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Molecular mechanism of the enzymatic oxidation investigated for imidazoacridinone antitumor drug, C-1311

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Abstract

The imidazoacridinone derivative, C-1311, is an antitumor agent that has been under phase I of clinical trial. The work presented here aims to elucidate the molecular mechanism of the enzymatic oxidative activation of this drug in such a model metabolic system, where the covalent binding to DNA was previously demonstrated. The oxidative activation of C-1311 was performed with HRP/H_2O_2 and MPO/H_2O_2 systems. The obtained final products of such transformations were separated and analysed by HPLC. The structures of the products were identified by means of ESI-MS and NMR. It was demonstrated that C-1311 was oxidised with HRP and MPO in the manner dependent on the drug:H_2O_2 ratio and the drug was more susceptible to HRP oxidation than to MPO. Structural studies showed compounds C0 and C1 to be the result of dealkylation, which occurred in the amino groups of the side chain. The structures of C3 and C4 products were identified as dimers, whose monomers held the imidazoacridinone core. The activation of the imidazoacridinone ring system in position ortho to 8-hydroxyl group was necessary to form such dimers. We suggest that similar mechanism of C-1311 activation should occur in the presence of DNA when, instead of the dimer formation, the covalent binding to DNA, showed earlier for this drug, was formed. Since peroxidase-type enzymes are present in the cell nucleus of tumour cells the activation mechanisms of the C-1311 proposed here may be expected to take place in the cellular environment *in vivo*.

Keywords: C-1311; Imidazoacridinone antitumor agent; Mechanism of enzymatic activation; Metabolic activation; Peroxidase-mediated activation; Products of enzymatic oxidation

1. Introduction

Imidazoacridinones are a group of antitumor compounds developed in our laboratory [1–3]. The most promising analogue, which contains the hydroxyl group in position 8 of the imidazoacridinone core, compound C-1311 presented in Scheme 1 [4], has been under phase I of clinical trials. C-1311 exhibits strong cytotoxic properties *in vitro* against cells of solid tumours and high antitumor activity against experimental tumours in animals: P-388 leukemia [2], MAC 15A and MAC 29 adenocarcinomas of the colon and human xenograft HT-29 in nude mice [5]. This drug is pharmacologically attractive being unlike other antitumor agents. It expresses preferential cytotoxic and antitumor activity against solid tumours [5–7], only limited mutagenic potential [5] and low potency to generate oxygen free radicals [4], which suggests the lack of cardiotoxic properties. Furthermore, C-1311 is rapidly transported in tumour cells, accumulating in the cell nucleus [5,7–9].

The first biological effect observed in the cells treated with imidazoacridinones seems to be the induction of cell cycle arrest in the G_2 phase of tumour cells [10], which is followed by apoptosis [11,12]. The studies on the molecular mechanism of imidazoacridinone antitumor action indicated that DNA is a target for the drugs which were shown to intercalate into DNA [13,14] and to trap topoisomerase II cleavage complexes [9]. We also demonstrated that antitumor imidazoacridinones underwent oxidative activation with the model enzymatic system of horseradish peroxidase (HRP), and their ability to be transformed

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Abbreviations: DQF-COSY, double-quantum-filtered correlation NMR spectroscopy; ESI-MS, electrospray ionisation mass spectrometry; H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase; MPO, myeloperoxidase; ROESY, rotating frame-enhanced NMR spectroscopy.

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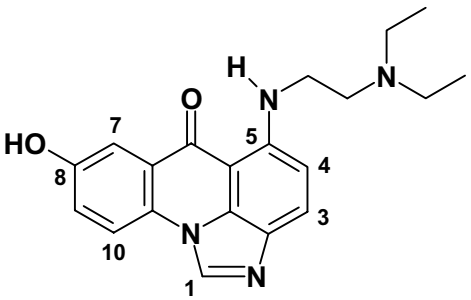


Scheme 1. Chemical structure of C-1311 antitumor drug.

under such conditions was in reasonable correlation with the antitumor activity of these compounds [15].

At the same time, C-1311 was shown to bind irreversibly, presumably covalently, to DNA of the tumour cells [16] as well as to DNA in the cell free system [17]. This irreversible binding in the cell free system was observed only in the case of the drug being enzymatically activated. Furthermore, even C-1311 intercalated previously into DNA was transformed in the presence of the model activation system. Thus, we concluded that the intercalation of C-1311 into DNA was followed by its enzyme-mediated activation, giving rise to the intercalated species that might irreversibly bind to DNA [18]. The recent results confirmed the real importance of metabolic activation in the molecular mechanism of imidazoacridinone biological action.

In our preliminary studies on the enzymatic transformation of C-1311, several metabolites were observed under *in vitro* conditions in the presence of HRP [18] and after incubation with microsomal enzymes of rat liver [19]. Similarly, Calabrese *et al.* found: (i) two metabolites of C-1311 formed with the microsomal fraction of mice and human liver cells *in vitro*, and (ii) one metabolite in human urine *in vivo* [20]. However, the chemical structure of any metabolite was still not proved. Therefore, neither the molecular mechanism of enzymatic activation of C-1311 *in vitro* nor the pathway of the metabolic transformation of this drug *in vivo* is known.

The present work aims to elucidate the molecular mechanism of the enzymatic activation of the imidazoacridinone drug *in vitro* performed under such conditions where the covalent binding to DNA was shown [17], that is, in the presence of peroxidase enzymes. Firstly, we investigated the reaction of C-1311 enzymatic activation with the model activation system, HRP/H_2O_2, and with animal myeloperoxidase (MPO). Secondly, chemical structures of several metabolites found under the studied conditions were determined.

