

Metabolic transformations of antitumor imidazoacridinone, C-1311, with microsomal fractions of rat and human liver

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The imidazoacridinone derivative C-1311 is an antitumor agent in Phase II clinical trials. The molecular mechanism of enzymatic oxidation of this compound in a peroxidase model system was reported earlier. The present studies were performed to elucidate the role of rat and human liver enzymes in metabolic transformations of this drug. C-1311 was incubated with different fractions of liver cells and the reaction mixtures were analyzed by RP-HPLC. We showed that the drug was more sensitive to metabolism with microsomes than with cytosol or S9 fraction of rat liver cells. Incubation of C-1311 with microsomes revealed the presence of four metabolites. Their structures were identified as dealkylation product, M0, as well as a dimer-like molecule, M1. Furthermore, we speculate that the hydroxyl group was most likely substituted in metabolite M3. It is of note that a higher rate of transformation was observed for rat than for human microsomes. However, the differences in metabolite amounts were specific for each metabolite. The reactivity of C-1311 with rat microsomes overexpressing P450 isoenzymes, of CYP3A and CYP4A families was higher than that with CYP1A and CYP2B. Moreover, the M1 metabolite was selectively formed with CYP3A, whereas M3 with CYP4A. In conclusion, this study revealed that C-1311 varied in susceptibility to metabolic transformation in rat and human cells and showed selectivity in the metabolism with P450 isoenzymes. The obtained results could be useful for preparing the schedule of individual directed therapy with C-1311 in future patients.

Keywords: antitumor drug, C-1311, imidazoacridinone, *in vitro* metabolism, microsomes, P450 isoenzymes, Symadex

INTRODUCTION

5-Diethylaminoethylamino-8-hydroxyimidazoacridinone, C-1311 (Symadex^R), Fig. 1, is an antitumor agent developed in our Department (Cholody *et al.*, 1990; 1992; 1996). It is currently undergoing Phase II clinical trials for colon and breast cancers. Unlike other antitumor agents, C-1311 expresses only limited mutagenic potential (Berger *et al.*, 1996) and low potency to generate oxygen free radicals, which suggests a lack of cardiotoxic properties. Cellular uptake of this agent occurs rapidly (Burger *et al.*, 1996) and most of the drug accumulates in the

nucleus (Składanowski *et al.*, 1996), which enables its fast interaction with DNA.

Previous studies on the biological action of C-1311 showed that at low doses this drug induced arrest of the cell cycle progression in the G2 phase and subsequent apoptosis of murine leukemia L1210 cells (Augustin *et al.*, 1996; Lamb & Wheatley, 1996). In ovarian and osteogenic sarcoma cells, G2 arrest resulted only in low level of apoptosis (Zaffaroni *et al.*, 2001), while in human colon carcinoma HT-29 cells, drug-treated cells after initial G2 arrest progressed into mitosis but were unable to undergo cytokinesis and died in a process resembling mitotic catastrophe (Hyzy *et al.*, 2005).

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Abbreviation: CYP, cytochrome P450 isoenzyme

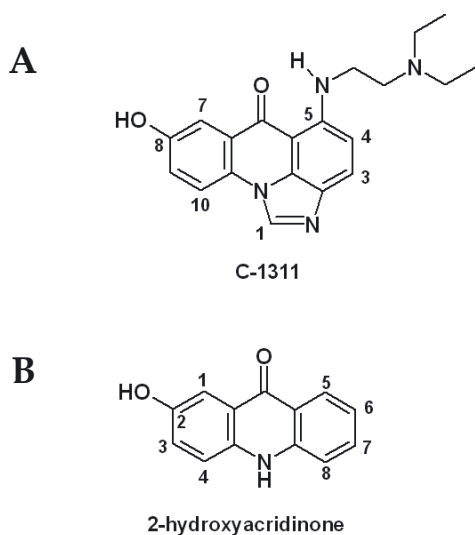


Figure 1. Chemical structure of C-1311 antitumor agent (Symadex^R) (A) and its reference compound 2-hydroxyacridinone (B)

