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Title page

Title:

Imidazoacridinone antitumor agent C-1311 as a selective mechanism-based inactivator of human cytochrome P450 1A2 and 3A4 isoenzymes.

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Abstract

Background: 5-Diethylaminoethylamino-8-hydroxyimidazoacridinone (C-1311), a promising antitumor agent that is also active against autoimmune diseases, was determined to be a selective inhibitor of the cytochrome P450 (CYP) 1A2 and 3A4 isoenzymes. Therefore, C-1311 might modulate the effectiveness of other drugs used in multidrug therapy. The present work aimed to identify the mechanism of the observed C-1311-mediated inactivation of CYP1A2 and CYP3A4.

Methods: The inactivation experiments 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) and testosterone 6β-hydroxylation (CYP3A4). The concentrations of CYP-specific substrates and their metabolites formed by CYP isoenzymes were measured by RP-HPLC with UV-Vis detection.

Results: The inhibition of CYPs by C-1311 was time-, concentration- and NADPH-dependent, which suggested a mechanism-based mode of action. Using a 10-fold dilution method and potassium ferricyanide we demonstrated the irreversible nature of the inhibition. In addition, the inhibition was attenuated by the presence of alternate substrates (alternative active site ligands) but not by a nucleophilic trapping agent (glutathione) or a reactive oxygen scavenger (catalase), which further supported a mechanism-based action. Substrate depletion partition ratios of 299 and 985 were calculated for the inactivation of CYP1A2 and CYP3A4, respectively.

Conclusions: Our results indicated that C-1311 is a potent mechanism-based inactivator of CYP1A2 and CYP3A4. This finding provided new insights into the mechanism of C-1311 antitumor action, particularly in relation to potential pharmacokinetic drug-drug interactions between C-1311 and/or its derivatives and the substrates of CYP isoforms.

Keywords: antitumor agent, C-1311, drug-drug interactions, cytochrome P450 isoenzymes, mechanism-based inhibition

Introduction

Imidazoacridinones are a novel class of antitumor agents developed in our department [1-2]. 5-Diethylaminoethylamino-8-hydroxyimidazoacridinone (C-1311) (Fig. 1) is the most active compound in this group. As an inhibitor of both topoisomerases and certain receptor kinases, including FMS-like tyrosine kinase FLT3 [3,4], C-1311 has shown activity against experimental models of murine and colorectal cancer *in vitro* and in animals [5]. In phase I and II of clinical trials, C-1311 exhibited activity against advanced solid tumors, and it was effective in women with metastatic breast cancer [6,7]. It was also tested for the treatment of autoimmune diseases. Moreover, C-1311 in combination with paclitaxel was shown to be efficacious against human bladder cancer in the *in vivo* hollow fiber assay [8]. Unlike other antitumor agents, C-1311 expresses only limited mutagenic potential and has a low potency to generate oxygen free radicals, which suggests that it has reduced cardiotoxic properties [9]. Cellular uptake of this agent occurs rapidly, and most of the drug accumulates in the nucleus, which is believed to enable its myeloperoxidase-mediated metabolism and fast interaction with DNA [10].

Multiple studies on the molecular mode of C-1311 antitumor action revealed that the metabolic activation of this drug by intracellular enzymes might be a prerequisite step in the biochemical mechanism of its action [10]. Activation under enzymatic oxidative conditions resulted in the intercalation and the following covalent binding of the drug to DNA and other cellular macromolecules [11]. Thus, studies on the molecular mechanism of the enzymatic oxidative activation of C-1311 with different liver drug-metabolizing enzymes were investigated. We showed that under *in vitro* conditions C-1311 underwent metabolic transformations in the presence of rat and human liver microsomes [12]. Thus, C-1311 was a good substrate for microsomal and the selected human recombinant flavin-containing monooxygenases [13] and UDP-glucuronosyltransferases [14]. In contrast, no products of C-1311 were observed with any tested human recombinant cytochrome P450 (CYP) isoenzymes, which should be emphasized. However, it was demonstrated that CYP1A2 and CYP3A4 were significantly inhibited by C-1311, but no inhibition was observed in the case of

CYP2C19 and CYP2D6 [13]. Nevertheless, these studies were rather limited in scope, and no attempt was made to elucidate the type and the mechanism of the observed inhibition.

Many drugs that are substrates of CYP1A2 and CYP3A4 can be the modulators of their catalytic activity and consequently might have a strong potential to influence the metabolic transformation of other therapeutics. Thus, undesirable drug-drug interactions may result in problems of clinical significance during multidrug therapy. A number of substances with structural similarities to C-1311 (*i.e.*, compounds containing an imidazole and dialkylaminoalkylamino functional groups) were shown to be irreversible inhibitors of CYPs [15-17]. It was believed that they formed a reactive intermediate (RI) that could irreversibly modify critical active site moieties and thus inactivate the enzyme in a process characterized as mechanism-based inactivation (MBI) [18]. Thus, it was reasonable to expect that such interactions between C-1311 and CYPs may also occur.

