

Amsacrine as a Topoisomerase II Poison: Importance of Drug–DNA Interactions

Adam C. Ketron,^{†,§} William A. Denny,^{||} David E. Graves,^{*,⊥} and Neil Osheroff^{*,†,§,‡}

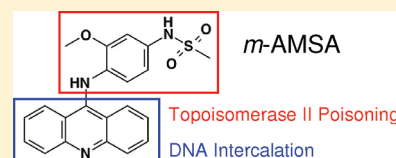
[†]Department of Biochemistry, [‡]Department of Medicine (Hematology/Oncology), and [§]Vanderbilt Institute of Chemical Biology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146, United States

^{||}Auckland Cancer Society Research Centre, Faculty of Medical and Health Science, The University of Auckland, Private Bag 92019, Auckland 1142, New Zealand

[⊥]Department of Chemistry, University of Alabama at Birmingham, Birmingham, Alabama 35294, United States

S Supporting Information

ABSTRACT: Amsacrine (*m*-AMSA) is an anticancer agent that displays activity against refractory acute leukemias as well as Hodgkin's and non-Hodgkin's lymphomas. The drug is comprised of an intercalative acridine moiety coupled to a 4'-amino-methanesulfon-*m*-aniside headgroup. *m*-AMSA is historically significant in that it was the first drug demonstrated to function as a topoisomerase II poison. Although *m*-AMSA was designed as a DNA binding agent, the ability to intercalate does not appear to be the sole determinant of drug activity. Therefore, to more fully analyze structure–function relationships and the role of DNA binding in the action of *m*-AMSA, we analyzed a series of derivatives for the ability to enhance DNA cleavage mediated by human topoisomerase II α and topoisomerase II β and to intercalate DNA. Results indicate that the 3'-methoxy (*m*-AMSA) positively affects drug function, potentially by restricting the rotation of the headgroup in a favorable orientation. Shifting the methoxy to the 2'-position (*o*-AMSA), which abrogates drug function, appears to increase the degree of rotational freedom of the headgroup and may impair interactions of the 1'-substituent or other portions of the headgroup within the ternary complex. Finally, the nonintercalative *m*-AMSA headgroup enhanced enzyme-mediated DNA cleavage when it was detached from the acridine moiety, albeit with 100-fold lower affinity. Taken together, our results suggest that much of the activity and specificity of *m*-AMSA as a topoisomerase II poison is embodied in the headgroup, while DNA intercalation is used primarily to increase the affinity of *m*-AMSA for the topoisomerase II–DNA cleavage complex.



Amsacrine (*m*-AMSA) is an acridine derivative with anti-neoplastic activity.^{1,2} The drug is in multiple clinical trials for the treatment of hematological cancers in the United States³ and is used to treat refractory acute lymphocytic and non-lymphocytic leukemias as well as Hodgkin's and non-Hodgkin's lymphomas in other countries.^{1,4–6} *m*-AMSA kills cells by acting as a topoisomerase II poison and increases levels of covalent enzyme-cleaved DNA complexes primarily by decreasing rates of ligation.^{1,7–13} In vitro, *m*-AMSA displays similar activity toward the two isoforms of human topoisomerase II, α and β .^{14,15} However, evidence suggests that the β isoform may be the more important target for the cytotoxic actions of the drug.^{16–19}

m-AMSA is a historically significant topoisomerase II-targeted anticancer drug. In a pioneering study published by Zwelling et al. in 1981,²⁰ the authors proposed that *m*-AMSA targeted a topoisomerase based on the ability of the drug to induce protein-associated DNA strand breaks in treated human cells. Three years later, *m*-AMSA was the first drug demonstrated to poison mammalian topoisomerase II in vitro or in human cells.^{7,21}

Whereas some topoisomerase II-targeted drugs, such as etoposide, have little if any interaction with DNA in the absence of enzyme,^{22–24} *m*-AMSA was designed to be a DNA binding agent.^{25,26} To this point, *m*-AMSA is one of the most widely studied intercalative topoisomerase II poisons.^{27,28} The drug is

comprised of an acridine moiety coupled to a 4'-amino-methanesulfon-*m*-aniside headgroup. It has long been known that moving the aniside methoxy group from the *meta* (3') to the *ortho* (2') position (see Figure 1) attenuates drug activity against mammalian topoisomerase II, despite the fact that the resulting *o*-AMSA is a stronger intercalator than *m*-AMSA.^{7,20,29–31}

The relative activity of *m*-AMSA versus *o*-AMSA against topoisomerase II indicates that DNA binding cannot be the sole determinant of drug function. Moreover, it raises a number of questions regarding the precise role of DNA intercalation in the action of *m*-AMSA and the contributions of headgroup substituents to topoisomerase II poisoning. Therefore, to more fully analyze structure–function relationships and the role of DNA binding in the action of *m*-AMSA, a series of derivatives was analyzed. Results indicate that much of the activity and specificity of *m*-AMSA as a topoisomerase II poison is embodied in the headgroup. DNA intercalation also is important for optimal drug function, being used primarily to increase the affinity of *m*-AMSA for the topoisomerase II–DNA cleavage complex.

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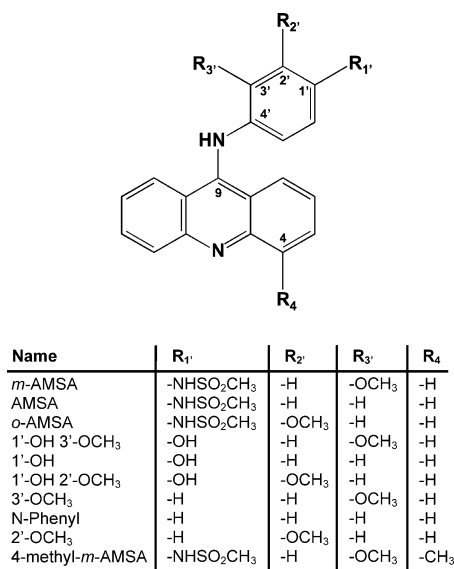


Figure 1. Structure of *m*-AMSA and its derivatives.

