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SHORT COMMUNICATIONS

Microsomal activation and increased production of 4′-(9-acridinylamino)-3-methanesulfon-*m*-anisidide (m-AMSA)-dependent, topoisomerase-associated DNA lesions in nuclei from human HL-60 leukemia cells

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The compound 4′-(9-acridinylamino)-3-methanesulfon-*m*-anisidide (Amsacrine or m-AMSA) is an acridine-containing antitumor drug which has been widely investigated for the treatment of several human malignancies, including acute myelocytic leukemia [1]. The drug is one of a class of structurally diverse compounds that inhibit DNA topoisomerase II [2, 3], a nuclear enzyme which catalyzes double-stranded DNA cleavage and religation reactions which result in changes in the topology of DNA. These topological alterations are thought to function in the regulation of DNA replication and transcription [4–8]. There is significant evidence to suggest that the mechanisms for the inhibitory effects of drugs such as m-AMSA involve the formation of an inhibited complex in which topoisomerase II is covalently linked to a DNA strand break. This lesion is believed to give rise to the cytotoxicity of m-AMSA and other topoisomerase-interactive drugs [3, 4, 9, 10]. In addition, the formation of the drug- and enzyme-dependent protein-linked DNA lesions, which are precipitable in the presence of sodium dodecyl sulfate (SDS) and potassium, represents one basis for *in vivo* measurements of the intranuclear concentrations of the DNA topoisomerases [9, 10].

Several aspects of the pharmacology of m-AMSA have been investigated already (for review, see Ref. 1). Hepatic metabolism and biliary excretion have been identified as significant pathways of m-AMSA elimination in the human [11], and the major biliary metabolite in the rat is the m-AMSA–glutathione (GSH) conjugate with the thioether linkage at the 5′-position of the anilino ring [12, 13]. Studies by Wong *et al.* [14] have also demonstrated that the GSH conjugate of m-AMSA is capable of binding to DNA. Aspects of the metabolism of m-AMSA at the subcellular level have been reported by Shoemaker *et al.* [15], who found that rat hepatic microsomes could catalyze the metabolism of m-AMSA to 3′-methoxy-4′-(9-acridinylamino)-2′,5′-cyclohexadien-1′-one (m-AQI) and $N^{1\prime}$-methanesulfonyl-$N^{4\prime}$-(9-acridinyl)-3′-methoxy-2′,5′-cyclohexadiene-1′,4′-diimine (m-AQDI). It was noted that each of these m-AMSA metabolites was significantly more cytotoxic towards mouse L1210 cells than was the parent compound [15].

To study the cellular determinants involved in the production of cytotoxic DNA lesions involving m-AMSA and topoisomerase II, we have measured their formation in nuclei isolated from human HL-60 promyelocytic leukemia cells incubated in the presence of m-AMSA plus microsomal fractions, in which one or more different members of the cytochrome P450 family would be expected to interact with the drug. We have found that: (1) the NADPH-dependent microsomal activation of m-AMSA can result in a 2-fold increase in the production of drug-dependent, topoisomerase-associated DNA lesions, (2) GSH (up to 1 mM) neither inhibits nor enhances the microsomal activation of m-AMSA, and (3) the activated product of the microsomal metabolism of m-AMSA is relatively short-lived.

Materials and methods

Materials and cell growth. HL-60 cells were obtained at low passage number (passage 17) from Dr Robert C. Gallo (National Institutes of Health, Bethesda, MD). m-AMSA was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. [^{3}H-*methyl*]Thymidine was purchased from DuPont-New England Nuclear (Boston, MA), and all other chemicals were of reagent or analytical grade and purchased from the Sigma Chemical Co. (St Louis, MO). HL-60 cell cultures were grown in RPMI-1640 to which only 20% iron-supplemented, heat-inactivated calf serum (Hyclone Laboratories, Logan, UT) was added, as described previously [16–18].

Measurement of drug-dependent, topoisomerase-associated DNA lesions. m-AMSA-induced protein-associated DNA lesions were measured essentially as described by others [9, 10] with some modifications [18]. HL-60 cells (4×10^5 cells/mL) were labeled for 24 hr with 0.3 μCi/mL of [^{3}H]thymidine. After the labeling period, nuclei were prepared by the method described by Covey *et al.* [19] with minor modifications. Microsomes from HL-60 cells were prepared by the method of Coon *et al.* [20], and stored at −20° in a buffer containing 50 mM Tris–HCl, 1 mM EDTA, 20% glycerol, pH 7.5. Microsomes from control, acetone-, phenobarbital- and arachlor-pretreated New Zealand White rabbits were also prepared as described by Coon *et al.* [20], and were provided by Dr D. R. Koop of Case Western Reserve University (Cleveland, OH).

