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DNA Interaction and Topoisomerase II Inhibition by the Antitumor Agent 3′-(9-Acridinylamino)-5′-hydroxymethylaniline and Derivatives

ALEX SCARBOROUGH,* TSANN-LONG SU,* F. F. LETEUTRE,† YVES POMMIER,† AND TING-CHAO CHOU*,‡,[1]

**Molecular Pharmacology and Therapeutics Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021; ‡Cornell University Graduate School of Medical Sciences, New York, New York 10021; and †Laboratory of Molecular Pharmacology, National Cancer Institute, Bethesda, Maryland 28892*

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Cleavage and cytotoxicity assays have been used to show that 3′-(9-acridinylamino)-5′-hydroxymethylaniline (AHMA) is a potent antileukemic agent that inhibits topoisomerase II-mediated relaxation of supercoiled DNA and promotes the topoisomerase II-mediated cleavage of DNA at a subset of the cleavage sites of 4′-(9-acridinylamino)-methanesulfon-*m*-anisidide (*m*-AMSA). Equilibrium binding data show a larger binding constant for the more cytotoxic derivatives for binding to poly(dA-dT)$_2$ as compared to poly(dG-dC)$_2$, but greater cooperativity for binding to poly(dG-dC)$_2$ and a steric barrier to binding caused by the size of the 5′-hydroxymethyl substituent. Circular dichroism shows a more intercalated binding geometry on poly(dG-dC)$_2$ for 1′-substituted derivatives when compared to AHMA and derivatives with a free 1′-amino group, which is absent with poly(dA-dT)$_2$ and calf thymus DNA and is indicative of specific interactions with GC-rich areas in natural DNA for AHMA and derivatives with free 1′-amino groups which may stabilize the drug–DNA complex.

INTRODUCTION

It has long been known that 9-aminoacridines bind to DNA as intercalators. Among these compounds, 4′-(9-acridinylamino)-methansulfon-anisidide (*m*-AMSA, a 9-anilinoacridine derivative) and its analogues have been extensively studied as potential antitumor agents for the treatment of a variety of tumors and leukemia (*1*). It is thought that this agent stabilizes the topoisomerase II–DNA cleavage complex resulting in the cleavage of DNA (*2*, *3*), and leading to cytotoxicity. The drug intercalates into DNA (*4*), but because of its rapid dissociation (*5*) footprinting studies have been unable to provide any sequence specificity of binding; this has been estimated only by equilibrium binding studies which have revealed no significant preference for poly(dA-dT)$_2$ or poly(dG-dC)$_2$ (*6*). Footprinting analysis has been achieved only by modifying the acridine ring to obtain amsacrine-4-

[1] To whom correspondence should be addressed at Memorial Sloan-Kettering Cancer Center, RRL-617, 1275 York Avenue, New York, NY 10021.

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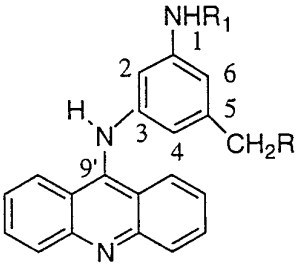


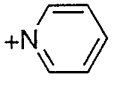
Compound	R_1	R
1	H	OH
2	Ac	OH
3	H	Ac
4	SO_2Me	OH
5	SO_2Me	Ac
6	SO_2Me	 Cl-
7	m-AMSA	

FIG. 1. Structure of 3′-(9-acridinylamino)-5′-hydroxymethyl-aniline (AHMA), and its derivatives.

carboxamides (*7*). In addition, it has not been possible so far to study the drug–DNA complexes by X-ray crystallography. Nevertheless, X-ray crystallography of *m*-AMSA alone shows that the anilino ring forms an angle of 70° with the planar acridine ring (*8*); the study of 9-aminoacridine–RNA complexes (*9*) as well as theoretical studies (*10*) suggest that the anilino ring of *m*-AMSA lies in the minor

groove when the acridine ring is intercalated. Thermodynamic studies have revealed that there are distinct differences in the binding process for the relatively cytotoxic *m*-AMSA as compared to the inactive isomer *o*-AMSA and other 9-anilinoacridines. *m*-AMSA binds to DNA through an enthalpy driven process while less effective compounds bind by an entropy driven process (*11*). Therefore the cytotoxicity of acridine-based topoisomerase II poisons may indeed be linked to steric or electronic factors controlled by anilino ring substituents projecting into the minor groove which interact with DNA or the enzyme, or both, and which promote the formation of the ternary complex.

