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Enzymatic activation of a new antitumour drug, 5-diethylaminoethylamino-8-hydroxyimidazoacridinone, C-1311, observed after its intercalation into DNA

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Abstract

The imidazoacridinone derivative, C-1311, is a new antitumour agent that exhibits strong antitumour activity against experimental colorectal cancer and has been selected for entry into clinical trial. The compound has previously been shown to have DNA non-covalent binding properties *in vitro* and to bind irreversibly to DNA of tumour cells. The latter effect has also been observed in a cell-free system, but only in the presence of activated enzymes. The present studies were aimed at finding out whether and in what way the enzymatic activation of C-1311 and its non-covalent binding to DNA influence or depend on each other. Enzymatic activation was performed with a model system containing horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) and was followed by UV-VIS spectroscopy and by HPLC with UV-VIS and electrospray ionisation mass spectrometry detection. DNA non-covalent binding was studied in the cell-free system by means of an unwinding assay and UV-VIS spectroscopy. It was shown that C-1311 was oxidised by the HRP/H_2O_2 system in a manner dependent on the drug:H_2O_2 ratio. In the case of ratios of 1:3 and 1:5, the reaction gave highly reactive species that were quickly transformed into the further products p2 and p3 that were unable to intercalate into DNA. In the presence of DNA, C-1311 first intercalated into DNA and the intercalated compound was then oxidised. This oxidation was directed to only one product. Therefore, DNA seems to play the role of a "scavenger" of the reactive oxidation product(s) yielded from the intercalated drug and prevents its further deactivation. We conclude that, under the conditions studied, intercalation of C-1311 into DNA is followed by its HRP-mediated activation, giving rise to the intercalated species that might irreversibly bind to DNA. Since peroxidase-type enzymes are present in the cell nucleus, the proposed sequence of events may also be expected to take place in the cellular environment *in vivo*.

Keywords: Antitumour compounds; DNA intercalation; Enzymatic activation; Imidazoacridinone derivatives; Metabolic activation; Mode of antitumour action

1. Introduction

5-Diethylaminoethylamino-8-hydroxyimidazoacridinone, C-1311 (Fig. 1), is a highly active antitumour compound, developed in our Department [1,2], which is currently undergoing phase I clinical studies. It exhibits strong cytotoxic properties against the cells of solid tumours [3,4] and high antitumour activity against transplantable tumours in animals: adenocarcinomas of the colon, MAC 15A, MAC 29, and the human xenograft HT29 [4]. This drug also shows some other attractive pharmacological properties: it does not generate oxygen free radicals and therefore is not expected to display strong cardiotoxicity [2]. C-1311 exhibits only limited mutagenic potential [5]. Cellular transport of this agent occurs rapidly and most of the drug accumulates in the nucleus [4,6], which enables its fast interaction with DNA.

In an attempt to determine the factors underlying the antitumour properties of this drug, studies concerning several aspects of its biological activity have been carried out. It has been found that C-1311 as well as other imidazoacridinones induce the arrest of cell cycle progression in G_2 phase [3,7]. This arrest seems to be the first biological effect observed in cells treated with these agents. It has been shown that C-1311 induces apoptosis in tumour cells [8]. It is also able to inhibit the catalytic activity of DNA topo-

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Abbreviations: ESI–MS, electrospray ionisation mass spectrometry; HRP, horseradish peroxidase; H_2O_2, hydrogen peroxide; and SV40, simian virus 40.

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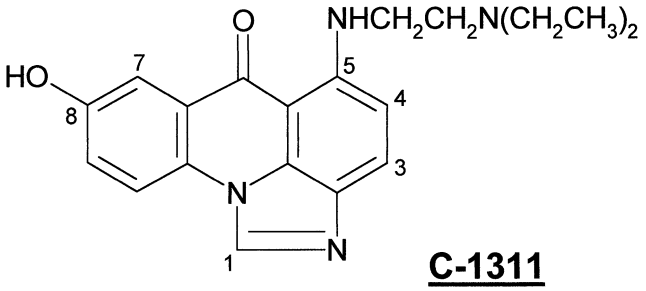


Fig. 1. Chemical structure of C-1311.

isomerase II and stimulate the formation of covalent DNA–topoisomerase complexes. The latter effects are observed in a cell-free system as well as in whole cell [6].

Imidazoacridinones bind non-covalently to calf thymus DNA [9,10], and this effect seems to be necessary but not sufficient for the biological action of these compounds [9]. C-1311 has been shown to bind irreversibly, presumably covalently, to DNA of tumour cells as well as to DNA in a cell-free system. This irreversible binding in the cell-free system has been observed only when the drug is enzymatically activated [11]. It has also been demonstrated that C-1311 forms interstrand cross-links in DNA of tumour cells, and that this effect is not detected in the case of lysates in which enzymes have been inactivated [12]. The above results allowed us to conclude that the metabolic activation of the imidazoacridinone agent, C-1311, is a prerequisite for its biological activity.

In a previous study, we showed that several imidazoacridinone derivatives underwent enzymatic oxidation in the presence of HRP and that the level of this transformation was related to the antitumour activity of these compounds [13]. The rate as well as the type of products of enzymatic oxidation in the case of highly potent 8-hydroxyimidazoacridinones differed from those observed for less active non-hydroxy derivatives. Therefore, the results obtained confirmed that metabolic activation may represent the crucial step in the biological action of these compounds and indicated that imidazoacridinones underwent specific oxidative activation.

