridar et al. 2002; Zhao et al. 2002), and irinotecan (Hanioka et al. 2002).

The partition ratio gives an idea of the efficiency of the inactivator. We demonstrated that C-1305, with an estimated P value of 197, is a moderately efficient inactivator of CYP3A4, and it is much stronger than CYP1A2, which has a P value of 1087 (Figure 8). This finding allows

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us to presume that C-1305-mediated inhibition of CYP1A2 and CYP3A4 activities may take place in slightly different ways. The differences in the strength of inhibition may result from the binding of an inhibitor in different pockets of the active sites of the two CYPs studied. On the other hand, the different structures and properties of the CYP1A2 and CYP3A4 active centers should be considered. Specifically, the CYP1A2 active site is opened directly above the set of four pyrrole rings of heme, while one of the pyrrole rings is protected by a protein helix in CYP3A4 and many other CYP isoforms. Thus, a larger and flexible active site of CYP3A4 enables interactions with substrates far from the heme and may take different spatial conformations depending on the type of the substrate. This ability would explain a wide range of substrate specificities of this isoenzyme (De Groot & Ekins 2002; Sevrioukova & Poulos 2013). It is also possible that, aside from the main mechanism of MBI, additional interactions between C-1305 and CYP (mainly CYP1A2) can influence the observed inhibition effect. These include reversible inhibition of CYP by C-1305 and its metabolites, and interactions of C-1305 with its metabolites.

Cytochrome P450 isoenzymes have been proposed as one of the targets of the reactive intermediates (Kalgutkar et al. 2007; Macherey & Dansette 2008). The precise mechanism by which C-1305 is transformed by CYP1A2 or CYP3A4 into a reactive intermediate, which inactivates the enzyme, remains unknown. MBI of CYPs by drugs can be due to the chemical modification of the heme or the apoprotein in a manner that destroys heme function or binding (Ortiz de Montellano & Correia 1995; Kalgutkar et al. 2007). The structure of C-1305 molecule strongly suggests that CYPs could catalyse hydroxylation of the aromatic ring or dealkylation of dialkylaminoalkyl side chain, as it has previously been confirmed for a number of drugs with a triazole moiety like anastrozole (Grimm & Dyroff 1997) and azamulin (Stresser et al. 2004), and with a diaminoalkyl side chain like fluoxetine (Murray & Murray 2003).

We suspect that the inhibitory effect of C-1305 on CYP monooxygenases may operate by several mechanisms. Some of the observations give a strong suggestion that CYP-mediated oxidation of the compound phenyl ring would lead to the formation of unstable epoxide intermediate (Potęga, unpublished results). The resulting epoxide metabolite may be further

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transformed to electrophilic diol or *ortho*-quinone derivatives, which can react with nucleophilic groups on macromolecules or conjugate with GSH (Fontana et al. 2005). On the other hand, the primary amines, from CYP-mediated N-demethylation of C-1305 may also be related to C-1305 toxicity. Further studies are suggested to determine the potential reactive intermediates responsible for the inactivation of CYP1A2 and CYP3A4 by anti-tumour TA. The probable similarity in mechanism of CYP1A2 and CYP3A4 inactivation by two TAs, C-1305 and C-1299, with different substituents at the 8C on the phenyl ring, indicates that this structural element is presumably not responsible for the inactivation of CYP isoenzymes, which should be emphasized.

It is interesting to add that C-1305 was shown earlier to be a selective agonist of pregnane X receptor (PXR) (Niemira et al. 2013). Given that both PXR and CYP3A4 have a large, hydrophobic, and flexible ligand-binding domain (Hosea et al. 2000; Ekins et al. 2009), it is possible that a ligand of PXR may be also a substrate, direct inhibitor, or mechanism-based inactivator of CYP3A4. Our current findings indicated that C-1305, which is a PXR agonist, inactivated CYP3A4, thereby giving rise to the opposing effects on the catalytic activity of the enzyme. Such dual and opposing effects of activating PXR and inhibiting CYP3A4 activity were known earlier for ritonavir (Luo et al. 2002) and meclizine (Foo et al. 2015).

