Long-Term Inhibition of DNA Synthesis and the Persistence of Trapped Topoisomerase II Complexes in Determining the Toxicity of the Antitumor DNA Intercalators mAMSA and Mitoxantrone

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ABSTRACT

The cytotoxic actions of several classes of antitumor DNA intercalators are thought to result from some disturbance to DNA metabolism following trapping of the nuclear enzyme DNA topoisomerase II as a covalent complex on DNA. Here we have studied topoisomerase II trapping and DNA synthesis patterns in relation to the acute cytotoxic actions of 4′- (9-acridinylamino)methanesulfon-m-anisidide (mAMSA) or mitoxantrone on SV40 transformed human fibroblasts. These two DNA intercalators differed significantly in their cytotoxic potential, mitoxantrone being 24-fold more toxic than mAMSA when assayed by the inhibition of clonogenicity. Although both drugs induced G2 delay at cytotoxic concentrations, mAMSA-treated cells recovered normal cell cycle phase distributions within 24 h of removal of drug, while mitoxantrone-treated cells continued to accumulate in G2 up to 48 h following drug treatment with evidence of complete inhibition of entry into mitosis. Compared with mAMSA, mitoxantrone showed a similar capacity to induce cleavable complexes in cellular DNA, and only a 2-fold greater ability to inhibit DNA synthesis. Within a 4-h posttreatment period, mAMSA-treated cells recovered normal rates of DNA synthesis, whereas a continued depression of DNA synthesis was observed in mitoxantrone-treated cells. The recovery patterns of DNA synthesis correlated with the rapid disappearance of mAMSA-induced complexes (<27% lesions remaining 2 h after drug removal) and the persistence of mitoxantrone-induced complexes during a 4-h posttreatment period. This difference in complex longevity was observed in other human transformed fibroblast cell lines irrespective of differences in the absolute levels of complexes induced by either agent. We suggest that the results provide evidence that DNA intercalators may differ in the forms of complexes induced and that the comparatively high cytotoxicity of mitoxantrone relates to the ability of the drug to trap topoisomerase II complexes in a form which effects a long-term inhibition of DNA replication and G2 traverse.

INTRODUCTION

Several types of antitumor DNA intercalating agents, including anthraquinone (e.g., mitoxantrone) and aminoacridine (e.g., mAMSA) derivatives, have been shown to be capable of trapping the nuclear enzyme DNA topoisomerase II in the form of a “cleavable complex” on cellular DNA (for a review see Ref. 1). This common target has raised the possibility that the cytotoxicity of apparently unrelated intercalating agents is effected through a single cellular pathway. The mechanism by which cleavable complexes constitute biologically deleterious lesions is not clear but presumably involves some disruption of DNA metabolism (1). A common feature of these intercalators is the ability to inhibit DNA synthesis (2). This paper addresses the relationship between DNA synthesis inhibition and topoisomerase II trapping in the cytotoxic actions of the intercalators mAMSA and mitoxantrone.

DNA topoisomerase II (for a review see Ref. 3) is the eukaryotic counterpart of bacterial gyrase and can alter the topology of DNA in a reaction involving the introduction of a transient double strand break into DNA, through which an intact helix can be passed (4). This reaction involves the formation of a noncovalent enzyme-DNA complex which is in rapid equilibrium with a covalent cleavable complex (5). This covalent complex can be cleaved by strong protein denaturants (6) such as alkali or sodium dodecyl sulfate to yield strand breaks in which one subunit of the homodimeric enzyme is covalently bound through a tyrosine residue to the 5′ end of each nicked DNA strand. Topoisomerase II poisons such as mAMSA and mitoxantrone stabilize the cleavable complex, preventing relaxation of the DNA strands (5). There is evidence that the accumulation of these drug-stabilized cleavable complexes, sequestering strand breaks, is an important factor in the cytotoxic action of these drugs (7).

