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Deoxyribonucleic Acid Breaks Produced by 4′-(9-Acridinylamino)methanesulfon-*m*-anisidide and Copper†

Angela Wong,* Cheng-Hsiung Huang, and Stanley T. Crooke

ABSTRACT: We have demonstrated that 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (mAMSA), in the presence of Cu(II) ion, causes the breakage of plasmid pDPT275 and pBR322 superhelical form I DNA. In neutral pH, the degradative product was nicked, relaxed form II DNA, resulting from single-stranded DNA breakage. The extent of DNA breakage was both mAMSA concentration and Cu(II) concentration dependent. DNA breakage increased with increasing time of drug treatment. The mAMSA–Cu(II)-induced DNA breakage varied with pH values and also with the nature of the buffer systems. In both Tris-HCl and borate buffers the extent of DNA breakage increased with increasing pH. In Tris-HCl buffer (pH 7–9), only single-strand breaks were obtained, whereas in borate buffer (pH 9–10.5), linear form III DNA was obtained. At equivalent pH, the optimum buffer was borate. No breakage was observed at pH values below 6. The interaction of Cu(II) with mAMSA was examined by using absorption and fluorescence spectroscopies. Interaction of Cu(II) with mAMSA was characterized by a decrease in the absorption at 435 and 420 nm with a simultaneous increase at 330 nm. A highly fluorescent product was obtained upon reacting mAMSA with Cu(II), with an emission spectrum (excitation at 400 nm) showing a doublet at 430 and 450 nm and a shoulder around 480 nm. The spectral changes are also dependent similarly on the pH and the nature of buffer. Other divalent metal ions such as Co(II), Cd(II), Ni(II), and Zn(II) do not induce DNA breakage or spectral changes. The oAMSA isomer, which has no antitumor activity, is less effective in inducing DNA breakage than the mAMSA.

4′-(9-Acridinylamino)methanesulfon-*m*-anisidide (mAMSA)[1] is a promising synthetic anticancer agent. It is currently in phase II–III clinical evaluation. Clinical studies suggested that mAMSA is active in acute leukemia (Slevin et al., 1981). Its mechanism of action is still unclear, but the target seems to be DNA. mAMSA intercalates into DNA (Waring, 1976),

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[1] Abbreviations: dsb, double-strand break; ssb, single-strand break; EDTA, ethylenediaminetetraacetic acid; EB, ethidium bromide; mAMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; mAQDI, N^1-(methylsulfonyl)-N^4-(9-acridinyl)-3-methoxy-2,5-cyclohexadiene-1,4-diimine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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and the interaction with DNA may lead to the inhibition of nucleic acid synthesis (Gormley, 1978). It has been reported that mAMSA induces DNA single-strand breaks, double-strand breaks, and DNA protein cross-links in L1210 leukemia cells (Zwelling et al., 1981). However, it has not been demonstrated to degrade isolated DNA.

In the present studies, using superhelical plasmid DNA, we demonstrate that mAMSA, in the presence of Cu(II) ion, produces single-strand breakage of superhelical DNA. Absorption and fluorescence spectroscopic data showed that mAMSA interacts with Cu(II) ion. This suggests that mAMSA appears to be similar to two other antitumor drugs, bleomycin (Sausville et al., 1976) and adriamycin (Someya & Tanaka, 1979), and to 1,10-phenanthroline (Sigman et al., 1979; Downey et al., 1980). All can form coordinate complexes with a transition metal ion and induce DNA breakage in vitro by facilitating the redox reaction of a metal ion close to a site where damage to DNA may readily occur.

Cu(II) is relatively abundant in biological fluids, and micromolar levels of weakly bound Cu(II) are present in serum. Therefore, the activity observed may be important in the mechanism of cytotoxicity of mAMSA. oAMSA, in which the methoxy group is on the ortho instead of the meta position, is inactive as an antitumor agent, even though it intercalates into superhelical DNA (Waring, 1976). It has been reported that oAMSA failed to induce any DNA single-strand break in the cell (Burr-Furlong et al., 1978; Ralph, 1980). Alkaline elution studies showed that oAMSA did not produce protein-associated DNA breakage (Zwelling et al., 1981). In the present studies we demonstrate that oAMSA is less effective in inducing in vitro DNA breakage than mAMSA. This suggests that the drug–Cu(II)-induced DNA cutting activity may correlate with cytotoxic activities. Furthermore, because of the simplicity of the superhelical DNA assay system, this in vitro system may serve as a useful tool in studying the primary sequence specificity of the mAMSA-induced DNA breakage.

Materials and Methods

Materials

mAMSA and blenoxane were supplied by Bristol Laboratories, Syracuse, NY. Blenoxane contained approximately 60% bleomycin A_2, 30% bleomycin B_2, and 10% other bleomycins. oAMSA was a generous gift from Dr. Leonard Zwelling, Laboratory of Molecular Pharmacology, National Cancer Institute. Both mAMSA and oAMSA solutions were prepared by dissolving the drugs in dimethyl sulfoxide (3 mg/mL) and diluting with distilled water to 1 mg/mL. pDPT275 plasmid is a second step copy number mutant derived from R100(NR1) plasmid, and pDPT275 DNA was isolated according to the procedure of Clewell & Helinski (1970). Dimethyl sulfoxide, Na_2EDTA, ethidium bromide, 2-mercaptoethanol, $CuSO_4 \cdot 5H_2O$, $ZnCl_2$, $CdCl_2$, $CoCl_2 \cdot 6H_2O$, $NiCl_2$, sodium borate, sodium citrate, sodium phosphate, and Tris were obtained from Sigma Chemical Co., St. Louis, MO, and agarose-ME was from Bethesda Research Laboratories, Gaithersburg, MD.