EXPERIMENTAL PROCEDURES

Enzymes and Materials. Human topoisomerase II α and topoisomerase II β were expressed in *Saccharomyces cerevisiae*³² and purified as described previously.³³ Human topoisomerase I was purchased from Topogen. Negatively supercoiled pBR322 DNA was prepared from *Escherichia coli* using a Plasmid Mega Kit (Qiagen) as described by the manufacturer. [γ -³²P]ATP (~6000 Ci/mmol) was obtained from Perkin-Elmer. *m*-AMSA and its derivatives were synthesized as described previously.^{25,26} *N*-(4-Amino-3-methoxyphenyl)methane-sulfonamide hydrochloride (*m*-AMSA headgroup) and etoposide were obtained from Sigma. The *m*-AMSA headgroup was stored at -20 °C as a 0.5 M stock solution in 100% DMSO. All other drugs were stored at 4 °C as 20 mM stock solutions in 100% DMSO. Other chemicals were analytical reagent grade.

Plasmid DNA Cleavage. DNA cleavage reactions were carried out using the procedure of Fortune and Osheroff.³⁴ Topoisomerase II DNA cleavage assays contained 220 nM human topoisomerase II α or human topoisomerase II β and 10 nM negatively supercoiled pBR322 in a total of 20 μ L of DNA cleavage buffer [10 mM Tris-HCl (pH 7.9), 5 mM MgCl₂, 100 mM KCl, 0.1 mM EDTA, and 2.5% (v/v) glycerol]. Unless stated otherwise, reaction mixtures were incubated at 37 °C for 6 min, and enzyme-DNA cleavage complexes were trapped by the addition of 2 μ L of 5% SDS followed by 2 μ L of 250 mM EDTA (pH 8.0). Proteinase K (2 μ L of a 0.8 mg/mL solution) was added, and samples were incubated at 45 °C for 30 min to digest the enzyme. Samples were mixed with 2 μ L of agarose gel loading buffer [60% sucrose in 10 mM Tris-HCl (pH 7.9), 0.5% bromophenol blue, and 0.5% xylene cyanol FF], heated at 45 °C for 5 min, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris-acetate (pH 8.3) and 2 mM EDTA containing 0.5 μ g/mL ethidium bromide. DNA bands were visualized with long-range ultraviolet light and quantified using an Alpha Innotech digital imaging system. DNA cleavage was monitored by the conversion of supercoiled plasmid DNA to linear molecules.

Assays were carried out in the absence of compound, in the presence of 0–50 μ M *m*-AMSA or its derivatives, or in the presence of 0–3.5 mM *m*-AMSA headgroup. In some cases,

assays were carried out in the presence of 3 mM dithiothreitol (DTT).

Molecular Modeling. The Calculate Energy Protocol within the Minimization Module of Discovery Studio 2.1 (Accelrys, Inc.) was used for conformational space searching for *m*-AMSA and *o*-AMSA. Initially, *m*-AMSA and *o*-AMSA were input into Discovery Studio 2.1 using the Builder module. Atoms were assigned using the CHARMM force field. Geometries for each of the compounds were optimized using the minimization protocol within the simulation tool. Lowest-energy structures for each of the compounds were derived using the conjugate gradient algorithm, 2000 steps, and a root-mean-square (rms) gradient of 0.001. A dielectric of 1.0, a nonbond list radius of 14.0, and spherical cutoff electrostatics were applied.

In the lowest-energy structures, torsion angles 1 (angle of rotation between C9 and the linking N) for *m*-AMSA and *o*-AMSA (-101.78° and -102.82°, respectively) were similar. The lowest-energy torsion angles 2 (angle of rotation between the linking N and C4') were -11.69° for *m*-AMSA and -3.84° for *o*-AMSA. Using these rotation angles in the starting structures for *m*-AMSA and *o*-AMSA, changes to torsion angle 1 and torsion angle 2 were evaluated for their contributions to the overall potential energy of the drugs using the energy calculation module within the simulation protocol. Each of the torsion angles was modulated in $\pm 5^\circ$ increments from its lowest-energy value, and the energy was calculated for each torsion angle change. This method allows the relative stability of the drug to be determined with respect to the lowest-energy structure associated with each change in torsion angle.

DNA Cleavage Site Utilization. DNA cleavage sites were mapped using a modification³⁵ of the procedure of O'Reilly and Kreuzer.³⁶ The pBR322 DNA substrate was linearized by treatment with *Hind*III. Terminal 5'-phosphates were removed by treatment with calf intestinal alkaline phosphatase and replaced with [³²P]phosphate using T4 polynucleotide kinase and [γ -³²P]ATP. The DNA was treated with *Eco*RI, and the 4332 bp singly end-labeled fragment was purified from the small *Eco*RI-*Hind*III fragment by passage through a CHROMA SPIN+TE-100 column (Clontech). Reaction mixtures contained 1 nM labeled pBR322 DNA substrate and 90 nM human topoisomerase II α in 50 μ L of DNA cleavage buffer supplemented with 1 mM ATP in the absence or presence of *m*-AMSA or its derivatives. Reaction mixtures were incubated at 37 °C for 30 s, and enzyme-DNA cleavage complexes were trapped by the addition of 5 μ L of 5% SDS followed by 3.75 μ L of 250 mM EDTA (pH 8.0). Proteinase K (5 μ L of a 0.8 mg/mL solution) was added, and samples were incubated at 45 °C for 30 min to digest the enzyme. DNA products were precipitated with ethanol and resuspended in 5 μ L of polyacrylamide gel loading buffer (40% formamide, 10 mM NaOH, 0.02% xylene cyanol FF, and 0.02% bromophenol blue). Samples were subjected to electrophoresis in denaturing 6% polyacrylamide sequencing gels. Gels were dried in vacuo, and DNA cleavage products were visualized with a Bio-Rad Molecular Imager FX.

DNA Intercalation. DNA intercalation was monitored as described previously.^{34,37} When used as a substrate, relaxed plasmid DNA was generated by incubation with topoisomerase I.^{34,37} Intercalation reaction mixtures contained 20 nM topoisomerase I, 5 nM relaxed or negatively supercoiled pBR322 DNA, and 0–150 μ M *m*-AMSA or its derivatives. Ethidium bromide (10 μ M) and etoposide (100 μ M) were included as positive and negative controls, respectively. Assays were carried out in a total of 20 μ L of 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA,

50 mM KCl, 10 mM MgCl₂, and 0.5 mM DTT. Mixtures were incubated at 37 °C for 10 min, extracted with a phenol/chloroform/isoamyl alcohol mixture (25:24:1), and added to 3 μ L of 0.77% SDS and 77 mM EDTA (pH 8.0). Samples were mixed with 2 μ L of agarose gel loading buffer, heated at 45 °C for 5 min, and subjected to electrophoresis in a 1% agarose gel in 100 mM Tris-borate (pH 8.3) and 2 mM EDTA. Gels were stained with 1 μ g/mL ethidium bromide, and DNA bands were visualized as described for plasmid DNA cleavage.