Three earlier observations incited us to undertake the present study: (a) non-covalent binding to DNA was necessary for the biological action of imidazoacridinones; (b) irreversible binding to DNA in the cell-free system occurred only in the presence of the HRP/H_2O_2 system; and (c) metabolic activation of C-1311 was a prerequisite for biologically important DNA cross-linking. Hence, non-covalent binding to DNA and metabolic activation were found to be the initial steps of imidazoacridinone action, and both these phenomena were necessary for the biological activity of C-1311. Therefore, we wondered what the sequence of events was: did the products of metabolic activation interact with DNA or did the oxidative metabolism of C-1311 take place after DNA non-covalent binding of the drug. Determining this sequence was the aim of the present studies.

Here, the HRP/H_2O_2 system was chosen as a model of metabolic activation because HRP-mediated oxidation had been shown to be relevant to the biological activity of imidazoacridinones [13]. The enzymatic oxidation of C-1311 was followed by UV-VIS spectroscopy and by HPLC with UV-VIS and ESI–MS detection. The interaction with DNA was studied in the cell-free system by a DNA unwinding assay as well as spectroscopically.

2. Materials and methods

2.1. Drugs, enzymes, and chemicals

The imidazoacridinone C-1311 was synthesised in the Department of Pharmaceutical Technology and Biochemistry of the Technical University of Gdańsk [1]. HRP, calf thymus DNA, H_2O_2, SDS, agarose, Tris–HCl, and methanol (HPLC grade) were purchased from Sigma. SV40 viral DNA, human topoisomerase I, and cell culture chemicals were from GIBCO Life Technologies. Proteinase K was obtained from Serva. All other chemicals used were of analytical grade. Ultrapure water (18 MΩ) was used in all experiments. Stock solutions of C-1311 (10 mM), HRP (1 mg/mL), and calf thymus DNA (2 mg/mL) were prepared in 0.05 M phosphate buffer, pH 7.4, and aliquots were stored at −20°.

2.2. C-1311 oxidation mediated by HRP

Incubation mixtures containing C-1311 (0.05–0.1 mM) and H_2O_2 (0.05–0.5 mM) were prepared by dilution of the stocks with 0.05 M phosphate buffer [13]. In some experiments, calf thymus DNA was added to a final concentration of 0.6-mM base pairs. All experiments were performed at room temperature. Oxidation was initiated by the addition of the appropriate amount of the stock HRP solution to a final concentration of 5 μg/mL.

2.3. Spectrophotometric analysis

The spectra of reaction mixtures were recorded in the range of wavelengths characteristic of imidazoacridinone chromophore (350–600 nm). The samples (2 mL) in 1-cm quartz cuvettes were placed in a LambdaBio UV-VIS spectrophotometer (Perkin Elmer), and the spectra were collected after 0.5, 1, 2, 5, 10, 15, 30, and 60 min of the reaction.

2.4. HPLC monitoring of HRP oxidation

The HRP-mediated oxidation of C-1311 was followed by reversed-phase HPLC using a Spherisorb ODS2 analytical column (25 × 0.4 mm, C18, Jones) and isocratic elution at 1 mL/min with methanol: 0.05 phosphate buffer, pH 2.5 (7:3) v/v containing 0.01% of triethylamine. An HPLC

system including 600K system controller, U6K pump system, and 991 multidiode array detector (Waters-Millipore) was employed in these experiments. The MS analysis of the products was carried out by electrospray ionisation with positive ion detection performed on a Finnigan MAT TSQ 700 tandem mass spectrometer equipped with a Finnigan electrospray ionisation source interfaced with the HPLC system and a Dionex GP40 gradient pump. N_2 was used as a sheath gas (386 kPa/56 psi).

2.5. DNA unwinding assay

The imidazoacridinone-induced DNA unwinding was examined using a DNA topoisomerase I assay [14]. Supercoiled SV40 DNA (1 μg) was incubated with 5 U topoisomerase I in 30 μL of reaction buffer (10 mM Tris–HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 μg/mL of BSA). After 5 min at 37°, the compound or a mixture of its metabolites obtained during 3 hr of HRP-mediated oxidation (as described above) was added. Assuming complete reaction between drug and HRP, the summary concentrations of reactive metabolites produced were adjusted to 1, 3, 6, and 10 μM. After 60-min incubation, the reactions with DNA were stopped by the addition of SDS and proteinase K (final concentrations 1% and 0.5 mg/mL, respectively) and the samples were allowed to remain for 15 min at 37°. Then, the samples were extracted with phenol: chloroform:isoamyl alcohol (25:24:1) and electrophoresed overnight at 1 V/cm in 1% agarose gel using Tris/acetate/EDTA buffer. DNA bands were visualised with ethidium bromide and photographed under UV light.

3. Results

3.1. Enzymatic oxidation in the presence of the HRP/H_2O_2 system

The HRP-mediated oxidation of C-1311 was performed in the presence of HRP and 1:1, 1:2, 1:3, and 1:5 drug:H_2O_2 ratios. The samples were subjected to HPLC analysis and the chromatograms obtained are shown in Fig. 2. For equimolar ratio of the substrates, one main product, p1, and small amounts of p2 and p3 were observed. The time-course of the reaction (data not shown) revealed that the concentration of C-1311 dropped twice during the first 10 min and then reached the plateau state, which was close to the plateau level observed for the final concentration of p1. In the case of a twofold excess of H_2O_2, the HPLC peak of the substrate went down much faster, and peaks of p2 and p3 were clearly observed. The chromatograms for samples containing a greater excess of H_2O_2 exhibited products p1 and p2 and a greater proportion of p3.