Methods

Determination of Drug and DNA Concentrations. Drug concentrations were determined spectrophotometrically. For mAMSA, a molar extinction coefficient of 1.20×10^4 cm^{-1} M^{-1} at 434 nm (in water) was used (Wilson et al., 1981). A molar extinction coefficient of 6600 cm^{-1} M^{-1} was used for pDPT275 plasmid DNA in aqueous solutions.

Reaction of mAMSA with pDPT275 and pBR322 DNA. The reaction mixture (60 μL) contained 10 mM Tris-HCl, pH 7.5, 0.74 μg of pDPT275 DNA or 0.74 μg of pBR322 DNA, and varying amounts of mAMSA in the presence or absence of Cu(II). The mixture was incubated at room temperature for various times (15–120 min), and 20 μL of a solution containing 50% glycerol (v/v), 40 mM EDTA, and 0.05% bromophenol blue (w/v) was then added before subjection to agarose gel electrophoresis.

Treatment of pDPT275 DNA with Blenoxane. The pDPT275 DNA was incubated with varying amounts of blenoxane in a buffer solution (final volume 50 μL) containing 50 mM borate (pH 9.5), 66 mM NaCl, and 25 mM 2-mercaptoethanol for 30 min at room temperature.

Agarose Gel Electrophoresis. Electrophoresis was performed as described by Huang et al. (1981). Agarose slab gels (1.1%) were run at 4 V/cm for 14 h in 40 mM Tris-HCl buffer containing 5 mM sodium acetate and 1 mM EDTA, pH 7.8. After electrophoresis, gels were stained with 1 μg/mL ethidium bromide in the electrophoresis buffer and photographed with a Polaroid CU-5 Land Camera equipped with a No. 8 Kodak Wratten gelatin filter (Eastman Kodak Co., Rochester, NY) and type 665 Land films while excited with a transilluminator (Ultraviolet Products, Inc.). The negative films of gels were used for densitometric scannings.

Quantitation of DNA Bands. The negative film of the gel was scanned with a DU-8B UV/vis spectrophotometer, equipped with a slab gel scan compuset module (Beckman Instrumetns, Fullerton, CA). The amount of form II and form III DNA was used to calculate the extent of single-strand and double-strand breaks, respectively, according to the procedures described previously (Lloyd et al., 1978; Huang et al., 1981). In this procedure the difference in the intensity of the maximal EB staining between form I and form II DNA is corrected (the stainability of form I DNA was 70% of that of form II or form III DNA).

Spectrophotometric Measurements. The absorption spectra were measured with a Beckman DU-7 UV/vis spectrophotometer, equipped with a Hewlett Packard 26716 graphic printer, in 1-mL, 1-cm quartz cuvettes. The incubation mixtures contained 10 μM mAMSA and 100 μM $CuSO_4 \cdot 5H_2O$ or other divalent metal salts ($ZnCl_2$, $NiCl_2$, $CdCl_2$, and $CoCl_2 \cdot 6H_2O$) in 1 mL of 10 mM Tris-HCl buffer, pH 7.5.

Fluorometric Measurements. Fluorescence measurements were performed in a 1-mL, 1-cm quartz cuvette with an Aminco SPF-500 ratio spectrofluorometer, equipped with a Hewlett Packard 9815A/S calculator and a HP 7225A graphic plotter. For measurements of emission at an excitation of 400 nm, 9 μM mAMSA and 100 μM $CuSO_4 \cdot 5H_2O$ or other divalent metal salts ($ZnCl_2$, $NiCl_2$, $CdCl_2$, and $CoCl_2 \cdot 6H_2O$) were added to 1 mL of 10 mM Tris-HCl buffer, pH 7.5. Uncorrected fluorescence spectra were reported.

Results

DNA Breakage Induced by mAMSA in the Presence of Cu(II). In the presence of Cu(II), mAMSA induced single-strand DNA breakage, converting superhelical plasmid pDPT275 DNA as well as pBR322 DNA to circular nicked relaxed form II DNA. Figure 1 shows the ethidium bromide stained DNA banding pattern of mAMSA-treated pDPT275 DNA in an agarose gel. Lane a represents the untreated pDPT275 DNA form I preparation. Lanes b–e show that, as a control, treatment of form I DNA with blenoxane at increasing concentrations (1–30 nM) caused a gradual decrease in the banding intensity of form I and form I oligomer and a simultaneous increase in that of both the form II (single-strand breaks) and form III DNA (double-strand breaks). Lanes g–l show the DNA-breakage activity of increasing