Competition with DNA Intercalators. A 50 bp oligonucleotide duplex was designed using a previously identified topoisomerase II cleavage site from pBR322.³⁸ Oligonucleotide sequences were generated using an Applied Biosystems DNA synthesizer. The 50-mer top and bottom sequences were 5'-TT-GGTATCTGCGCTCTGCTGAAGCC↓AGTTACCTTCG-GAAAAAGAGTTGGT-3' and 5'-ACCAACTCTTTTTCG-GAAGGT↓AACTGGCTTCAGCAGAGCGCAGATACCAA-3', respectively (arrows denote cleavage sites). The bottom strand was labeled on the 5'-terminus with [γ -³²P]ATP using T4 polynucleotide kinase. Following labeling and gel purification, complementary oligonucleotides were annealed by incubation at 70 °C for 10 min and cooling to 25 °C.

DNA cleavage by human topoisomerase II α was assessed by a modification of the procedure of Fortune et al.³⁸ Reaction mixtures contained 220 nM human topoisomerase II α and 100 nM double-stranded oligonucleotide in 10 μ L of DNA cleavage buffer. Assays were carried out in the absence or presence of 25 μ M *m*-AMSA and 0–50 μ M acridine, 9-aminoacridine, or ethidium bromide. Reaction mixtures were incubated for 10 min at 37 °C. DNA cleavage products were trapped by the addition of 2 μ L of 10% SDS, followed by 1 μ L of 375 mM EDTA (pH 8.0). Samples were digested with proteinase K, and DNA products were precipitated with ethanol and resuspended in 5 μ L of polyacrylamide gel loading buffer. Samples were subjected to electrophoresis in denaturing 14% polyacrylamide sequencing gels. Gels were dried in vacuo, and DNA cleavage products were visualized as described above.

RESULTS AND DISCUSSION

Contributions of *m*-AMSA Headgroup Substituents to Drug-Induced DNA Cleavage by Human Type II Topoisomerases. It has long been known that the activity of *o*-AMSA as a topoisomerase II poison is dramatically lower than that of *m*-AMSA (see Figure 1 for drug structures).^{7,20,29–31} As seen in Figure 2, *m*-AMSA enhanced DNA cleavage mediated by human topoisomerase II α or topoisomerase II β ~7–8-fold as compared to reactions without drug, whereas *o*-AMSA displayed almost no ability to poison either enzyme. This difference exists despite the fact that the only change between *m*- and *o*-AMSA is the position of the methoxy group (3' and 2', respectively). The molecular basis underlying this difference in drug activity has not been delineated, and several questions have yet to be addressed. For example, does the low activity of *o*-AMSA reflect the loss of a critical interaction between the 3'-methoxy of *m*-AMSA and topoisomerase II or DNA, or does the 2'-methoxy of *o*-AMSA sterically hinder interactions of the drug in the enzyme–DNA complex (or a combination of both)?

Therefore, we characterized the ability of AMSA, a derivative of *m*-AMSA that is lacking the methoxy substituent, to stimulate topoisomerase II-mediated DNA cleavage. If the activity of AMSA were similar to that of *m*-AMSA, it would suggest that the methoxy group does not enhance drug activity when in the

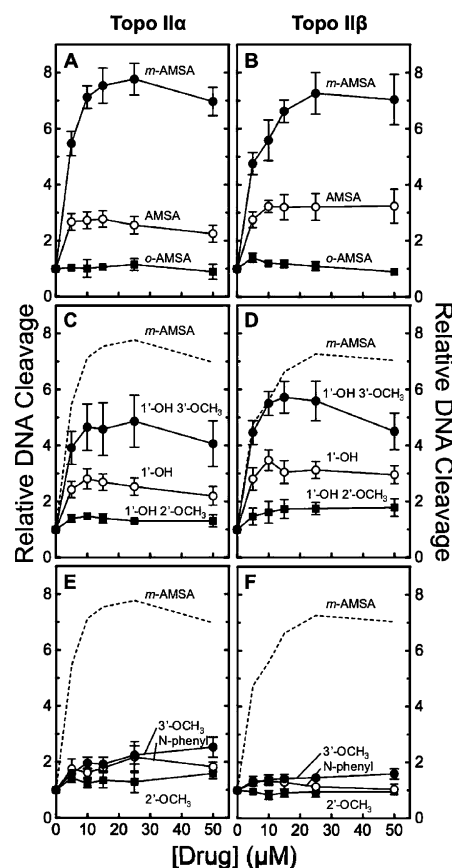


Figure 2. Enhancement of topoisomerase II-mediated DNA cleavage by *m*-AMSA and its derivatives. The effects of *m*-AMSA (●), AMSA (○), and *o*-AMSA (■) (A and B); 1'-OH 3'-OCH₃ (●), 1'-OH (○), and 1'-OH 2'-OCH₃ (■) (C and D); and 3'-OCH₃ (●), N-phenyl (○), and 2'-OCH₃ (■) (E and F) on the cleavage of negatively supercoiled plasmid DNA by human topoisomerase II α (A, C, and E) and topoisomerase II β (B, D, and F) were determined. Error bars represent the standard deviation of three independent experiments. Data for *m*-AMSA are included as dashed lines in panels C–F for the sake of comparison.

3'-position, but rather inhibits activity when in the 2'-position. Alternatively, if the activity of AMSA were similar to that of *o*-AMSA, it would imply that the 3'-methoxy is critical for topoisomerase II poisoning. Results are shown in panels A and B of Figure 2. The activity of AMSA was intermediate between those of *m*-AMSA and *o*-AMSA, increasing levels of DNA cleavage ~3-fold. This finding indicates that the presence of the 3'-methoxy positively affects drug function and is necessary for optimal activity, while the presence of the 2'-methoxy impairs drug interactions.