The aminoacridine derivative mAMSA has been shown to bind to DNA through weak intercalation (8) with an orientation such that the anilino ring is located in the minor groove of the DNA and is one of few intercalators which bind preferentially to A+T-rich sequences (9). The production of topoisomerase-associated strand breaks, presumably as a result of complex formation, by a range of mAMSA-related aminoacridines has been shown to correlate well with drug cytotoxicity (7). Mitoxantrone also binds to nucleic acids by intercalation, however, the long alky side chains of mitoxantrone are believed to bind electrostatically with the anionic exterior of the helix and prevent complete intercalation (10, 11). Unlike mAMSA, mitoxantrone is one of several intercalators which can cause condensation and compaction of chromatin (12), and there is the additional possibility of DNA damage due to free radical production (13).

The actions of mitoxantrone and mAMSA on cultured cells are similar in that both act as topoisomerase poisons, inducing the formation of cleavable complexes. In addition, both drugs inhibit DNA synthesis and cause an arrest of cells in G2 phase of the cell cycle, typical cellular effects of topoisomerase poisons. However, despite a common intracellular target, mitoxantrone has been shown to be significantly more toxic than mAMSA to human cells (14, 15). Furthermore, while topoisomerase-associated damage induced by both mAMSA and mitoxantrone in breast cancer cells is strongly enhanced by estrogen stimulation of topoisomerase II availability, potentiation of cytotoxicity occurs for mAMSA alone (15), suggesting that the dependence of cytotoxicity upon topoisomerase II may be different for the two drugs. It is recognized that intercalators may interact with topoisomerase II in more than one way (16); thus the observed differences in cytotoxicity of mitoxantrone and mAMSA may not be related to the initial formation of complexes but instead may reflect differences between the form of the complexes induced and the ability of cells to resolve such damage.

The precise mechanism by which trapped topoisomerase...
complexes kill tumor cells is still not understood but appears to be through interference with DNA metabolism. Both mitoxantrone and mAMSA inhibit DNA synthesis and are most cytotoxic to cells in the S phase of the cell cycle (17, 18). It has been proposed that the cytotoxic action of the topoisomerase I poison camptothecin is due to collision of the replication fork with drug-stabilized topoisomerase I-DNA complexes (19). Given the involvement of topoisomerase II in DNA replication (20), and the preferential localization of drug-induced topoisomerase II-DNA complexes on newly replicated DNA (21), a parallel model can be proposed for the action of topoisomerase II poisons.

The difference in cytotoxicity observed between different topoisomerase poisons provides a model system for investigating the role of trapped complexes in determining cytotoxicity. Here we have studied the relationship between the persistence of mAMSA- or mitoxantrone-induced protein-DNA complexes and the ability of human cells to recover from the inhibition of DNA synthesis and execute cell cycle traverse. A preliminary report of our findings has been presented previously (22).

**MATERIALS AND METHODS**

Cell Culture. The origins and sources of the SV40 transformed normal and ataxia-telangiectasia-derived human fibroblast lines are shown in Table 1. Cultures were maintained as monolayers in Eagle's minimal essential medium, supplemented with 10% fetal calf serum, 10 mM glutamine, and antibiotics and were incubated at 37°C in an atmosphere of 5% CO2 in air. Cell stocks were routinely screened for Mycoplasma contamination.

Drug Preparation and Cell Survival Assay. Mitoxantrone (Novantron; a gift from Lederle Laboratories, Gosport, United Kingdom) was stored at −20°C as a 2 mM aqueous stock. mAMSA (amsacrine; Park Davies, Eastleigh, United Kingdom) was stored at −20°C as a 10 mM stock in dimethyl sulfoxide. Drug cytotoxicity was assessed by measuring the clonogenic potential of drug-treated cells. Monolayer cultures in exponential growth phase were detached using trypsin/Versene, plated at a density of 5 x 10⁴ cells/well for 48 h prior to drug treatment. After a 1-h exposure to drug, cells were either washed twice with Versene and frozen or washed with PBS and incubated in fresh medium for various recovery periods before freezing. The frozen cells were thawed slowly in an isotonic salt solution and aliquots containing 1 x 10³ cells were lysed in SDS prior to precipitation of protein-DNA complexes with KCl. These precipitates were washed three times and their radioactivity was expressed as a percentage of the total radioactivity in the lysate.