Studies on the molecular mechanism of the antitumor action of C-1311 indicate that C-1311 traps the cleavable complexes between DNA and topoisomerase II (Skladanowski *et al.*, 1996) and intercalates into DNA (Dziegielewski *et al.*, 2002). Under oxidative enzymatic conditions, intercalation of C-1311 into DNA is followed by peroxidase-mediated activation of the drug, giving rise to intercalated species that might irreversibly bind to DNA (Dziegielewski & Konopa, 1998; Mazerska *et al.*, 2001). Therefore, it was postulated that not only the intercalation but also the covalent binding to DNA preceded by metabolic activation of C-1311, were significant steps in the biochemical mechanism of its action. Consequently, the molecular mechanism of the enzymatic oxidative activation of this drug was investigated in the same model system for which the covalent binding to DNA was demonstrated (Mazerska *et al.*, 2003). Structural studies of the obtained metabolites showed that two of them were products of dealkylation of amino groups of the side chain, whereas the next two were identified as dimers.

All the above described elements of the molecular mechanism of the antitumor action determined for C-1311 in an enzymatic model system were expected to take place also in the cellular environment *in vivo*. For that reason the subject of the present work is the metabolic transformation of the studied drug with a range of human and rat fractions of liver enzymes. We aimed to examine the specificity of the enzymatic transformation of C-1311 with human and rat liver protein fractions as well as with microsomal fractions characterized by various levels of selected families of cytochrome P450 isoenzymes. Comparison of the transformation rates

and of the amount of selected metabolites is presented and metabolite structures are proposed. The obtained results will allow the prediction of metabolic pathways of C-1311 in future patients differing in the level of cytochrome P450 isoenzymes and, in turn, will help to design directed individual antitumor therapy with this drug.

MATERIALS AND METHODS

Chemicals. C-1311 was synthesized in the Department of Pharmaceutical Technology and Biochemistry, Gdańsk University of Technology, as described earlier (Cholody *et al.*, 1990). The following chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA): NADPH, MgCl₂, and EDTA. Amonium formate, mono- and disodium phosphate (AnalaR), HPLC grade, were from BDH Ltd (Poole, England). Formic acid and methanol HPLC grade were obtained from Lab-Scan Analytical Sciences (Dublin, Ireland). All chemicals were used without further purifications. Ultra pure water, 18 MΩ, used in all experiments, was obtained with NANOPure water system. Phosphate buffer contained 0.05 M Na₂HPO₄, 1 mM EDTA and 3 mM MgCl₂, pH 7.4. Stock solutions of NADPH (20 mM) and C-1311 (2 mM) were freshly prepared with water or phosphate buffer.

Enzymes. The following protein fractions were purchased from TEBU-Bio (Paris, France): rat liver cellular enzyme fraction, S9, R1000.S9; rat liver cytosol, R1000.C; rat liver microsomes, R1000; human liver microsomes, H0610; rat liver microsomes phenobarbital-treated, R1078; rat liver microsomes 3-methylcholanthrene-treated, R1084; rat liver microsomes clofibrac acid-treated, R1063, and rat liver microsomes dexamethasone-treated, R1078.

Metabolic transformations with various fractions of liver enzymes. Incubation of C-1311 with the liver enzyme fractions listed above was carried out in 0.05 M phosphate buffer, pH 7.4. The incubation mixture consisted of 2×10^{-3} M NADPH, enzyme protein (2 mg/ml) and 2 or 1×10^{-4} M C-1311. The incubation was carried out at 37°C in air. Preincubation of proteins and NADPH for 5 min was followed by addition of the drug. After appropriate period of time the incubation mixture was placed in ice and equal volume of ice-cold methanol was added. Then the solution was centrifuged for 15 min at $12\,000 \times g$. The supernatant was analyzed directly by HPLC/UV/Vis and HPLC/ESI-MS. All experiments were performed in triplicates and two options were applied for the result presentation (i) standard deviations of the HPLC peak height were marked in the figures or (ii) results representative for three independent experiments were shown.