Products p1, p2, and p3 were identified by ESI–MS operating in the positive ion mode. The obtained MS spectra are presented in Fig. 3. The peak with the retention time of 10.2 min (p1) exhibited one ion in the mass spectrum at m/z 323.2, whereas two ions were observed for both peaks at 14.5 and 20.5 min (p2 and p3, respectively). These were determined at m/z 338.2 and 675.3 for peak p2 and 572.2 and 600.2 for peak p3. The results indicated that the mass ion of p1, m/z 323.2, was formed by the loss of 28 mass units of the substrate molecule. Therefore, p1 might be a deethylation product of C-1311. The mass ion for p2 at m/z 675.3 probably resulted from the dimer-like structure, whereas the ion at m/z 338.2 could relate to the [1/2M + 1] value. Product p3 gave two ions at m/z 572.2 and 600.2, which could also result from dimer-like compounds in the p3 fraction.

The results showed that product p1 dominated in the reaction mixture with the equimolar drug:H_2O_2 ratio, whereas p1, p2, p3, and other products were observed with 3- and 5-fold excess of H_2O_2. Therefore, the following studies on HRP-mediated oxidation affected by DNA intercalation were performed with the 1:1 and 1:5 drug:H_2O_2 ratios.

3.2. Intercalation into DNA and HRP-mediated oxidation

A method taking advantage of the unwinding of supercoiled SV40 DNA by topoisomerase I observed in the presence of DNA intercalators [14] was used to detect DNA intercalation of C-1311 and of products of its enzymatic activation. The electrophoregrams obtained are presented in Fig. 4. Typical patterns of relaxed DNA bands were observed for the parent drug and for the incubation mixture activated by the HRP with the 1:1 drug/H_2O_2 ratio (lanes 3–6 and 8–11, respectively). The products of C-1311 enzymatic oxidation with the fivefold excess of H_2O_2 displayed no detectable effect on topoisomerase I-mediated relaxation of DNA (lanes 13–16). These results indicated first that DNA non-covalent binding of C-1311 consists of intercalation and second that the intercalation levels observed in the reaction mixture after “mild” oxidation of C-1311 and observed for the parent compound were similar. However, none of the drug metabolites obtained at higher H_2O_2:drug ratios turned out to be a DNA intercalator.

The results of the spectrophotometric monitoring of HRP-mediated oxidation in the presence of calf thymus DNA for C1311:H_2O_2 ratios 1:1 and 1:5 are presented in Figs. 5 and 6, respectively. Each of the figures contains two sets of spectra. The first (A) was recorded during the HRP oxidation of C-1311, while the second (B) was obtained when the enzyme was added after preincubation of the drug with DNA for 15 min, i.e. the time sufficient to reach the equilibrium state. The DNA excess was used, which assured intercalation of all drug molecules into DNA.

Fig. 5A shows that the spectrum of C-1311 changed rapidly during the reaction with HRP for the 1:1 drug:H_2O_2 ratio. The subsequent addition of DNA resulted in the next significant changes in the spectrum (dash-dotted line), which confirmed our previous result from the unwinding