RESULTS

Comparative Toxicity. The long- and medium-term cellular responses of MRC5CV1 cells to the two drugs were assayed by the measurement of clonogenic potential and cell cycle perturbation, respectively. Computer analyses of the clonogenic survival data (1-h drug exposures, Fig. 1a) gave D0 values of 121.8 ± 0.5 for mitoxantrone compared with 2.9 ± 0.5 for mAMSA, indicating that this human cell line is 40-fold more sensitive to mitoxantrone than mAMSA. This difference in toxicity is similar to that shown by cultured human breast cancer cells grown under conditions of estrogen deprivation (40-fold) or stimulation (21-fold) (15). Cell cycle analyses of cultures treated with drugs for 1 h (Fig. 1b) showed that, although both agents induced G2/M delay at concentrations which are cytotoxic, the time dependence of cell cycle delay differed. The degree of G2 delay induced by mAMSA was maximal 12 h after removal of DNA synthesis after exposure to drug was determined as above, except that the cells were washed with PBS at the end of drug treatment and incubated in fresh medium for various recovery periods. The H pulse was added throughout the final 10 min of each recovery period, followed by immediate freezing.

Cells were prepared for harvesting by slow thawing in 1% SDS, followed by a 30-min precipitation in 10% trichloroacetic acid at 4°C. Precipitates were harvested onto Whatman GF-C filters using a microplate cell harvester, and filters were washed twice with 5% trichloroacetic acid, once with 95% ethanol, and air dried prior to the measurement of radioactivity using liquid scintillometry. The rate of DNA synthesis was calculated as follows: 100 x [H](drug treated)/[H](control)/[C](drug treated)/[C](control).

K-SDS Precipitation of Protein-DNA Complexes. The method used was essentially a modification of that of Rowe et al. (7) as described by Arndel et al. (27). Briefly, cells were plated at a density of 8 x 10⁵ well in 24-well plates. DNA was labeled with 0.05 μCi/ml [3H]thymidine (specific activity, 61 μCi/μmol) for 48 h, followed by a 4-h chase period in fresh medium. After 1 h exposure to drug, cells were either washed twice with Versene and frozen or washed with PBS and incubated in fresh medium for various recovery periods before freezing. The frozen cells were thawed slowly in an isotonic salt solution and aliquots containing 1 x 10³ cells were lysed in SDS prior to precipitation of protein-DNA complexes with KCl. These precipitates were washed three times and their radioactivity was expressed as a percentage of the total radioactivity in the lysate.

![Fig. 1. Toxicity of 1-h exposure to mitoxantrone (●) or mAMSA (○) determined by clonogenic potential (a) and by retention of cells in the G2/M phase of the cell cycle (b). Points, arithmetic means (range, approximately ±5%) of values from two independent experiments. Posttreatment incubation periods for cell cycle analysis (b) were: ● and ○, 6 h; □ and ▲, 12 h; ▼ and ▼, 24 h.](image-url)
drug and negligible by 24 h, while that induced by mitoxantrone increased with time up to at least 48 h (Fig. 1b).

Inhibition of DNA Synthesis and Induction of DNA Damage. DNA synthesis rates were measured by pulse labeling of cells following acute (1 h) exposure to drug. Fig. 2a shows that both mAMSA and mitoxantrone inhibited DNA synthesis in a dose-dependent, biphasic manner. Considering either the initial or final slopes of the inhibition curves, mitoxantrone was approximately 3- to 5-fold more potent than mAMSA.