The identification of the metabolite structures aimed not only to elucidate the molecular mechanism of C-1311 oxidative activation but the oxidation products with known structures will also be applied in our further studies as the reference compounds for the comparable identification of the metabolites formed with low concentrations under *in vivo* conditions.

In this study, the transformation of C-1311 with HRP and MPO was followed by UV-Vis spectroscopy and by HPLC with multidiode array detection. Structural studies of the oxidation products were accomplished by employing ESI-MS with positive ion detection and NMR techniques of DQF-COSY and ROESY.

2. Materials and methods

2.1. Chemicals and enzymes

C-1311 was synthesised in the Department of Pharmaceutical Technology and Biochemistry, Gdansk University of Technology as described earlier [1]. Horseradish peroxidase and hydrogen peroxide were purchased from Sigma. Myeloperoxidase and methanol HPLC grade were obtained from Fluka. Ammonium formate, sodium mono- and di-phosphate (AnalaR), HPLC grade were from BDH Ltd. and sodium mono- and di-phosphate from Serva Feibiochemica. All chemicals were used without further purifications. Ultra pure water, 18 MΩ, used in all experiments, was obtained with NANOpure water system. Stock solutions of HRP (1 mg mL^{-1}) and MPO (0.5 mg mL^{-1}) were prepared in 0.05 M phosphate buffer, pH 7.4, and aliquots were stored at −20°. Ten millimolar stock solution of C-1311 was freshly prepared.

2.2. Incubation of C-1311 with HRP and MPO

Incubation mixtures containing C-1311 (0.05–0.1 mM) and H_2O_2 (0.025–0.5 mM) were prepared by dilution of the stocks with 0.05 M phosphate buffer, pH 7.4. The experiments were performed at room temperature for HRP and at 37° for MPO-mediated oxidation. Reactions were initiated by the addition of the appropriate amount of the stock solution of the enzyme to a final concentrations from 0.1 to 0.5 μg mL^{-1} for HRP and from 0.1 to 0.6 μM for MPO.

2.3. Spectrophotometric analysis of MPO-mediated activation

The spectra of the reaction mixture was recorded in the range of wavelengths characteristic for imidazoacridinone chromophore (350–600 nm). The samples (2 mL) in 1 cm quartz cuvettes were placed in CARY300-Bio UV-Vis Spectrophotometer, model 635, and the spectra were collected every 1 min of 30 min reaction time.

2.4. HPLC monitoring of C-1311 oxidation with HRP and MPO

The reaction mixture obtained after HRP oxidation was prepared for HPLC analysis by solid phase extraction, SPE: 2–5 mL of the solution was passed through Super Clean LC-18, 6 mL cartridge (Supelco). Subsequently, the

cartridge was washed with 5 mL of water. After that the mixture of products was eluted with 0.5 mL of 1% water solution of formic acid and then with 5 mL 3:1 methanol:1% water solution of formic acid. The reaction with MPO was stopped by freezing and the incubation mixture underwent HPLC analysis after centrifugation. The 5–20 μL of the obtained solutions were analysed by a 5 μm Suplex pKb100 analytical column, 25 cm × 0.46 cm (Supelco) with a Waters Associates HPLC system equipped with a model 600K system controller, Rheodyne injector 7725i and 996 UV-Vis multidiode array detector controlled with Millenium software. HPLC analyses were carried out at a flow rate of 1 mL min^{-1} with the following system: a linear gradient from 35 to 40% methanol in ammonium formate for 5 min, followed by a linear gradient from 40 to 90% methanol in ammonium formate for 10 min.

2.5. Isolation of HRP oxidation products by preparative HPLC

The 20 mL solution of 10^{-4} M C-1311 in pH 7.4, 0.05 M phosphate buffer was incubated with 2 μg mL^{-1} HRP and 2×10^{-4} M H_2O_2 at room temperature for 10 min. Then, the incubation mixture was concentrated by SPE procedure: 20 mL solution volume was passed through previously prepared a Super Clean LC-18, 18 mL cartridge (Supelco). Subsequently, the cartridge was washed with 20 mL of water. After that the mixture of products was eluted with: 0.5 mL of 1% water solution of formic acid, next 2 mL of 50% methanol, and then 5 mL of 80% methanol in 1% water solution of formic acid. 0.5–1.5 mL of the selected fraction was injected onto HPLC Waters instrument described above and was separated on 25 cm × 1 cm Suplex pKb100 reversed phase column (Supelco) with flow rate 1 mL min^{-1}.

The individual eluent system was employed for HPLC isolation of each product destined for NMR experiments; for *product C0*: an isocratic elution at 45% methanol in ammonium formate (50 mM, pH 3.2); *products C1 and C2*: a linear gradient from 70 to 80% methanol in ammonium formate for 5 min, followed by an isocratic elution at 80% methanol in ammonium formate for 10 min; *product C3*: an isocratic elution at 30% methanol in ammonium formate for 5 min, followed by a linear gradient from 30 to 95% methanol in ammonium formate for 15 min. The isolation experiments were repeated 10–20 times and fractions collected in this way were lyophilised on Freeze Dry System (Lymph, Lock 4.5, Labconco) with previous evaporating of methanol. The purity of the products were analysed by HPLC under the above conditions, point 4, and then the products underwent NMR experiments.

The following eluent system was employed for HPLC separation of the products, fractions of which was analysed by ESI-MS: a linear gradient from 42 to 70% methanol in ammonium formate (50 mM, pH 3.2) for 15 min, followed by a linear gradient from 70 to 90% methanol in ammonium formate for 15 min. The fractions were analysed directly without lyophilisation.