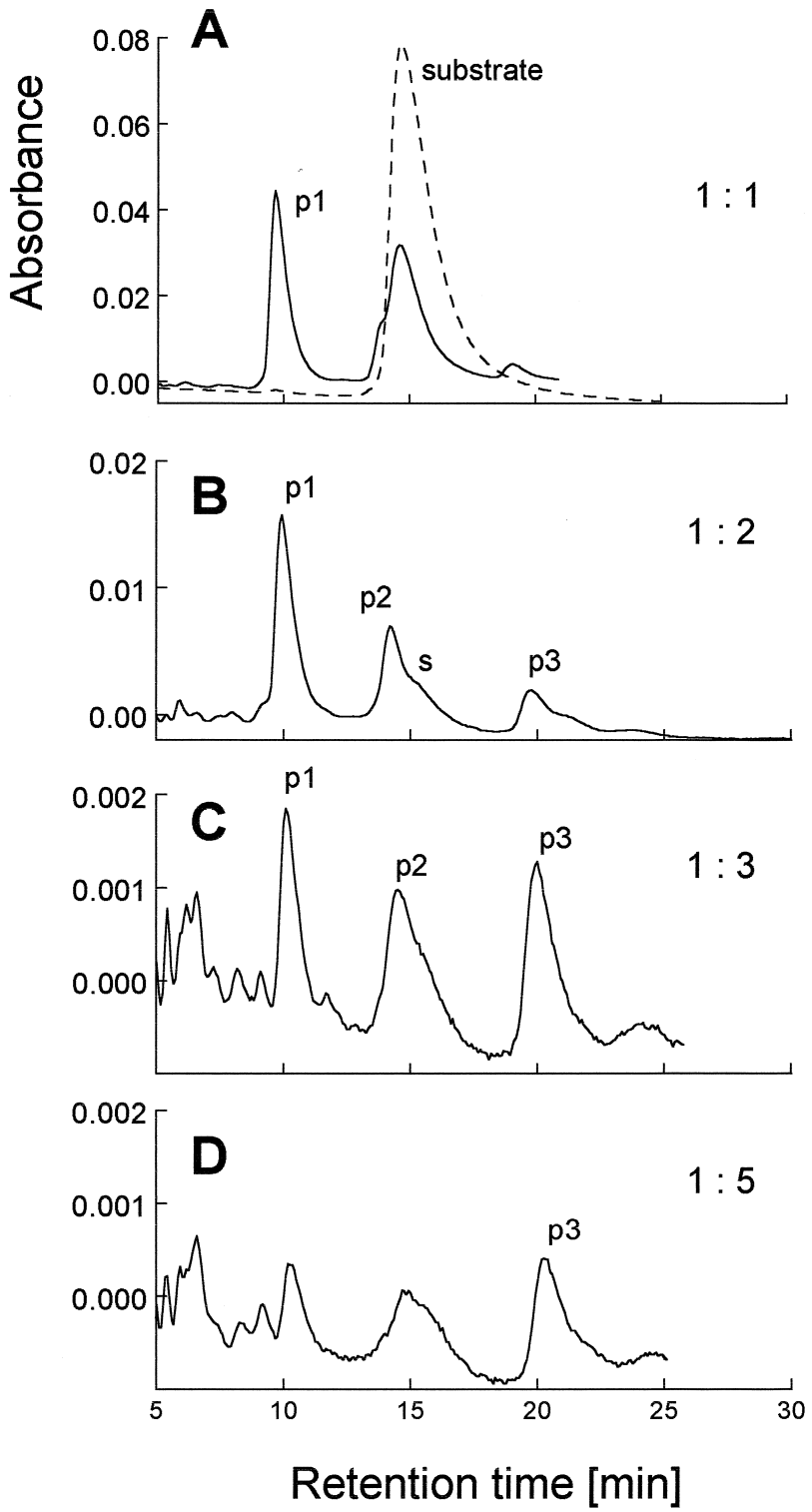


Fig. 2. HPLC chromatograms (reversed-phase, methanol:0.05 M phosphate buffer, pH 2.5, 7:3 supplemented with 0.01% of triethylamine, detection at 265 nm) taken after 15-minute oxidation of C-1311 by the HRP/H_2O_2 system. The incubation mixture contained 0.1 mM imidazoacridinone drug, 5 μg/mL of HRP, and (A) 0.1 mM, (B) 0.2 mM, (C) 0.3 mM, and (D) 0.5 mM H_2O_2 in 0.05 M phosphate buffer. The dashed line in panel A represents the chromatogram of the substrate(s).

assay (Fig. 4) that intercalation occurred in this case. The addition of HRP to the reaction mixture after drug intercalation into DNA (Fig. 5B) led to the enzymatic transformation reflected by a new set of spectra (solid lines). However, these new spectra resembled neither the ones obtained directly after HRP oxidation (Fig. 5A, solid lines) nor those