Another factor which influences the cytotoxicity of drugs may be the plasma half-life. *m*-AMSA possesses a methanesulfonyl and a methoxy function at C-1′ and C-3′ of the 9-anilino ring (Fig. 1) and readily undergoes reversible oxidation giving a quinonediimine intermediate, which forms an adduct with glutathione through nucleophilic attack at a cysteine residue. More than 50% of the dose was excreted as the glutathione conjugate in the bile when mice were treated with *m*-AMSA. The half-life of *m*-AMSA in the presence of fresh mouse blood at 37°C is approximately 30 min (*12*). Recently, we have synthesized and evaluated the cytotoxicity of a series of 3-(9-acridinylamino)-5-hydroxymethyl aniline (AHMA) derivatives (*12*) (Fig. 1). Unlike *m*-AMSA, the substituents on the anilino ring of AHMA are in the meta position to each other, and therefore cannot form an iminoquinone intermediate via biooxidation, thus AHMA possesses a longer plasma half-life (1.5 h) than *m*-AMSA (*12*). We also found that AHMA has greater efficacy against murine leukemia and solid tumors (Lewis lung carcinoma, E0771 mammary adenocarcinoma and B-16 melanoma) than *m*-AMSA (*12*). In our preliminary report, we showed that AHMA inhibited Topo II-mediated DNA decatenation and relaxation (*13*).

The intriguing antitumor activity and chemical structure of this agent provides several features for structure–activity relationship studies using the amino and hydroxymethyl substituents on the anilino ring. We have synthesized a series of AHMA derivatives by introducing various substituents at the NH_2 and/or CH_2OH functions (*12*) and found that these substituents, with only a few exceptions, do not greatly affect the cytotoxicity of the parent compound. Therefore, formation of a stable ternary structure between the drug (AHMA derivatives), DNA, and the degree of cleavage of DNA by Topo II was not adversely affected; this indicates that the position of the substituent is more important than the kind. In this report we compare cleavage patterns of AHMA and *m*-AMSA and determine how varying the nature of substituent(s) at NH_2 and/or CH_2OH of the anilino ring of AHMA derivatives influences affinity for, and binding geometry on, DNA and determine if there is a correlation between these factors and cytotoxicity.

MATERIALS AND METHODS

Drug Preparations

m-AMSA was obtained from the Drug Synthesis and Chemistry Branch, NCI. AHMA derivatives, compounds 1–6 (Fig. 1), were synthesized in this laboratory (*12*). Drugs were dissolved in DMSO for use.

DNA Preparations

Poly(dG-dC)$_2$ and poly(dA-dT)$_2$, sodium salts, were purchased lyophilized (Sigma Chemical Co.) and dissolved in buffer. Extinction coefficients were 1.32×10^4 M^{-1} cm^{-1} at 262 nm for poly(dA-dT)$_2$ (*14*) and 1.68×10^4 M^{-1} cm^{-1} at 254 nm for poly(dG-dC)$_2$ (*15*).

Equilibrium constant determination. Only *m*-AMSA gave a clear isosbestic point with calf thymus DNA. To circumvent this problem, the homopolynucleotides poly(dG-dC)$_2$ and poly(dA-dT)$_2$ were used. These gave clear isosbestic points with the derivatives; unfortunately, AHMA (hydrochloride salt) did not with poly(dG-dC)$_2$, only with poly(dA-dT)$_2$.