2.6. Spectral characteristic of HRP oxidation products

UV-Vis spectra were extracted from the HPLC chromatograms collected with multidiode array detector in ammonium formate, pH 3.2/methanol mixture. ESI-MS analysis of the product fractions was accomplished by electrospray ionisation with positive ion detection performed on a Mass Spectrometer LC-MSD, Agilent 1100, USA. ^{1}H NMR measurements with COSY, an ROESY techniques were carried out at 500.13 MHz in deuterated dimethyl sulfoxide (DMSO-d_6) using a Varian 500 spectrometer. Chemical shifts are reported in parts per million for ^{1}H relative to the internal standard $(CH_3)_4Si$.

2.6.1. Product C0

UV-Vis spectrum: λ_{max} 370 nm, 422 nm; ESI-MS: $[M + 1]^+$, *m/z* 323.1, ESI-MS/MS fragment ions at *m/z* 72.1, 252.0, 278.1; ^{1}H NMR: δ (ppm) 5.42 (s, 1H, H–O7), 6.86 (d, 1H, H-4, $I = 8.79$ Hz), 7.37 (dd, 1H, H-9, $I_1 = 8.79$ Hz, $I_2 = 2.93$ Hz), 7.54 (d, 1H, H-7, $I = 2.93$ Hz), 7.99 (d, 1H, H-3, $I = 8.79$ Hz), 7.29 (d, 1H, H-10, $I = 8.79$ Hz), 9.03 (t, 1H, H–N5, $I = 5.37$ Hz), 9.12 (s, 1H, H-1).

2.6.2. Product C1

UV-Vis spectrum: λ_{max} 365 nm, 414 nm; ESI-MS: $[M + 1]^+$, *m/z* 252; ^{1}H NMR: δ (ppm) 6.78 (d, 1H, H-4, $I = 8.79$ Hz), 7.33 (dd, 1H, H-9, $I_1 = 8.79$ Hz, $I_2 = 2.93$ Hz), 7.68 (s, 1H, H–O7), 7.71 (d, 1H, H-7, $I = 2.93$ Hz), 7.85 (d, 1H, H-3, $I = 8.79$ Hz), 8.25 (dd, 1H, H-10, $I = 8.79$ Hz), 8.34 (s, 2H, H–N5), 9.06 (s, 1H, H-1).

2.6.3. Product C2

UV-Vis spectrum: λ_{max} 380 nm; ESI-MS: $[M + 1]^+$, *m/z* 280; ^{1}H NMR: δ (ppm) 7.45 (dd, 1H, H-9, $I_1 = 8.79$ Hz, $I_2 = 2.93$ Hz), 7.75 (d, 1H, H-7, $I = 2.93$ Hz), 8.24 (d, 1H, H-4, $I = 8.79$ Hz), 8.34 (dd, 1H, H-10, $I = 8.79$ Hz), 8.38 (s, 1H, H–O7), 8.71 (s, 1H, H–N5), 8.73 (d, 1H, H-3, $I = 8.79$ Hz), 9.44 (s, 1H, H-1), 11.58 (s, H-CN5). COSY conjugated signals at δ 8.71 and 11.58 ppm and ROESY conjugated signals at δ 8.34 and 9.44 ppm were observed.

2.6.4. Product C3

UV-Vis spectrum: λ_{max} 370 nm, 422 nm; ESI-MS: $[M + 1]^+$, *m/z* 671.2, 643.4 $[(M + 2)/2]^+$, *m/z* 336.3, 323.1; ^{1}H NMR: δ (ppm) 6.69 (m, 1H, H-9′ or H-10′), 6.72 (s, 1H, H-7′), 6.80 (d, 1H, H-4, $I = 9.28$ Hz), 6.92 (d, 1H, H-4′, $I = 9.28$ Hz), 7.25 (s, 1H, H-7), 7.79 (d, 1H, H-3′, $I = 9.28$ Hz), 7.88 (d, 1H, H-9, $I = 8.79$ Hz), 8.00 (s, 1H, H-1′), 8.01 (d, 1H, H-3, $I \approx 9$ Hz), 8.60 (d, 1H, H-10, $I = 8.79$ Hz), 8.83 (t, 1H, H–N5, $I = 5.37$ Hz), 9.24 (s, 1H, H-1). ROESY conjugated signals at δ 9.24 and 8.60 ppm, at δ 6.80 and 3.39 ppm, and 6.92 and 3.63 ppm were observed.

2.6.5. Products C4

UV-Vis spectrum: λ_{max} 366 nm, 414 nm; ESI-MS $[M + 1]^+$, *m/z* 572.2, 588.2, 600.3, 616.0.

3. Results

3.1. HRP-mediated metabolism of C-1311

Our previous studies [18] showed that C-1311 was oxidised by the HRP/H_2O_2 system in the manner dependent on the drug:H_2O_2 ratio, and the reaction gave highly reactive species that were quickly transformed into further products. Here, closer examination of the HRP-mediated activation of C-1311 is presented. This work was aimed at finding out products of the HRP-mediated C-1311 oxidation. The reaction was performed under various incubation conditions and was monitored under new HPLC conditions.