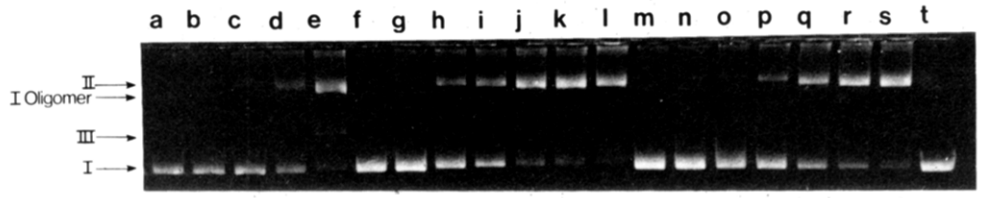

FIGURE 1: Agarose gel electrophoretic pattern of ethidium bromide stained pDPT275 DNA after treatment with blenoxane and with mAMSA and Cu(II). DNA migrated from top to bottom in the order of decreasing distance of form I, form II, form I oligomer, and form II DNA. Lanes a and t: untreated DNA. Lanes b–e: 1, 3, 10, and 30 nM blenoxane. Lane f: 100 μM Cu(II). Lanes g–l: 5, 25, 50, 100, 150, and 250 μM mAMSA, in the presence of 100 μM Cu(II). Lane m: 100 μM mAMSA. Lanes n–s: 1, 5, 25, 50, 75, and 100 μM Cu(II), in the presence of 100 μM Cu(II).

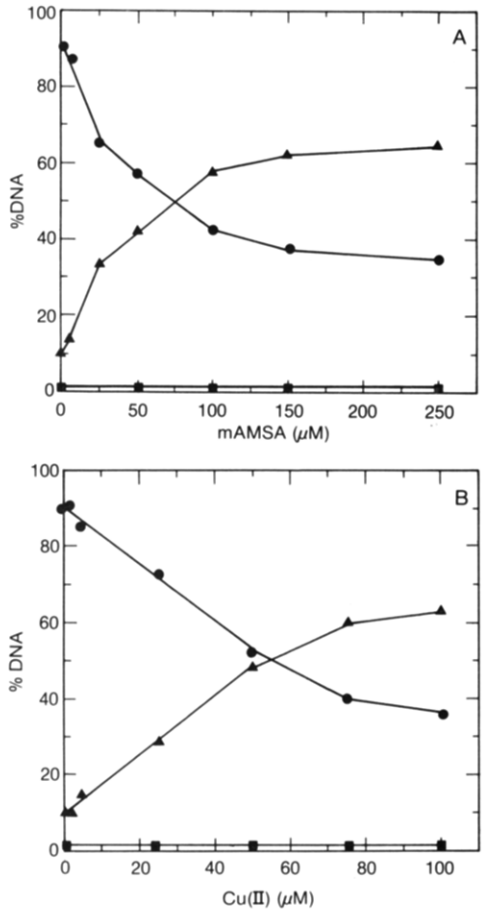

FIGURE 2: (A) Percentage distribution of DNA conformational isomers after treatment with mAMSA and Cu(II). Data were obtained from the gel pattern shown in Figure 1, lanes g–l. DNA was treated with increasing concentrations of mAMSA in the presence of 100 μM Cu(II). (●) Form I DNA; (▲) form II DNA; (■) form III DNA. (B) Percentage distribution of DNA conformational isomers after treatment with mAMSA and Cu(II). Data were obtained from the gel pattern shown in Figure 1, lanes n–s. DNA was treated with varying amounts of Cu(II) in the presence of 100 μM mAMSA. (●) Form I DNA; (▲) form II DNA; (■) form III DNA.

concentrations of mAMSA in the presence of 100 μM Cu(II). The decrease in form I DNA was accompanied by an increase in form II DNA. No form III DNA was produced. Thus, a combination of mAMSA and Cu(II) induced single-strand breakage but no detectable double-strand breakage. Cu(II) when used alone, at concentrations up to 100 μM, induced no DNA breakage (lane f). In the absence of Cu(II), there was no detectable DNA breakage produced at an mAMSA concentration as great as 270 μM, which equals to an mAMSA/DNA ratio of 7.2/1 (data not shown).

The DNA breakage induced by mAMSA and Cu(II) is also dependent on the Cu(II) concentration. In the presence of 100 μM mAMSA (Figure 1, lanes n–s), the percentage of form

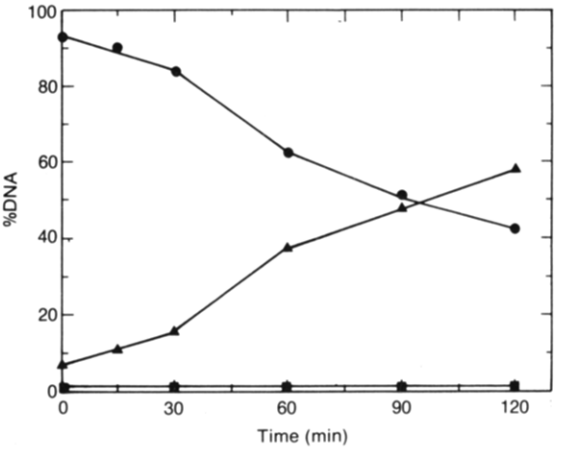

FIGURE 3: Time dependency of breakage induced by mAMSA and copper. Reaction mixtures contained 50 μM mAMSA and 50 μM Cu(II). (●) Form I DNA; (▲) form II DNA; (■) form III DNA.