The reactions were initiated by the addition of up to 30 μM m-AMSA and incubated at 37° for 1 hr, after which separate 100-μL aliquots were transferred into microfuge tubes containing 900 μL of lysis buffer (1.38% SDS, 5.5 mM EDTA and 0.44 mg/mL salmon sperm DNA). The samples were heated to 65° in a Techne Dri-Block 3 heater, an aliquot (250 μL) of 325 mM KCl was added to the lysed cells, and the tubes were vortexed at the highest setting for 30 sec to shear the DNA [18]. The lysates were then placed at 4° for 15 min, and the precipitates were pelleted in a Sorvall Microspin 24S microcentrifuge (DuPont, Sorvall Instruments, Wilmington, DE) at 15,000 *g*. The precipitates were washed twice in a wash buffer (10 mM Tris, pH 7.5, 100 mM KCl, 2 mM EDTA and 0.1 mg/mL salmon sperm DNA) at 65° followed by further incubation for 10 min at 4°. The samples were again subjected to centrifugation, the resultant pellets were resuspended in 500 μL of water, and the fraction of ^{3}H-labeled DNA which precipitated in the presence of SDS and K^+ was determined by scintillation counting.

Results and discussion

Alterations in the amount of topoisomerase II which associates with endogenous DNA to form m-AMSA-dependent precipitable complexes were first evaluated in isolated nuclei to determine a useful range of drug concentrations. The formation of m-AMSA-dependent, K^+-SDS precipitated DNA was measured in the absence and presence of exogenous ATP (Fig. 1). The data demonstrate the dose- and ATP-dependent generation of topoisomerase II–DNA complexes in HL-60 cell nuclei during a 1-hr exposure to m-AMSA. At the lower concentrations of the drug, it is evident that not all of the available topoisomerase II was bound in a drug-dependent complex. At a con-

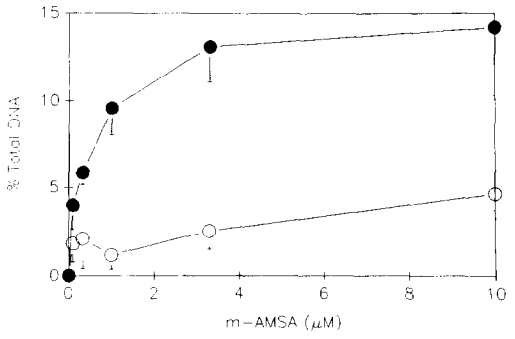

Fig. 1. Dose-dependent and ATP-dependent formation of precipitable DNA lesions in HL-60 cell nuclei. HL-60 cell nuclei were prepared from cells prelabeled with [^{3}H]thymidine as described [19], and incubated in the indicated concentrations of m-AMSA for 1 hr at 37° in the absence (○) or presence (●) of 1 mM ATP. The nuclei were lysed, and the ^{3}H-labeled DNA was sheared and precipitated as described in the text. The proportion of DNA present in the K^+–SDS precipitates was calculated on the basis of the amount of labeled DNA present in equivalent aliquots of intact nuclei. Data are means ± standard deviations of the mean from one representative experiment (N = 4).

centration of m-AMSA of 10 μM and higher, the extent of formation of DNA lesions was maximal for the parent compound, but the formation of this lesion could be modified by the addition of ATP (Fig. 1) or by incubation of the parent compound with microsomes and NADPH (Table 1). The ATP dependence of this lesion has been described by others in some detail [19]. Our results are consistent with the concept that supplementation with ATP is required because the intranuclear pool of the nucleotide is significantly depleted when nuclei are isolated. It is noteworthy that, at the highest concentrations of drug utilized, only a small fraction of the total nuclear DNA is involved in the formation of the m-AMSA-dependent complex. Similar results were obtained with drug concentrations as high as 30 μM (data not shown). The use of isolated nuclei, and not intact cells, decreases the likelihood that alterations in drug permeability via metabolism by exogenously added microsomes contributed to the results described below.

