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INTERACTION OF THE PEROXIDASE-DERIVED METABOLITE OF MITOXANTRONE WITH NUCLEIC ACIDS

EVIDENCE FOR COVALENT BINDING OF ^{14}C-LABELED DRUG

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Abstract—The antitumor agent mitoxantrone undergoes horseradish peroxidase-catalyzed oxidation by hydrogen peroxide to an identifiable cyclic metabolite which is a substituted hexahydronaphtho[2,3-f]-quinoxaline-7,12-dione. Binding of mitoxantrone to DNA inhibited enzymatic oxidation of the drug. The metabolite of mitoxantrone, derived from the action of the HRP/H_2O_2 system on the drug, bound non-covalently to DNA oligomers. Spectrophotometric analyses of such complexes showed formation of a new, blue-shifted, metachromatic absorption band which was observed when the DNA base pair to drug ratio was close to 1. Measurements of DNA unwinding angles suggest that the metabolite, in contrast to mitoxantrone, did not intercalate but rather bound externally to DNA. Experiments with ^{14}C-labeled mitoxantrone confirmed that peroxidase-activated drug binds covalently to DNA.

Mitoxantrone (MXH_2,‡ 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione) (Fig. 1) is a synthetic anticancer agent which shows activity against a broad spectrum of malignancies [1, 2]. In contrast to the clinically useful anthracyclines, daunorubicin and doxorubicin, which are highly cardiotoxic [3], mitoxantrone appears to exhibit significantly lower cardiotoxicity [4, 5]. The exact mechanism(s) of action of MXH_2 has not been elucidated; however, the diminished cardiotoxicity may be related to its more negative reduction potential in comparison to anthracyclines [4–6]. This prevents efficient enzymatic reduction of the drug to its semiquinone free radical and/or hydroquinone forms and subsequent redox cycling in the presence of molecular oxygen [4, 5]. In contrast, anthracyclines readily engage in this type of reaction and, in the presence of oxygen, produce oxygen-derived species ($O_2^{\cdot-}$, H_2O_2, $OH^{\cdot}$) which are considered to be lethal to cells.

Recently, it was demonstrated that mitoxantrone readily undergoes enzymatically-catalyzed oxidation [7, 8]. For example, in the presence of horseradish peroxidase (HRP) and hydrogen peroxide, mitoxantrone undergoes oxidation and structural rearrangements yielding a metabolite, MH_2 (a substituted hexahydronaphtho[2,3-f]quinoxaline-7,12-dione) (Fig. 1). The reaction proceeds via formation of free radical species from MXH_2 and/or MH_2, as demonstrated using EPR spectroscopy [7, 8]. The

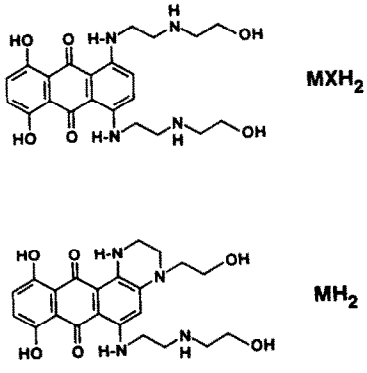

Fig. 1. Structure of mitoxantrone (MXH_2) and its peroxidase-derived metabolite (MH_2).

oxidized form of the metabolite, MH_2^{2+} shows electrophilic character [7, 8].

Certain oxidatively activated anticancer agents have been shown to bind covalently to cellular macromolecules, including DNA [9, 10]. Although mitoxantrone undergoes oxidative activation, the ability to form such metabolite–DNA conjugates has not been explored. Mitoxantrone has features which are similar to anthracyclines and essential for DNA intercalation, such as a planar and electron-rich chromophore. Additionally, the presence of side chains with basic amino groups, can enhance the complexing capabilities via electrostatic binding to the phosphate groups of DNA [11]. Mitoxantrone stabilizes double helical DNA to thermal denaturation [12] and extends its length as shown by electron microscopy [13]. Spectrophotometric measurements showed that absorption maxima of the drug exhibit hypochromic and bathochromic shifts upon complexation with double-stranded DNA [14, 15]. Determinations using a topoisomerase I assay, and independently by viscosity measurements, show that mitoxantrone unwinds DNA by 17° [16]. These observations are in agreement with inter-

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‡ Abbreviations: MXH_2, mitoxantrone; 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]9,10-anthracenedione; MH_2, mitoxantrone metabolite; AH^-, ascorbic acid; HRP, horseradish peroxidase; and GSH, reduced glutathione.