II production increased with increasing concentrations (5–100 μM) of Cu(II).

Quantitative analysis by densitometry of the single-strand break production by mAMSA and Cu(II) was performed. The untreated pDPT275 DNA preparation contained approximately 80–85% form I DNA, 10% form I oligomers, and 5–10% form II DNA. Form III DNA was not detected. The form I oligomers were more resistant to breakage than the monomeric form I DNA. Since the form I oligomers contributed to only a small percentage (10%) of the total plasmid DNA and also because of the small changes induced by drug treatment, they were not considered in the calculation of the percentage DNA distribution.

Parts A and B of Figures 2 show the densitometric scanning results of lanes g–l and n–s, respectively, of Figure 1. In the presence of 100 μM Cu(II), DNA degradation was observed to increase with increasing concentrations of mAMSA (5–100 μM). A maximum of 60–65% conversion of form I DNA to form II occurred at 150–250 μM of mAMSA (Figure 2A). In the presence of a constant amount of mAMSA (100 μM), increasing the concentration of Cu(II) also resulted in increased DNA breakage, with a maximum of 60–65% form II DNA produced (Figure 2B).

Time Dependency of DNA Breakage Induced by mAMSA and Cu(II). The kinetic behavior of the reaction of mAMSA and Cu(II) with DNA was shown in Figure 3. The conversion of form I to form II DNA increased with increasing time of incubation and was reasonably linear during the assay period (0–120 min). Form III DNA was not detected at pH 7.5 at any tested time periods.

Cleavage of pBR322 DNA by mAMSA and Cu(II). The DNA breakage is not restricted to pDPT275 plasmid DNA. In the presence of Cu(II), mAMSA readily cleaved superhelical pBR322 DNA to produce form II DNA. As with pDPT275 DNA, no double-strand breakage was observed in

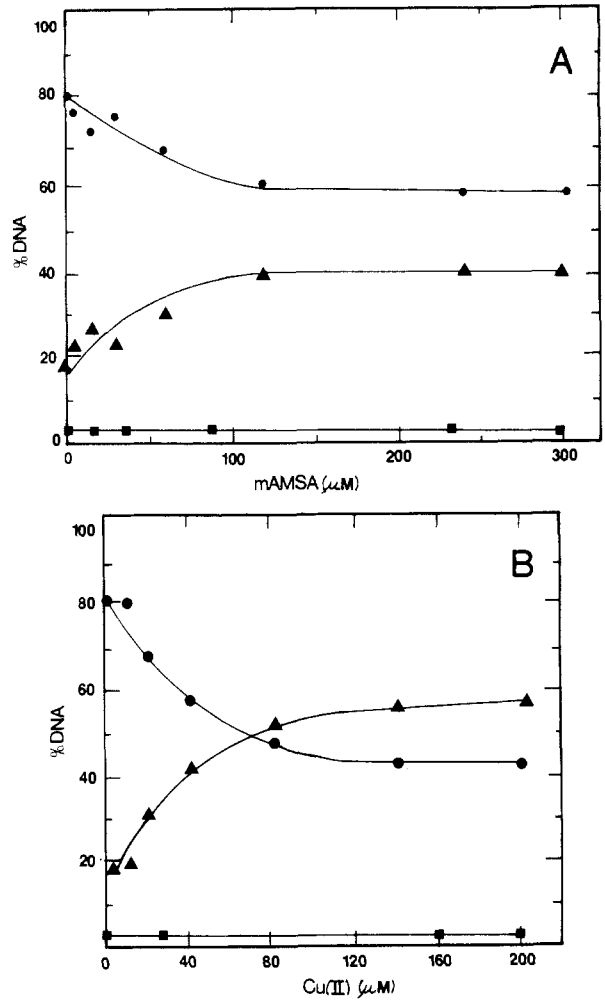

FIGURE 4: (A) Percentage DNA distribution after treatment of pBR322 DNA with varying amounts of mAMSA in the presence of 50 μM Cu(II). (●) Form I DNA; (▲) form II DNA; (■) form III DNA. (B) Percentage DNA distribution after treatment of pBR322 DNA with increasing concentrations of Cu(II) in the presence of 120 μM mAMSA. (●) Form I DNA; (▲) form II DNA; (■) form III DNA.

10 mM Tris-HCl buffer, pH 7.5. Figure 4A shows that, in the presence of Cu(II) (50 μM), a maximum of 40% form II DNA was produced at 100 μM of mAMSA. Figure 4B shows that, in the presence of 120 μM mAMSA, a maximum of 50–55% form II DNA was produced at 80 μM of Cu(II).

Effects of Several Divalent Metal Ions on the Degradation of DNA by mAMSA. None of the other divalent metal ions tested [Ni(II), Cd(II), Co(II), and Zn(II)] at concentrations up to 100 μM induced DNA breakage, either in the presence (90 μM) or in the absence of mAMSA (data not shown). Thus, Cu(II) is the only metal ion found to be effective in participating in mAMSA-induced DNA breakage.