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Conclusions

In the present *in vitro* study, we demonstrated the existence of specific interactions between C-1305 and/or its metabolites, and human recombinant CYP1A2 and CYP3A4, which led to a decrease in the isoenzyme activities. A major conclusion is that the C-1305-mediated inactivation of CYP1A2 and CYP3A4 exhibits a number of characteristics consistent with mechanism-based inactivation. This work opens new avenues for understanding the mechanism of bioactivation and cytotoxicity induced by this anticancer compound. Our findings indicate that pharmacokinetic interactions between C-1305 and the substrates of CYP1A2 or CYP3A4 are likely to occur, which should be carefully considered in the optimal chemotherapy schedules designed for individual patients. Considering pharmacological aspect, inhibitory effect of C-1305 on CYP1A2 and CYP3A4 activity gives also the possibility for planning effective anti-tumour therapy in patients with genetic polymorphisms in CYP genes.

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Declaration of interest statement

The authors confirm that this article content has no conflicts of interest.

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Tables with captions

Table 1

Kinetic parameters for the inactivation of 7-ethoxycoumarin O-deethylation (CYP1A2) and 6β-testosterone hydroxylation (CYP3A4) activities by C-1305.

Inhibitor	Kinetic parameters of CYP-specific reactions					
	7-Ethoxycoumarin O-deethylation (CYP1A2)			Testosterone 6β-hydroxylation (CYP3A4)		
	K_I [μM]	k_{inact} [min^{-1}]	$t_{1/2inact}$ [min]	K_I [μM]	k_{inact} [min^{-1}]	$t_{1/2inact}$ [min]
C-1305	10.8±2.14	0.016±0.0015	43.3±4.09	9.1±2.82	0.022±0.0031	31.5±4.53

The presented values of kinetic parameters were calculated using GraphPad Prism 6.05 (GraphPad Software, Inc., San Diego, CA) and are based on the data shown in Figure 3. K_I: the concentration required for half-maximum inactivation; k_{inact}: the maximum rate of inactivation at saturation; $t_{1/2inact}$: inactivation half-life.

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Figure captions

Figure 1

Chemical structures of anti-tumour triazoloacridinones, C-1305 and C-1299.

Figure 2

Scheme of mechanism-based inactivation experiments.

Figure 3

Time- and concentration-dependent inactivation of 7-ethoxycoumarin O-deethylation (CYP1A2) (**A**) and testosterone 6β-hydroxylation (CYP3A4) (**B**) activities by C-1305. Assay conditions were described in Materials and methods section. The concentrations of C-1305 were: (■) 0, (◆) 5, (□) 10, (▲) 25, (△) 50, and (◇) 100 μM. The insets show corresponding Kitz-Wilson plots (the inverse of the observed inactivation rate constant, $1/k_{obs}$, *versus* the inverse of C-1305 concentration). Each point represents the mean ± SD for three independent experiments. The K_I, k_{inact} and $t_{1/2inact}$ values are shown in Table 1.

Figure 4

Effect of NADPH on inactivation of human recombinant CYP1A2 (**A**) and CYP3A4 (**B**) by C-1305. Assay conditions were described in Materials and methods section. The concentrations of C-1305 were: 5, 10, 20, 50, and 100 μM. Data are normalized to the enzymatic activity of the C-1305-free control incubations and expressed as the mean ± SD for three independent experiments. Comparisons were made using two-way ANOVA followed by Bonferroni's multiple comparisons test. *Indicates significance of 0.5 or 1 mM NADPH results with respect to no NADPH results at the same preincubation time. Levels were considered significant at ****p<0.0001, ***p<0.001, **p<0.01, and *p<0.05; ns, not significant.

Figure 5

Substrate protection against inactivation of human recombinant CYP1A2 (**A**) and CYP3A4 (**B**) by C-1305. Assay conditions were described in Materials and methods section. Aliquots were removed from the primary reaction mixture at the indicated time points and assayed for remaining enzyme activity in a secondary incubation mixture. The primary reaction mixtures

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contained the following molar ratios of C-1305 to 7EC (TT): (■) 1:0, (◆) 1:1, (□) 1:2, and (△) 1:4. Each point represents the mean ± SD for three independent experiments.