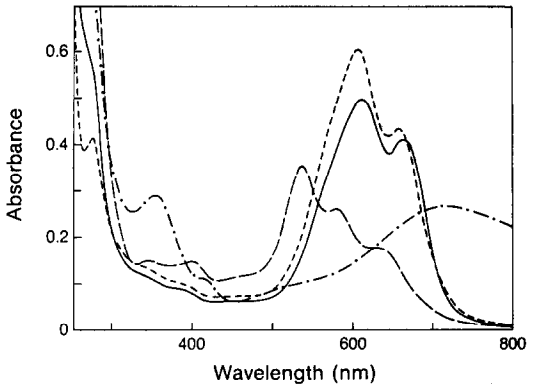

Fig. 2. Absorption spectra of MXH_2 (48 μM) (----); MXH_2 (48 μM) in the presence of sonicated ct DNA (48 μM, in base pairs) (——); as above + HRP (10 μg/ml) + H_2O_2 (100 μM) (-·-·-); as above + ascorbic acid (1 mM) (— —). Reactions were carried out in Tris/HCl buffer, pH 8.

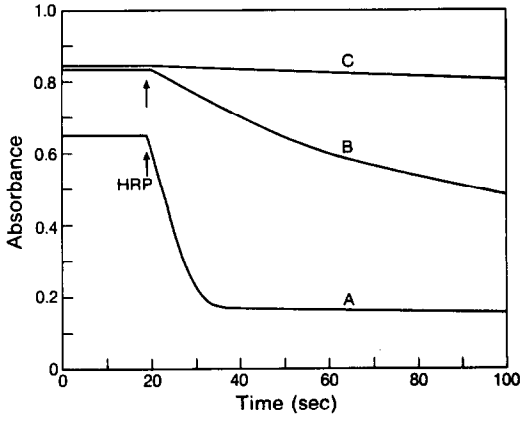

Fig. 3. Kinetics of the oxidation of MXH_2: effects of the presence of ct DNA and salt concentration (A) [MXH_2] = 51 μM, DNA absent, NaCl (100 mM); (B) same as A in the presence of ct DNA, DNA bp/drug = 5.3; (C) same as B but in the presence of 5 mM NaCl. Reactions were in phosphate buffer, pH 7. [H_2O_2] = 135 μM. Oxidation was initiated by injection of HRP as indicated by arrows. Measurements were performed by recording the decrease in the absorption maxima for a given sample: (A) at 608 nm; (B) 680 nm; and (C) 682 nm.

calation of mitoxantrone into the DNA macromolecule. Similar results were found for other diamino-substituted anthraquinones [17]. Mitoxantrone is able to induce a destabilization of the secondary structure of nucleic acids in solutions causing their condensation [18, 19]. In addition, DNA transcription and RNA processing are affected by mitoxantrone [13, 14, 20]. Such biochemical evidence indicates that nucleic acids are among the principal cellular targets of the drug. It must be borne in mind, however, that mechanisms other than, or in addition to, DNA binding may be significant in the strong antitumor activity of aminoanthraquinones [12].

Binding of a dye to a macromolecule usually changes its physiochemical properties, such as redox and photosensitizing capabilities [21, 22]. For example, it was demonstrated recently that certain

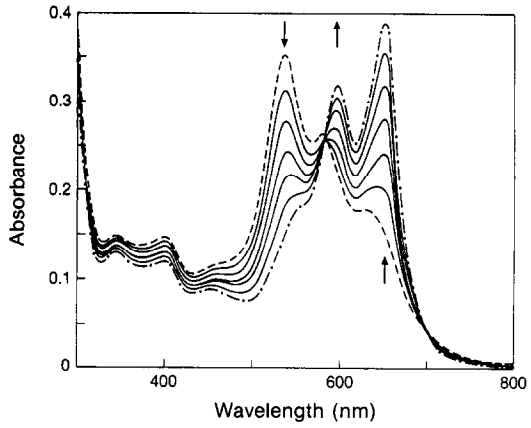

Fig. 4. Absorption spectra of the purple product P with sonicated ct DNA. To a 1-ml solution of the purple product (---), obtained as in Fig. 2, 20-μl aliquots of DNA were added (stock solution 2.5 mM in base pairs) and relevant spectra were recorded. The final spectrum (-·-·-) corresponds to a DNA bp/MH_2 ratio of 6.5. Reaction was in Tris/HCl buffer, pH 8.