The representative chromatograms obtained after the HRP oxidation of C-1311 are presented in Fig. 1. The contents of the reaction mixture were analysed with regard to the HRP amount. Three metabolites C0, C1 and C2 yielded in the presence of the low concentration of peroxidase (Fig. 1A), whereas the higher concentration of HRP gave rise to next products, C3 and the group of C4 (Fig. 1B). It was demonstrated that under the extremely high enzyme concentration (Fig. 1C) or with high excess of H_2O_2 (data not shown) the concentration of all products except C0 significantly decreased. The results allowed us to determine the optimal conditions of C-1311 enzymatic oxidation in respect of the number and amount of the products formed, namely, the 1:2 C-1311:H_2O_2 ratio, 2 μg mL^{-1} of HRP and 10 min of the incubation time.

The UV-Vis spectra of the observed metabolites were extracted from HPLC chromatograms and are presented in Fig. 2. Fig. 2A shows the spectral characteristics of the activation products C0, C1 and C2, whereas Fig. 2B shows the spectra of the metabolites, C3 and one of C4. Products C0 and C1 displayed the spectra close to that of the substrate. In the case of the spectrum of metabolite C2, the absorption band was shifted towards lower wavelength in comparison with that of the substrate. On the other hand, products C3 and C4 gave the spectra which were only slightly different from the spectrum of the substrate.

3.2. Oxidative metabolism in the presence of MPO

The studies on the susceptibility of C-1311 to metabolic transformation with peroxidases were extended by applying MPO. It is an animal enzyme and is present in neutrophiles [21]. MPO was shown to catalyse the oxidative activation of xenobiotics, several antitumor agents included in [22,23]. What is more, MPO was demonstrated to associate with DNA in the nuclei of HL-60 cells and neutrophils [24].

Preliminary results of the MPO-mediated oxidation of imidazoacridinones demonstrated earlier (data not shown) show that the susceptibility to enzymatic oxidation with

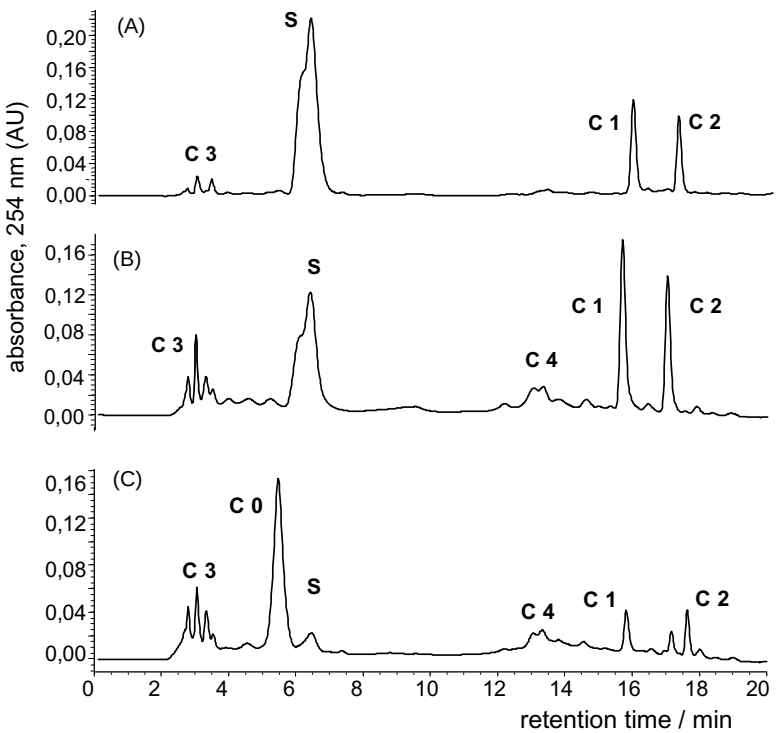


Fig. 1. HPLC chromatograms (reversed-phase, methanol:0.05 M ammonium formate pH 3.2, gradient system, 254 nm) taken after 10-min oxidation of C-1311 with HRP/H_2O_2 system. The incubation mixture contained 0.1 mM C-1311, 0.2 mM H_2O_2 and HRP: (A) 0.2 μg mL^{-1}, (B) 1 μg mL^{-1}, (C) 5 μg mL^{-1}, in 0.05 M phosphate buffer pH 7.4.