HPLC analysis of the reaction mixtures. The supernatant obtained according to the procedure described above was analyzed by a reversed phase 5 μm Suplex pKb100 analytical (0.4×25 cm) or semi-preparative (1×25 cm) column (Supelco, Bellefonte, PA, USA) with Waters Associates HPLC systems. One system was equipped with a model 600K system controller, Rheodyne injector 7725i and 996 UV-Vis multidiode array detector controlled with Millennium software. The second Waters HPLC system contained: binary pump 1535, Rheodyne injector 7725i and dual lambda absorbance detector 2487 controlled with Breeze software. The HPLC analyses were carried out at a flow rate of $1 \text{ mL} \times \text{min}^{-1}$ with the following eluent system: a linear gradient from 15 to 80% methanol in ammonium formate (0.05 M, pH 3.2) for 25 min, followed by a linear gradient from 80 to 100% methanol in ammonium formate for 3 min.

HPLC/ESI-MS analyses of the products were carried out by electrospray ionization with positive ion detection performed on an LC/MSD/Agilent 1100 mass spectrometer.

RESULTS

Metabolism with different enzyme fractions of rat liver cells

Studies on the metabolism of C-1311 were carried out with liver protein S9 fraction, cytosol and microsomes. The reactions were performed under a wide range of incubation conditions and were monitored by RP-HPLC. The rates of substrate transformations were calculated as the ratio of the substrate HPLC peak height after a given time of incubation to the initial peak height of the substrate. A comparison of the reactivity of C-1311 observed after incubation with different liver fractions as well as of the amounts of the main metabolic products are presented in Figs. 2 and 3, respectively. The diagrams in these figures revealed that the rate of metabolism with microsomal enzymes was significantly higher than that with cytosol and S9 fraction. Similarly, the amounts of the main metabolite were the highest with microsomes (not shown), particularly after shorter incubations.

Considering the above results, HPLC analyses of the reaction mixture obtained after incubation of C-1311 with rat liver microsomes are presented in Fig. 3. One can notice that four metabolites resulted from the reaction. Three of the chromatographic peaks related to metabolites M1, M2 and M4 were of significantly lower intensity than the peak of M3. The contour graph revealed that the UV-Vis spectra of the metabolites were not identical to that of the

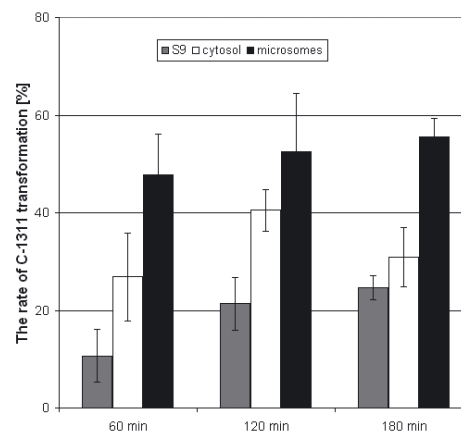


Figure 2. A comparison of C-1311 reactivity with different fractions of rat liver enzymes.

Rates of substrate transformation were calculated as ratio of substrate HPLC peak height, 380 nm, after given time of incubation to initial peak height of the substrate. The incubation mixture contained 0.1 mM C-1311, 2 mM NADPH and 2 mg/ml proteins in 0.05 M phosphate buffer, pH 7.4. Standard deviations were calculated on the basis of three independent experiments.

substrate. This indicates that a modification of the imidazoacridinone moiety occurs in the structures of the metabolites.