To further explore the role of the methoxy group in drug function, we compared the activities of compounds that contained a 3'-methoxy, a 2'-methoxy, or no methoxy in two additional series: one that replaced the 1'-methanesulfonamide with a 1'-hydroxy moiety (Figure 2C,D) and another that lacked a 1'-substituent (Figure 2E,F). Results were similar to those described above for the 1'-methanesulfonamide series. Compounds with a 3'-methoxy always induced the highest levels of DNA cleavage, compounds with a 2'-methoxy had little effect on enzyme activity, and compounds lacking the methoxy had an intermediate effect.

Although the above relationships regarding the methoxy group were consistent across the three series, the nature of the

1'-substituent had a profound effect on drug activity. Comparing compounds with a 3'-methoxy, the activity of that with a 1'-methanesulfonamide was greater than that with a 1'-hydroxy, which was much greater than that with no substituent at the 1'-position. Thus, it appears that the ability of the 1'-substituent to form hydrogen bonds (or other interactions) is important for drug activity against human type II topoisomerases.

These results indicate that the 3'-methoxy and 1'-methanesulfonamide positively impact the ability of *m*-AMSA to poison topoisomerase II, while the 2'-methoxy of *o*-AMSA impairs this process. However, they do not provide an understanding of the underlying mechanism by which these substituents affect drug activity. Therefore, modeling studies were carried out with *m*-AMSA and *o*-AMSA to address this issue (Figure 3).

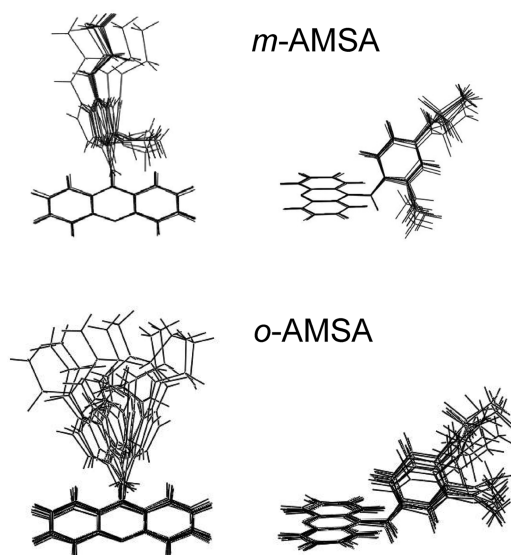


Figure 3. Energy minimization models of *m*-AMSA and *o*-AMSA. Low-energy structures (within ± 5 kcal/mol of the optimized structures) for *m*-AMSA (top) and *o*-AMSA (bottom) are shown. Front and side views are shown at the left and right, respectively.

As determined by energy minimization calculations, the orientation of the headgroup in *m*-AMSA appears to be much more constrained than it is in *o*-AMSA (Figure 3). The lowest potential energy for *m*-AMSA (8.85 kcal/mol) was observed when torsion angle 1 [angle of rotation between C9 and the linking N (see Figure 1)] was set to -101.78° . Torsion angle 1 could be changed by -15° to $+30^\circ$ (a total of 45°) without significant changes in energy (range of 12.63 at -15° to 12.1 kcal/mol at $+30^\circ$). Rotations past -15° and $+30^\circ$ resulted in a marked increase in the potential energy of the structure. Similar energy profiles were observed for changes to torsion angle 2 (angle of rotation between the linking N and C4'). The lowest energy (8.85 kcal/mol) was observed at an angle of -11.69° . Incremental changes to torsion angle 2 followed by energy calculations revealed a significant increase in the potential energy of the molecule if torsion angle 2 was rotated more than -25° and $+60^\circ$ from the starting value of -11.69° .

In contrast to *m*-AMSA, *o*-AMSA appears to have a much broader low-energy conformational space. Starting with the lowest-energy conformation (-102.82°), we found torsion angle 1 could be changed by -40° to $+45^\circ$ (a total of 85°) with relatively small fluctuations in the potential energy of the molecule (range of 14.4 kcal/mol at -40° to 14.8 kcal/mol at $+45^\circ$). The lowest energy for torsion angle 2 was observed at

-3.84° , and the angle could be rotated with nearly full rotational freedom.

On the basis of these modeling studies, we propose the following: the 3'-methoxy enhances drug activity by restricting the headgroup to a narrow range of favorable conformations. Conversely, when the 3'-methoxy is missing in AMSA or moved in *o*-AMSA, drug activity decreases because the headgroup is no longer constrained to a favored orientation. Finally, the activity of *o*-AMSA is even lower than that of AMSA because, in addition to the unrestricted headgroup, the presence of the 2'-methoxy may impose steric constraints that further inhibit interactions of the 1'-substituent or other portions of the headgroup with the protein or DNA.

To determine whether changes in the substituents mentioned above affect the specificity of the drug class, sites of DNA cleaved by human topoisomerase II α in the presence of *m*-AMSA and several derivatives were determined (Figure 4).

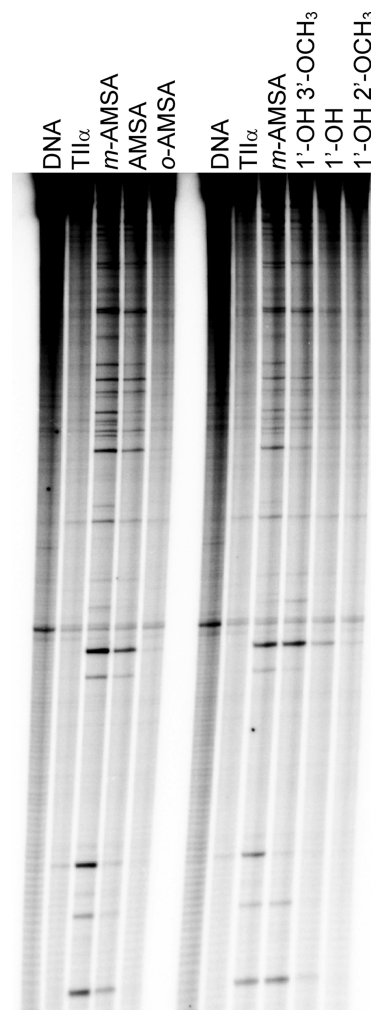


Figure 4. DNA cleavage site specificity and utilization by human topoisomerase II α in the presence of *m*-AMSA and its derivatives. A singly end-labeled linear 4332 bp fragment of pBR322 was used as the cleavage substrate. An autoradiogram of a polyacrylamide gel is shown. DNA cleavage reactions were conducted in the absence of drug (TII α) or in the presence of 10 μ M *m*-AMSA; 25 μ M AMSA, 1'-OH 3'-OCH₃, or 1'-OH; or 100 μ M *o*-AMSA or 1'-OH 2'-OCH₃. DNA standards (DNA) also are shown. Results are representative of three independent experiments.