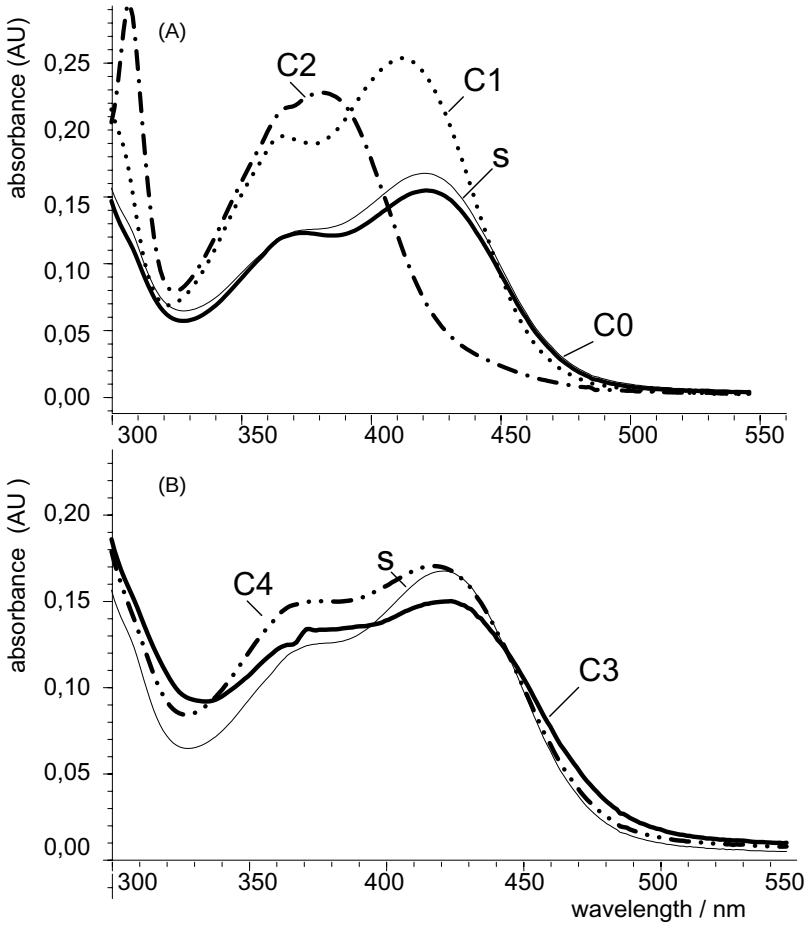


Fig. 2. The UV-Vis spectra of the main products obtained from the HRP oxidative activation of C-1311. The spectra were extracted from the HPLC chromatograms in ammonium formate pH 3.2/methanol mixture: (A) C-1311: thin line, C0: bold line, C1: dotted line, C2: dashed-dotted line. (B) C-1311: thin line, C3: bold line, C4: dashed-dotted-dotted line.

MPO depended on the substituent in position 8 of the imidazoacridinone core. The 8-hydroxy derivatives underwent oxidative transformations far more readily than 8-methoxy and the analogues devoid of substituents in position 8.

The spectrophotometric monitoring of the reaction catalysed by MPO and performed for the imidazoacridinone antitumor agent, C-1311, selected earlier is presented in Fig. 3. The decrease of the drug absorbance at 425 nm and the simultaneous appearance of a wide flat band near 500 nm were observed in this set of spectra.

The HPLC analysis of C-1311 activation performed in the presence of MPO and 1:1, 1:2 and 1:4 drug:H_2O_2 ratios is shown in Fig. 4. The preliminary identification of the products was made by the comparison of the UV-Vis spectra extracted from the above chromatograms with those extracted from the chromatograms collected earlier for the HRP-mediated oxidation (Fig. 2). A new product, which was not found with HRP, and small amounts of C1 and C2 known from HRP transformation were observed at the equimolar ratio of the substrate amounts (Fig. 4A). The chromatogram of the samples obtained with the greater excess of H_2O_2 (Fig. 4B and C) exhibited an additional group of C4 metabolites, which were also found earlier after the HRP oxidation. However, the rate of the MPO activation was lower than that of the HRP activation.

3.3. Products from the peroxidase-mediated metabolism of C-1311

The significant similarity in the final oxidation products formed after the incubation with HRP and with MPO shown above, allowed us to study the structure of the products obtained after oxidation with one of the two enzymes. HRP was selected here as it was a stronger activator of C-1311 than MPO, and it is a more common enzyme.

3.3.1. Enzymatic synthesis, separation and isolation of the oxidation products

The synthesis of the HRP-mediated metabolites of C-1311 was performed under the optimal conditions selected above: the 1:2 C-1311:H_2O_2 ratio, 2 μg mL^{-1} of HRP and 10 min of incubation time. The reaction mixture was extracted using the solid phase extraction, and then the fractions of the products were separated by HPLC under the preparative mode. A lot of fractions were collected for each

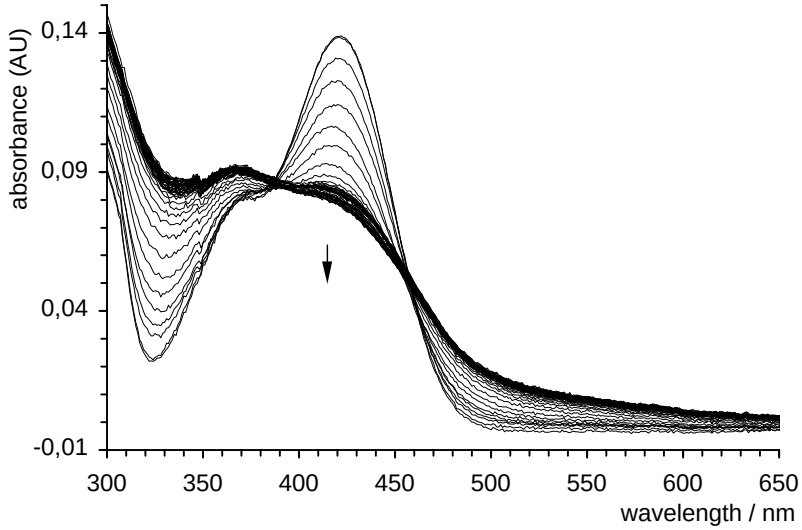


Fig. 3. The sequence of the absorbance spectra taken every 60 s during the incubation of 0.025 mM C-1311, 0.125 mM H_2O_2 and 0.1 μM MPO in 0.05 M phosphate buffer pH 7.4.