Comparison of metabolic rates between rat and human microsomes

Incubation of C-1311 with human liver microsomes gave chromatographic peaks characterized by retention times and UV-Vis spectra identical to those formed with rat microsomes (not shown). Next, the transformation rates observed with human and rat enzymes were compared in Fig. 4A. As shown in this figure, C-1311 was much more susceptible to the action of rat than of human liver enzymes. Furthermore, amounts of the metabolites formed, compared in Fig. 4B, indicated that transformation of C-1311 to products M2 and M3 was more efficient in the presence of rat microsomes. In contrast, the amounts of M1 and M4 metabolites were higher after incubation with human than with rat enzymes.

Chemical structures of metabolites

Optimal chromatographic conditions were determined in order to perform ESI-MS analysis of the metabolites represented by the HPLC peaks. ESI-MS spectra of these metabolic products were performed directly on HPLC fractions. Firstly, we examined the spectrum of the substrate peak. It is presented in Fig. 5. The mass ion m/z 351.1 represents the structure of C-1311. We also found the mass ion, m/z 323.1 of low intensity, which was not observed in



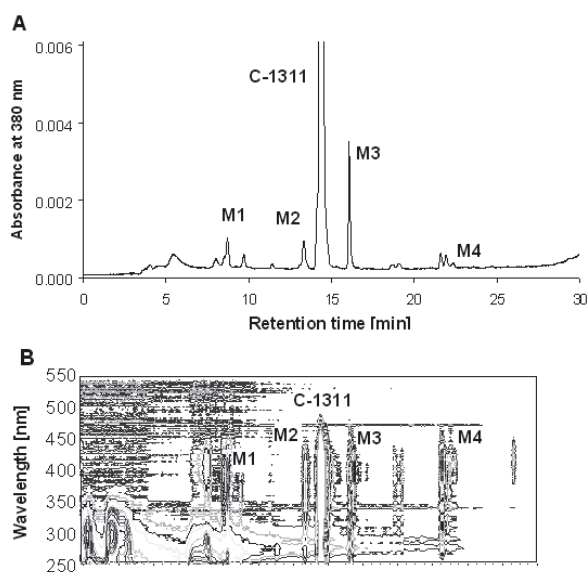


Figure 3. HPLC chromatogram taken after 60 min of incubation of C-1311 with rat liver microsomes.

Panel (A). The chromatogram (reversed-phase, methanol/0.05 M ammonium formate pH 3.2, gradient system) at 380 nm. Panel (B). Contour plots of the chromatogram collected from 250 to 550 nm. The incubation mixture contained 0.2 mM C-1311, 2 mM NADPH and 2 mg/ml proteins in 0.05 M phosphate buffer, pH 7.4.

the MS spectrum of native C-1311. An identical value of m/z was identified earlier for the metabolite C0 formed after incubation of C-1311 with peroxidase (Mazerska *et al.*, 2003). The structure of metabolite C0 determined on the basis of ESI-MS/MS and NMR spectra indicated that it was a product of C-1311 deethylation in the aminoalkyl side chain. Therefore, the mass ion m/z 323.1 that was found in this work demonstrates that the metabolite M0 identical to C0 was present inside the HPLC peak denoted C-1311 in Fig. 3. M0 structure is shown in the inset in Fig. 5.

The HPLC peak M1 contained mass ions identified at m/z 350.1 and 699.3. The ESI-MS spectrum is presented in Fig. 6A. The 699.3 m/z value indicated the dimer structure of this product. On the other hand, this molecular mass value was an even number. Therefore, an even number of nitrogen atoms should be present in the dimer molecule. The mass ion m/z 350.1 was equal to the value of $(M+2)/2$ and resulted from the formation of a double charged molecule. This indicates the presence of two aminoalkyl side chains in the M1 dimer structure. It should be emphasized that the ESI-MS spectrum of C-1311 contained only the mass ion equal to $M+1$, m/z 351.1, but not one equal to m/z 350 or of the double value. Summing up, the M1 metabolite is proposed to be a dimer-like molecule, the two components of which having the structure of the native compound. Dimer-like structures of C-1311 metabolites were