aminoanthraquinones, structurally related to mitoxantrone, are devoid of photosensitizing properties after binding to DNA [23].

The objective of the present study was to examine the effect of binding of mitoxantrone by DNA on the oxidation of the drug by the HRP/H_2O_2 system and to study the interaction between the mitoxantrone metabolite (MH_2) and DNA.

MATERIALS AND METHODS

Mitoxantrone and [^{14}C]mitoxantrone (sp. act. 35 Ci/mol) were supplied by Dr F. E. Durr (Lederle Laboratories, Pearl River, NY). Calf thymus DNA (ct DNA), L-cysteine reduced glutathione (GSH) and *m*-cresol were obtained from the Aldrich Chemical Co. (Milwaukee, WI) and sonicated ct DNA (average size 3000 base pairs, range 200–6000 base pairs) from Pharmacia (Uppsala, Sweden). Bacteriophage PM2 DNA was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Poly(dA)·poly(dT), poly(dG)·poly(dC) and horseradish peroxidase, type VI (EC 1.11.1.7) were from the Sigma Chemical Co. (St Louis, MO). Oligonucleotides (10 base pairs) were prepared by Dr R. Pon, Regional DNA Synthesis Laboratory, Calgary, Alberta. Ascorbic acid (AH^-) was from American Chemicals, Ltd. (Montreal, Quebec). Phenol and Scinti-verse II were obtained from the Fisher Scientific Co., Fair Lawn, NY. Hydrogen peroxide (30%) was from Anachem. Metabolite (MH_2) was synthesized according to the method described in an earlier report [8].

The following buffers were used: Tris/HCl (200 mM KCl) pH 8, phosphate (100 mM) pH 7, 0.1 × SSC (1.5 mM sodium citrate, 15 mM NaCl) pH 7, and acetate (100 mM) pH 5. Stock solutions of mitoxantrone (5 mM) and H_2O_2 (8.8 mM) were prepared in distilled water and ct DNA (2.5 mM in base pairs) in 0.1 × SSC buffer. Oxidation was initiated by injection of 5 or 10 μl of a stock solution of HRP (1–2 mg/ml) to a solution (1 ml) containing

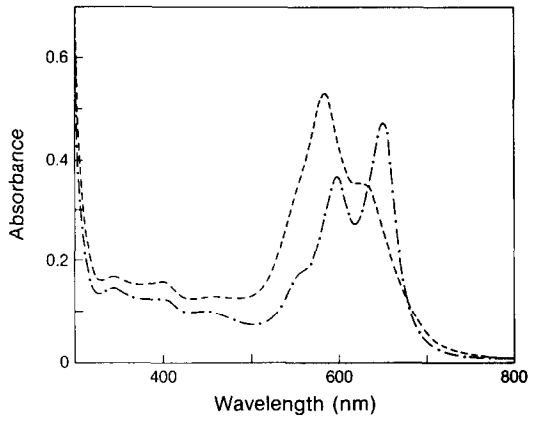

Fig. 5. Absorption spectra of the metabolite MH_2 recorded in the presence (–·–·–) and absence (– – –) of sonicated ct DNA. [DNA bp]/[MH_2] = 9. Reaction was in Tris/HCl buffer, pH 8.

Table 2. Relative rates of oxidation of MXH_2 for different concentrations of ct DNA in the sample ([MXH_2] = 48 μM; [HRP] = 10 μg/ml; [H_2O_2] = 88 μM in 0.1 M phosphate buffer, pH 7.0)

DNA base pair/MXH_2 ratio	$\Delta A/A$ (%)
0	63*
1	45.5
2.5	40
5	21
10	8.6

* This level corresponds to a plateau reached within 13 sec from the initiation of the reaction with HRP.