Using the K-SDS precipitation method to detect DNA-protein cross-linking revealed that both mAMSA and mitoxantrone induced significant levels of complexes (Fig. 2b). The dose-dependent generation of complexes, assayed immediately after drug treatment, was similar for both agents. Complex induction was biphasic with evidence of similar plateau levels of complexes generated at drug concentrations >2.5 μM. The ability of an epipodophyllotoxin, VP-16, to induce a level of complexes generated at drug concentrations >2.5 μM. The protein cross-linking revealed that both mAMSA and mitoxantrone (data not shown) indicates that the plateau observed here does not represent the detection limit of the assay used. Although both DNA synthesis inhibition and complex generation curves were biphasic, the data show that mitoxantrone is a more potent inhibitor of DNA synthesis than mAMSA when compared at equivalent initial levels of complex formation.

Recovery of DNA Synthesis and Disappearance of Protein-DNA Complexes. DNA synthesis rates (Fig. 3a) were measured after 1-h exposures to drugs at concentrations causing approximately 50% inhibition of DNA synthesis (2.5 μM mAMSA, 1.0 μM mitoxantrone; Fig. 2a). Recovery of DNA synthesis was consistently observed to occur within a 4-h posttreatment period for cells treated with mAMSA, DNA synthesis rates returning to levels similar to those of untreated cells. In contrast, the rate of DNA synthesis remained depressed throughout the 4-h period following removal of mitoxantrone from the external medium. Fig. 3b compares the levels of drug-induced protein-DNA complexes throughout a 4-h recovery period following treatment at drug concentrations used in the DNA synthesis recovery experiments. We have consistently observed a rapid loss of mAMSA-induced complexes (80% of the complexes being repaired within 2 h of removal of the drug), while mitoxantrone-induced complexes persisted at a high level throughout a posttreatment period of at least 4 h. Persistence of mitoxantrone-induced complexes has been observed in similar experiments with other transformed fibroblast lines (Table 1). Although the initial levels of complexes vary between these cell lines, there is a consistent removal of 73–86% of mAMSA-induced complexes and persistence of >90% of those induced by mitoxantrone. The levels of complexes induced by either mAMSA or mitoxantrone were significantly elevated in an A-T-derived cell line (AT5BIVA) which is known to overproduce topoisomerase II (28). It should be noted that overproduction of topoisomerase II is not a general feature of A-T-derived cells.

![Figure 2](image_url)

Fig. 2. DNA synthesis inhibition (a) and DNA-protein complex formation (b) induced by 1-h exposure to mitoxantrone (•) or mAMSA (O). Points, arithmetic means (bars, ±SE) of 8-12 determinations.

**Table 1** Repair of intercalator-induced protein-DNA complexess in transformed human fibroblast cells

<table>
<thead>
<tr>
<th>Cell line</th>
<th>DNA-protein cross-linking a</th>
<th>% lesions remaining at t = 1 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>mAMSA treatment (2.5 μM × 1 h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRC5CVI b</td>
<td>Normal</td>
<td>10.4 ± 0.9 f</td>
</tr>
<tr>
<td>1Br3gn2 c</td>
<td>Normal</td>
<td>20.3 ± 1.6 e</td>
</tr>
<tr>
<td>GM0637 d</td>
<td>Normal</td>
<td>19.1 ± 1.4 e</td>
</tr>
<tr>
<td>AT5BIVA b</td>
<td>A-T</td>
<td>61.1 ± 6.9 116 ± 4.2</td>
</tr>
<tr>
<td>NEI-3/48 d</td>
<td>A-T</td>
<td>20.8 ± 1.5 3.3 ± 0.4</td>
</tr>
<tr>
<td>Mitoxantrone treatment (1.0 μM × 1 h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRC5CVI</td>
<td>Normal</td>
<td>7.3 ± 1.2 e</td>
</tr>
<tr>
<td>1Br3gn2</td>
<td>Normal</td>
<td>11.1 ± 0.8 e</td>
</tr>
<tr>
<td>GM0637</td>
<td>Normal</td>
<td>19.0 ± 1.0 e</td>
</tr>
<tr>
<td>AT5BIVA</td>
<td>A-T</td>
<td>25.3 ± 1.4 22.8 ± 1.3</td>
</tr>
<tr>
<td>NEI-3/48</td>
<td>A-T</td>
<td>18.2 ± 1.6 17.8 ± 0.7</td>
</tr>
</tbody>
</table>

- a Measurement of 14C-DNA-protein complexes as % total 14C-DNA precipitated in a K-SDS-containing solution (see "Materials and Methods") at the end of a 1-h drug treatment (t = 0) or after a 1-h posttreatment incubation (t = 1).
- b Kindly supplied by Dr. C. Arlett, University of Sussex, Sussex, United Kingdom.
- c Kindly supplied by Dr. L. Mayne, University of Sussex.
- d Means of 8 determinations (±SE).