Figure 6

Effects of glutathione (GSH) (**A**) and catalase (CAT) (**B**) on inactivation of human recombinant CYP1A2 and CYP3A4 by C-1305. Assay conditions were described in Materials and methods section. Each point represents the mean ± SD for three independent experiments.

Figure 7

Effect of potassium ferricyanide on inactivation of human recombinant CYP1A2 and CYP3A4 by C-1305. Assay conditions were described in Materials and methods section. Data are normalized to the enzymatic activity of the vehicle-treated control group that was not subjected to preincubation and expressed as the mean ± SD for three independent experiments. Comparisons were made using an unpaired *t*-test. *Indicates significance of 0.5 mM NADPH results with respect to no NADPH results at the same incubation time. Levels were considered significant at ****$p<0.0001$ and ***$p<0.001$.

Figure 8

Partition ratio for the inactivation of human recombinant CYP1A2 (**A**) and CYP3A4 (**B**) by C-1305. Assay conditions were described in Materials and methods section. The extrapolated partition ratio (P) was estimated from the intercept of the linear regression line from the lower ratios, and the straight line was obtained from higher ratios. Data are normalized to the enzymatic activity of the C-1305-free control incubations and expressed as the mean ± SD for three independent experiments.

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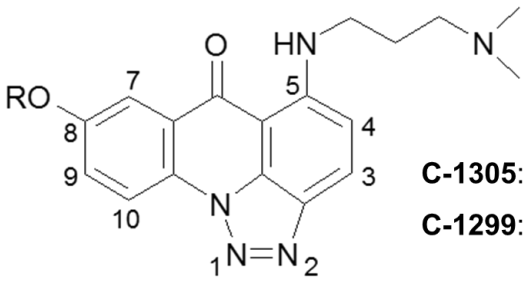
Figure 1