DNA-adhered mitoxantrone. The binding of [^{14}C]mitoxantrone to DNA was assessed through a modification of a procedure described previously by Irving and Veazey [24]. The reaction mixtures contained ^{14}C-labeled mitoxantrone (25 μM), and hydrogen peroxide (50 μM) in 0.1 M phosphate buffer pH 7.0, and the reaction was initiated by addition of horseradish peroxidase (final concentration 5 μg/ml). Three minutes was sufficient for the complete oxidation of the added drug. Then ct

Table 1. Absorption maxima measured for MXH_2, MH_2 and their complexes with sonicated ct DNA (Tris/HCl buffer, pH 8)

Compound or DNA complex	Absorption maxima (nm)
MXH_2	608*, 656
DNA/MXH_2	
(1:1)	612, 668
(10:1)	628, 680
MH_2	584, 628
	(588, 636 and threshold at 547)†
DNA/MH_2	
(1:1)	536, 580, 628 (threshold)
(9:1)	596, 652, 554 (threshold)
MH_2-GS	616, 664

* Wavelength for the highest peak is underlined.
† In MeOH.

all necessary substrates, unless stated otherwise. Absorption spectra were measured in 1 cm quartz cuvettes using an HP Diode Array spectrophotometer model 8452A. The radioactivity of [^{14}C]mitoxantrone and DNA-bound drug was measured with a Beckman LS 750 liquid scintillation counter.

Thermal denaturation studies were performed as follows. A solution of ct DNA was prepared in 0.1 × SSC buffer at 150 μM. Samples were heated at a rate of 1°/min in the absence and presence of drug at a drug:DNA base pair ratio of 0.1. The melting temperature midpoint (Tm) was determined from the absorbance profile produced on the Gilford 250 spectrophotometer equipped with a thermal programmer.

Determination of DNA unwinding angle using topoisomerase I was performed according to the procedure described previously [16].

Covalent binding of enzymatically activated mitoxantrone to DNA. The strong affinity of mitoxantrone for DNA [14–19] precludes the employment of molecular sieves or other chromatographic methods for the separation of the covalently bound DNA–[^{14}C]mitoxantrone product from non-covalently

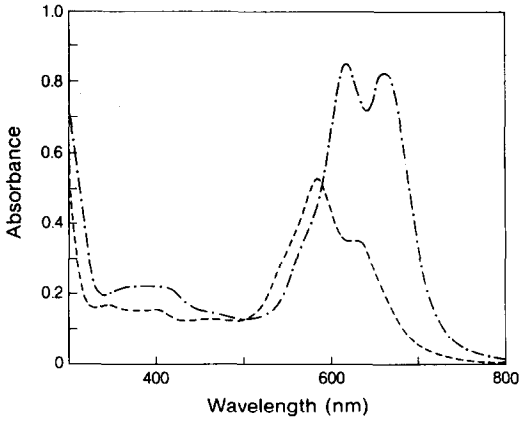

Fig. 6. Absorption spectra recorded upon addition of ascorbic acid (0.5 mM, – – –) and GSH (2.5, –·–·–) to a sample containing MH_2^{2+}. Reaction was in Tris/HCl buffer, pH 8.

Table 3. Measurements of radioactivity of [^{14}C]mitoxantrone binding to ct DNA*

Time (hr)	With HRP added		No HRP added	
	Radioactivity† (cpm)	Bound drug (pmol/mg DNA)	Radioactivity† (cpm)	Bound drug (pmol/mg DNA)
0	177	178	168	170
0.25	1620	1636	216	218
0.50	2376	2400	270	272
1.0	2502	2527	360	363
2.0	2846	2872	268	270
4.0	4693	4727	356	360
6.0	5732	5781	359	362
24.0	6559	6617	450	454

* See Materials and Methods for exact experimental conditions.

† The radioactivity measurements were performed using 1/5 parts of total volume described in Materials and Methods. Respective measurement for labeled drug gave the value of 450,000 cpm.