Considering the above, the aim of the present study was to evaluate the interactions between C-1311 and human CYPs. We intended to identify the mechanism(s) of C-1311-mediated CYP1A2 and CYP3A4 enzyme inhibition, and we particularly intended to know whether C-1311-CYP interaction is MBI. To verify this hypothesis, we used a two-step incubation scheme to measure the effects of C-1311 on the following CYP-specific reactions: 7-ethoxycoumarin O-deethylation (CYP1A2) and testosterone 6β-hydroxylation (CYP3A4) [19,20]. The elucidation of the mechanism of CYPs' inhibition by C-1311 may help to evaluate the role of C-1311-CYP interactions in the antitumor action of this drug, particularly considering that the metabolism of C-1311 by CYPs was not observed.

Materials and methods

Chemicals and enzymes

An imidazoacridinone derivative, C-1311, was synthesized at the Gdańsk University of Technology [1]. Catalase (CAT; from bovine liver), 7-ethoxycoumarin (7EC), L-glutathione reduced (GSH), potassium ferricyanide ($K_3[Fe(CN)_6]$), and testosterone (TT) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Methanol (gradient grade for liquid chromatography) and NADPH were obtained from Merck KGaA (Darmstadt, Germany). Ammonium formate was from Fisher Scientific (Loughborough, UK). All other chemicals and solvents were of the highest purity available. The human recombinant CYP1A2 and CYP3A4 isoenzymes, co-expressed with human NADPH-cytochrome P450 oxidoreductase in *Escherichia coli* cells (Bactosomes), were purchased from Tebu-bio (Le Perray-En-Yvelines, France).

Enzyme inactivation assays

The activities of CYPs were studied by measuring the rate of the CYP-specific reactions, 7-ethoxycoumarin O-deethylation (CYP1A2) and testosterone 6β-hydroxylation (CYP3A4).

The inactivation experiments involved two steps: a primary (preincubation) reaction with the inhibitor and a secondary (incubation) reaction with the substrate (Fig. 2). The preincubation mixtures consisted of CYP (1 μM) and increasing concentrations of C-1311 (0.05, 0.1, 0.2, 0.5, and 1 mM) in 0.1 M potassium phosphate buffer, pH 7.4 (assay buffer). These primary reaction mixtures were preincubated at 37°C for 3 min and were initiated by adding NADPH (1 mM). Negative control incubations lacked NADPH. After incubation at 37°C for the times indicated, the secondary reactions were started by transferring 10 μl of the primary reaction mixtures to 90 μl of assay buffer containing 7-ethoxycoumarin or testosterone (0.02 mM) and NADPH (0.5 mM). Incubations were conducted at 37°C for 30 min, and the reactions were terminated by adding ice-cold methanol (1:1, v/v). The incubation mixtures were placed in ice for 10 min and centrifuged for 5 min at 10000 x g.

Concentrations of CYP-specific substrates and their metabolites, formed in Bactosomes, were assessed by the HPLC method with UV-Vis detection, as described previously [13].

Effects of a competing CYP-specific substrate on CYP1A2 and CYP3A4 inactivation

CYP1A2 or CYP3A4 inactivation (0.5 μM) by C-1311 (0.05 mM for CYP1A2, 0.2 mM for CYP3A4) in the absence and presence of a CYP-specific substrate was investigated by adding a 2- and 4-fold molar excess of 7EC or TT, respectively, over C-1311 to the primary reaction mixture, which was initiated by adding NADPH (1 mM). At the end of the incubation time, aliquots in duplicate were removed for the determination of remaining CYP1A2 and CYP3A4 activity as described above.

Effects of GSH and catalase on CYP1A2 and CYP3A4 inactivation

GSH (1, 2, and 5 mM) was included in the primary reaction mixture with CYP1A2 or CYP3A4 (0.5 μM), C-1311 (0.05 mM for CYP1A2, 0.2 mM for CYP3A4), and NADPH (1 mM). Aliquots in triplicate were taken to determine the remaining enzyme activity in the secondary incubations as described above. In control samples, a vehicle was added in place of GSH. In a separate study, CYP1A2 or CYP3A4 was incubated with C-1311 and NADPH in the absence and presence of catalase (1000, 2000, and 5000 U/ml).

Irreversibility of inactivation

The experiment was divided into three parts: the inactivation assay (preincubation), the restoration of activity assay, and the remaining activity assay. The primary reaction mixtures containing CYP1A2 or CYP3A4 (0.5 μM) and C-1311 (0.05 mM for CYP1A2, 0.2 mM for CYP3A4) in the absence and presence of NADPH (1 mM) were preincubated at 37°C for 30 min. An aliquot (10 μl) was removed and added to the restoration of activity plate, which had 50 μl of the potassium ferricyanide (2 mM; assay 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 the secondary incubations as described above.

Partition ratio of inactivation

In this experiment, C-1311 (concentrations range 0.0125-2.5 mM) was added to the primary reaction mixtures containing CYP1A2 or CYP3A4 (0.5 μM). After adding NADPH (1 mM), the reaction mixtures were incubated at 37°C for 30 min (CYP1A2) or 60 min (CYP3A4) to allow the inactivation to reach completion. Negative control incubations lacked NADPH. At

the end of the incubation time, 10 µl aliquots in triplicate were removed from the primary reaction mixtures and assayed for remaining enzyme activity as previously described [21].