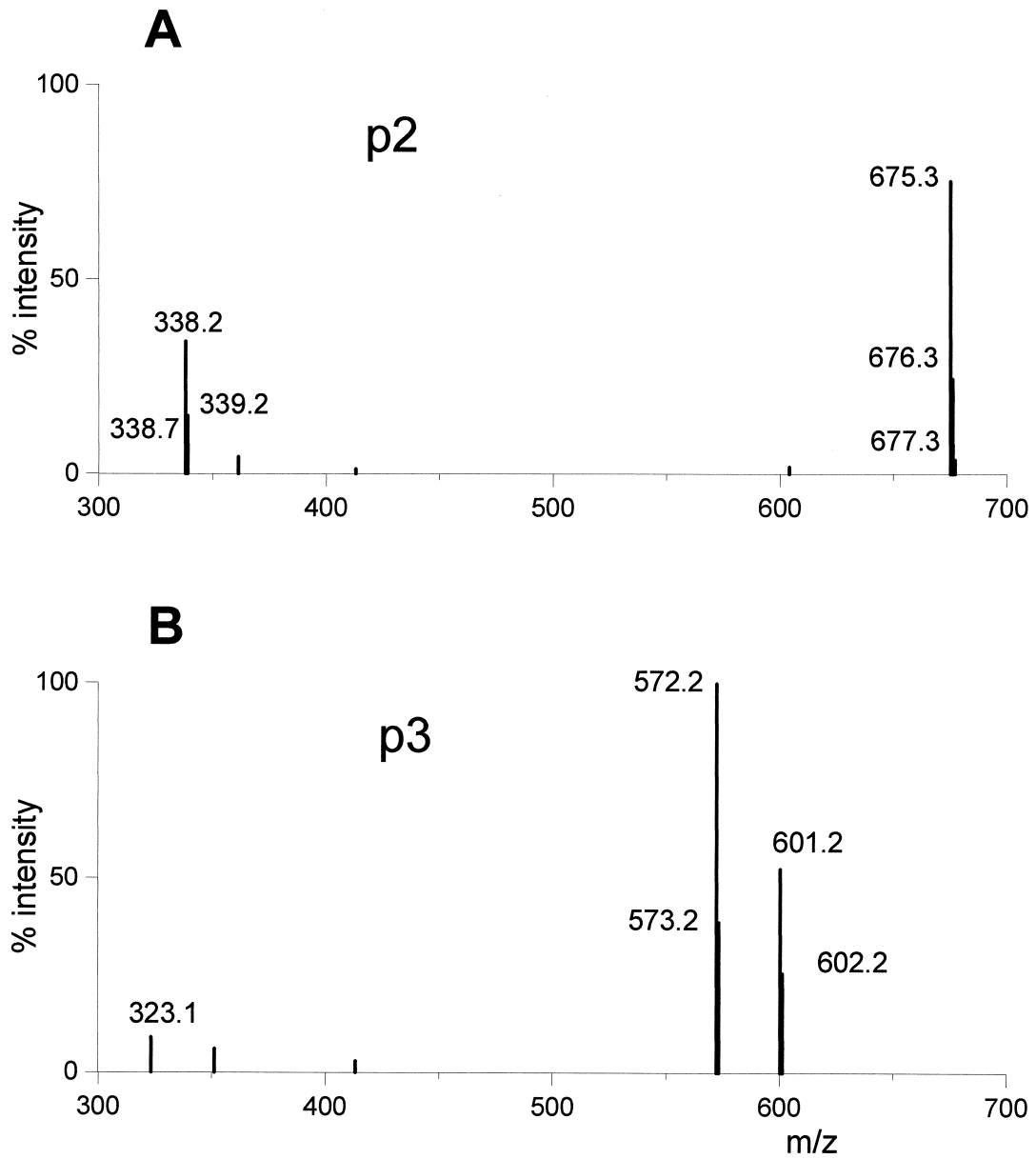


Fig. 3. Positive ion ESI–MS spectra for (A) p2 and (B) p3 HPLC peaks.

obtained when HRP oxidation was followed by drug intercalation (Fig. 5A, dash-dotted line).

The spectra recorded during oxidation of C-1311 with HRP and the fivefold excess of H_2O_2 are presented in Fig. 6. The most significant difference between the two sets of spectra presented in panels A and B consists in the fact that two isosbestic points, at 406 and 457 nm, appeared in Fig. 6B, i.e. when the HRP was added to the incubation mixture after intercalation of C-1311 into DNA. The presence of the isosbestic points indicates that no more than two species exist in the reaction mixture. On the other hand, as isosbestic points were not observed during HRP oxidation of the drug without DNA (Fig. 6A), a multicomponent mixture of the products was formed under these conditions. Nevertheless, noticeable changes in the spectra were seen in this case as well. For instance, the intensity ratio of the band at 370 and 425 nm increased. This reflects the significant chemical transformations that took place in the enzymatically transformed imidazoacridinone core.

Fig. 6, A and B also presents, for comparison, a spectrum of the reaction mixture in which DNA was added after enzymatic activation of C-1311 (dash-dotted line). It was identical in character to the final spectrum recorded during HRP oxidation without DNA (panel A, final solid line) and different from that observed when intercalation of the drug preceded its HRP oxidation (panel B, final solid line).

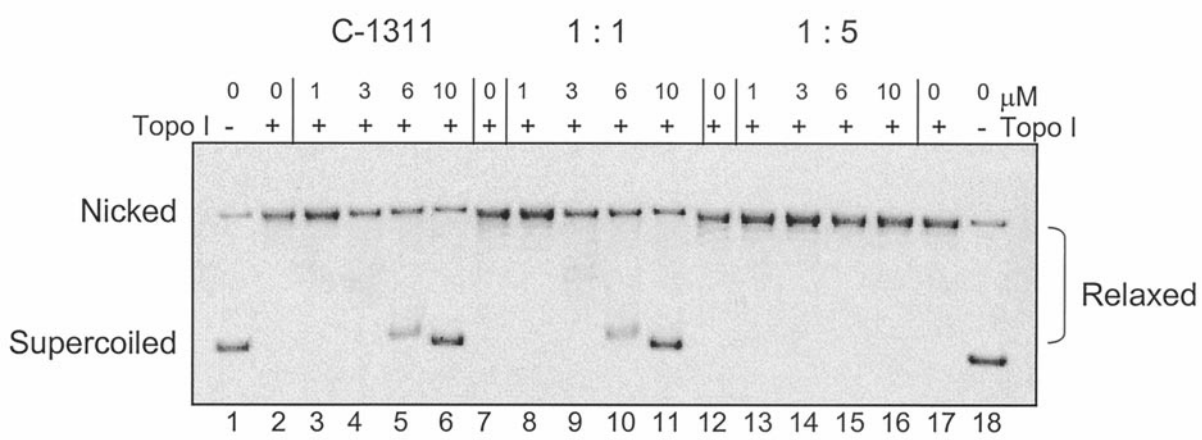


Fig. 4. Intercalation of C-1311 and its HRP oxidation products into DNA. Native supercoiled SV40 DNA, 1 μg, (*lanes 1 and 18*) was incubated with 5 U topoisomerase I in 30 μL of reaction buffer in the absence of the drug (*lanes 2, 7, 12, and 17*), in the presence of the indicated concentrations of C-1311 (*lanes 3–6*), or in the presence of C-1311 metabolite mixture obtained after 3 hr of HRP-mediated oxidation with the 1:1 drug:H_2O_2 ratio (*lanes 8–11*) or the 1:5 drug:H_2O_2 ratio (*lanes 13–16*). The incubations were carried out at 37° and stopped after 30 min by proteinase K/SDS digestion. The DNA was extracted from the samples with phenol:chloroform:isoamyl alcohol (25:24:1) and analysed in 1% agarose gel.

