Formation of DNA adducts by formaldehyde-activated mitoxantrone

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ABSTRACT

Recent studies with the anthracycline Adriamycin have demonstrated its activation by formaldehyde and subsequent binding to DNA in vitro. Since formaldehyde levels are known to be higher in cells of myeloid origin and the structurally related drug mitoxantrone is most effective against cancers of myeloid origin, this indicates a possible role of formaldehyde in the activation of mitoxantrone. In vitro studies revealed that the activation of mitoxantrone by formaldehyde leads to the formation of drug–DNA adducts. These adducts stabilised DNA such that they functioned as virtual interstrand crosslinks. The interstrand crosslinks were formed in the presence of mitoxantrone and formaldehyde in a time- and concentration-dependent manner. In the absence of formaldehyde no crosslinks were formed, indicating a key role in drug activation and DNA binding. The adducts (virtual crosslinks) were relatively unstable with 50% crosslinks remaining after 10 min at 60°C in 45% formamide. Like Adriamycin, the mitoxantrone–formaldehyde–DNA crosslinks are heat labile and do not display the stability associated with covalent interstrand crosslinks.

INTRODUCTION

Mitoxantrone is a synthetic anthraquinone drug which belongs to the anthracenedione class of compounds (1,2). These drugs have a symmetrical structure comprising a tricyclic planar chromophore and two basic side chains (3). Mitoxantrone shows significant activity in patients with acute nonlymphocytic leukemia, advanced breast cancer, non-Hodgkins lymphoma (2), non-small cell lung cancer and melanoma (4). This drug offers comparable efficacy but lower acute toxicity than the most active currently available single agents used in the treatment of advanced breast cancer (4). The primary side effect associated with mitoxantrone is nausea and vomiting and the major delayed toxicity is myelosuppression (5).

Because of its structure, mitoxantrone may be viewed as an anthracycline analog with the daunosamine sugar of Adriamycin and other anthracyclines being replaced with two identical aminoalkyl side chains (1,5) while the aromatic ring structure of the anthracyclines that permit intercalation into DNA is retained. The major dose-limiting side effect associated with Adriamycin treatment is cardiotoxicity (1,3,5), which has prompted the search for new derivatives. Adriamycin cardiotoxicity is associated with the generation of oxygen-derived free radicals by redox cycling of the anthracycline quinone. The quinone is readily reduced to the semiquinone which reacts rapidly with oxygen and eventually leads to production of hydroxyl and other oxygen radicals (5). Since heart tissue does not possess detoxifying enzymes such as catalase and superoxide dismutase (6), it is sensitive to oxygen radical damage. In contrast, mitoxantrone is unable to be reduced to the semiquinone intermediate due to its more negative reduction potential than the anthracyclines and therefore does not exhibit the cardiotoxicity associated with the anthracyclines Adriamycin and daunomycin (5,7).

There is still a considerable degree of conjecture regarding the mechanism of action of mitoxantrone and little is known of the mechanism by which mitoxantrone binds to DNA. Mitoxantrone intercalates into DNA and can be oxidatively activated to bind to nucleotides on DNA (7–9). It has been observed that there is a fast cellular uptake of mitoxantrone whereby the intracellular concentration exceeds by 80-fold its extracellular concentration in human erythroleukemic K562 cells (2) and this cell accumulation is concentrated particularly in the nucleus in the form of complexes with nucleic acids (2,10). It has been suggested that mitoxantrone is activated by oxidation by myeloperoxidase, the most abundant protein in neutrophils and also found in monocytes (11). Myeloperoxidase has subsequently been shown to oxidise mitoxantrone to metabolites which bind covalently to DNA and RNA (12).

Recently, substantial evidence has revealed that formaldehyde plays a role in the binding of anthracyclines to DNA (13–17). Elevated levels of formaldehyde have been found in lymphocytes of chronic lymphocytic leukemia compared to normal lymphocytes. In addition, formaldehyde levels are higher in normal myeloid cells than in normal granulocytes (13). Studies with daunorubicin and Adriamycin have found that stable covalent adducts in DNA are formed in the presence of formaldehyde (14–18). Koch and co-workers synthesised conjugate forms of these anthracyclines with formaldehyde and showed that the Adriamycin–formaldehyde derivatives have enhanced cytotoxicity towards tumour cells, presumably because they do not require drug-induced intracellular production of formaldehyde (15).