Effects of pH and Various Buffers on the DNA Breakage Induced by mAMSA and Cu(II). The extent of DNA breakage induced by mAMSA and Cu(II) varied with pH values and also with the nature of the buffer systems used in the reaction (Figure 5). The reaction mixtures contained 100 μM mAMSA, 50 μM Cu(II), and 0.74 μg of pDPT275 DNA in various buffers. In citrate buffer, at or below pH 6, mAMSA and Cu(II) induced virtually no DNA breakage. In phosphate buffer, only a slight increase in the percentage of form II DNA was observed as the pH was increased from 6 to 7. However, when Tris-HCl buffer was used, 20–25% form II DNA was produced at pH 7. There was a maximum production of form II DNA at pH 7.5 and 8 (form II:

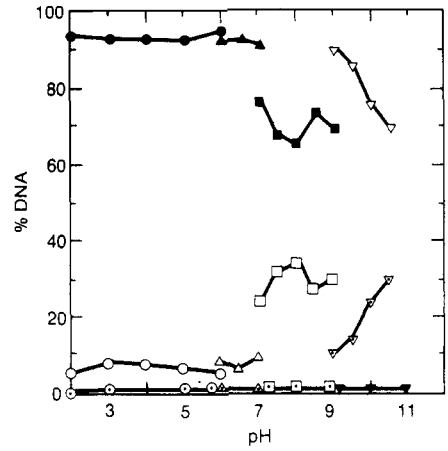

FIGURE 5: Percentage DNA distribution after treatment of pDPT275 DNA (0.74 μg) with mAMSA (100 μM) and Cu(II) (50 μM) in various pH and buffer compositions. For treatment in citrate buffer: (●) form I DNA; (○) form II DNA; (dotted open circle) form III DNA. For treatment in phosphate buffer: (▲) form I DNA; (△) form II DNA; (dotted open triangle) form III DNA. For treatment in Tris-HCl buffer: (■) form I DNA; (□) form II DNA; (dotted open box) form III DNA. For treatment in borate buffer: (▼) form I DNA; (▽) form II DNA; (dotted open inverted triangle) form III DNA.

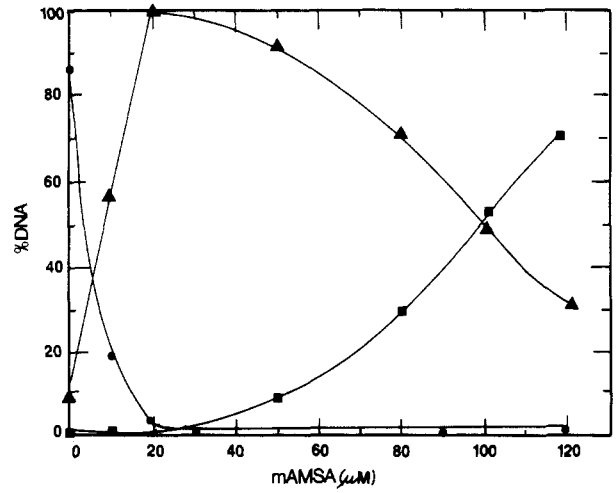

FIGURE 6: Percentage DNA distribution after treatment of pDPT275 DNA with varying amounts of mAMSA in the presence of 50 μM Cu(II) in 10 mM borate buffer, pH 9.5. (●) Form I DNA; (▲) form II DNA; (■) form III DNA.

30–35%) with a subsequent decline at pH 8.5 and 9.0, with the production of 25% and 30% of form II DNA, respectively. No form III DNA was detected. In borate buffer, at pH ranges from 9.0 to 10.5, all the form I DNA was degraded into form II and form III DNA. As the pH was increased, there was a gradual decrease in the percentage of form II DNA and a simultaneous increase in form III DNA. In the experiment at pH 10.5, 30% form III DNA was produced.

In other experiments, the DNA breakage activity of mAMSA was studied between pH 8 and pH 10.5 in borate buffer. In the pH range 7–8, DNA cutting was more efficient in borate buffer than in Tris-HCl buffer. In borate buffer, approximately 40% form II DNA was produced at pH 8, and this increased to greater than 90% at pH 9.0. However, the extent of production of form III DNA varied slightly (±10%) from experiment to experiment. Neither mAMSA nor Cu(II) alone was active in breaking DNA over the entire pH range (pH 3 to 10.5, data not shown).

Figure 6 shows the DNA-breakage activity of increasing concentrations of mAMSA [in the presence of 50 μM Cu(II)]

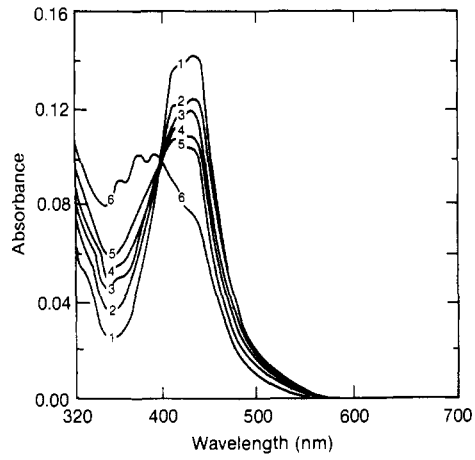

FIGURE 7: Time course of absorption spectral changes for mAMSA induced by the addition of Cu(II). mAMSA and Cu(II) concentrations were 10 and 100 μM, respectively, in 1 mL of 10 mM Tris-HCl buffer, pH 7.5. Absorption spectra were recorded at different time periods after the addition of Cu(II): (1) 0 time, (2) 20 min, (3) 60 min, (4) 2 h, (5) 4 h, and (6) 24 h.