Similar cleavage maps were observed for *m*-AMSA, AMSA (which lacks the methoxy group), and 1'-OH 3'-OCH₃ (which

contains a hydroxy in place of the 1'-methanesulfonamide group of the parent drug). However, minor differences with regard to site specificity and utilization were observed. This result suggests that portions of the *m*-AMSA headgroup may have interactions with DNA as well as the protein in the ternary enzyme–drug–DNA complex.

DNA Intercalation. *m*-AMSA was originally designed as a DNA binding drug.^{25,26} However, there is no clear correlation between the strength of DNA binding (as determined by intercalation) and drug activity against topoisomerase II. As discussed earlier, *o*-AMSA, which intercalates more strongly than *m*-AMSA, displays little ability to poison the type II enzyme.^{7,20,29–31} Therefore, to more fully explore relationships between DNA binding and topoisomerase II poisoning, the ability of the compounds described in Figure 1 to intercalate was determined. Representative intercalation assay gels for *m*-AMSA, AMSA, *o*-AMSA, and 9-aminoacridine are shown in Figure 5.

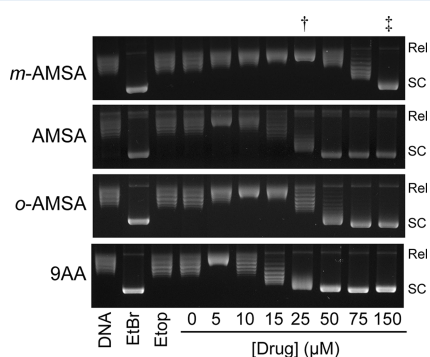


Figure 5. DNA intercalation by *m*-AMSA and its derivatives. The abilities of 0–150 μM *m*-AMSA, AMSA, *o*-AMSA, and 9-aminoacridine (9AA) to intercalate into DNA were determined using a topoisomerase I-based supercoiling assay. Representative ethidium bromide (EtBr)-stained agarose gels are shown. The effects of 10 μM EtBr and 100 μM etoposide (Etop) are included as positive and negative controls, respectively. Relaxed DNA standards (DNA) also are shown. Results are representative of three independent experiments. As described in Table 1, concentrations of intercalators required to yield “fully relaxed” (Rel) and “fully supercoiled” (SC) plasmid are used for comparative purposes. Lanes that include these concentrations for *m*-AMSA are denoted with a dagger (†) and double dagger (‡), respectively.

Representative gels for the other compounds employed are shown in Figure S1 of the Supporting Information.

The DNA intercalation assay is based on the fact that intercalative agents induce constrained negative supercoils and compensatory unconstrained positive superhelical twists in covalently closed circular DNA. Therefore, as the concentration of an intercalative compound increases, a plasmid that is negatively supercoiled or relaxed (i.e., contains an equilibrium distribution of topoisomers whose mean number of superhelical twists is zero) appears to become positively supercoiled. Treatment of an intercalated plasmid with topoisomerase I removes the unconstrained positive DNA supercoils. Subsequent extraction of the compound allows the local drug-induced unwinding to redistribute in a global manner and manifest itself as a net negative supercoiling of the plasmid. Thus, in the presence of an intercalative agent, topoisomerase treatment converts plasmids (through a completely relaxed intermediate population) to a distribution of negatively supercoiled molecules.

In order to compare the relative abilities of compounds to intercalate, two data points were employed: the concentration

of drug that converts the population of plasmids to the most relaxed form (i.e., the highest band seen on the gels in Figure 5) following treatment with topoisomerase I and the concentration of drug that converts the initial population to the fully supercoiled form (i.e., the lowest band seen on the gels in

Table 1. DNA Intercalation by *m*-AMSA and Its Derivatives^a

| compound | fully relaxed concentration (μM) | fully supercoiled concentration (μM) |
|---------------------------|---|---|
| <i>m</i> -AMSA | 25 | 150 |
| AMSA | 5–10 | 50 |
| <i>o</i> -AMSA | 10 | 75 |
| 1'-OH 3'-OCH ₃ | 15 | 150 |
| 1'-OH | 5 | 25 |
| 1'-OH 2'-OCH ₃ | 5 | 25–50 |
| 3'-OCH ₃ | 50–75 | >150 |
| <i>N</i> -Phenyl | 15–25 | 75 |
| 2'-OCH ₃ | 15 | 150 |
| 9-aminoacridine | 5 | 50 |
| 4-methyl- <i>m</i> -AMSA | 15 | 75 |
| acridine | 75 | ≥150 |
| ethidium bromide | 1 | 5–7.5 |

^aThe concentrations of compounds required to convert the plasmid substrate to a fully relaxed or fully supercoiled population was assessed by the topoisomerase I DNA supercoiling assay described in the legend of Figure 5.

Figure 5). These values are given in Table 1 for all of the compounds used in this study.

The chemical nature of the 1'-substituent had a consistent effect on drug intercalation into DNA, with the strength of intercalation being: 1'-hydroxy series > 1'-methanesulfonamide series > no 1'-substituent series. The presence and position of the methoxy group also affected drug intercalation in a consistent manner, with the strength of intercalation being: no methoxy > 2'-methoxy > 3'-methoxy. Despite these findings, as originally observed for *m*-AMSA and *o*-AMSA,^{7,20,29–31} there appears to be little correlation between the strength of DNA binding and enhancement of topoisomerase II-mediated DNA cleavage. For example, while members of the 1'-hydroxy series are stronger intercalators than corresponding members of the 1'-methanesulfonamide series, they are weaker topoisomerase II poisons.