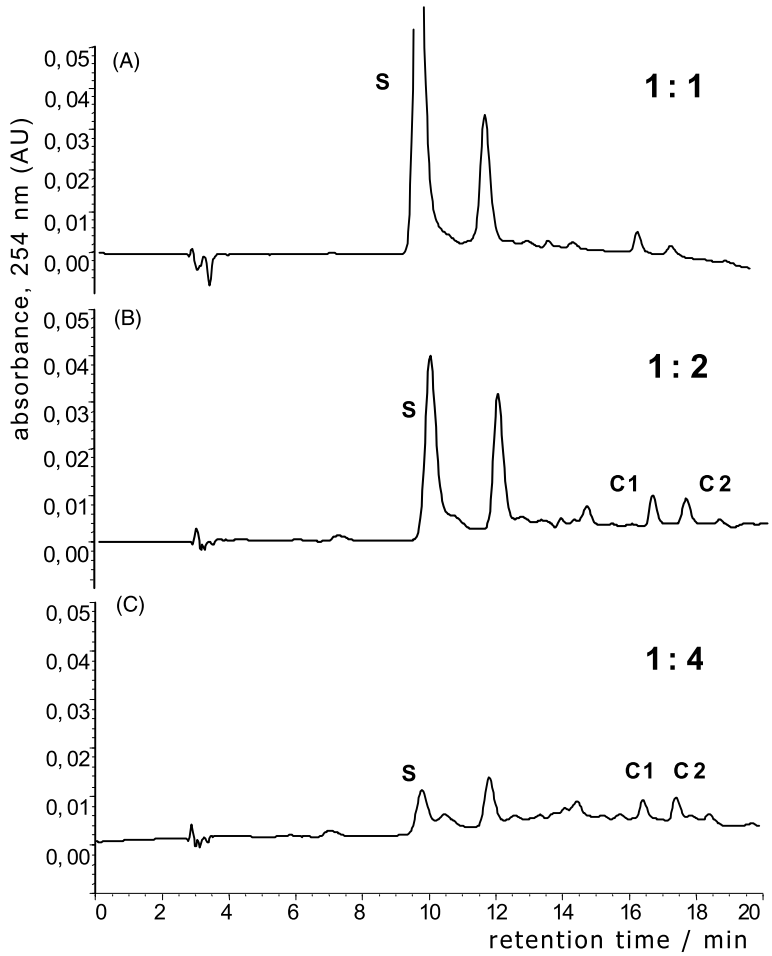
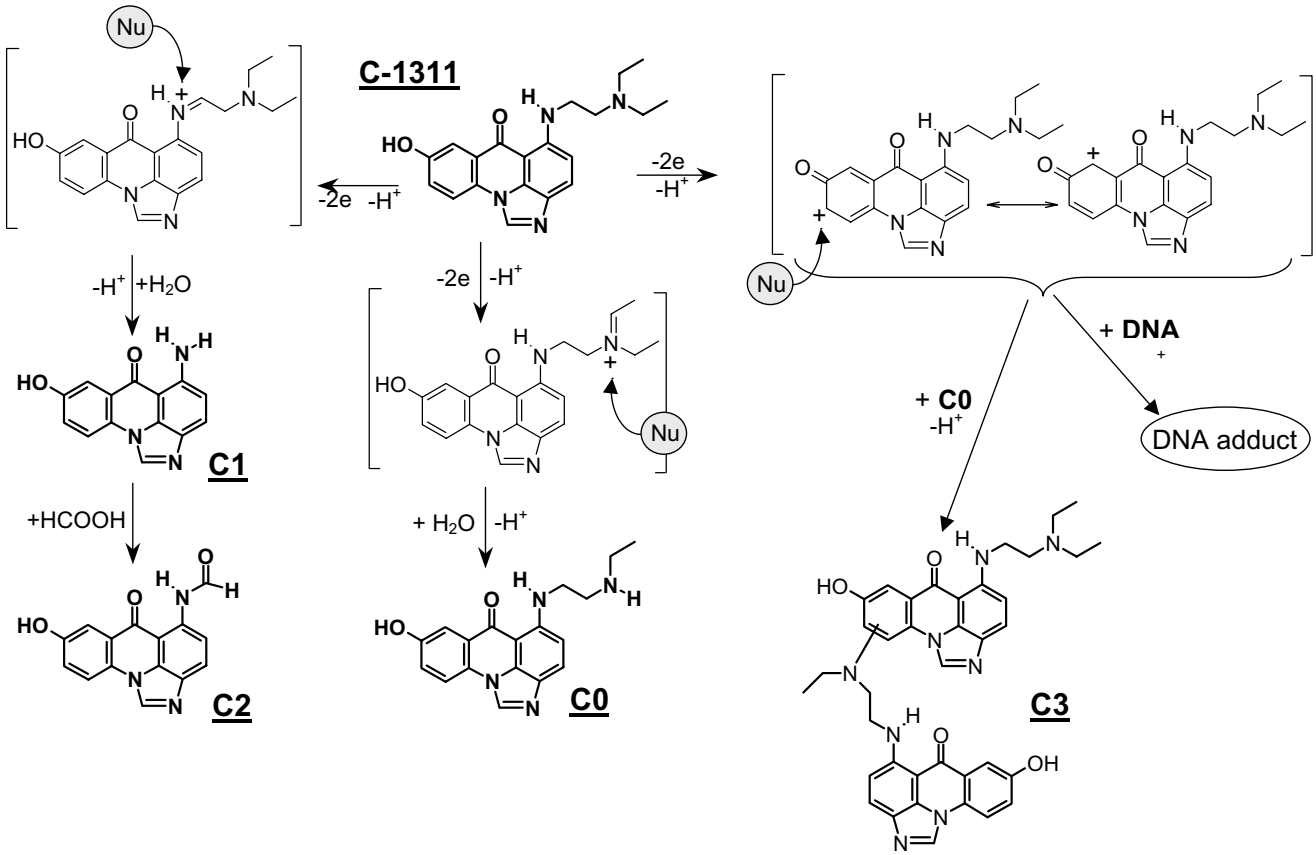


Fig. 4. HPLC chromatograms (reversed-phase, methanol:0.05 M ammonium formate pH 3.2, gradient system, 254 nm) taken after 10-min oxidation of C-1311 by MPO/H_2O_2 system. The incubation mixture contained 0.1 mM C-1311, 0.6 μM MPO and H_2O_2: (A) 0.1 mM, (B) 0.2 mM, (C) 0.4 mM, in 0.05 M phosphate buffer pH 7.4.