C-1305: R = H

C-1299: R = CH_3

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Figure 2

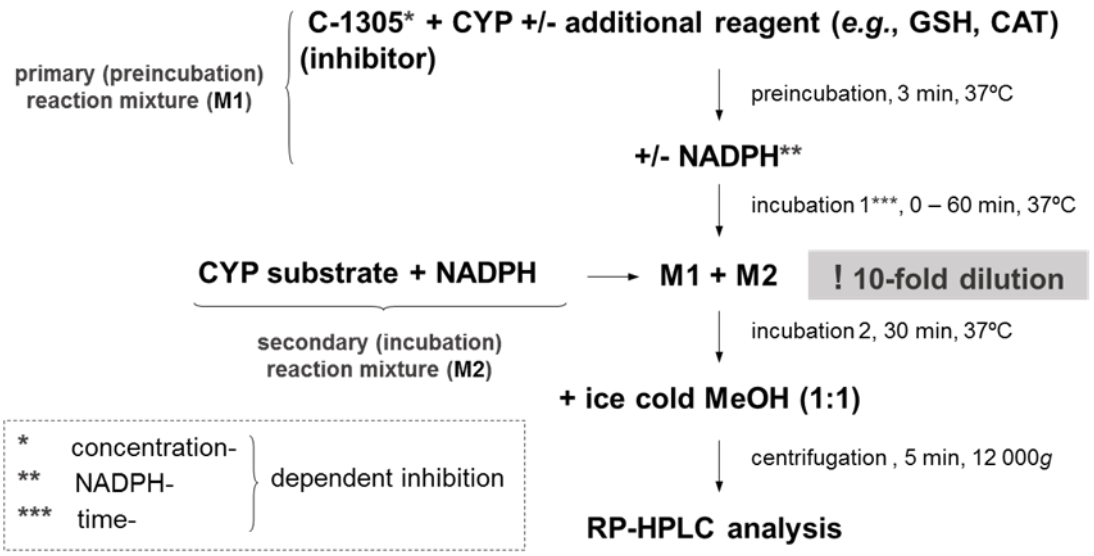

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Figure 3

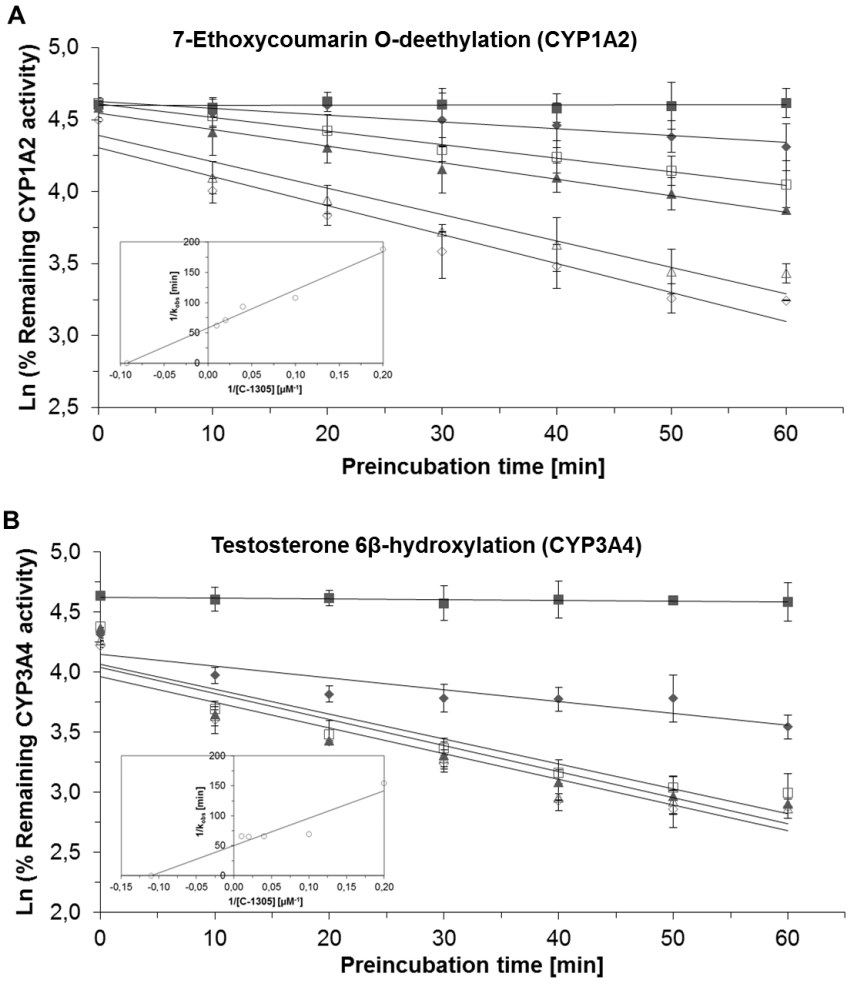