Beer's law titrations were carried out in 9 mM NaCl, 5 mM sodium phosphate, 0.1 mM EDTA with 5% DMSO (which was necessary to improve solubility), pH 7.3 (PBSE), at the wavelengths of maximum absorbance to determine molar absorptivities for the unbound compounds. Aggregates were removed by adding 1 mM solutions (stored at −10°C) of the compounds to a large volume of buffer (2–4 ml), sonicating the solution (Branson B-12 sonifier) for 10 mins, followed by centrifugation at 14,000 *g*, the top 80% of the solution was used and was at a concentration that was close to the maximum within the linear range of the Beer's law plot (generally 5–6 μM). To further increase accuracy an average of several optical density measurements were obtained from the IBM 9341 spectrophotometer. Molar absorptivities for bound drugs were obtained from solutions containing DNA and drug at a DNA/drug ratio of 100/1 ($R = 100$). Scatchard plots (*16*) were constructed (*17*) and processed using the cooperative binding equation of McGhee and Von Hippel (*18*).

Induced circular dichromism spectra. Solutions (1–5 mM) of the anilinoacridines were diluted in PBSE to yield solutions approximately 3 μM in concentration. Approximately 1 μM solutions were attained by dilution with PBSE in 10-mm cuvettes. Final concentrations were well below the point of aggregation as determined by Beer's Law plots. DNA was dissolved in PBSE without DMSO; the integrity of the DNA in 5% DMSO/PBSE was checked by comparison with the CD spectrum of the DNA with and without DMSO, there was no difference. Measurements were made on a Jasco J710 spectropolarimeter that was continually flushed with nitrogen. Each spectrum was the average of 8–12 scans.

DNA unwinding assay. The method of Fisher and coworkers (*19*) was modified. Supercoiled PBR322 (0.8 μg) was incubated with 2.5 units of calf thymus topoisomerase I in 80 μl of relaxation buffer (10 mM Tris, 50 mM KCl, 100 mM NaCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, and 30 μg/ml bovine serum albumin, pH 7.9) at 37°C for 2 h. Four microliters of the anilinoacridines at 1, 10, and 50 μM in DMSO was added to the relaxed plasmid; tubes were incubated for 0.5 h at 37°C. Five microliters of 20% SDS was added and the anilinoacridines were extracted twice with 40 μl of equilibrated (pH 8.0) phenol (*20*) and once with 80 μl of chloroform. Thirty microliters of aqueous phase was mixed with 6 μl of gel loading buffer (80% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanol in water; DNA was fractionated by electrophoresis through 1% agarose in TAE buffer at 1.5 V/cm for 16 h at room temperature. Gels were strained using 1 μg/μl ethidium bromide in TAE for 30 min and photographed under UV light.

Inhibition of relaxation of supercoiled DNA. Inhibition of topoisomerase II-induced relaxation of pBR322 was demonstrated as previously described (21).

Cleavage Assays

Teniposide (VM-26) was obtained from Bristol-Meyers Co. (Wallingford, CT). 5-iminodaunorubicin, mitoxantrone, and *m*-AMSA were obtained from the Drug Synthesis and Chemistry Branch, NCI (Bethesda, MD). Drug stock solutions were made in DMSO at 10 mM and diluted in water immediately before use. The final concentration of DMSO in enzymatic reactions did not exceed 1% (v/v), a concentration without detectable effect on *in vitro* Topo II reactions. Simian virus 40 (SV40) and human c-*myc* DNA were purchased from the American Type Culture Collection (Rockville, MD); restriction enzymes, T4 polynucleotide kinase, and Taq DNA polymerase were obtained from Life Technologies Inc. (Gaithersburg, MD) or New England Biolabs (Beverly, MA) and polyacrylamide/bis-acrylamide were purchased from Perkin–Elmer (Norwalk, CT). [γ-^{32}P]ATP and [γ-^{32}P]dATP were purchased from DuPont NEN (Boston, MA). DNA Topo II was purified from mouse leukemia L1210 cell nuclei as described previously (*22*) and was stored at −70°C in 40% (v/v) glycerol, 0.35 M NaCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM KH_2PO_4, 0.2 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride, pH 6.4. The purified enzyme yielded a single 170-kDa band after silver staining of SDS–polyacrylamide gels (*22*).