Site of metabolism prediction

C-1311 was submitted to MetaSite version 3.0.4 (Molecular Discovery Ltd., Italy) to predict the site(s) of metabolism that are likely to cause MBI. This method is a fully automated computational procedure that considers structural complementarity between the enzyme active site and the ligand and comes up with the most optimal orientation. The site of metabolism is described by a probability index that is a product of similarity between ligand and protein [22].

Statistical analysis

All data were expressed as 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 one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test, and $p \leq 0.05$ was accepted to be statistically significant.

Results

C-1311-mediated CYP1A2 and CYP3A4 inactivation

Time-dependent enzyme inhibition is one of the criteria to determine irreversible or mechanism-based inhibitors [17]. CYP1A2 and CYP3A4 inactivation by C-1311 was studied by monitoring their loss in the 7-ethoxycoumarin O-deethylation and testosterone 6β-hydroxylation activity, respectively, over time. The remaining CYP activities in relation to a negative control (without NADPH) were measured. As shown in Fig. 3A-B, C-1311 caused time- and concentration-dependent CYP1A2 and CYP3A4 inactivation. The remaining enzyme activities at the time of 0 min or at the C-1311 concentration of 0 mM were normalized to 100% to facilitate a visual comparison of the rates of inactivation at each time point or concentration. The potential of C-1311 to inhibit CYP1A2 activity was much higher than that of CYP3A4. The inactivation of CYP1A2 progressed rapidly with increasing preincubation time and concentrations of C-1311. In the case of CYP3A4, the observed changes in the enzyme activity were gradual.

NADPH-dependent inactivation

In order to determine whether metabolic biotransformation of C-1311 is required for enzyme inactivation, we performed experiments in which the appropriate CYP was incubated with C-1311 in the presence or absence of NADPH in the primary reaction mixtures. We observed a progressive loss of CYP1A2 (CYP3A4) activity as a function of time (Fig. 3A-B). Approximately 57±3.1% of CYP1A2 activity was lost after 10 min, and only 9±1.6% remained after 60 min. However, approximately 35±1.8% of CYP3A4 activity was lost after 20 min and 60±4.7% after 120 min of preincubation. These results suggested that NADPH-dependent activation of C-1311 to RI by cytochrome CYP1A2 or CYP3A4 was required for enzyme inactivation, though in the absence of NADPH a low level of inhibition was still observed.

Effects of a competing CYP-specific substrate on CYP1A2 and CYP3A4 inactivation

In the next step, we studied whether inactivation occurred in the active site of the enzyme. Thus, CYP1A2 (CYP3A4) was incubated with C-1311 simultaneously in the presence of a 2- or 4-fold molar excess of a CYP-specific substrate, 7-ethoxycoumarin or testosterone, over C-

1311 in the primary reaction mixture. Both alternate substrates reduced the ability of C-1311 to inactivate CYP1A2 or CYP3A4 in a time- and concentration-dependent manner (Fig. 4). The remaining CYP1A2 activity in samples co-incubated with C-1311 and 7EC was 46±5.8% and 61±6.5% higher at 10 and 30 min, respectively, compared to samples containing C-1311 and lacked 7EC. The remaining CYP3A4 activity in samples that had C-1311 co-incubated with TT increased 31±0.8% and 37±2.5% at 10 and 30 min, respectively, compared to testosterone-free incubations. These results indicated that 7EC and TT compete with C-1311 for metabolism by CYP1A2 or CYP3A4 (respectively), and they thereby protect against C-1311-mediated inactivation. They also suggested that the inactivation of CYP proceeded via the attachment of C-1311 RI to the active site.

Effects of GSH, catalase, and potassium ferricyanide on CYP1A2 and CYP3A4 inactivation

MBI is the case when the inactivating compound is catalytically transformed to a RI in order to inactivate the enzyme without leaving the active site. Inactivation is mechanism-based when the rate of inactivation is not reduced in the presence of exogenous scavenger nucleophiles, such as glutathione. Therefore, in order to determine the protective effect of GSH against the enzymatic inactivation caused by C-1311, the CYP1A2 or CYP3A4 inactivation incubations were performed in the presence or the absence of GSH (1, 2, and 5 mM). Fig. 5A shows that GSH had no effect on the rate of CYP1A2 inactivation. GSH alone did not reduce the activity of CYP1A2 (data not shown). A similar lack of protective effect was observed when catalase (1000, 2000, and 5000 U/ml), a scavenger of reactive oxygen species, was included in the primary incubation mixture with C-1311 (Fig. 5B). In turn, the presence of GSH resulted in about a 30% increase in the CYP3A4 activity remaining compared to the activity of the GSH-free incubation (Fig. 5A). Similarly, CAT only had a very little (~20%) protective effect against CYP3A4 inactivation by C-1311 (Fig. 5B). The assay to determine whether the catalytic function of CYP could be restored after oxidation by potassium ferricyanide was also performed. As expected, the addition of potassium ferricyanide to the inactivation mixture did not reverse the enzymes' inactivation significantly (Table 1).

Determination of the partition ratio

The partition ratio P, the average number of cycles of metabolism that the enzyme traverses before it is inactivated, was estimated graphically using the method described in [21]. CYP1A2 (CYP3A4) was incubated with various concentrations of C-1311, and the inactivation was allowed to progress for 30 min (CYP1A2) or 60 min (CYP3A4), until it was essentially complete. The percentage of remaining activity was plotted as a function of the molar ratio of C-1311 to CYP. The turnover number (P+1) was estimated from the intercept of the linear regression line obtained from lower ratios of C-1311 to CYP and with the straight line derived from the higher ratios of C-1311 to CYP. With this method, we estimated the turnover numbers of ~300 (CYP1A2) and ~986 (CYP3A4) (Fig. 6), and, consequently, the Ps were ~299 and ~985, respectively.