DNA (5.5 μg) was added to the reaction mixture. To increase the probability of the covalent binding of the enzymatically activated drug, the binding experiments were performed using a 400-fold excess of DNA base pairs over the drug concentration. The reaction was allowed to proceed and was sampled at 0.25, 0.5, 1, 2, 4, 6, and 24 hr. Blanks, i.e. incubations without enzyme, were used to follow any non-specific binding of radioactive mitoxantrone or its oxidation products and to correct for the possible carry-over of ^{14}C-labeled products bound to protein into the aqueous phase. The reactions were terminated by the addition of ascorbic acid (200 μM) and followed by 3-fold extraction with phenol-*m*-cresol (30 g phenol and 4.2 ml *m*-cresol saturated previously with 0.1 mM phosphate buffer, pH 7). The final aqueous phase was extracted twice with 1 ml of ether to remove traces of phenol and cresol. Residual ether was removed by passing nitrogen gas through the samples. The resulting aqueous phase and 1 ml of 2% potassium acetate in 95% ethanol were mixed in Eppendorf 1.5-ml conical microcentrifuge tubes and kept for 2 hr at −70°. The precipitate of DNA was centrifuged and washed three times with cold 95% ethanol. The pellets were then dried, dissolved in 100 μl of 0.1 mM phosphate buffer, pH 7.0, and, after addition of 10 ml of Scinti-Verse II scintillation mixture, were counted for radioactivity. The results are expressed as amount of labeled mitoxantrone bound per milligram of ct DNA (pmol/mg DNA) and are the average of duplicate determinations, which typically differed from each other by 5–10% in the complete system. Amount of bound drug was calculated using the radioactivity of [^{14}C]mitoxantrone as a reference. In a separate experiment (not shown), the recovery of radioactive material during the extraction procedure, identical with that employed in the binding experiment, was determined. It was found that 3-fold phenol—cresol extraction provided at least 99.9% recovery of radioactive material in the phenol phase.

RESULTS

Spectroscopic studies of metabolite–DNA interactions. Figure 2 shows the spectra of free (– – –) and DNA-bound (——) mitoxantrone (MXH_2) at a drug

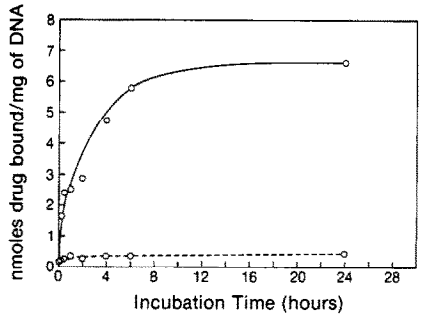

Fig. 7. Kinetics of the covalent binding of the ^{14}C-labeled mitoxantrone to ct DNA upon HRP/H_2O_2-activation. Key: (– –) reaction without HRP; (——) complete reaction system. Each point is the average value of two independent experiments. Experimental details are given in Materials and Methods.

to DNA base pair ratio of 1. In agreement with earlier reports [14, 15], it was found that the binding induced small bathochromic and hypochromic shifts in the UV–VIS spectrum of the drug. The red shift depended on the DNA base pair to drug ratio, and the positions of the absorption peaks identified are listed in Table 1.

Addition of hydrogen peroxide (0.1 mM) to the solution of MXH_2/sonicated ct DNA did not change the optical characteristics of the sample. Subsequent introduction of HRP, however, induced rapid oxidation of mitoxantrone and the formation of a new broad absorption band with maximum around 700 nm (Fig. 2). The rate of the oxidation depended on the DNA base pair to drug ratio for constant enzyme and H_2O_2 concentrations. Table 2 shows relative rates of the reaction, measured as a decrease of the absorbance at the highest peak ($\delta A/A$; A= absorbance) after 30 sec of incubation of MXH_2/DNA with the HRP/H_2O_2 system. Similar inhibition, again dependent on the DNA bp/drug ratio, was observed in the presence of non-sonicated ct DNA. This is demonstrated in Fig. 3 together with the effect of salt concentration on the oxidation. Figure 3 shows kinetic runs recorded at the same DNA bp/drug ratio (5.3), in phosphate buffer, pH 7, at two different salt concentrations: 100 mM NaCl (B) and 5 mM NaCl (C). For comparison, run A

illustrates kinetics recorded in the absence of DNA (pH 7.0, 100 mM NaCl). Similar measurements (no DNA) performed in the presence of 5 mM NaCl gave results which did not differ significantly from run A in Fig. 3. The results indicate that the electrostatic interaction between the drug and DNA profoundly affects the efficiency of the oxidation, although no such marked salt effect was found in the absorption spectra of the MXH_2/DNA system.