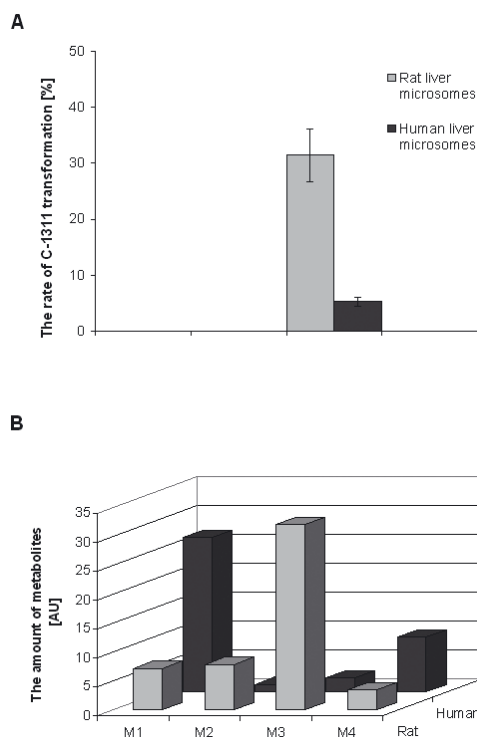


Figure 4. A comparison of C-1311 metabolic profile observed with rat and human liver microsomes.

Panel (A). Transformation rates were calculated as ratio of substrate HPLC peak height after 60 min of incubation to initial peak height of the substrate. Panel (B). Amounts of metabolites M1, M2, M3 and M4 were presented as HPLC peak area after 60 min of incubation. The incubation was performed with 0.2 mM C-1311, 2 mM NADPH and 2 mg/ml proteins in 0.05 M phosphate buffer, pH 7.4. The results are representative of three independent experiments with the mean standard deviations equal to 8.5%.

proven earlier for products formed with peroxidase enzymes (Mazerska *et al.*, 2003). Furthermore, the peroxidase-mediated metabolite (Mazerska, 2003) and the product of electrochemical oxidation (Mazerska *et al.*, 2002) found for the reference compound, 2-hydroxyacridinone (Fig. 1), were also shown to be dimer-like with monomers bound by carbon atoms at *ortho* positions to the hydroxyl group. Identical binding of monomers is proposed for metabolite M1 analyzed in these studies, the structure of which is proposed in the inset of Fig. 6.

The m/z values 308.1; 600.0; 644.2 observed for M2, similarly to m/z 318.1 for M4, were not found earlier in ESI-MS spectrum of any products of C-1311 transformation. The low intensity of M2 and M4 peaks did not allow us to obtain MS/MS or HR-MS spectra that would be useful for comparison with those obtained for the reference products. It was also impossible to synthesize these metabolites with liver enzymes in an amount, which would be sufficient for NMR structural studies.

HPLC peak M3 presented in Fig. 3 is of considerable intensity. Mass spectrum of M3 shown

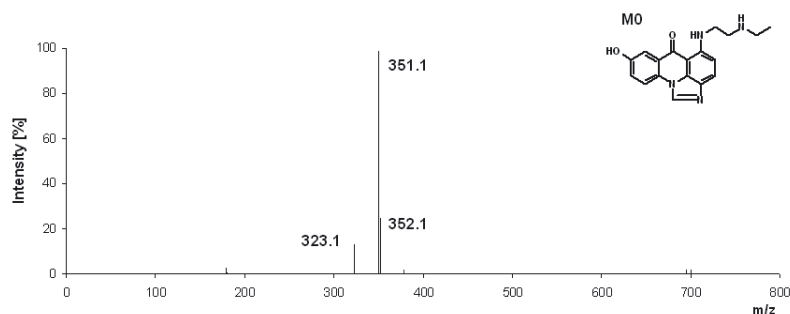


Figure 5. ESI-MS spectrum of chromatographic peak C-1311 and the structure proposed for M0 metabolite, m/z 323.1.