One caveat regarding the above observations should be noted: all of the *m*-AMSA derivatives that were examined contained altered substituents on the headgroup. Because portions of the headgroup are likely to interact with topoisomerase II or the scissile bond in the cleavage complex, it is possible that the same alterations that strengthen DNA intercalation also interfere with these critical interactions (or vice versa). This could explain the lack of correlation between drug activity and DNA intercalation. Therefore, to address the issue of DNA binding without changing substituents on the headgroup, the ability of 4-methyl-*m*-AMSA to intercalate DNA and poison topoisomerase II α was evaluated. The methyl substituent in this compound is on the acridine ring. A previous cross-linking study using a photoactivated *m*-AMSA analogue³⁹ demonstrated that the acridine moiety interacts with DNA in the ternary topoisomerase II–drug–DNA complex.⁴⁰

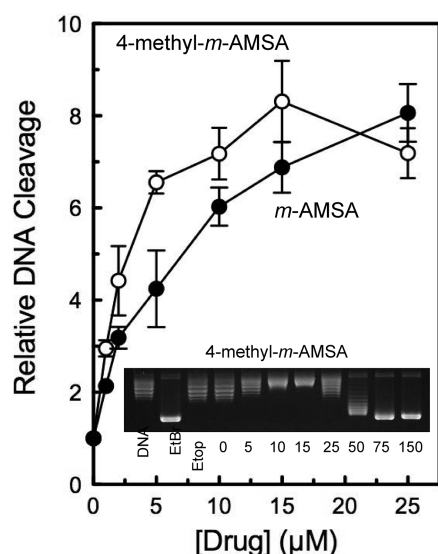


Figure 6. Enhancement of topoisomerase II α -mediated DNA cleavage by 4-methyl-*m*-AMSA. The effects of 4-methyl-*m*-AMSA (○) on the cleavage of negatively supercoiled plasmid DNA by human topoisomerase II α are compared to those of *m*-AMSA (●). Error bars represent the standard deviation of three independent experiments. The inset shows a representative topoisomerase I-based intercalation assay for 4-methyl-*m*-AMSA.

Intercalation studies (Figure 6, inset, and Table 1) indicate that 4-methyl-*m*-AMSA binds DNA with an affinity that is approximately twice that of *m*-AMSA. Similarly, the potency of 4-methyl-*m*-AMSA (as determined by a topoisomerase II α –DNA cleavage assay) was \sim 2-fold higher than that of *m*-AMSA (Figure 6). Despite the change in potency, the methylated and unmethylated compounds displayed comparable efficacies (maximal levels of DNA cleavage).

The correlation between DNA binding and cleavage observed for 4-methyl-*m*-AMSA suggests that intercalation plays an important role in drug action by increasing the affinity of *m*-AMSA for the ternary complex. If this suggestion is correct, inhibiting the ability of *m*-AMSA to intercalate should attenuate drug action. To test this prediction, we carried out competition experiments in the presence of acridine (a weak intercalator), 9-aminoacridine (a strong intercalator), and ethidium bromide (a stronger intercalator) (see Table 1, Figure 4, and Figure S1 of the Supporting Information for intercalation data).

As seen in Figure 7, acridine, 9-aminoacridine, and ethidium bromide inhibited topoisomerase II α -mediated DNA cleavage induced by 25 μ M *m*-AMSA to an extent proportional to their DNA binding strengths. Acridine showed little inhibition of DNA cleavage at 50 μ M. In contrast, 9-aminoacridine and ethidium bromide inhibited *m*-AMSA-induced DNA cleavage by 50% at \sim 21 and \sim 7 μ M, respectively.

It is notable that intercalators can decrease levels of topoisomerase II-mediated cleavage by interfering with DNA binding or by altering the apparent topology of DNA (making the substrate appear to be positively supercoiled) in a closed topological system.¹⁵ To minimize the effects of intercalators on baseline levels of DNA cleavage mediated by human topoisomerase II α , an oligonucleotide system was utilized for the competition experiments. Indeed, at 50 μ M ethidium bromide, the highest concentration of the strongest intercalator employed (Table 1),

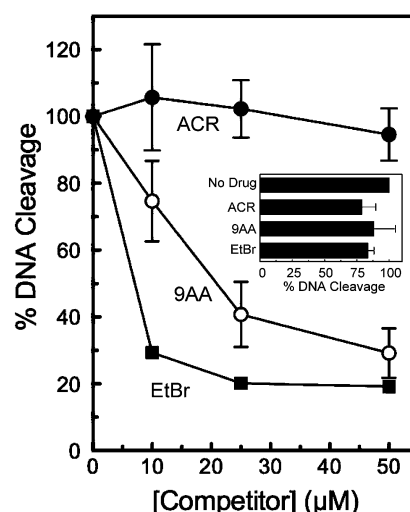


Figure 7. Inhibition of *m*-AMSA-induced topoisomerase II α -mediated DNA cleavage by intercalators. The abilities of 0–50 μ M acridine (ACR, ●), 9-aminoacridine (9AA, ○), and ethidium bromide (EtBr, ■) to inhibit DNA cleavage induced by 25 μ M *m*-AMSA were determined. A singly end-labeled 50-mer oligonucleotide substrate was used as the cleavage substrate. The level of DNA cleavage in the presence of 25 μ M *m*-AMSA and the absence of competitor was set to 100%. The inset shows the effects of 50 μ M ACR, 9AA, and EtBr on DNA cleavage mediated by human topoisomerase II α in the absence of *m*-AMSA. The baseline level of DNA cleavage in the absence of intercalators was set to 100%. Error bars represent the standard deviation of three independent experiments for both the main panel and the inset.

baseline levels of enzyme-mediated DNA cleavage decreased only 16% (Figure 7, inset).

Taken together, these findings support the conclusion that DNA intercalation plays an important role in the actions of *m*-AMSA as a topoisomerase II poison.

Activity of the *m*-AMSA Headgroup. A number of topoisomerase II-targeted drugs contain a multi-ring system attached to a headgroup.^{1,2} For example, the anticancer drug etoposide is comprised of a glycosylated polycyclic core (albeit nonintercalative) that is linked to a 3',5'-dimethoxy-4'-hydroxyphenyl headgroup (E-ring).^{1,2,24} A recent structure of a covalent human topoisomerase II β –DNA cleavage complex formed in the presence of etoposide indicates that the polycyclic drug core interacts primarily with DNA, while the headgroup is positioned at the interface between the enzyme and the cleaved scissile bond and interacts with both the protein and DNA.⁴¹ Saturation transfer difference nuclear magnetic resonance studies of the etoposide–topoisomerase II α binary complex coupled with activity studies also suggest that there are strong interactions between portions of the polycyclic core/glycosyl group and DNA and between the headgroup and the enzyme.^{42–44}

Consistent with the studies of etoposide, the findings described above for *m*-AMSA imply that the acridine core and headgroup of the drug may play different and complementary functions in stabilizing the topoisomerase II–DNA complex. We propose that the acridine portion of *m*-AMSA is largely responsible for DNA binding, while the headgroup interacts with the enzyme and the scissile bond in the ternary complex.