Scheme 2. The pathway for C-1311 oxidative enzymatic transformations proposed on the basis of the chemical structures of the products identified here (see footnote 1).

metabolite, and next they were lyophilised and submitted to NMR studies. ESI-MS analyses were performed directly in the solutions of the HPLC fractions.

3.3.2. Identification of the product structures

3.3.2.1. Products C0, C1 and C2. The chromatographic peak of product C0 is close to that of the substrate as it is shown in Fig. 1. The slightly lower retention time of C0 indicated its slightly higher hydrophilicity than that of the substrate. At the same time, C0 revealed the UV-Vis spectrum identical to that of the substrate (Fig. 2) and the aromatic range of its NMR spectrum was also close to that of C-1311. It means that the unchanged imidazoacridinone ring system was preserved in C0. The C0 mass ion, *m/z* 323.3, was found previously [18]. The ESI-MS/MS spectrum obtained here showed fragment ions at *m/z* 278, 252 and 72. The above results when taken together showed metabolite C0 to be the product of C-1311 deethylation in the aminoalkyl side chain, whose structure is presented in Scheme 2.[1]

The next activation product was C1. It gave the UV-Vis spectrum (Fig. 2) and the shifts of aromatic protons in NMR experiments close to those of the parent compound, C-1311. The C1 mass ion was identified at *m/z* 252.1 Therefore, the structure of C1 shown in Scheme 2 was determined as a product of the complete dealkylation of the substrate.

Product C2 differed significantly from C0, C1 and from the substrate in its UV-Vis spectrum (Fig. 2) as well as in NMR results. The maximum of the C2 UV-Vis band was shifted to 380 nm in comparison with 422 nm observed for C-1311, thus, the electron density of the heterocyclic ring system was modified in this product. The NMR experiments revealed that such modification took place close to the amino group in position 5. Furthermore, ROESY NMR spectra showed that the imidazole ring was preserved in the C2 molecule, whereas the aminoalkyl side chain was removed. Besides, the DQF-COSY experiment exhibited the presence of the polar group attached to amino substituent in position 5. Finally, the ESI-MS spectrum of C2 gave the mass ion at *m/z* 280. In conclusion, the structure of C2 was shown to be a 5-formylamido derivative of the imidazoacridinone and is presented in Scheme 2.

3.3.2.2. Products C3 and C4. Product C3 is unique compared to other products due to its high hydrophilicity

[1] The structures, which were drawn in bold lines were proven in this paper whereas, the remaining ones related to the possible intermediates. The most possible directions for the attack of cellular nucleophiles to C-1311 compound are marked with circles and arrows.

(Fig. 1). The DQF-COSY NMR experiment resulted in two couples of aromatic protons, therefore, the dimer-like structure was proposed for the product C3. The first set of protons was similar in the chemical shifts to that of the substrate, whereas the second set of protons was close to that of the substrate only in H3′ and H4′ shifts. The ROESY NMR spectrum demonstrated the presence of the aminoalkyl side chain in at least one component of the dimer. Furthermore, the presence of the mass ions, *m/z* 671.2 and 643 confirmed the dimer structure of C3. The *m/z* values proved the molecular mass of the dimer to be an even number, which indicated the presence of an even number of nitrogen atoms in the dimer molecule. The mass ions *m/z* 323.3 and 336.3 were also found in the C3 spectrum. They were equal to the value of (M + 2)/2 and resulted from the formation of the double charged molecule. These findings confirmed the NMR results indicating the presence of two aminoalkyl side chains in the C3 dimer structure. Summing up, the C3 activation product of C-1311 was determined to be a dimer-like molecule, two components of which were probably bound by the substitution of the aminoalkyl side chain in the position of imidazoacridinone ring close to the hydroxyl group. The proposed structure of C3 is presented in Scheme 2.

The wide unresolved chromatographic band presented in Fig. 1 relates to the group of products named C4. The ESI-MS spectrum obtained for this fraction gave four mass ions *m/z* 572.2, 588.8, 600.3 and 616.0. This result strongly indicated the dimer-like structure of products C4. Nevertheless, the mass ions of components C4 were significantly lower than the mass ion of C3, and the mass ions related to (M + 2)/2 described above for C3 were not observed for products C4. Furthermore, unlike product C3, the molecular masses of C4 took the odd numbers, indicating the odd number of nitrogen atoms in the structures of the dimers. All the above findings strongly suggested that C4 was a mixture of at least four dimer-like products, in which one of the two aminoalkyl side chains was devoid of one amino group. The place, in which the two monomers were combined was not identified, however, it is possible that it was the same as in the case of product C3.

4. Discussion

The metabolic activation of C-1311 [15,18] and its covalent binding to DNA [17] were shown previously to be the important steps in the biochemical mechanism of action. The present work aims to propose the molecular mechanism of the metabolic activation of C-1311. To achieve this aim we characterised the products obtained after C-1311 activation with the *in vitro* system, HRP/H_2O_2, where there was the covalent binding to DNA previously demonstrated [17]. The studies on the reaction of C-1311 with MPO were also described in this work.