4. Discussion

The present study was aimed at determining the relationship between the DNA non-covalent binding of imidazoacridinone drug and its capacity for metabolic activation in the model enzymatic system. It is known from previous studies that (a) DNA non-covalent binding of C-1311 is necessary for its biological activity [9] and (b) enzymatic oxidation is necessary for irreversible binding of this drug to DNA in a cell-free system [11]. The summary scheme of the results obtained in this paper is presented in Fig. 7.

The first stage of our investigations focussed on the oxidative transformation of C-1311 in the model HRP/H_2O_2 system. We demonstrated that the equimolar ratio of the drug and H_2O_2 resulted in the formation of one main product with a yield near 50%. A higher excess of H_2O_2 induced a higher reaction rate and led to the formation of at least three products, i.e. p1, p2, and p3, for which the mass spectra were determined. Although the exhibition of the metabolite structures was not possible, the results revealed that p2 and p3 may be dimer-like compounds. Their origins seemed to be strongly reactive, probably radical intermediates. This indicates that p2 and p3 are the after products of C-1311 activation.

Non-covalent binding of imidazoacridinones to DNA was demonstrated earlier by several methods [9,20]. In this study, we showed that C-1311 intercalates into DNA. The same level of intercalation was also observed after enzymatic oxidation of C-1311 with an equimolar drug/H_2O_2 ratio (Fig. 4). HPLC analysis showed that half of the parent compound was enzymatically transformed under these conditions, making it likely that metabolite p1 intercalated into DNA. It was also shown that the previous DNA intercalation of C-1311 did not abolish its ability to be transformed by HRP under the conditions of equimolar drug/H_2O_2 ratio.

DNA intercalation was not observed after enzymatic oxidation performed with a fivefold excess of H_2O_2. It seemed here that oxidation prevented the DNA intercalation of C-1311 metabolite(s). On the other hand, the intercalation of the parent compound into DNA did not disable the subsequent enzymatic transformation of the intercalated drug. Moreover, the pathway of this transformation changed completely in comparison with the reaction occurring without DNA. The presence of the two isosbestic points in the set of spectra suggests that we are dealing here with the mixture of only two species. Furthermore, the spectrum of the HRP-mediated product obtained in the presence of DNA (Fig. 6B, final solid line) was significantly different from that generated in the absence of DNA (Fig. 6A, final solid line). One possible explanation for these events is that DNA was able to "scavenge" the reactive intermediates of oxidation of the intercalated drug, with the reaction then being directed to only one product. The above suggestion was confirmed by the susceptibility of C-1311 to form dimer-like compounds. When reactive intermediates are formed in the reaction mixture, they probably recombine to the dimers very quickly. When the activation occurs with the previously intercalated drug, the reactive metabolites are in proximity to the DNA and succeed in "catching" it. As a result, in the first case covalent binding was not observed, whereas in the second case it was [11].

The mode of action proposed above would distinguish imidazoacridinone drugs from many other antitumour agents, especially from ellipticine and mitoxantrone. It was demonstrated for the latter [15,16] that the addition of DNA followed by the subsequent addition of myeloperoxidase did not induce significant oxidative transformation of mitoxantrone. Moreover, mitoxantrone was shown to bind covalently to DNA when peroxidase oxidation preceded interaction with DNA. Neither of these results was observed in the case of imidazoacridinone. These differences between the two agents could be explained on the basis of our