The spectrum was collected during RP-HPLC analysis in methanol/0.05 M ammonium formate pH 3.2, gradient system.

in Fig. 6B contains two main peaks for m/z values equal to 367.1 and 733.3. The 367 m/z value of a metabolite was also found earlier after incubation of C-1311 with murine and human cytosolic enzymes (Calabrese *at al.*, 1999). The m/z value 367.1 is 16 units higher than that of the substrate and was proposed to result from the binding of a hydroxyl group to the imidazoacridinone ring. A similar situation is also possible in relation to the M3 metabolite observed here. However, in our MS experiments, an intensive peak of a mass ion, of double value, m/z 733.3 was also found. In this case the value m/z 367.1 is equal to $(M+2)/2$ and reflects the presence of a double positively charged molecule. This, in turn, indicates the presence of two aminoalkyl side chains in the studied metabolite and allows us to postulate a dimer-like structure for M3.

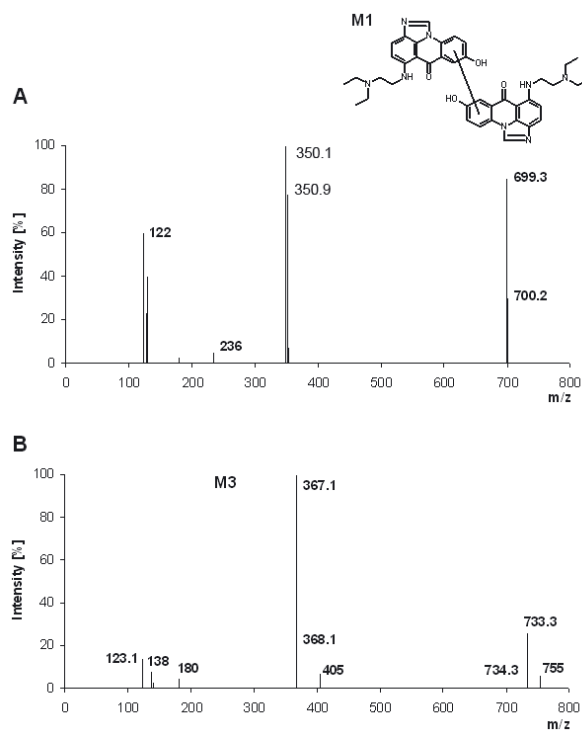


Figure 6. ESI-MS spectra of peaks M1 (A) and M3 (B) collected during RP-HPLC analysis in methanol/0.05 M ammonium formate pH 3.2, gradient system. Proposed M1 structure is presented in the inset.

Metabolism with microsomes overexpressing cytochrome P450 isoenzymes

The imidazoacridinone C-1311 was incubated with four sets of microsomes isolated from rat livers expressing increased levels of cytochrome P450

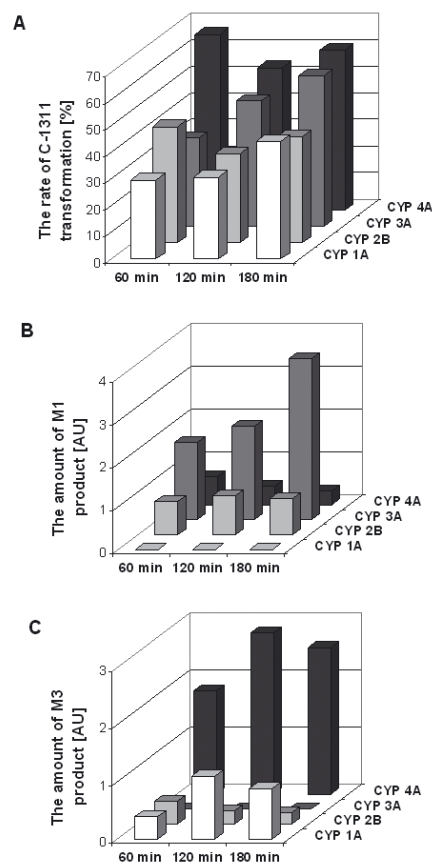


Figure 7. A comparison of C-1311 metabolic profile with microsomes expressing higher level of selected CYP families.