Site of metabolism prediction

The MetaSite program has been demonstrated to be able to predict the likely site metabolism within the top three predictions in 80% of the cases in structurally diverse compounds [22]. This program predicted some substructures in the C-1311 molecule that are supposed to associate with MBI, where they are metabolized to RIs that bind irreversibly to the enzyme (Fig. 7). The diethyloamino functional group was singled out as the top ranked site of compound metabolism with cytochrome CYP1A2 and CYP3A4. MetaSite also predicted the 2N on the imidazole ring and the 3C on the phenyl group of acridinone as sites of metabolism of C-1311 with the CYP3A4 isoenzyme in the top three rankings.

Discussion

Our previous studies on *in vitro* metabolism of C-1311 demonstrated that human recombinant CYPs do not give any metabolic products of this compound. In contrast, C-1311 was shown to inhibit the catalytic activity of human recombinant and microsomal CYP1A2 and CYP3A4 [13], but the actual mechanism of the inhibition was not assessed. These results suggest the existence of a catalysis-dependent inactivation of CYP upon oxidation of C-1311 to the RI. In accordance, the 8-hydroxyl group of C-1311 participated in peroxidase-mediated metabolism, which produced RIs susceptible to substitution in the presence of cellular nucleophiles [23]. The studies presented here intended to provide new data that would support our hypothesis concerning specific, mechanism-based inhibition of the C-1311 oxidative metabolism by CYPs. Thus, interaction specificity between representative drug-metabolism CYPs, 1A2 and 3A4 [24], and compound C-1311 was investigated.

The obtained results revealed that C-1311-mediated CYP1A2 and CYP3A4 inhibition was time- and concentration-dependent, and that it also required NADPH, which strongly suggests that the inhibition proceeds via a catalytic step(s) (Fig. 3). However, 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 the final observed inhibition effect. In turn, the decrease in inactivation obtained in the presence of a CYP-specific substrate, 7-ethoxycoumarin or testosterone (Fig. 4), indicates the transformation of C-1311 to RI, what is followed by its covalent binding to the active site of the enzyme. However, testosterone partially protected CYP3A4 against inactivation, presumably by competing with C-1311 for the binding site of the enzyme. Further evidence for the involvement of the CYP active site in the metabolic activation of C-1311 was provided by the observation that inactivation was not protected by a trapping agent such as glutathione (exogenous nucleophile) (Fig. 5A). The lack of GSH effect on the rate of inactivation of CYPs suggests that the RI(s), once it has been generated, does not leave the active site.

Furthermore, the addition of catalase did not prevent inactivation, suggesting that reactive oxygen species (hydrogen peroxides) were not involved in the inactivation event (Fig. 5B). We also considered that mechanism-based enzyme inactivators are often irreversible inhibitors. Irreversibility of inactivation was evaluated here, next to dilution method, by oxidation with potassium ferricyanide. Activities of both CYP isoforms could not be recovered by potassium ferricyanide, what indicated the irreversible nature of inhibition (Table 1). In sum, the above observations are largely consistent with the criteria for a mechanism-based inhibitor [17]. The MBI of CYPs was known earlier for several therapeutic drugs, including the chemotherapeutic alkylating agent thioTEPA [25], a signal transduction inhibitor, gefitinib [26], and a histamine H1 antagonist, meclizine [27].

The partition ratio is a measure of the efficiency of the mechanism-based inactivator. We demonstrated that C-1311 is a stronger inactivator of CYP1A2 than CYP3A4, with estimated P values of 299 and 985, respectively (Fig. 6). This finding allows us to presume that C-1311-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, the active site of CYP3A4 is very flexible 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 [28]. It's also possible that, aside from the main mechanism of MBI, additional interactions between C-1311 and CYP3A4 can influence the observed inhibition effect.

The precise mechanism by which C-1311 is transformed by CYP1A2 or CYP3A4 into a RI, which inactivates the enzyme, remains unknown. The intermediate species could react with heme nucleophiles or with apoprotein in a manner that destroys heme function or binding [17]. Considering the known chemical structures of imidazoacridinone metabolites and

MetaSite proposals of reactive substructures in a C-1311 molecule, we can suggest three possible sequences of events leading to C-1311-mediated CYP1A2 and CYP3A4 inactivation, which are shown in Fig. 8.