in borate buffer at pH 9.5. At 10 and 20 μM mAMSA, all the form I DNA was completely degraded into form II DNA. Form III DNA was not detected. However, at mAMSA concentrations above 20 μM, form III production was obtained at the expense of form II DNA. With an increasing concentration of mAMSA (50–120 μM), there was a gradual decrease in the percentage of form II DNA (100–30%) and a simultaneous increase of form III DNA (0–70%). At 120 μM mAMSA, a smear DNA pattern was observed, indicating an extensive DNA breakage.

Changes in Absorption of mAMSA upon Reaction with Cu(II). Figure 7 shows that mAMSA exhibits a visible absorption spectrum below 600 nm, with a major peak at 435 nm, a shoulder at around 420 nm, and a minor peak at 330 nm, as reported by Gormley et al. (1978). The addition of Cu(II) ion in Tris-HCl buffer, pH 7.5, resulted in a decrease in the absorption above 400 nm and a simultaneous increase below 400 nm. The absorption spectral changes increased with time, indicating an increase in the mAMSA–Cu(II) interaction. An isosbestic point at approximately 395 nm was obtained, indicating that mAMSA was converted to a single absorbing product. Other divalent metal ions [Ni(II), Cd(II), Co(II), and Zn(II)] (up to 100 μM) were tested and found to induce no spectral change of mAMSA (data not shown).

The absorption spectral changes were affected by the buffer and also by pH. Parts A and B of Figure 8 show the spectra which were recorded after incubating mAMSA and Cu(II) in Tris-HCl and borate buffer, respectively, for 2 h. In Tris-HCl buffer (Figure 8A), as the pH value was changed from 7 to 9, the decrease in the absorbance above 400 nm became larger. At pH 9.0, two new absorption peaks were observed at 356 and 377 nm. An isosbestic point at approximately 395 nm was obtained (Figure 8A). In borate buffer (Figure 8B), when the pH was varied from pH 9 to 11, the decrease in the characteristic mAMSA absorption above 400 nm became more significant, and the resulting spectra had peaks at 356 and 377 nm. The spectral changes in borate buffer (pH 9–11) were more extensive than those observed with Tris-HCl buffer at pH 7–9. Again, this conversion showed an isosbestic point at approximately 395 nm (Figure 8B). Thus, the absorption changes that occurred in Tris-HCl buffer and borate buffer have the same isosbestic point. The increase in the optical changes with increasing pH suggests an increase in the extent of Cu(II)–mAMSA interaction. The

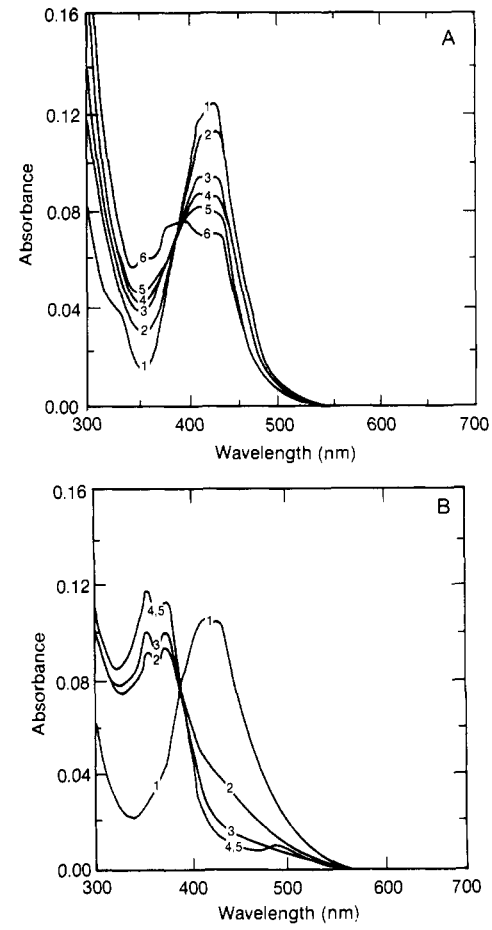

FIGURE 8: (A) pH dependency of mAMSA–Cu(II) interaction in Tris-HCl buffer. Reaction mixtures contained 10 μM mAMSA and 100 μM Cu(II) in 1 mL of 10 mM Tris-HCl buffer at various pH values. (1) 10 μM mAMSA at pH 7; (2) 10 μM mAMSA and 100 μM Cu(II) at pH 7, (3) pH 7.5, (4) pH 8, (5) pH 8.5, and (6) pH 9. (B) pH dependency of mAMSA–Cu(II) interaction in borate buffer. Reaction mixtures contained 10 μM mAMSA and 100 μM Cu(II) in 1 mL of 10 mM borate buffer at various pH values. (1) 10 μM mAMSA at pH 9.0; (2) 10 μM mAMSA and 100 μM Cu(II) at pH 9, (3) pH 9.5, (4) pH 10, (5) pH 10.5, and (6) pH 11.

well-defined isosbestic point at 395 nm in all buffers suggests the conversion of mAMSA to a single species.