Preparation of end-labeled DNA fragments. SV40 DNA was labeled at both termini of the BcII restriction site using 1 unit of *Taq* DNA polymerase and [γ-^{32}P]-dATP (*21*). Human c-*myc* DNA fragments were prepared by PCR using a 392 DNA synthesizer from Applied Biosystems (ABi, Foster City, CA) and purified using oligonucleotide purification cartridges (ABi). The 403-base pair DNA fragment from the junction between the first intron and first exon was amplified between positions 2671 and 3073 (numbers refer to GenBank genomic positions) using oligonucleotides: 5′-TGCCGCATCCACGAAACTTTGC-3′ as sense primer and 5′-GAACTGTTCAGTGTTTACCCCG-3′ as antisense primer. Single end-labeling of the DNA fragment was obtained by 5′ end-labeling of the sense strand primer oligonucleotide (*23*, *24*). Approximately 0.1 μg of the c-*myc* DNA that had been restricted by *Xho*I and *Xba*I was used as a template for the PCR (*23*, *24*).

Topo II-induced DNA cleavage reactions. DNA fragments were equilibrated with or without drug in 10 mM Tris–HCl, pH 7.5, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM ATP, and 15 mg/ml bovine serum albumin for 5 min before addition of purified topo II (40–70 ng) or HL-60 nuclear extracts (2 μl corresponding to approximately the extract from 10^6 nuclei) in 20 μl final reaction volume (*25*). Reactions were performed at 37°C for 30 mins and then stopped by adding sodium dodecyl sulfate (SDS) to a final concentration of 1% and proteinase K to 0.4 mg/ml, followed by incubation for 1 h at 42°C.

DNA sequence analysis. DNA was precipitated with ethanol and resuspended in 2.5 μl loading buffer (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue). After heating to 90°C for 3 mins, samples

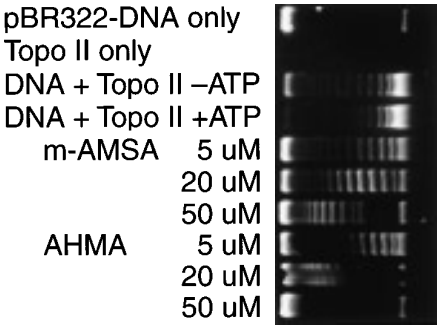


FIG. 2. Inhibition of topoisomerase II-mediated relaxation of supercoiled pBR322 DNA by *m*-AMSA and by AHMA.

were loaded onto DNA sequencing gels (7% polyacrylamide; 19:1, acrylamide: bisacrylamide) containing 7 M urea in TBE buffer. Electrophoresis was at 2500 V (60 W) for 4 h. Gels were dried on 3MM paper and exposed to Kodak XAR-2 film for autoradiography.

Agarose gel analysis for cleavage quantification. Three microliters of loading buffer (0.3% bromophenol blue, 16% ficoll, 10 mM NaH_2PO_4, pH 7.5) was added to each sample which was then heated at 65°C for 1–2 min before loading into a 1% agarose gel made in 0.1% SDS, 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8. Electrophoresis was at 2 V/cm overnight. Quantification of drug-induced DNA double-stranded breaks was done by scanning radioactive gels using a PhosphorImager (Molecular Dynamic, Sunnyvale, CA); for each lane, radioactivity was measured in DNA cleavage products (C) and in the total DNA present in the lane (T). Drug-induced cleavage was expressed as:

$$\% \text{ DNA cleaved} = 100 \times \frac{C/T - C_0/T_0}{1 - C_0/T_0}, \quad [1]$$

where C_0 and T_0 are the counts for cleaved and total DNA, respectively, in the presence of nuclear extracts without drug.

RESULTS AND DISCUSSION

Cleavage assays. Figures 2–5 all demonstrate that AHMA is a Topo II poison. Figure 2 shows that AHMA inhibits topoisomerase II-mediated relaxation of supercoiled pBR322 DNA in a dose-dependent manner, which is typical of the classical Topo II inhibitor *m*-AMSA. AHMA appeared to be somewhat more potent in this regard than *m*-AMSA. In Figure 3, where the cleavage pattern of the sense strand of a c-*myc* DNA fragment was examined, AHMA clearly showed a cleavage pattern different from that of other topo II inhibitors; however, similarities between the topo II cleavage sites induced by AHMA and *m*-AMSA are striking (lanes D and