First, compounds containing a dialkylaminoalkyl moiety, such as C-1311, very often cause MBI of CYPs. The proposed mechanism assumes that the nitroso derivative, which is the final product of N-dealkylation, oxidation, and hydrolysis catalyzed by cytochrome P450, creates a coordination complex with the Fe(II) ion of the enzyme prosthetic group. This type of complex, known as a metabolic intermediate complex (MIC), was proposed earlier as the intermediate product of catalysis with CYP1A2 for amiodarone [29] or amitriptyline [30], and with CYP3A4 for verapamil [31] or tamoxifen [32]. The possibility of dissolution of this adduct by treatment with potassium ferricyanide indicates a quasi-irreversible inhibition. The obtained results did not confirm the occurrence of this effect. Thus, we suppose that within the diethylaminoethylamino group of C-1311, metabolic activation gives RI, which probably inhibits the enzyme activity in an irreversible, not a quasi-irreversible, manner, but the formation of the MIC does not occur. On the other hand, following the results obtained for diltiazem, a dimethylamine, and nordiltiazem, a monomethylamine [33], it can be speculated that a possible cause of C-1311-mediated CYP inactivation may be a reactive iminium ion intermediate (**3**). It can be generated from the condensation of a secondary alkylamine (**1**) (a product of cytochrome CYP-mediated N-deethylation of C-1311) with an acetaldehyde molecule, a side product of the N-deethylation reaction (**2**) (Fig. 8, pathway A). Such a mechanism has not been well understood yet, but in our opinion it is more likely than a classic mechanism involving the nitroso derivative.

The second possibility of events assumes that C-1311-mediated inactivation of CYP1A2 and CYP3A4 may be initiated by aromatic oxidation on the phenyl ring leading to an unstable epoxide intermediate (**4**) (Fig. 8, pathway B). The resulting epoxide metabolite may be further transformed to diol (**5**) and *ortho*-quinone (**6**) derivatives [Potęga, unpublished results]. All of them are electrophiles and may potentially be implicated in the covalent apoprotein modification of CYP enzymes [34].

Finally, imidazole-containing compounds may also inhibit the activity of CYPs in a reversible manner. The major pathway reported for the bioactivation of imidazole derivatives, such as pyrimidineimidazole [18,19] and azole antifungal drugs like ketoconazole and clotrimazole, assumes the coordination of an imidazole nucleophilic nitrogen atom (N2 in C-1311 molecule) with the heme iron of ferrous CYP, which induces a type II spectral shift [35] (Fig. 8, pathway C). This type of inhibition, next to a MBI mechanism, is quite possible in the case of the observed CYP3A4 inactivation by C-1311, particularly because it would explain the partial recovery of enzyme activity in the presence of GSH or catalase.

In conclusion, to the best of our knowledge, this report presents the first description that C-1311 is enzymatically activated by CYP1A2 and CYP3A4, and it is characterized as a highly potent, irreversible inhibitor of these two human CYPs. We provided strong evidence that the inhibition involves a mechanism-based process, which results rather in the alkylation of the apoprotein than in MIC formation. In addition, it is possible that RI(s) generated from C-1311 would attack other cellular proteins. This work opens new avenues for understanding the mechanism of bioactivation and cytotoxicity induced by the anticancer compound C-1311. Our findings indicate that pharmacokinetic interactions between C-1311 and the substrates of CYP1A2 or CYP3A4 are likely to occur, which should be carefully considered in optimal chemotherapy schedules designed for individual patients.

Conflict of interest

The authors declare no conflict of interest.

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Figure captions

Figure 1

Chemical structure of antitumor imidazoacridinone C-1311.

Figure 2

Scheme of inactivation experiments.

Figure 3

Effect of C-1311 concentration, preincubation time, and NADPH on (**A**) CYP1A2 and (**B**) CYP3A4 activity. Values are expressed as the mean ± SD of duplicate experiments. The remaining CYP activities at time 0 min or at C-1311 concentration 0 mM were normalized to 100%. Comparisons were made using one-way ANOVA, followed by Bonferroni's multiple comparisons test, or an unpaired *t*-test. *(•) Indicates the significance of differences between results at different C-1311 concentration or at different preincubation time and control results at C-1311 concentration 0 mM or at preincubation time 0 min in the absence or presence of NADPH in the preincubation step**.** ◦ Indicates the significance of differences between results at the given C-1311 concentration or at the given preincubation time with added NADPH and control results with lacked NADPH in the preincubation step. Levels were considered significant at ****(••••, ◦◦◦◦) $p<0.0001$, ***(•••, ◦◦◦) $p<0.001$, **(••, ◦◦) $p<0.01$, and *(•, ◦) $p<0.05$; ns, not significant.

Figure 4

Substrate protection against (**A**) CYP1A2 and (**B**) CYP3A4 inactivation by C-1311. The concentration of NADPH in the primary reaction mixture was 1 mM. Each point represents the mean ± SD of duplicate experiments.

Figure 5

Effects of (**A**) glutathione (GSH) and (**B**) catalase (CAT) on C-1311-mediated CYP1A2 and CYP3A4 inactivation. Values are expressed as the mean ± SD of triplicate experiments. Comparisons were made using one-way ANOVA followed by Bonferroni's multiple comparisons test . *(•) Indicates the significance of differences between +/-GSH (or +/-CAT) results with added NADPH and control results with lacked NADPH in the preincubation step. ◦ Indicates the significance of differences between +GSH (or +CAT) results and -GSH (or -CAT) control results with added NADPH in the preincubation step. Levels were considered significant at ****(••••, ◦◦◦◦) $p<0.0001$, and ***(•••) $p<0.001$, and not significant at $^{ns}p\geq0.05$.

Figure 6

Loss of (**A**) CYP 1A2 and (**B**) 3A4 activity as a function of the [C-1311]/[CYP] molar ratio. 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. Each point represents the mean ± SD of triplicate experiments.