Fig. 3. Recovery of DNA synthesis (a) and drug-induced DNA-protein complexes (b) during a 4-h posttreatment period. •, 1.0 μM mitoxantrone; O, 2.5 μM mAMSA. All drug treatments were for 1 h. Points, arithmetic means (bars, ±SE) of 8-12 determinations.
Stathmokinetic Cell Cycle Analysis. Stathmokinetic experiments were performed in the presence of colcemid to block cell division permitting the analysis of the early effects of the drugs on cell cycle progression. The concentrations of drug used were chosen in order to relate cell cycle perturbations to the DNA synthesis rates measured throughout a 4-h posttreatment incubation period (Fig. 3a). As expected for an asynchronously growing control cell population, the presence of colcemid revealed a continuous progression of cells from G1 into S and subsequently into G2 (Fig. 4). Contour-gating analysis of the LSM (Fig. 5d) also revealed a progressive accumulation of cells in M (Fig. 4), 25% of cells entering the LSM phase within 18 h. Acute exposure to mAMSA had little effect on exit of cells from G1 (Fig. 4) but caused a slight S phase accumulation over a 12-h period, eventually achieving control G2 levels by 18 h and control levels for the mitotic fraction at 24 h (Figs. 4 and 5e). In comparison, mitoxantrone caused a slight delay (approximately 3 h) in G1 exit, all cells exiting by 18 h (Fig. 4), and a significant slowing of S phase traverse with entry into G2 not completed until 24 h. In contrast to mAMSA, acute exposure to mitoxantrone blocked the entry of cells into the LSM phase (Figs. 4 and 5f). The slow progression through the S phase and subsequent retention of cells in G2 correlates with the observed inability of mitoxantrone-treated cells to recover normal DNA synthesis rates.

DISCUSSION

This study demonstrates that the high cytotoxicity of mitoxantrone to normal human transformed fibroblasts, compared with that of mAMSA, could not be attributed to enhanced DNA-protein complex formation or a greater initial degree of DNA synthesis inhibition. Both drugs induced similar initial levels of complexes and the enhanced degree of DNA synthesis inhibition induced by mitoxantrone did not account for the 24-fold difference in cytotoxicity. However, we provide evidence that the elevated cytotoxicity of mitoxantrone may be associated with the persistence of the drug-induced complexes on the DNA imposing a long-term depression of DNA replication. The inability of mitoxantrone-treated cells to recover normal DNA synthesis rates correlates with the observed slowing of progression of cells through the S phase of the cell cycle. Importantly, cells treated with cytotoxic concentrations of mitoxantrone can eventually complete DNA synthesis but become arrested at a point prior to the G2/M transition; therefore persistence of complexes is not a reflection of metabolically inactive cells.

Although measurement of complex generation by the K-SDS precipitation method showed that the initial levels of complexes were the same for the two agents, we note that this method is relatively insensitive to differences in the distribution or turnover of complexes throughout the genome during the treatment time. Indeed, it is likely that there are “hot-spots” on the DNA for complex formation, and given the sequence preferences of mitoxantrone and mAMSA for G+C- and A+T-rich regions, respectively, it is reasonable to assume that these would differ for the two drugs. For example, matrix-associated regions of DNA are A+T rich and are hypersensitive to cleavage by topoisomerase II (29, 30). Thus the lesions induced by mitoxantrone or mAMSA may have a different spatial relationship with respect to the nuclear matrix and, therefore, to sites of DNA replication (31). Although mAMSA-induced complexes have been shown to be preferentially located on nascent DNA (21), preliminary experiments indicate no preferential location of mitoxantrone on newly replicated DNA. Thus we suggest that the enhanced capacity of mitoxantrone to inhibit the rate of DNA synthesis is not due to an unusually high level of complex trapping on nascent DNA.