Panel A. Transformation rates of C-1311 calculated as a ratio of substrate HPLC peak height, 380 nm, after given time of incubation to initial substrate peak height. Panel B and C. Amounts of metabolite M1 and M3, respectively, calculated as HPLC peak height at 380 nm. The incubation mixture contained 0.2 mM C-1311, 2 mM NADPH and 2 mg/ml protein in 0.05 M phosphate buffer, pH 7.4. The results are representative of three independent experiments with the mean standard deviation 5.5%.

isoenzymes: CYP1A, CYP2B, CYP3A and CYP4A. The differences in CYP expression levels were obtained by treatment of animals with 3-methylcholanthrene, phenobarbital, dexamethasone and clofibrac acid, respectively (XenoTech-TEBU). The reaction mixtures obtained after incubation of the drug with each set of microsome enzymes were analyzed by HPLC. The obtained chromatograms were close to that presented in Fig. 3 (not shown). The rate of C-1311 transformations performed under such conditions was compared in Fig. 7A. It is clear that higher concentrations of the CYP3A and CYP4A isoenzymes resulted in a higher rate of metabolism of the studied drug. This effect was intensified, particularly, after longer incubation. As shown in Fig. 7B and 7C, the selectivity in the action of CYPs towards C-1311 is strongly evident when the amounts of M1 and M3 metabolites are compared. M1 yielded in the highest concentration when the CYP3A family was overexpressed, whereas M3 dominated when the participation of CYP4A was principal.

DISCUSSION

The objective of the current investigations was to examine the specificity of the enzymatic transformation of an antitumor agent, C-1311, with a range of human and rat fractions of liver enzymes. It was postulated earlier that metabolic activation of C-1311 and its covalent binding to DNA were important steps in the biochemical mechanism of action of this drug (Dzięgielewski & Konopa, 1998). We studied previously the transformation of C-1311 in *in vitro* systems containing peroxidases, where the covalent binding to DNA was demonstrated. Structural studies on the oxidation products formed under such conditions allowed us to reveal that two reactive regions of the drug molecule (Fig. 1) were formed during metabolic transformations (Mazerska *et al.*, 2003). They are the *ortho* position to the hydroxyl group and both amino groups of the side chain. We postulated that the proposed molecular mechanism of enzymatic transformations of C-1311 might also take place in the cellular environment *in vivo*. The present studies were carried out to verify this hypothesis.

Metabolic transformations of the drug were studied here with rat and human liver enzymes. We found five metabolites in the reaction mixture and two of them were identical to those identified in the enzymatic model system. The first one, M0, was the product of dealkylation. It was formed by the oxidation of the ethyl group to hydroxyalkyl intermediate followed by the release of the related aldehyde. Dealkylation is a common type of *in vitro* and *in vivo* metabolic transformation catalyzed by

cytochrome P450 enzymes of microsomal fraction and is usually considered as an element of detoxication pathway of xenobiotics (Guengerich, 1990; Constantino *et al.*, 1992; Mani *et al.*, 1993). Surprisingly, we did not find in microsomes another common product of detoxication pathway formed by glucuronidation, which was observed previously in mouse urine and plasma (Calabrese *et al.*, 1999). Most likely the activity of UDP-glucuronosyltransferases towards C-1311 in rat and human liver microsomes was too low to yield sufficient amount of the corresponding metabolite.