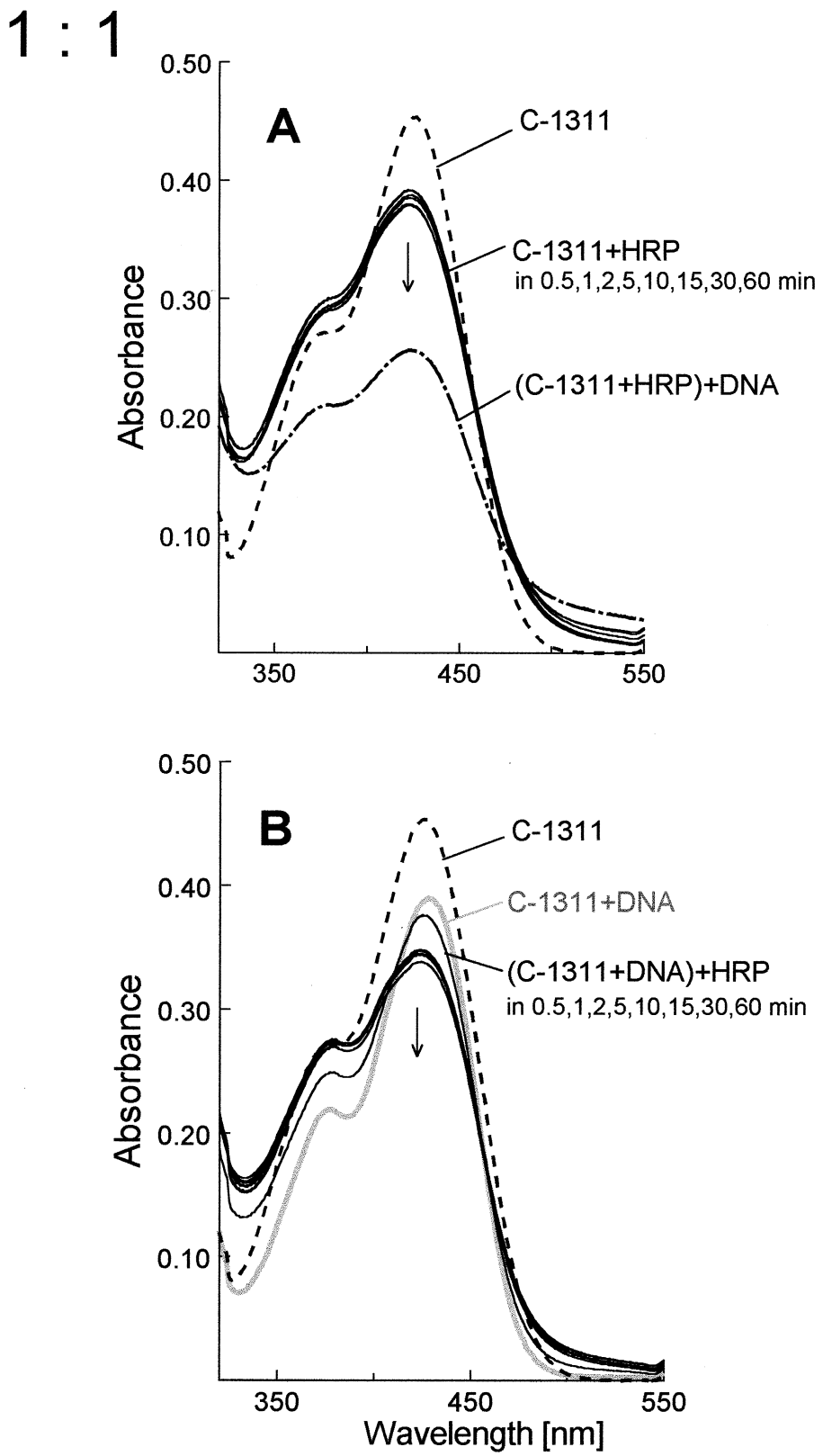
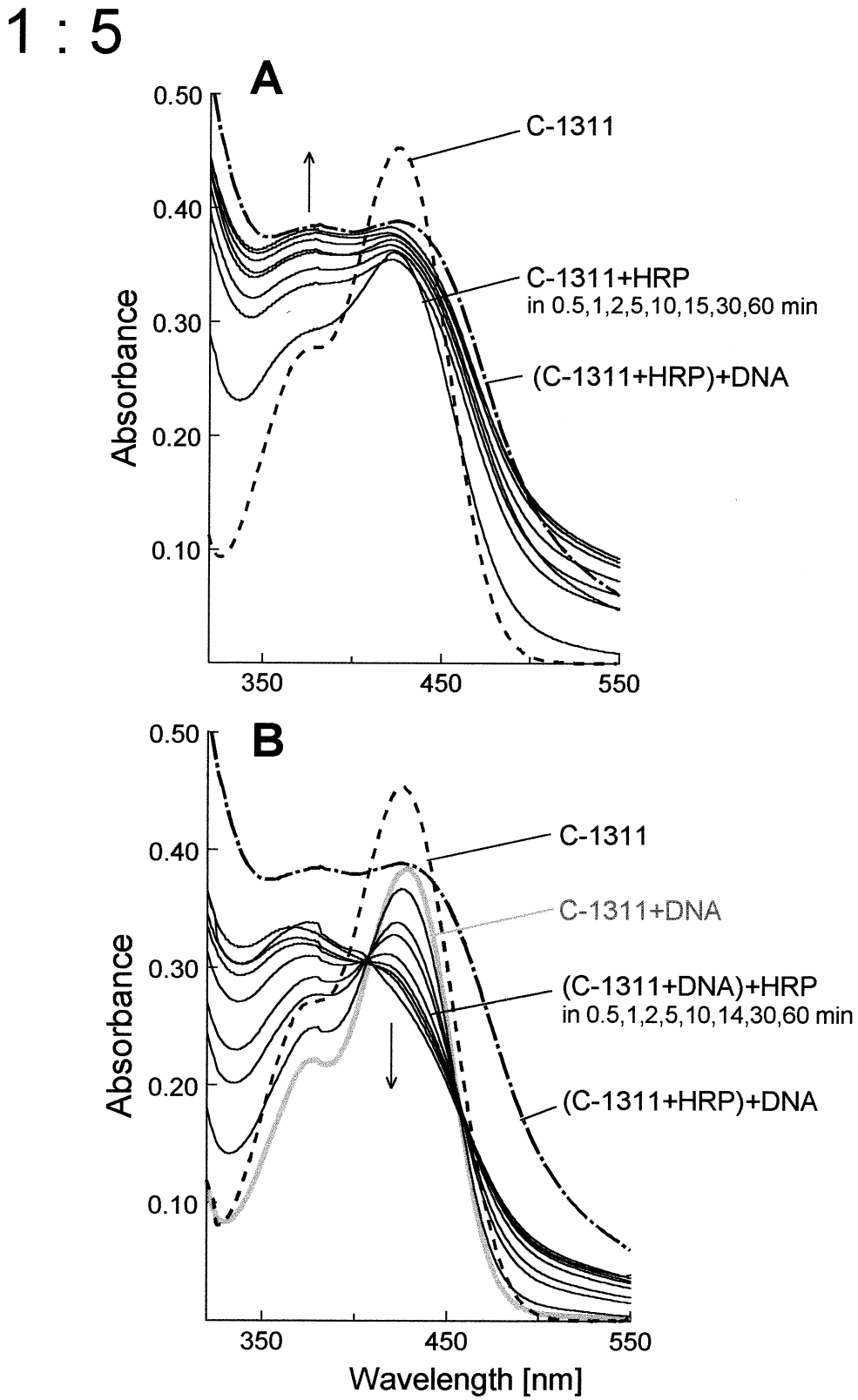