In order to address this hypothesis, the ability of the isolated *m*-AMSA headgroup to enhance DNA cleavage mediated by

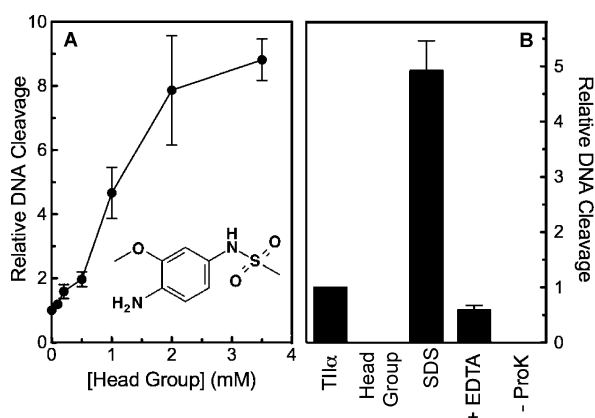


Figure 8. Enhancement of topoisomerase II α -mediated DNA cleavage by the detached *m*-AMSA headgroup. Panel A shows the effects of the isolated *m*-AMSA headgroup (structure shown as an inset) on the cleavage of negatively supercoiled plasmid DNA by human topoisomerase II α . Panel B shows a series of control experiments that confirmed that DNA cleavage induced by the isolated headgroup is mediated by human topoisomerase II α . Reaction mixtures contained DNA and enzyme in the absence of *m*-AMSA headgroup (TII α), DNA and 3 mM headgroup in the absence of enzyme, or complete reaction mixtures treated with SDS prior to addition of EDTA (SDS). The reversibility of DNA cleavage induced by 3 mM headgroup was determined by incubating reaction mixtures with EDTA prior to trapping cleavage complexes with SDS (EDTA). To determine whether DNA cleavage induced by 3 mM headgroup was protein-linked, proteinase K treatment was omitted (-ProK). Error bars for both panels represent standard deviations for three independent experiments.

human topoisomerase II α was assessed (unfortunately, the *o*-AMSA headgroup was unavailable for testing). As seen in Figure 8 (left panel), the detached headgroup stimulated DNA scission ~8–9-fold, which is comparable to levels observed with ~25 μ M *m*-AMSA. In contrast to the parent drug, however, ~3.5 mM headgroup was required to induce this level of cleavage. Thus, the efficacy of the isolated headgroup is similar to that of the parent compound, but the potency is >2 orders of magnitude lower.

To ensure that the detached headgroup was inducing DNA cleavage through an effect on topoisomerase II α , several control experiments were carried out (Figure 8, right panel). No DNA cleavage was observed in the absence of enzyme. In addition, DNA scission was reversed by the addition of EDTA, which chelates the essential divalent cation,⁴⁵ and no free linear DNA was seen in reaction mixtures that were not treated with proteinase K to digest the topoisomerase II. Thus, the isolated headgroup induces DNA cleavage through its effects on the type II enzyme.

Studies described above predict that the intercalation of *m*-AMSA is mediated by the acridine portion of the drug. If this is the case, the isolated headgroup would not be expected to intercalate. Indeed, even at concentrations as high as 4 mM, no intercalation by the headgroup was observed (Figure 9).

Both the headgroup and the intercalative acridine moiety are required for the high potency of *m*-AMSA as a topoisomerase II poison. Because intercalation alters the structure of DNA, acridine may increase the potency of *m*-AMSA by a specific or a general mechanism. In the first case, intercalation by the acridine moiety of an individual *m*-AMSA molecule increases the affinity of the attached headgroup for the enzyme and the

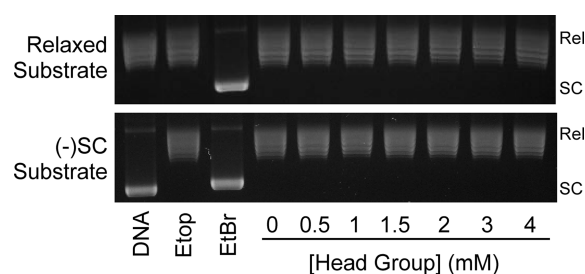


Figure 9. Isolated *m*-AMSA headgroup does not intercalate in DNA. Representative topoisoemerase I-based DNA intercalation assay gels are shown for the isolated *m*-AMSA headgroup. Assays starting with either relaxed or negatively supercoiled DNA plasmid substrates are included. Etoposide (Etop, 100 μ M) and ethidium bromide (EtBr, 10 μ M) are included as positive and negative controls, respectively. DNA standards (DNA) also are shown. Results are representative of three independent experiments.

scissile bond. In the second case, intercalation by *m*-AMSA molecules outside of the topoisomerase II active site alters the local DNA structure in a manner that increases the affinity of a different *m*-AMSA headgroup for the scissile bond. To distinguish between these two possibilities, we characterized the importance of the linkage between the acridine moiety and the headgroup for drug function. As seen in Figure 10, the activity

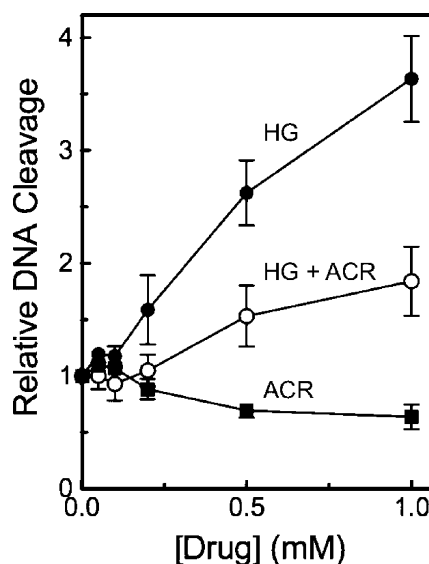


Figure 10. Covalent linkage of the headgroup to the acridine moiety is necessary for the high potency of *m*-AMSA as a topoisomerase II poison. The ability of the detached headgroup (HG, ●) or a 1:1 mixture of headgroup and acridine (HG + ACR, ○) to stimulate topoisomerase II α -mediated DNA cleavage is shown. A control experiment assessing the effects of acridine alone on the DNA cleavage activity of topoisomerase II α (ACR, ■) also is shown. Error bars represent the standard deviation of three independent experiments.

of a 1:1 detached headgroup:acridine mixture was lower than that of the headgroup alone, and the potency of the mixture was considerably lower than that of *m*-AMSA (in which the headgroup and acridine moiety are linked). This finding provides strong evidence that the linkage between the acridine

moiety and the headgroup is critical for the potent activity of *m*-AMSA.