As previously reported (Gormley et al., 1978), the addition of calf thymus DNA to mAMSA resulted in an immediate decrease in the mAMSA absorption spectrum due to intercalation of mAMSA into the DNA. In the presence of increasing Cu(II) concentrations (0–15 μM), the higher DNA concentrations were required to give equivalent quenching. However, the maximum quenching observed was equivalent at all Cu(II) concentrations (data not shown). Although these data might suggest a reduction in affinity of mAMSA for DNA in the presence of Cu(II), the analysis is complicated by the multiple possible reactions. Consequently, firm conclusions cannot be drawn.

Fluorescent Product Produced by mAMSA–Cu(II) Interactions. mAMSA does not exhibit detectable fluorescence. Addition of Cu(II) to mAMSA in Tris-HCl buffer, pH 7.5, resulted in the formation of a highly fluorescent product (Figure 9). The intensity of the emission spectra (excitation at 400 nm) of the mAMSA–Cu(II) reaction product increased with the time of incubation. The characteristics of the fluorescence spectra taken at different periods of time were similar, although increased in intensity. The fluorescence spectra displayed a doublet at 430 and 450 nm and a shoulder around 480 nm. None of the other divalent metal ions tested

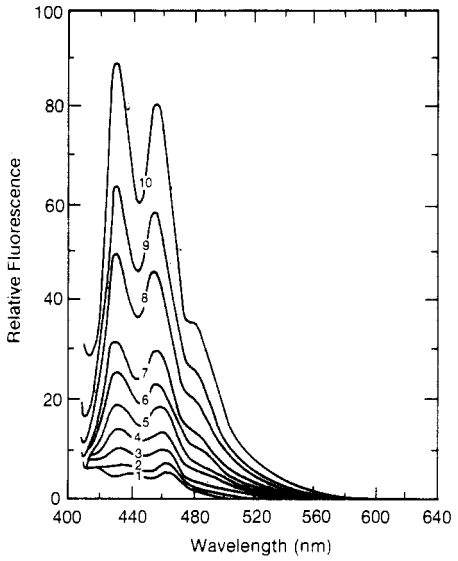

FIGURE 9: Induction of fluorescent product from mAMSA–Cu(II) interaction. Reaction mixture contained 9 μM mAMSA and 100 μM Cu(II) in 1 mL of 10 mM Tris-HCl buffer, pH 7.5. Fluorescence spectra were recorded at 0 min (1), 25 min (2), 50 min (3), 75 min (4), 100 min (5), 2 h (6), (7), 3 h (8), 3.5 h (9), and 4 h (10).

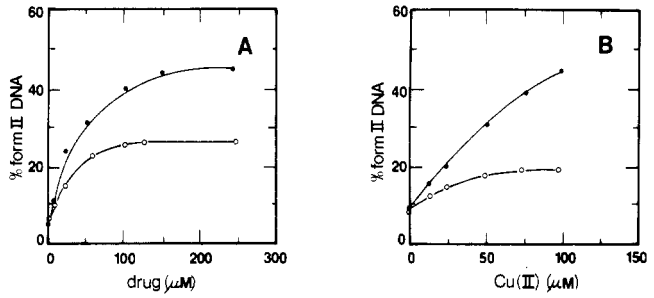

FIGURE 10: Percentage form II DNA produced from treatment of pDPT275 DNA with mAMSA + Cu(II) or oAMSA + Cu(II) in 10 mM Tris-HCl buffer, pH 7.5. (A) pDPT275 DNA was treated with increasing concentrations of drug in the presence of 100 μM Cu(II). (●) Treatment with mAMSA; (○) treatment with oAMSA. (B) pDPT275 DNA was treated with various amounts of Cu(II) in the presence of 100 μM of drug. (●) Treatment with mAMSA; (○) treatment with oAMSA.

[Ni(II), Cd(II), Co(II), and Zn(II)] was effective in producing a fluorescent product (data not shown).

Effect of oAMSA in DNA Breakage. The oAMSA isomer exhibited a lower DNA cutting activity than that of mAMSA (Figure 10A). In the presence of 100 μM Cu(II) in 10 mM Tris-HCl buffer, pH 7.5, the maximal production of form II DNA (25 percent) occurred at approximately 100 μM oAMSA. However, a maximum of 45 % form II DNA (no detectable form III DNA) was obtained at 150–200 μM mAMSA. mAMSA was also more effective in inducing DNA breakage when varying amounts of Cu(II) were added to a fixed concentration (100 μM) of drug (Figure 10B). The production of form II DNA induced by oAMSA reached a maximum (15–20%) at 50 μM Cu(II). The mAMSA-induced production of form II DNA increased with increasing concentration of Cu(II) and did not reach a maximum until 100 μM Cu(II) was present, at which it produced 45% of form II DNA.