Addition of ascorbic acid to the incubation medium containing mitoxantrone oxidized in the presence of DNA (DNA bp/drug = 1) generated a purple product (P) the spectrum of which is shown in Fig. 2 (——). It shows absorption maxima at 536 nm (the most intense peak), 580 and 628 nm (Table 1). Figure 4 shows the effect of subsequent additions of DNA on the spectrum of the sample. Arrows indicate the direction of changes. The titration caused a change in the color of the solution from purple to blue. The spectral lines intersect in two isosbestic points: 581 and 698 nm. The final blue product had absorption maxima at 596 and 652 nm and a threshold at 554 nm (Table 1). From this observation we conclude that interaction between the metabolite and DNA is predominantly physical in nature. Further confirmation of this suggestion comes from the observation that the addition of sodium dodecyl sulfate (SDS) to P turned its color to blue, apparently due to dissociation of the metabolite antrone metabolite, MH_2 (produced by oxidation of MXH_2 with HRP/H_2O_2 system and reduction by ascorbic acid), in the absence of DNA (peaks at 584 and 628 nm, line – – –) and upon addition of DNA to a final concentration of 480 μM, giving the DNA base pair to drug ratio = *ca.* 9 (peaks at 596 and 652 nm and a threshold at 554 nm, line –·–). The latter spectrum was identical with the spectrum produced following the titration of P with DNA (Fig. 4). Moreover, P could also be obtained when small aliquots of ct DNA were added after the production of MH_2 in solution (under conditions as in Fig. 2 but when DNA was added last).

These observations indicate that the formation of P involves both the mitoxantrone metabolite, MH_2, and the DNA. The optimal drug to DNA base pair ratio for formation of P was close to 1. Compound P was observed in Tris buffer pH 8, phosphate buffer pH 7, and acetate buffer pH 5. It was found that ct DNA could be replaced by sonicated ct DNA (average size 3000 base pairs), PM2 closed-circular supercoiled DNA, poly(dG)·poly(dC), or poly(dA)·poly(dT) (data not shown). However, short synthetic oligonucleotides (10 base pairs) or single-stranded DNA at the same concentrations did not produce P in the presence of MH_2. Ascorbic acid could be replaced by $NaBH_4$, NADH, but not by L-cysteine or reduced glutathione. The thiols, however, were able to reduce the metabolite because they changed the color of the sample to blue. Two types of absorption spectra were observed in the presence of GSH, depending on the drug to GSH ratio: for $[MH_2^{2+}] = [GSH]$ absorption maxima were identified at 600 and 656 nm (not shown), while for $[GSH] \geq [MH_2^{2+}]$ peaks were at 616 and 664 nm. Figure 6 shows the latter spectrum (line –·–) superimposed on the spectrum of the metabolite obtained in the presence of AH^- (– – –). The products could be subsequently reoxidized with the HRP/H_2O_2 system and reduced reversibly with AH^- or GSH, but again they produced spectra different from the one ascribed to MH_2. The difference between those two absorption curves implies that GSH not only reduces MH_2^{2+} (as ascorbic acid does) but it is likely that the thiol binds covalently to the metabolite, forming a MH_2–SG conjugate. The presence of DNA had no effect on this reaction. Titration of the blue solution with DNA did not influence the spectrum, implying that the thiol-reduced metabolite does not bind effectively to DNA.

Measurements of DNA unwinding angle suggested exterior binding of MH_2 to ct DNA because the curves obtained in the presence and absence of topoisomerase I did not form a crossover point (not shown). In contrast, mitoxantrone gives a distinct crossover point resulting in an unwinding angle of 17° [16], indicating intercalative binding. The elevation in melting temperature midpoint (ΔTm) for ct DNA in the presence of MH_2 (drug to DNA bp ratio = 0.1) = 3.5° compared to a value of 13.5° for the parent compound mitoxantrone under identical conditions, again suggesting differences in binding mechanism.