Fig. 5. The absorption spectra (A) of C-1311 (*dashed line*) taken: at 0.5, 1, 2, 5, 10, 15, 30, and 60 min of the HRP-mediated oxidation of C-1311 with the 1:1 drug/H_2O_2 ratio (*solid lines*); taken after 60 min of the HRP-mediated oxidation of C-1311 and the subsequent addition of ctDNA (*dash-dotted line*). The absorption spectra (B) of C-1311 (*dashed line*) taken: after C-1311 intercalation into DNA (*grey line*), and after C-1311 intercalation and subsequent HRP oxidation at 0.5, 1, 2, 5, 10, 15, 30, and 60 min (*solid lines*). The incubation mixture contained 0.1 mM imidazoacridinone drug, 5 μg/mL of HRP, 0.1 mM H_2O_2, and 0.6-mM base pairs of DNA in 0.05 M phosphate buffer.



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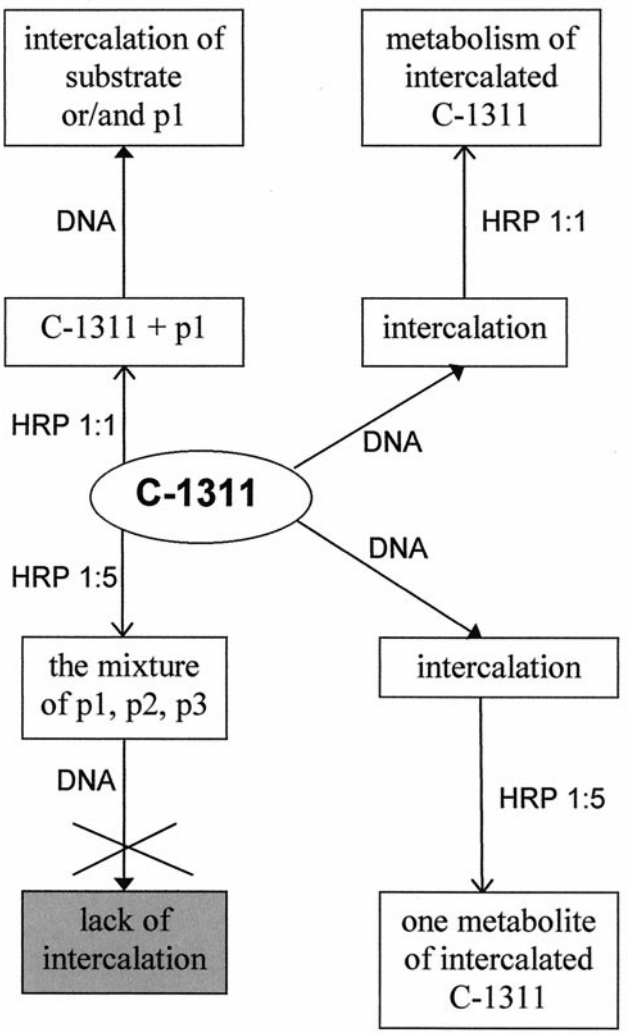

Fig. 7. The summary scheme of the results obtained: *HRP 1:1* and *HRP 1:5*, HRP-mediated activation of C-1311 with 1:1 and 1:5 drug:H_2O_2 ratios, respectively; *DNA*, incubation of the drug with ctDNA and SV40 DNA.

previous study [13], in which the stability of mitoxantrone radical products of HRP oxidation was compared with that observed for the imidazoacridinone, C-1311. Mitoxantrone radicals were more stable, probably stable enough to "wait for" the DNA target, whereas imidazoacridinone radicals were such highly reactive species that in the absence of DNA they were immediately involved in the further transformation. Thus, the subsequent addition of DNA could not result in its reaction with C-1311 metabolite(s).

In conclusion, the present study has shown that the imidazoacridinone antitumour drug, C-1311, was oxidised by the HRP/H_2O_2 system in a manner dependent on the drug:H_2O_2 ratio. In the case of the 1:5 drug:H_2O_2 ratio, the reaction gave highly reactive unknown intermediates that were quickly transformed into a mixture of p1, p2, and p3 products not intercalating into DNA. In the presence of DNA, the drug first intercalated into DNA before oxidation of the intercalated compound occurred. This oxidation was directed to only one product. Therefore, we postulate that intercalation of C-1311 drug might be followed by its HRP-mediated oxidation, giving rise to product(s) capable of irreversible binding into DNA. Considering that C-1311 binds irreversibly to DNA both in the cell-free system containing HRP/H_2O_2 as well as in the cell [11], we hypothesise that such oxidative activation of previously intercalated drug may also occur in the cell, as peroxidase-type enzymes are present in the cell nucleus and express strong affinity to DNA [17].

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