The data presented in table 1 illustrate that microsomal activation resulted in an increase in the production of m-AMSA-dependent, protein-associated DNA lesions over those observed in isolated nuclei which were not incubated with microsomes. Separate preparations of microsomes isolated from untreated rabbits or rabbits pretreated with acetone, phenobarbital, or arachlor each catalyzed an increased production of DNA lesions. This suggests that several different members of the cytochrome P450 family [20] are capable of metabolizing m-AMSA to a more active form. In addition, microsomes isolated from HL-60 cells catalyzed an equivalent (approximately 2-fold) increase in the formation of drug-dependent, protein-associated precipitable DNA lesions. The data in Fig. 1 demonstrated the ATP-dependence of the topoisomerase II component of the formation of DNA lesions. In a series of additional experiments (data not shown) it was found that: (1) ATP was also required for a comparable formation of the m-AMSA-dependent precipitable complexes in the presence of microsomes; (2) heat-inactivated microsomes (from phenobarbital-pretreated rabbits) did not increase the production of DNA lesions in isolated nuclei; (3) microsome-catalyzed increases in the production of DNA lesions were entirely dependent on the presence of NADPH; and (4) substitution of albumin (0.1 mg/mL in microsomal storage buffer) for the microsomes did not result in an increase in the formation of DNA lesions. Taken together, these data suggest that the increased formation of DNA lesions in nuclei supplemented with microsomes is due to an enzyme-mediated, NADPH-dependent metabolic activation of m-AMSA.

We have carried out experiments in which m-AMSA was utilized to measure the activity of DNA topoisomerase II *in vivo* after treatment of HL-60 cells with phorbol diesters [18]. It was found that treatment with phorbol myristate acetate (PMA) does not increase the amount of topoisomerase II that could be trapped by the formation of these drug-dependent cytotoxic lesions. One possible

Table 1. Formation of m-AMSA-dependent precipitable DNA lesions in the presence of rabbit hepatic microsomes and HL-60 cell microsomes

Microsomes	% Total precipitated DNA	% Control
None	9.4 ± 1.6	100
Rabbit		
Control	22.1 ± 9.6	235
Acetone	14.7 ± 1.4	156
Phenobarbital	14.1 ± 2.2	150
Arachlor	14.2 ± 1.7	151
HL-60	18.1 ± 6.3	193

Nuclei were resuspended to a final concentration of approximately 5×10^6 nuclei/mL in 150 mM NaCl, 1 mM KP_i, 5 mM $MgCl_2$, 1 mM ethylene glycol bis (2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, and 0.1 mM dithiothreitol, pH 7.4. Microsomes were isolated from New Zealand White rabbits pretreated with the indicated compounds or from untreated HL-60 cells. ATP (final concentration of 1 mM), NADPH (1 mM), and aliquots of microsomes (0.1 mg/mL) or an equivalent amount of microsomal storage buffer [20] were added to the nuclei. The reactions (100 μL) were initiated by the addition of m-AMSA to a final concentration of 10 μM and incubated at 37° for 1 hr. Data are the means ± one standard deviation from several experiments (N = 8).

explanation for the lack of effect on the formation of DNA lesions, in light of the data presented in Table 1, is that treatment of HL-60 cells with phorbol diesters such as PMA may alter the metabolic activity of the microsomes, such that activation of the m-AMSA does not occur or occurs to a lesser extent than in untreated cells. To address this point, microsomes were isolated from HL-60 cells after treatment with 100 nM PMA for 3 hr. When the studies described above were repeated using this microsome preparation, the results indicated that there were equivalent increases in the formation of DNA lesions (fraction of precipitable DNA) when nuclei were supplemented with microsomes derived from either untreated or from PMA-treated HL-60 cells in the presence of 20 μM m-AMSA (data not shown). Thus, it appeared that the generation of a more active metabolite of m-AMSA was a characteristic shared by microsomes isolated under several different conditions from different tissues, including untreated and PMA-treated HL-60 leukemia cells.

It has been reported that hepatic metabolism and the formation of a GSH adduct of m-AMSA represents a major pathway of elimination of the drug [11–13]. Furthermore, it has been observed that the GSH adduct of m-AMSA can interact with DNA [14]. It was of interest, therefore, to determine whether the addition of GSH to nuclei incubated with either rabbit hepatocyte-derived or HL-60 cell-derived microsomes would alter the formation of drug-dependent, topoisomerase-associated DNA lesions. No differences were found in the fractions of precipitated DNA when we compared the formation of these lesions in nuclei supplemented with microsomes and exposed to 20 μM m-AMSA in the absence or presence of up to 1 mM GSH (GSH present in a 50-fold excess relative to the concentration of m-AMSA). The results of these studies revealed that GSH did not promote the formation of the more active drug species in the presence of HL-60 cell or rabbit hepatic microsomes (93 ± 21% of the DNA lesions formed in the absence of GSH).