Given the structural similarities between Adriamycin and mitoxantrone, the activity of mitoxantrone against cells of myeloid origin (which have elevated levels of formaldehyde)
and that formaldehyde can be formed in the presence of myeloperoxidase and hydrogen peroxide, this suggests a possible critical role of formaldehyde in the formation of mitoxantrone–DNA adducts. In order to gain an understanding of this process, the present studies were directed towards investigating the role of formaldehyde in the formation of DNA adducts and interstrand crosslinks by mitoxantrone in vitro.

MATERIALS AND METHODS

Materials

Mitoxantrone was kindly provided by Lederle Laboratories (Pearl River, NY). The drug stock solution was prepared by dissolving in Milli-Q water (Millipore, MA) to a final concentration of 2 mM and stored at −20°C. Formaldehyde was purchased from BDH and formamide was from Sigma Chemical Co. Bovine serum albumin (BSA), the Klenow fragment of Escherichia coli DNA polymerase and deoxynucleotides were obtained from Pharmacia and [γ-32P]dCTP (specific activity 3000 Ci/mmol) was from Amersham. The restriction enzyme SalI was purchased from Promega and calf thymus DNA was from Worthington Biochemical Corporation (NJ). Nensorb 20 columns were obtained from NEN Research Products and the plasmid purification maxi kits were purchased from Qiagen. All other chemicals and reagents were of analytical grade and all solutions were prepared using water from a Milli-Q four stage purification system.

DNA isolation

The plasmid pCC1 (19) was isolated using a Qiagen maxi purification kit. The plasmid was then linearised by restriction digestion with SalI. This 3496 bp DNA fragment was used for all subsequent crosslinking studies.

Crosslinking assay

The linearised DNA from pCC1 was 3'-end-labelled using the Klenow fragment of E.coli DNA polymerase I and [γ-32P]dCTP (specific activity 3000 Ci/mmol) and was then purified using a Nensorb 20 chromatography column. The labelled DNA was then vacuum dried and resuspended in 300 µM bp sonicated calf thymus DNA in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

Mitoxantrone concentrations were measured spectrophotometrically at 608 nm. For studies of the dependence of mitoxantrone concentration on crosslink formation, end-labelled DNA (25 µM bp) was incubated with mitoxantrone (0–24 µM) and formaldehyde (3 mM) in phosphate-buffered saline (PBS). For studies of crosslink formation dependence on formaldehyde concentration, 25 µM bp labelled DNA was incubated with 20 µM mitoxantrone and 0–3 mM formaldehyde in PBS. Samples were incubated at 37°C for 2 h and then extracted once with phenol and once with chloroform and precipitated in ethanol. Samples were resuspended in 15 µl TE buffer and denatured in an equal volume of loading dye (90% formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) at 60°C for 5 min.

Electrophoresis and crosslink analysis

Samples were loaded onto a 0.8% agarose gel and subjected to electrophoresis overnight at 45 V in TAE buffer. The agarose gel was then vacuum dried on a BioRad Model 583 gel drier and exposed to a phosphor screen overnight. The gel was analysed and the bands quantitated using a Model 400B PhosphorImager and ImageQuant software (Molecular Dynamics, CA).

RESULTS

Concentration dependence of crosslink formation

When drug-treated DNA contains an interstrand crosslink it is resistant to strand separation and therefore runs slower through a gel matrix due to its higher molecular weight. Two DNA controls were included in the following experiments to establish the initial integrity of the DNA and to confirm that the denaturing procedures were appropriate.

Crosslinks generated in the presence of mitoxantrone and formaldehyde were dependent on the formaldehyde level, as shown by the increase in crosslinks associated with an increase in formaldehyde concentration (Fig. 1). At 3 mM formaldehyde and 20 µM mitoxantrone, DNA was totally crosslinked after 2 h incubation (Fig. 1A). In contrast, the control lane, where DNA was incubated with 3 mM formaldehyde but no mitoxantrone, shows no crosslink formation. The percentage of crosslinked DNA was quantitated in each lane and is shown in Figure 1B. At 1.5 mM formaldehyde ~50% of DNA was crosslinked under these reaction conditions and crosslink levels began to plateau at ~3 mM formaldehyde.

Crosslink formation was also dependent on mitoxantrone concentration (Fig. 2), indicated by the increase in interstrand crosslink production with increasing mitoxantrone concentration. The control sample, incubated in the absence of formaldehyde, contained single-stranded, non-crosslinked DNA, indicating the requirement for both mitoxantrone and formaldehyde in crosslink formation. The results were quantitated (Fig. 2B) and reveal that at 20 µM mitoxantrone and 3 mM formaldehyde DNA was completely crosslinked, consistent with the results observed for the formaldehyde concentration dependence (Fig. 1). At 10 µM mitoxantrone 50% of the DNA was crosslinked.