Measurements of the absorbance at 588 nm versus the metabolite (MH_2) concentration showed that the compound obeyed the Beer–Lambert law for $[MH_2] < 116\ \mu M$ (in monomers). The UV–VIS absorption spectra of MH_2, however, retained the pattern characteristic of a highly dimerized compound even in dilute solutions. By contrast, the spectra of MH_2 in methanolic solutions indicated a higher proportion of monomers of the metabolite (Table 1).

Covalent binding of enzymatically activated ^{14}C*-labeled mitoxantrone to DNA.* Figure 7 shows the results of the measurements of binding in samples incubated up to 24 hr. Values for complete reaction containing enzyme were in the range of 1636 to 6617 pmol/mg DNA for incubation times of 15 min and 24 hr respectively (Table 3), while blank values (enzyme omitted) were 9 and 25 pmol/mg DNA accordingly. Since the former values were significantly above the blank values and the binding yields were time dependent (Fig. 7), covalent binding to DNA is indicated. However, it has to be pointed out that the binding of HRP-activated mitoxantrone, although clearly evident, did not exceed 1.5% of total drug employed under the conditions described above.

DISCUSSION

Enzymatic oxidation of mitoxantrone is a multistep process involving generation of free radicals and structural rearrangements [7, 8]. The detailed mechanism of this process is still not elucidated completely, but it can be described generally by the equations below (for $[H_2O_2] > [MXH_2]$):

$$MXH_2 \xrightarrow{HRP/H_2O_2} MXH_2^{\cdot+}/MX_2^{\cdot+} \rightarrow MH_2^{2+} \quad (1)$$

$$MXH_2 + MH_2^{2+} \rightarrow 2MH_2^{\cdot+} \quad (2)$$

$$MH_2 + MH_2^{2+} \rightarrow 2MH_2^{\cdot+}. \quad (3)$$

It is not clear at the present time if the radicals are formed during enzymatic oxidation (HRP catalyzed one-electron oxidation, Eqn 1) or via comproportionation-disproportionation of the fully reduced and oxidized forms of the substrates (Eqns 2 and 3) [8]. It is known, however, that the first step (i.e. the oxidation of mitoxantrone to its metabolite) is irreversible, although the metabolite itself can participate in reversible oxidation and reduction reactions:

$$MXH_2 \underset{AH^-}{\overset{HRP/H_2O_2}{\rightleftharpoons}} MH_2^{2+} \underset{AH^-}{\overset{HRP/H_2O_2}{\rightleftharpoons}} MH_2. \quad (4)$$

Upon addition of ascorbic acid the oxidized metabolite is reduced as evidenced by the absorption spectrum characteristic of MH_2 shown in Fig. 5 [7, 8]. The reaction most probably proceeds as described by Eqn 5 because EPR measurements of samples

$$MH_2^{2+} + 2AH^- \rightarrow MH_2 + 2A^{\cdot -} + 2H^+ \quad (5)$$

containing MH_2^{2+}, to which AH^- was added, showed the presence of the ascorbyl radical (data not shown). Attempts to examine the effects of DNA binding on the generation of the drug-derived radicals were unsuccessful. To detect an EPR signal from the $MXH_2/HRP/H_2O_2$ system, a rather high concentration of the drug is required (*ca.* 3 mM), and the pH of the medium must be kept below 5 [7]. Under these conditions, the addition of DNA at a ratio of 1 and above resulted in an immediate precipitation of the solute.

In this report we have demonstrated that complexation of mitoxantrone with DNA inhibited the process of enzymatic oxidation of the drug. The greatest inhibition was observed for the DNA bp/drug ratio of 10 (Table 2). This can be explained assuming that under these conditions most of the drug molecules are intercalated into the DNA and are thereby not readily accessible to the enzyme. As the DNA concentration decreases relative to the drug concentration, the fraction of unbound drug increases, becoming accessible to the enzyme, and the oxidation rate increases.