The effect of hepatic microsomal activation of m-AMSA on the activity of the drug in extrahepatic tissues may depend, in part, on the half-life of the putative activated metabolite. To determine the relative stability of the metabolite, the following experiments were performed. Preincubations of m-AMSA with HL-60 cell-derived microsomes were carried out for 1 hr in the absence of nuclei. At the end of the preincubation period, aliquots of the microsomal incubations were added to suspensions of nuclei, and the precipitable DNA lesions formed at 10 min were measured. The results of additional experiments (data not shown) indicated that the formation of these lesions in the absence of microsomes was extremely rapid, in that maximal fractions of precipitated DNA were obtained within 10 min of incubation with m-AMSA. We found that there were no significant increases in the DNA lesions formed after 1 hr of microsomal metabolism of m-AMSA (109 ± 27% of the formation of DNA lesions seen without microsomal metabolism). In addition, no increase or decrease in the formation of precipitable DNA lesions was observed when GSH was added to the preincubation mixture. These results suggest that the putatively activated m-AMSA metabolite was relatively short lived and was generated by the concurrent incubation of microsomes plus nuclear components to yield the DNA lesions measured in these experiments. Moreover, the increase in DNA lesions observed in the presence of HL-60 cell-derived microsomes (Table 1) suggests that the metabolic activation in nonhepatic tissues may contribute significantly to the effects of m-AMSA. The identities of the microsomal enzymes (e.g. one or more of the cytochromes P450 or other NADPH-dependent drug-metabolizing enzymes) which are responsible for the GSH-independent activation of m-AMSA at the leukemic target cell level are currently under investigation.

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Inhibitory effects of cytotoxic disulfides on membrane Na^+,K^+-ATPase

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Four decyl and phenyl disulfide analogs of 6-mercaptopurine (6-MP) and 6-thioguanine (6-TG) (Fig. 1) have been reported to be effective against L1210 and S180 *in vivo* [1]. Inomata *et al.* [1] proposed that the cytotoxicity displayed by these compounds might be the result of the release of the purine portion of the molecules in the cellular milieu. We recently reported on the activity of these analogs against the EMT6 cell line [2]. Our investigation involved the examination of the hypoxic and aerobic cell cytotoxicity of this series of compounds. No differential in activity was observed; however, we did find that glutathione (GSH) was depleted from EMT6 cells after treatment with cytotoxic concentrations of the disulfides. Moreover, when cellular GSH was depleted prior to exposure to these agents, their cytotoxicity was potentiated.

A recent study suggested that the GSH depletion produced by the disulfides was the result of their interaction with the tripeptide [3]. This kinetic study reported that the disulfide–GSH interaction occurs spontaneously in a 1:1 manner and that the rates of the reaction could be enhanced in the presence of GSH-*S*-transferase. The products of the reactions were isolated and identified as the GSH-thiol mixed disulfide, the probable cause for the depletion of GSH from cells treated with the disulfides.

The *in vitro* cytotoxicity displayed by these agents was far greater than that which would be expected due to the release of the purine portion of the drugs [2] as was suggested by Inomata *et al.* [1]. Observations made during the cytotoxicity studies suggested that the membrane may be the critical target of these agents, as cells which were treated with toxic concentrations of compounds 1–4 detached from the culture flask surface and appeared swollen and distorted.* In an attempt to explain their cytotoxic action, the effects of compounds 1–4 on the membrane enzyme Na^+,K^+-ATPase were measured. Inhibition of this enzyme, which mediates active transport across the membrane and is sensitive to thiol active agents [4], has been reported to affect cell replication [5]. A recent report [6] also suggests that porphyrin-induced photosensitization may contribute to cell cytotoxicity through inhibition of Na^+,K^+-ATPase. The enzyme has a number of thiol groups essential for activity [4]. Alkylating agents [7] and nitroimidazoles, which have been found to form complexes with GSH [8], inhibit Na^+,K^+-ATPase [9]. On the basis of these reports, it was anticipated that compounds 1–4, shown recently to react with the thiol group of GSH [3], might inhibit membrane Na^+,K^+-ATPase through thiol interactions, contributing to the death of the cell.

Materials and methods

The decyl and phenyl disulfides of 6-MP and 6-TG (compounds 1–4) were synthesized as previously described [10, 11] and recrystallized prior to use.

These along with *N*-ethylmaleimide (NEM, Sigma Chemical Co., St Louis, MO), dicyclohexylcarbodiimide (BDH Chemicals Canada Ltd., Saskatoon, Sask.), 6-mercaptopurine and thiophenol (Sigma) were dissolved in dimethyl sulfoxide (reagent grade, BDH). Ouabain (Sigma) was dissolved in 95% ethanol:Tris–HCl (10 mM, pH 7.5), 50:50. Acid molybdate, Fiske & Subbarow

	R	R'
1	$-(CH_2)_9CH_3$	H
2	$-(CH_2)_9CH_3$	NH_2
3		H
4		NH_2

Fig. 1. Structures of compounds 1–4.

* Kirkpatrick DL, unpublished observations.