Time dependence of crosslink formation

End-labelled plasmid DNA was incubated with formaldehyde and mitoxantrone for times up to 60 min. The results shown in Figure 3 reveal a time dependence for crosslink formation where the DNA is completely crosslinked at 20 µM mitoxantrone, 3 mM formaldehyde after 30 min at 37°C. The results for time dependence of crosslink formation at three formaldehyde concentrations are shown in Figure 3B. The absolute level of crosslinking was dependent on formaldehyde concentration with maxima of 12, 55 and 90% double-stranded DNA at 1, 2 and 3 mM formaldehyde, respectively. Maximum crosslinking was detected after ~30 min, irrespective of the
formaldehyde concentration. Increasing concentrations of formaldehyde led to faster reaction rates, as indicated by the increased gradients of each slope on the graph. Figure 3C summarises the time dependence of crosslink formation at varying mitoxantrone concentrations. Maximal crosslink levels were reached by ~30 min for all drug concentrations, with 90% interstrand crosslinking at 20 µM, 45% at 10 µM and 16% at 5 µM drug. The extent of crosslinking varied as a function of drug concentration and an increase in reaction rate at higher concentrations was observed.

Thermal stability of interstrand crosslinks
As the DNA crosslinks formed by Adriamycin are known to exhibit thermal instability, it was necessary to investigate the stability of the mitoxantrone-induced DNA crosslink. Partially crosslinked DNA samples were incubated at varying temperatures (60–72°C) in TE buffer for 5 min. The melting curve (Fig. 4) indicates that 50% of crosslinks are denatured at 65°C and after 5 min at 72°C all of the crosslinks were lost.

Time dependence of crosslink stability
DNA was treated with mitoxantrone and formaldehyde to induce an initial level of crosslinks. The samples were then incubated at 37, 60 (Fig. 5A) and 90°C for 0–120, 0–70 and 0–2.5 min, respectively. The percentage crosslinks remaining is expressed as a function of incubation time in Figure 6B and illustrates the lability of crosslinks over time. At 90°C, interstrand crosslinks were totally lost within 1 min, while at 60°C the crosslinks were stable for 5 min, with a 50% loss after 10 min. When the crosslinked DNA was incubated at 37°C half of the crosslinks remained after 50 min but were totally lost after 80 min.

DISCUSSION
Activation of mitoxantrone by formaldehyde
Recent studies have shown that Adriamycin forms virtual DNA interstrand crosslinks both in vitro and in tumour cells (20–22) (Cullinane et al., unpublished data). These lesions were shown in vitro to be dependent on formaldehyde (14–17). Due to the
structural similarity between mitoxantrone and Adriamycin, the formation of formaldehyde-mediated DNA interstrand crosslinks by mitoxantrone was investigated. The results indicate that mitoxantrone forms similar crosslinks and the formation of these lesions is dependent on both formaldehyde concentration, with crosslinks being detected as low as 0.5 mM, and on mitoxantrone concentration, with crosslinks being detected at <5 µM drug. This mitoxantrone concentration is similar to that used in the clinic, where the dosage administered intravenously is typically 14 mg/m² (~7 µM) (1), with an effectively intracellular concentration that is approximately 80-fold more than the extracellular level (2).

The time dependence of interstrand crosslinking revealed that the reaction between mitoxantrone and formaldehyde resulted in rapid binding of the drug to DNA, with the reaction reaching completion within 30 min at all concentrations studied. The absolute level of interstrand crosslinks generated increased when faster reaction rates were achieved at higher concentrations of mitoxantrone and formaldehyde. In comparison, time dependence studies of daunorubicin crosslinking to DNA by formaldehyde also exhibited a rapid approach to equilibration, but at considerably higher concentrations of reactants [350 µM DNA, 42–420 mM (0.125–1%) formaldehyde and 35 µM daunorubicin] (16). The reaction reached completion after 90 min at 42 mM formaldehyde, much slower than the reaction observed with 20 µM mitoxantrone, 3 mM formaldehyde and 25 µM DNA. Studies with barminomycin (essentially a preactivated form of Adriamycin) also exhibited slower reaction rates, with 50% of DNA being crosslinked in 1 h at 5 µM drug (23).