It is known that both mitoxantrone and mAMSA are capable of damaging DNA independently of topoisomerase II involvement (10, 13, 32). However, measurement of DNA strand breaks by an alkaline-denaturing method which detects both DNA replication.
protein-associated and direct strand breaks showed a similar level of break induction for the two drugs. Furthermore, we have observed that the production of both DNA-protein cross-links (Table 1) and DNA strand breaks (28) by both agents is significantly elevated in an A-T fibroblast line which overproduces topoisomerase II (ATS5BIVA), consistent with the majority of the damage induced by these agents being due to the action of topoisomerase II.

The rapid disappearance of the mAMSA-induced complexes described here compares with a previously measured half-life of 10–15 min in mouse leukemic L1210 cells (33). We have measured the level of intercalator-induced complexes during a posttreatment period for a range of human transformed fibroblast lines (Table 1). In all fibroblast lines tested we have observed a persistence of mitoxantrone-induced complexes compared with a rapid removal of those induced by mAMSA, irrespective of the initial levels of complex generation.

Mitoxantrone was also shown to be significantly more toxic than mAMSA for each of these lines (data not shown). There is little mention in the literature concerning the resolution of complexes induced by mitoxantrone and as far as we are aware this represents the first demonstration of the unexpected persistence of mitoxantrone-induced complexes.

The persistence of mitoxantrone-induced complexes relative to those induced by mAMSA may reflect differences in the forms of complexes induced by the two drugs, due to differences in either the mode of drug-DNA binding responsible for complex trapping or the DNA sequences involved in the complex. Such differences in the form or distribution of the complexes induced may result in differences in the longevity of the complexes. It is also possible that the complexes induced by mitoxantrone disappear but are continually reformcd, thus giving the overall impression of a long half-life when total complex levels are measured at any one time. Alternatively, long-term retention of mitoxantrone or one of its active metabolites may contribute to persistent trapping of topoisomerase molecules and we are currently investigating these possibilities.

As noted in the "Introduction," the mechanism by which drug-stabilized topoisomerase II-DNA complexes effect cell killing is not clear. The high cytotoxicity of mitoxantrone is associated with a marked slowing of cells during the S phase traverse at concentrations which are cytotoxic, implicating an involvement of inhibition of DNA replication in the cytostatic action of the drug. Furthermore, the results presented here show that inhibition of DNA synthesis in the presence of mitoxantrone or mAMSA occurs at concentrations which induce the generation of cleavable complexes. However, the lack of a correlation between the initial level of complexes induced and the differential cytotoxicity of the drugs implies that additional factors must be involved. Clearly, in the case of mitoxantrone, additional stress is imposed on the cell by the persistence of the DNA-protein complexes. We therefore propose that the high cytotoxicity of mitoxantrone to human cells is associated with this persistence of the cleavable complexes on the DNA, resulting in a long-term block to recovery from DNA synthesis inhibition. Whether complex persistence results in an enhanced probability of the complexes being converted into actively lethal lesions (34) remains a possibility which merits further study.

The results presented here suggest that the form of cleavable complex induced may differ, depending on the intercalator involved, and that the persistence of these complexes can determine the cytotoxicity of the drug. Structural studies of the features of the drug molecules which give rise to such differences would have implications for the future development of more potent antitumor agents. Finally, it is possible that the use of DNA synthesis inhibitors on cells containing long-lived cleavable complexes may act to modify the lethal action of drugs such as mitoxantrone in a manner not predicted from previous studies using topoisomerase poisons which trap complexes that disappear rapidly. Such interactions would have implications for the use of these agents in combination therapy.

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REFERENCES


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