Figure 7

Site of metabolism prediction in C-1311. The top three sites of metabolism predicted by MetaSite are indicated by arrows, with the position ranked first circled.

Figure 8

Proposed potential mechanisms for the oxidation of C-1311 leading to irreversible CYP1A2 and CYP3A4 inactivation. MBI: mechanism-based inactivation.

Tables

Table 1

Effect of potassium ferricyanide on C-1311-mediated CYP1A2 and CYP3A4 inactivation. Values are expressed as the mean ± SD of triplicate experiments. Comparisons were made using an unpaired *t*-test. * Indicates the significance of differences between control experiments with C-1311 and +/-NADPH in the preincubation step for the same CYP and those with added or lacked potassium ferricyanide. Levels were considered significant at ***$p<0.001$ and *$p<0.05$; ns, not significant.

	% Remaining CYP activity	
	CYP1A2	**CYP3A4**
-$K_3[Fe(CN)_6]$ -NADPH	99±6.5	103±5.6
+$K_3[Fe(CN)_6]$ -NADPH	93	98±9.7
+$K_3[Fe(CN)_6]$ +NADPH	66±8.5	59±1.3
-$K_3[Fe(CN)_6]$ +NADPH	69±8.4	56±2.1

Figure 1

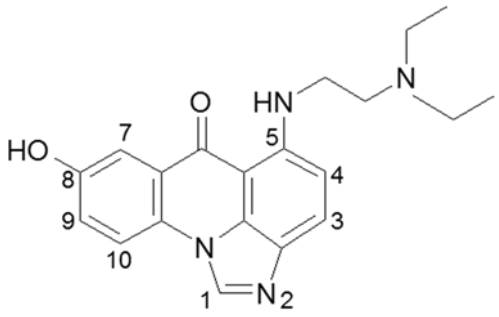

Figure 2

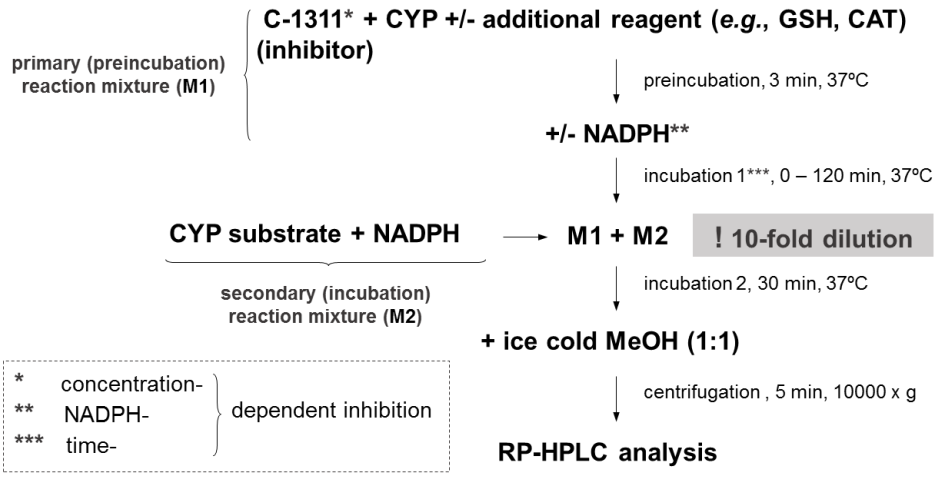

Figure 3

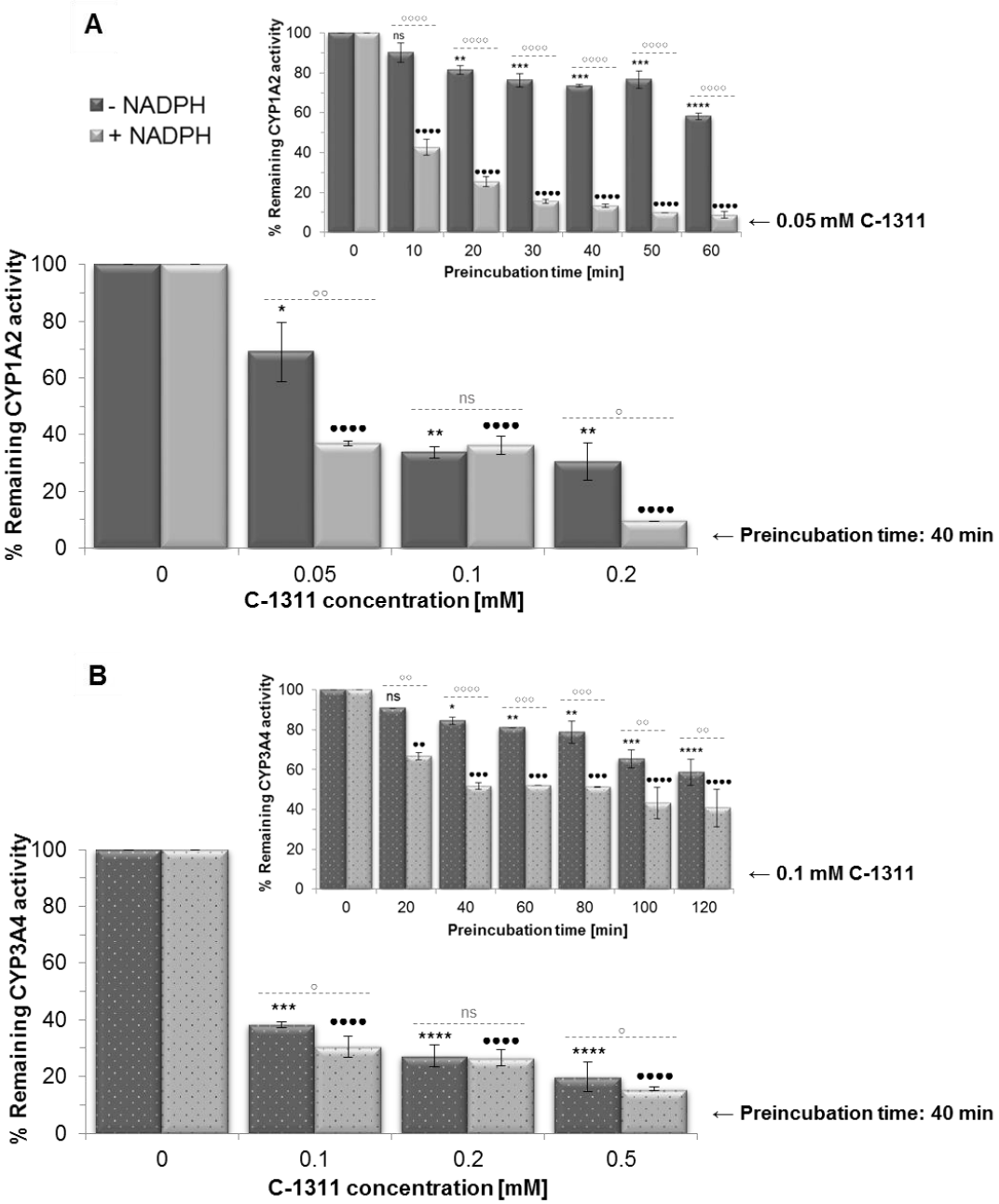

Figure 4

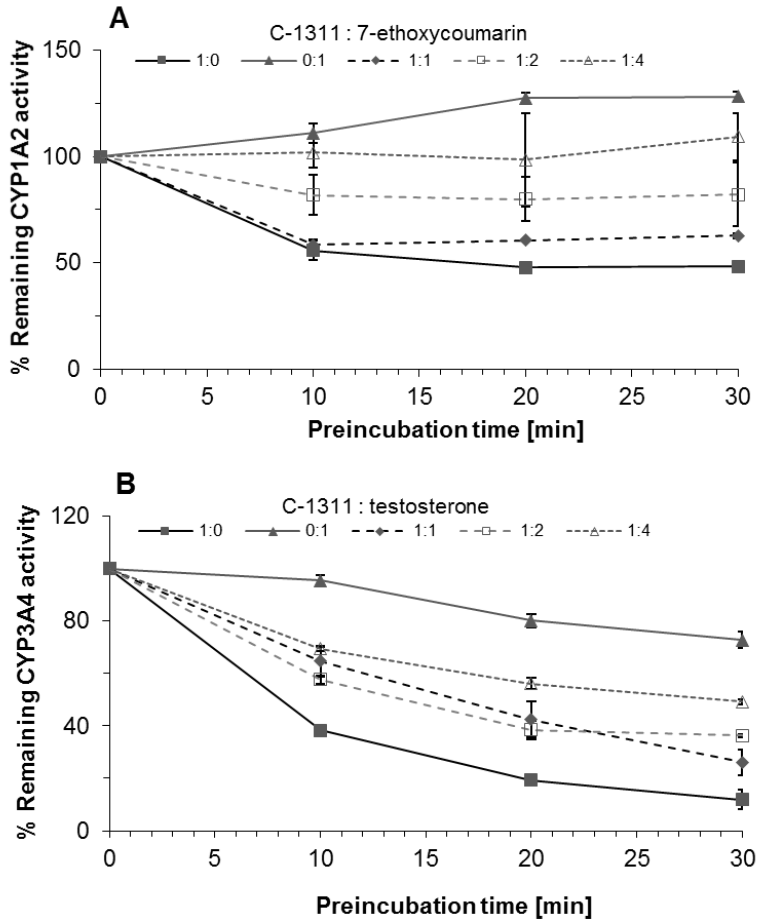

Figure 5

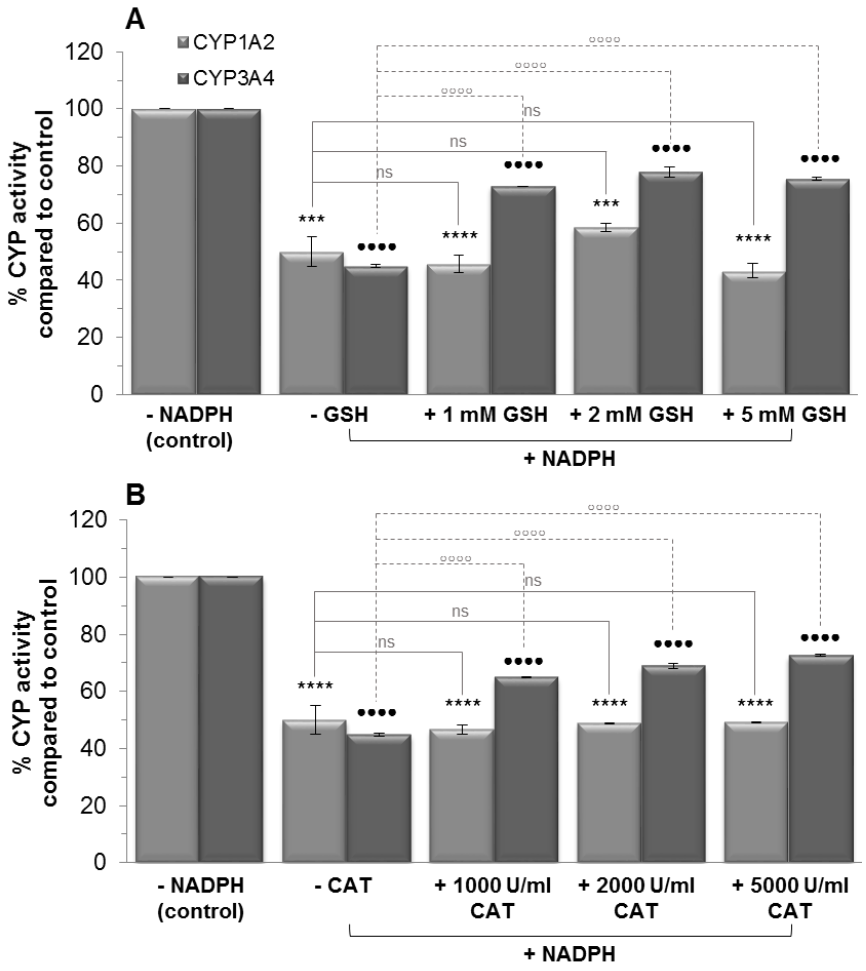

Figure 6

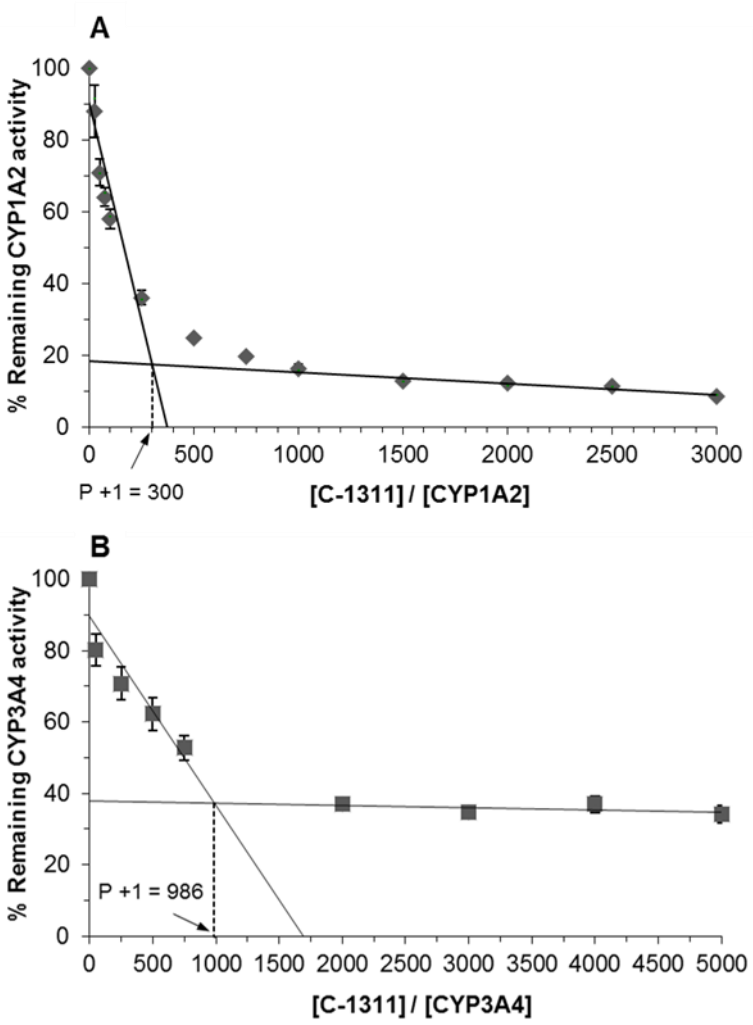

Figure 7

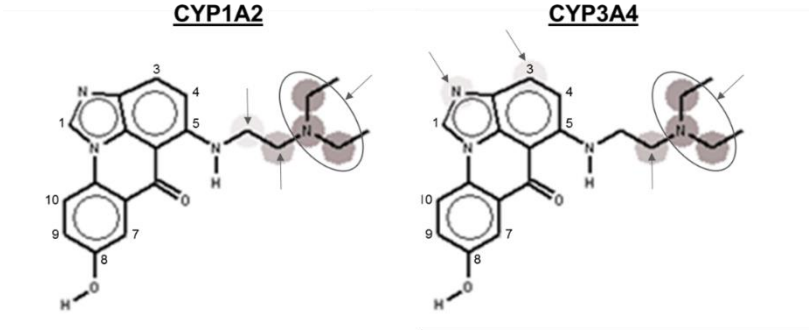

Figure 8