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Figure 4

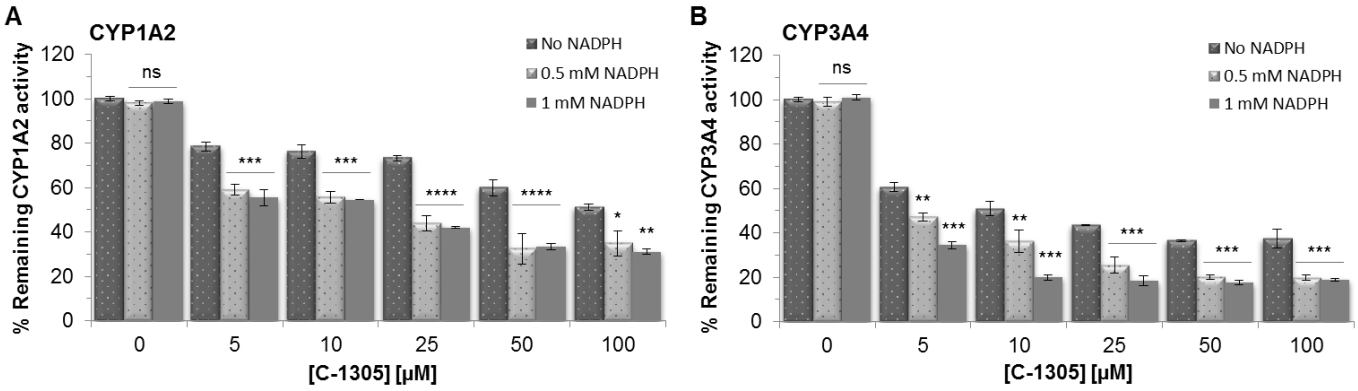

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Figure 5

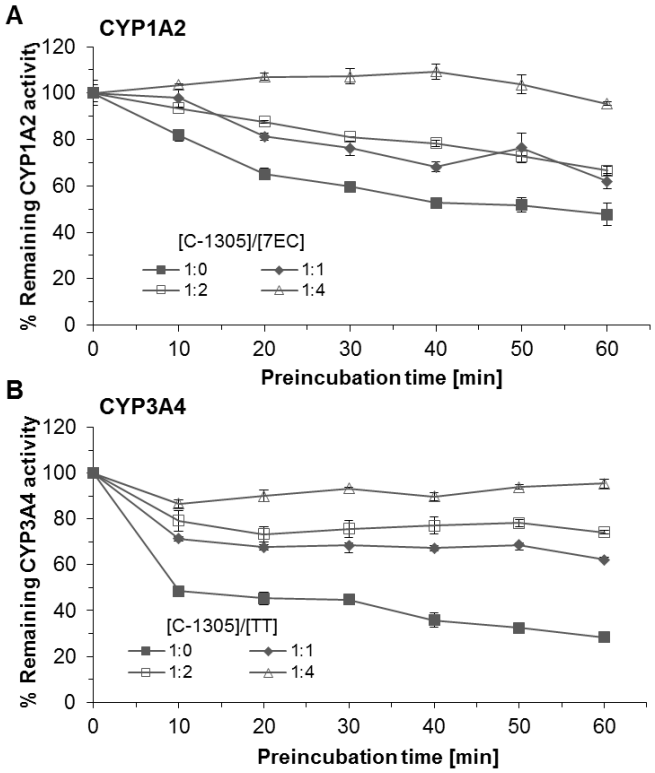

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Figure 6

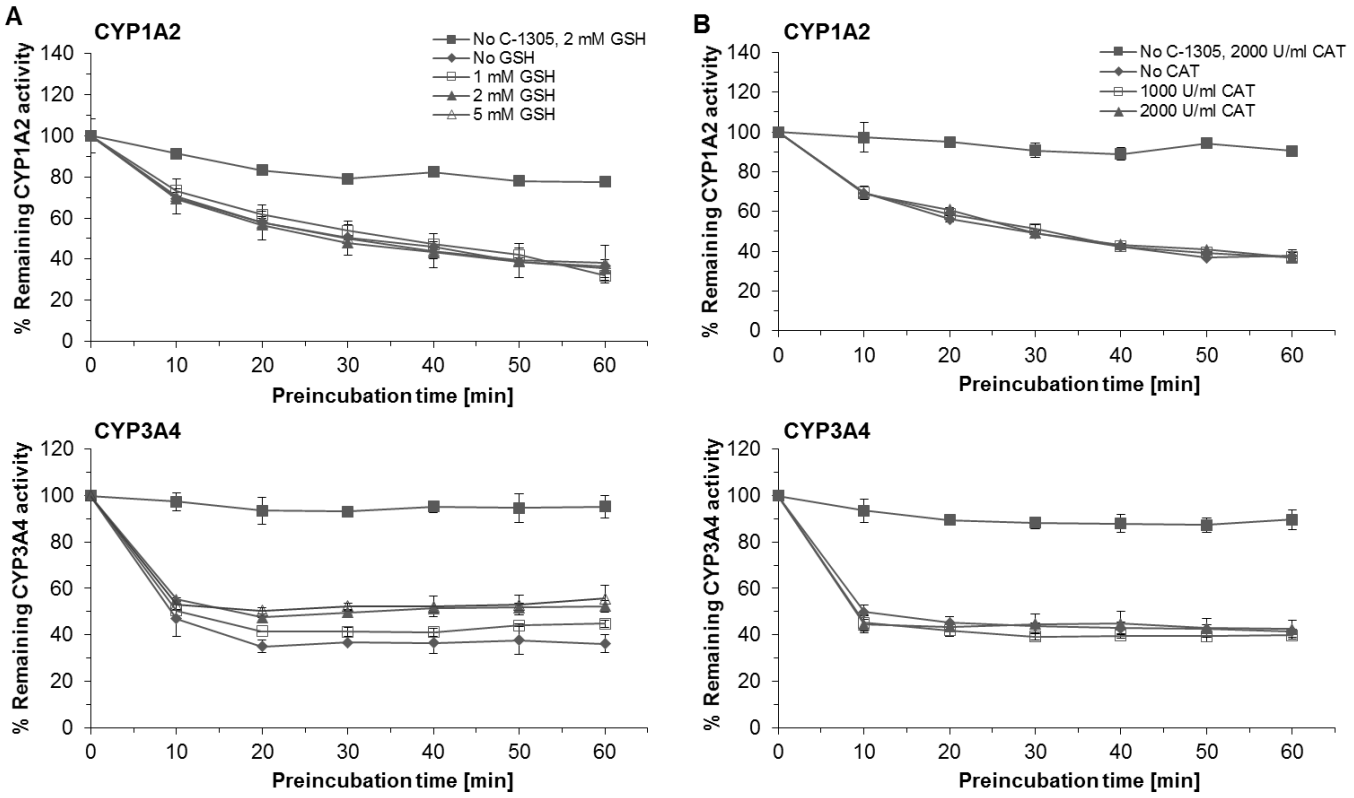