Two metabolites described in this work were proposed to have a dimer-like structure. Formation of dimer structures seems to be a general characteristic feature of acridinone oxidative transformations. We identified earlier such a structure for one of the products of electrochemical, photochemical (Mazerska *et al.*, 2002) as well as of enzymatic oxidation (Mazerska, 2003) of 2-hydroxyacridinone, the model compound of C-1311, presented in Fig. 1. One can add that several dimer-like products of enzymatic oxidation have also been found earlier for various biologically active compounds with aromatic hydroxyl groups (Michon *et al.*, 1997; Favretto *et al.*, 1998). Likewise, peroxidase-mediated oxidation of C-1311 gave rise to a dimer-like product (Mazerska *et al.*, 2003). There are two reasons of the strong tendency of imidazoacridinones to form dimers. First, this molecule was shown to express high susceptibility to aggregation in water solutions. Physicochemical interactions between the planar heterocyclic rings were responsible for the formation of such structures (Dzięgielewski *et al.*, 2002). Secondly, as it was mentioned above, oxidation of C-1311 took place also in the imidazoacridinone ring, probably by the reactive carbocation formed in the *ortho* position to the hydroxyl group. The carbocation could be stabilized by covalent binding to nucleophiles present in the reaction environment. They might be nucleic acids or proteins in the cell environment or a second molecule of the drug under the enzymatic experimental conditions applied here. In the latter case, a dimer-like metabolite is formed and this fact indicates that C-1311 undergoes not only the above mentioned detoxication but also metabolic activation.

The present results allowed us to compare the metabolism of C-1311 carried out with various fractions of liver enzymes. We demonstrated that microsomal fraction containing enzymatic proteins and fragments of endoplasmic reticulum were more active in transformation of the studied drug than proteins of the cytosol fraction. Because cytochromes P450 are the major enzymatic constituent of microsomes, this group of enzymes seems to be responsible for metabolism of C-1311. Moreover, a comparison between human and rat fractions of mi-



osomes demonstrated a significantly lower rate of drug transformation in the former. This is consistent with other reports (Hariparsad *et al.*, 2006) and indicates that kinetic data obtained for rat metabolism are not applicable to human enzymes. However, in this work, the metabolic products were found to be identical for both enzyme sources. This fact was very useful for evaluation of metabolite structures and allowed an insight into the possible metabolic pathway of C-1311 in humans.

Although the total level of liver microsomal P450s does not vary considerably among humans, genetic polymorphism and susceptibility to induction of a given P450 isoenzyme result in interindividual variations in levels of P450 isoforms (CYPs) (Guengerich, 1995). What is more, a variability in expression of CYPs has been observed between various tissues of animal and human organism. Significant differences in CYP expression has been also found between healthy and tumor tissues (Murray, 2000; Oyama *et al.*, 2004). In this light, all data concerning a selectivity in catalytic properties of various CYPs towards a potent drug are desirable (Deeken, 2007). C-1311 presented different rates of transformations with rat liver microsomes expressing diverse levels of CYP families (Fig. 7). Furthermore, the CYP3A and CYP4A families were apparently selective in the formation of metabolites M1 and M3, respectively. These results might be applied in at least two fields according to earlier reports (Gustafson *et al.*, 2005; De Jong *et al.*, 2006). Firstly, the metabolic pathway of C-1311 can be predicted for individual patients with specific expression of CYP3A and CYP4A isoforms. However, further studies on relationships between expression levels of CYP3A or CYP4A and antitumor activity and/or toxicity of C-1311 are needed to confirm the role of specific CYPs in the biological action of the drug. On the other hand, the selectivity of CYP metabolism might allow selective direction of the drug action towards tumor cells because CYP isoenzymes are expressed in different levels in normal and tumor tissues (Murray, 2000; Oyama *et al.*, 2004). It has been reported that the expression of CYP1B1 was higher, while of CYP1A1 and CYP3A4 lower, in tumor breast cells than in healthy breast tissue (Osborne 1998; Husbeck & Powis, 2002). Such differences indicate that active metabolites would be formed only within tumor tissue with an elevated level of a particular CYP. The above discussed possibilities of selective metabolism of C-1311 dependent on the expression of P450 isoenzymes build up a background of individualized directed therapy with this drug, which is particularly critical in the case of cancer chemotherapy.

Acknowledgements

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