Besides intercalation, ionic interactions also contribute to the strength of binding and may, therefore, affect the oxidation process. This is illustrated clearly in Fig. 3. This is in agreement with the two modes of the drug binding, which may occur simultaneously [14]. At low salt concentration the binding is enhanced by electrostatic attraction of the two alkylamino side chains to the outside of the double helix, probably holding the drug chromophore in a more rigid position and/or bound more firmly between the DNA bases, making it again less accessible to the enzyme. At high salt concentration the drug cannot react with DNA as easily and is therefore more likely to be in solution and accessible to enzyme.

Results of measurements presented in this report demonstrate that the mitoxantrone metabolite, MH_2, is capable of binding covalently and non-covalently to DNA. The absorption spectrum of the reduced metabolite (Fig. 5) shows a pattern characteristic of a solute existing in highly dimerized/aggregated forms: the absorption peak at lower wavelength (584 nm) is more intense than at higher wavelength (628 nm). Addition of DNA to the sample markedly changed its absorption spectrum. For a DNA bp/drug ratio of 1 a blue shift in the absorption maximum was observed and the color of the sample turned purple (Fig. 2). The same complex was formed if the oxidized form of the metabolite was produced in the presence of DNA and ascorbic acid was added subsequently.

Formation of a new, blue-shifted, absorption band is characteristic of many cationic dyes, such as methylene blue, crystal violet and pyronine, adsorbed on polyelectrolytes including DNA [25–28]. The phenomenon is called "metachromasia" and was first described by Michaelis and his coworkers [29, 30]. The effect was ascribed to different modes of interaction between the dye molecules: they can bind to a polymer as monomers, or they interact with each other on the polyelectrolyte to form dimers or aggregates similar to those in concentrated solutions. A simple model explains this behavior in terms of dilution of the dye molecules along the polymer chain as the ratio of [binding sites]/[dye] increases [31]. The following kinds of behavior were observed in the case of MH_2/DNA systems: (i) the blue shift of the absorption maxima versus the absorption peaks of MH_2 alone, which corresponds to the formation of higher aggregates of the metabolite on DNA (complex P), and (ii) subsequent gradual transformation of the spectrum, from the "highly concentrated" type to a "more dilute" form, i.e. with higher content of monomeric form of the metabolite, during titration with DNA (Fig. 4).

The binding study with the ^{14}C-labeled drug indicated that the oxidized metabolite, which possesses a highly reactive diimino function in its structure, which renders it strongly electrophilic, bound covalently to DNA. However, the yield of the binding was low, not exceeding 1.5% under the applied experimental conditions. The interpretation of this result must be done with caution, because earlier experiments showed that prolonged incubation of the drug under oxidative conditions leads to degradation products of undetermined structures [8]. Therefore, the nature of the newly-formed DNA adduct remains to be established.

Addition of GSH or L-cysteine to a solution of MH_2^{2+} yielded a product which is different from the one obtained with ascorbic acid. This conclusion is based on the following observations: (i) the UV–VIS spectrum of the sample containing the thiols was distinctly different from the spectrum recorded in the presence of ascorbic acid, both in the shape of the absorption lines and in the intensities of the peaks; (ii) the product of the reaction between MH_2^{2+} and the thiols did not bind to DNA, as judged by the lack of change in the absorption spectrum upon addition of DNA. Many oxidatively activated xenobiotics, including aromatic amines and anticancer agents, form covalently bound conjugates with GSH [32, 33]. It is likely that the oxidized form of the metabolite, MH_2^{2+}, which contains very reactive diimine functions [8], also reacts with GSH and L-cysteine in this way although this possibility requires further investigation. It is conceivable that the bulky moiety from the thiols attached to the

metabolite could hinder its binding to DNA. Participation of GSH in this type of reaction in cells is believed to be related to its protective and detoxifying role [32].

In conclusion, the oxidative enzymatic activation of the anticancer agent mitoxantrone was affected by the presence of DNA macromolecules in the incubation mixture. The degree of the inhibition depended on the DNA base pair to drug ratio and the concentration of salt since both these parameters determine the extent of intercalation of the drug molecules into DNA. The metabolite bound externally to DNA, and for low DNA bp/drug ratios produced new metachromic absorption bands in the visible spectrum of the complex. In addition, time-dependent covalent binding of the enzymatically activated drug to DNA was confirmed. The possible implications of those results for the cytotoxic action and general toxicity of mitoxantrone and related structures are under active investigation in our laboratories.

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