Because the *m*-AMSA headgroup has *para* amino substituents, it has the potential to undergo redox cycling with a concomitant amino–imino transition. Previous studies indicate that quinone-based compounds can poison topoisomerase II by a mechanism that differs from that of interfacial poisons such as *m*-AMSA.^{46–49} This redox-dependent mechanism involves covalent attachment of the drug to the enzyme.^{1,47–50} It is not known whether imino-based compounds can also act as redox poisons of topoisomerase II. Therefore, to determine if a proportion of the activity of the *m*-AMSA headgroup against human topoisomerase II α reflects a redox-dependent mechanism, the reducing agent DTT was added to DNA cleavage reaction mixtures. DTT blocks redox cycling and reduces quinones to the corresponding hydroquinones and imines to the corresponding amines. Thus, it abrogates the effects of redox-dependent poisons on topoisomerase II.

No significant decrease in the activity of *m*-AMSA was seen in the presence of DTT, but incubation of the headgroup with the reducing agent did lower drug activity (Figure 11).

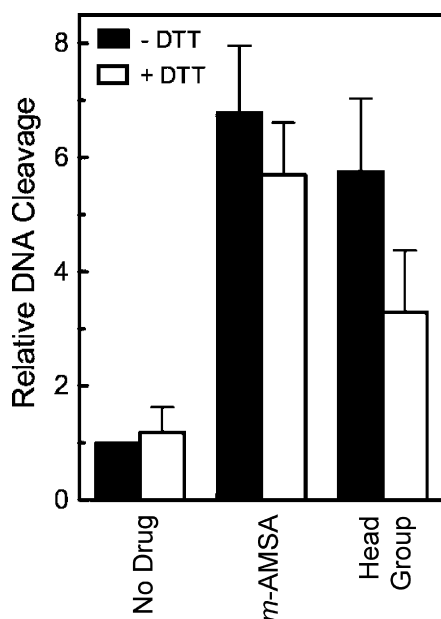


Figure 11. Partial redox dependence of the isolated *m*-AMSA headgroup as a topoisomerase II poison. The effects of 25 μ M *m*-AMSA or 3 mM *m*-AMSA headgroup on the cleavage of negatively supercoiled plasmid DNA by human topoisomerase II α in the absence (black) or presence (white) of 3 mM dithiothreitol (DTT) are shown. A control experiment conducted in the absence of drugs also is shown (No Drug). DNA cleavage levels are relative to those induced by the enzyme in the absence of drug or DTT. Error bars represent the standard deviation of three independent experiments.

However, because the headgroup retained the majority of its activity in the presence of DTT, we conclude that at least part of its activity toward topoisomerase II α reflects a redox-independent mechanism like that of the parent drug.

To further explore the properties of the headgroup, sites of topoisomerase II α -mediated DNA cleavage induced by the compound were determined. Sites cleaved in the presence of the headgroup represented a subset of those induced by

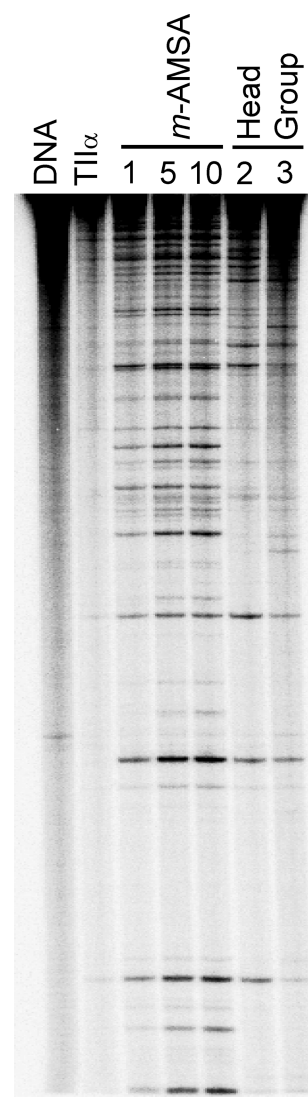


Figure 12. DNA cleavage site specificity and utilization by human topoisomerase II α in the presence of the *m*-AMSA headgroup. A singly end-labeled linear 4332 bp fragment of pBR322 was used as the cleavage substrate. An autoradiogram of a polyacrylamide gel is shown. DNA cleavage reactions were conducted in the absence of drug (TII α) or in the presence of 1, 5, or 10 μ M *m*-AMSA or 2 or 3 mM headgroup. A DNA standard (DNA) is also shown. Results are representative of three independent experiments.

m-AMSA (Figure 12). Therefore, the headgroup appears to be responsible for much of the specificity of the drug.

CONCLUSIONS

Although *m*-AMSA was the first compound shown to poison eukaryotic topoisomerase II, the specific functions of the individual components of the drug are still undefined. Taken together, our findings suggest that the activity and specificity of *m*-AMSA reside largely in the headgroup. Both the 3'-methoxy and 1'-methanesulfonamide substituents contribute positively to drug efficacy. Finally, the linkage between the headgroup and the intercalative acridine moiety provides a strong DNA anchor for the drug and, consequently, dramatically increases the affinity of *m*-AMSA for the topoisomerase II–DNA cleavage complex.

■ ASSOCIATED CONTENT

■ Supporting Information

Representative DNA intercalation gels for many of the compounds included in Table 1 (Figure S1). This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*Correspondence regarding amsacrine should be addressed to D.E.G.: telephone, (205) 975-5381; fax, (205) 975-2543; e-mail, dgraves@uab.edu. Correspondence regarding DNA topoisomerase II should be addressed to N.O.: telephone, (615) 322-4338; fax, (615) 343-1166; e-mail, neil.osheroff@vanderbilt.edu.

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Notes

The authors declare no competing financial interest.

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