Lability of the mitoxantrone interstrand crosslink

Adriamycin forms heat-labile crosslinks. The covalent bonds between Adriamycin or daunorubicin and DNA are the result of Schiff base chemistry linking the 3'-amino group of the anthra- cycline to the 2'-amino substituent of deoxyguanosine. There is also strong hydrogen bonding to the opposing strand and intercalation between both strands (14,15,18). The DNA adduct has been termed a virtual crosslink because it behaves functionally as an interstrand crosslink (18). The similarity in structure
between mitoxantrone and Adriamycin prompted examination of the stability of the apparent mitoxantrone crosslink. The results indicate that, like Adriamycin, formaldehyde-mediated mitoxantrone interstrand crosslinks are relatively labile. Adriamycin–DNA crosslinks exhibit a half-life of 4.7 h at 37°C (24) and are therefore more stable than mitoxantrone–DNA crosslinks, where half the number of crosslinks are lost in 50 min. The instability indicates that the drug–DNA interaction is not a classical stable covalent crosslink but rather an adduct which stabilizes DNA and acts as a ‘virtual crosslink’. This therefore suggests that mitoxantrone is likely to be coupled to DNA by formaldehyde at only one of the pendant amino groups, since coupling at both ends would be expected to lead to a more stable complex.

Mitoxantrone–DNA adducts and interstrand crosslinks

The formation of mitoxantrone–DNA adducts has previously been demonstrated in the presence of myeloperoxidase and hydrogen peroxide and attributed to oxidative activation of the drug (12). However, the reason for adduct formation may actually be the ability of hydrogen peroxide to form formaldehyde (18), which in turn converts mitoxantrone to its activated form. Interstrand DNA crosslink formation has also been shown in vitro by neutrophil activation of mitoxantrone (11,25). In this case the crosslinks were attributed to the increased amounts of myeloperoxidase in these cells (25), but since these cells also contain elevated levels of formaldehyde, this may account for activation of the drug and the subsequent binding to DNA.

Konopa and colleagues have reported DNA interstrand crosslink formation by mitoxantrone in vivo (26). Since some cancer cells have elevated levels of formaldehyde (13), this may have enabled drug activation and subsequent adduct formation with DNA. If the DNA adducts and DNA interstrand crosslinks are one and the same lesion, as they appear to be for Adriamycin (27), then it is highly likely that the comparable mitoxantrone–DNA adducts and interstrand crosslinks are also one and the same lesion and are also mediated by reaction with formaldehyde.
Possible structure of the virtual interstrand crosslink

The dependence of crosslink formation on both mitoxantrone and formaldehyde indicates that both mitoxantrone and formaldehyde are required to produce interstrand crosslinks in DNA in vitro. Mitoxantrone alone cannot form significant levels of interstrand crosslinks and therefore requires an extrinsic activator to enable covalent interactions with DNA. Although formaldehyde has been reported to crosslink proteins in cells (28), it does not form interstrand crosslinks with DNA in similar concentrations. Reaction of formaldehyde and mitoxantrone is expected to generate an active form of the drug which can bind to DNA. It is presumed that the drug binds to DNA via a methylene group provided by formaldehyde. The methylene group most likely associates with amino groups on the alkylamino side chain, forming an activated intermediate similar to that of formaldehyde-activated Adriamycin (15,17). The binding of formaldehyde to these sites would form a Schiff base which is reactive and would provide at least one potential binding site to nucleotides (Fig. 6), yielding adducts which would be comparable to that described for DNA–Adriamycin adducts and which function as virtual interstrand crosslinks (18). However, it is possible that both amino groups on the mitoxantrone side chains might be activated, therefore potentially leading to a conventional interstrand crosslink. Work is currently underway to confirm the chemical nature of these adducts/interstrand crosslinks and to establish if both amino groups on mitoxantrone can be activated and contribute to two aminal linkages to DNA.

Medical implications

The activation of mitoxantrone by formaldehyde may explain why mitoxantrone is more effective against specific tumors. It is known that formaldehyde is elevated in cells of both lymphoid and myeloid origin. For this reason cancers of these systems may have elevated amounts of formaldehyde which may enable enhanced activation of mitoxantrone (13). Solid tumors such as advanced breast cancers attract neutrophils which release formaldehyde following a respiratory burst (29) and in these tumors the release of formaldehyde would enable activation of mitoxantrone and subsequent cytotoxicity towards such solid tumors. The known activity of mitoxantrone against both myeloid tumors, as well as solid tumors, is therefore consistent with a possible activation mechanism of the drug by formaldehyde or other cellular aldehydes (30) and may also account for the known myelosuppression induced by mitoxantrone.

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REFERENCES