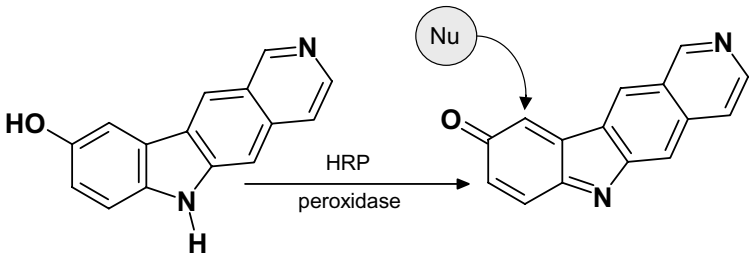
We showed that the peroxidase-mediated enzymatic oxidation of C-1311 occurred in the manner dependent on the drug:H_2O_2 ratio and on the amount of the enzyme. The character of the reaction was similar in the case of HRP and MPO, however the drug was more sensitive to the former enzyme than to the latter one. In general, three products, C0, C1 and C2 were observed under the moderate oxidation conditions, whereas, products C3 and a group of products C4, were obtained with a higher amount of the enzymes or with the excess of H_2O_2.

Scheme 2 presents the structures of the final metabolites identified in this paper (bold lines) and those of the reactive intermediates (thin lines). The intermediate structures were drawn on the basis of the well known mechanism of peroxidase-mediated activation [25–27], and taking into consideration the structures of the metabolites identified here for C-1311, and for the metabolites obtained earlier for ellipticine [28–30], anthrapyrazole [31] and mitoxantrone [32–34] antitumor agents.

You can notice in Scheme 2 that two sites of the drug molecule underwent the enzymatic activation. The first site was the aminoalkyl side chain, particularly its two amino groups, where the oxidative dealkylation occurred. Such dealkylation resulted in products C0 and C1. They were formed by the oxidation of the alkyl groups to hydroxyalkyl intermediates to be followed by the release of the related aldehyde. Dealkylation is a common type of *in vitro* and *in vivo* metabolic transformation catalysed by peroxidases as well as by cytochrome P-450 enzymes [25,35–37]. It is usually considered as an element of the detoxication pathway of xenobiotics.

The second site of the oxidation of the C-1311 molecule (presented in Scheme 2) was the aromatic ring with a hydroxyl substituent in position 8. This hydroxyl group is located in para position to the heterocyclic nitrogen atom. The identical structural motif exists also in the ellipticine molecule presented in Scheme 3. Therefore, the same mechanism of enzymatic oxidation might be expected for both compounds. However, ellipticine is oxidatively transformed to an iminoquinone derivative (Scheme 3), which is impossible in the case of the C-1311 compound, because the heterocyclic nitrogen atom is engaged in the structure of the C-1311 imidazole ring. As a consequence, the enzymatic oxidation of C-1311 yielded dimer-like products instead of the iminoquinone derivative. We suggest the oxidation of the two compounds run by the reactive carbocation formed in the ortho position to the hydroxyl group. In the case of ellipticine, the carbocation is transformed to a relatively stable iminoquinone derivative, whereas the C-1311 carbocation is stabilised by a reaction with nucleophiles present in the reaction environment. This stabilisation gave rise to the dimer-like compounds when C-1311 is alone in the reaction mixture.

The differences between ellipticine and C-1311 seem to be clearer when the formation of the covalent binding to DNA is considered. The iminoquinone metabolite of



Scheme 3. Chemical structure of ellipticine antitumor drug and of its HRP-mediated metabolite.

ellipticine was shown to bind covalently to DNA [38], whereas which we demonstrated earlier, the products obtained after the HRP oxidation of C-1311 did not interact with DNA [18]. The formation of the covalent binding in the case of C-1311 required the simultaneous presence of the drug and DNA in the reaction mixture [17]. In the absence of DNA and other biologically important nucleophiles, the carbocation intermediate was stabilised by the formation of the dimer-like products C3 and the C4 group. While, in the presence of DNA, the stabilisation could run with the covalent binding to DNA (Scheme 2).

The present results allowed us to explain the significant role of the 8-hydroxyl group in the antitumor activity of imidazoacridinones. We reported earlier [15] and showed in this paper that the susceptibility to enzymatic oxidation with HRP as well as with MPO depended on the substituent in position 8 of the imidazoacridinone core and correlated well with the antitumor activity of these compounds. Highly active antitumor 8-hydroxy derivatives underwent oxidative transformations far more readily than the less active 8-methoxy derivative and the analogues devoid of substituents in position 8. The studies presented here revealed that the presence of the hydroxyl group opened the possibility for easy oxidative activation of the aromatic ring system. Therefore, the earlier statement that the metabolic activation is the necessary condition for the antitumor action of imidazoacridinones [15] should be now extended by indicating that the activation of the heterocyclic ring system is necessary for the high antitumor activity of these compounds.

In conclusion, the structural studies on the oxidation products obtained after the enzymatic activation of C-1311 with peroxidase enzyme allowed us to indicate that two reactive regions of the drug molecule can be transformed during metabolic transformations. They are the ortho position to the hydroxyl group and the amino groups of the side chain. Because the peroxidase enzymes are present in the cell nucleus of tumour cells [24], the mechanism of the enzymatic activation proposed here may be expected to take place also in the cellular environment *in vivo*. We postulate that this mechanism belongs to those responsible for the antitumor action of C-1311, unfortunately it is probably also responsible for its toxicity. On the other hand, the activation leading to the dealkylated products gives probably the less toxic metabolites.

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