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Figure 7

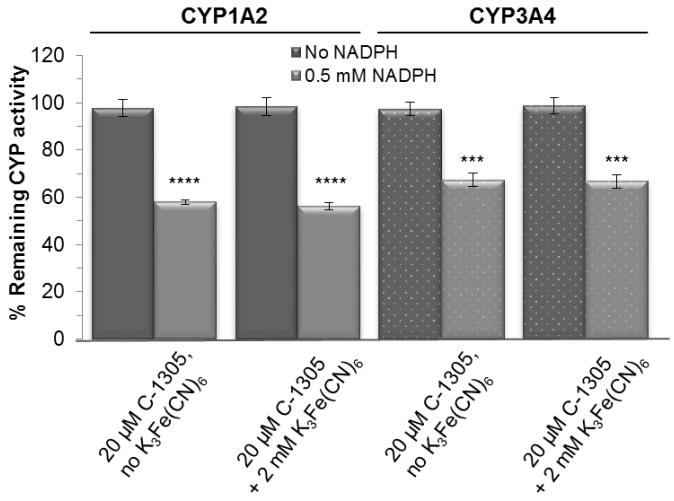

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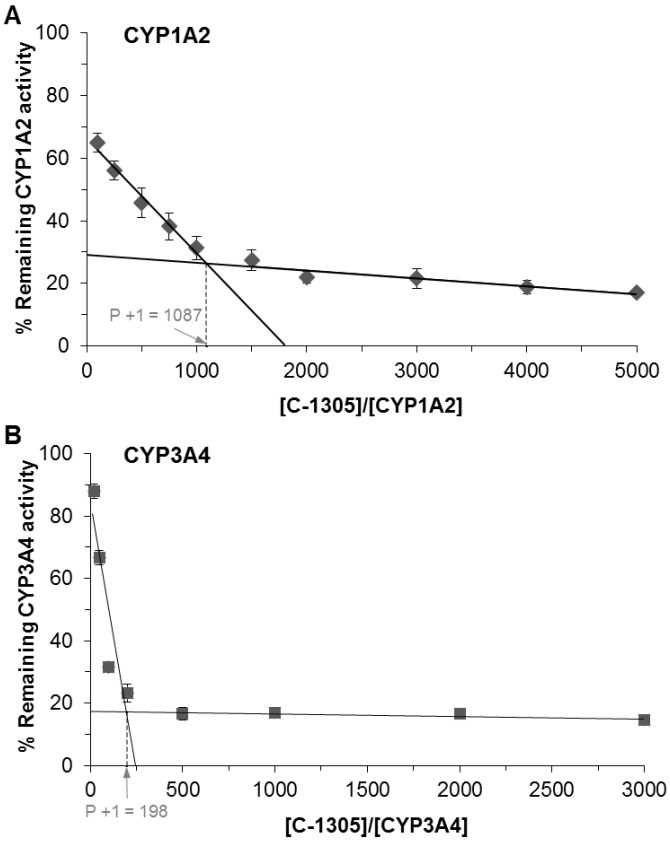
Figure 8



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