Discussion

The following important features of the mAMSA–Cu(II)-induced DNA breakage are reported here: (1) the presence of both mAMSA and Cu(II) is necessary; (2) the breakage is both mAMSA concentration and Cu(II) concentration dependent; (3) none of the other divalent metal ions tested [Ni(II, Co(II), Cd(II), and Zn(II)] is effective in inducing DNA breakage with mAMSA; (4) the breakage increases with increasing time of incubation; (5) the breakage varies with changes in pH and buffer systems; (6) at pH 7–9 in Tris-HCl buffer, single-strand breakage (formation of form II DNA) predominated, whereas at high pH values (pH 9–11) in borate buffer, extensive single-strand breakage results in the production of form III DNA; (7) at neutral pH, oAMSA is less effective than mAMSA.

That mAMSA interacts with Cu(II) is evident from the observations that (1) Cu(II) induced characteristic changes in the mAMSA absorption spectrum and (2) addition of Cu(II) to mAMSA induced the production of fluorescent product(s). The spectral change was Cu(II) specific. Other divalent metal ions such as Ni(II), Cd(II), Co(II), and Zn(II) induced no spectral change of mAMSA. The increase in the spectral changes with increasing time of incubation suggests an increase in the extent of Cu(II)–mAMSA interaction, which may result in an increase in the production of an mAMSA–Cu(II) complex or a complex of Cu(II) with modified mAMSA. Since a well-defined isosbestic point was obtained between 300 and 500 nm during the mAMSA spectral shifts, it is suggested that mAMSA was converted to a single product, which may be the complex between Cu(II) and mAMSA or modified mAMSA.

The interaction between mAMSA and Cu(II) may lead to DNA breakage. In other experiments (data not shown), we have found that partial removal of molecular oxygen suppressed the mAMSA–Cu(II)-induced DNA breakage (Wong et al., 1984). Therefore, the DNA breakage induced by mAMSA and Cu(II) can be compared to that induced by bleomycin and 1,10-phenanthroline; all require the presence of a transition metal and oxygen. However, unlike bleomycin and 1,10-phenanthroline, the mAMSA–Cu(II)-induced DNA breakage does not require the presence of a reducing agent. The role of the reducing agent is to facilitate the redox reaction of the drug–metal complexes, which may lead to the generation of free radicals of oxygen. Since the DNA breakage induced by mAMSA and Cu(II) does not require the addition of a reducing agent, it is possible that mAMSA itself may act as a reducing agent, which reduces Cu(II) to Cu(I). It has been demonstrated (Shoemaker et al., 1982) that mAMSA is biotransformed in vivo by oxidation to a quinoidal diimine, mAQDI [N^1-methylsulfonyl-N^4-(9-acridinyl)-3-methoxy-2,5-cyclohexadiene-1,4-diimine]. Also, mAQDI can be obtained in the in vitro system by using MnO_2 as an oxidant (Gaudich & Przybylski, 1983). It may be that same redox reaction takes place in the vitro reaction between mAMSA and Cu(II).

DNA was subject to damage by mAMSA and Cu(II) over a wide pH range (pH 6–10.5). The activity at pH values below pH 6 was insignificant. The pH of the system may modify the mAMSA–Cu(II) interaction by affecting the ability of the metal ion [Cu(II)] to form a complex with the ligand binding sites of mAMSA. It is known that, in acidic and neutral pH, metal ions will not compete effectively with protons at ligand binding sites, whereas in alkaline pH, more protons can be displaced from the ligand sites, resulting in an increased interaction with metal ions. Moreover, it has been reported that mAMSA has a pK_a at 7.19 which is due to the presence of the secondary amine group in the 9-aminoacridine nucleus (Atwell et al., 1977). Since its DNA cutting activity increased markedly in the pH range above 7 and was negligible below pH 7, it is possible that this unprotonated secondary amine is essential for DNA cutting activity.

The production of form III DNA may be a result of direct double-strand breaks occurring as a single event or of two closely spaced single-strand breaks at complementary strands. Our data (Figure 5B) suggest that the form III DNA produced by mAMSA in borate buffer (pH 9.5) may result from an accumulation of random single-strand breaks. This is because at a low drug concentration, form I DNA was completely degraded into form II DNA. Form III DNA was not detected. Form III production occurred only at high mAMSA concentrations. The production of form III DNA was associated with a corresponding decrease of form II DNA production. Alkaline elution studies with intact L1210 leukemia cells showed that mAMSA produced mainly single-strand breaks (the reported ssb/dsb ratio was 3 to 25; Zwelling et al., 1981). In the present studies, we demonstrate an analogous result using the in vitro pDPT275 DNA system. At both physiological and alkaline pH, single-strand breaks predominated. Although we have not further characterized the reaction products, no barbituric acid positive material was detected. It is thus unlikely that malondialdehyde was produced (data not shown).

oAMSA binds to purified DNA and has the same unwinding angle as mAMSA (Waring, 1976). Yet, oAMSA is essentially inactive in killing L1210 leukemia cells. In L1210 cells it is at least 200 times less potent in producing single-strand breaks as revealed by alkaline elution studies (Zwelling et al., 1981). This lack of antitumor activity of oAMSA appears to derive from the presence of the methoxy group specifically at the ortho position, where it may be involved in a hydrogen bonding with the $-NHSO_2CH_3$ group that may affect its interaction with Cu(II) ions. To what extent these variations with respect to interactions with Cu(II) and in vitro DNA cutting can account for changes in biological activity will only be clarified when additional analogues are studied. We have extended our drug/in vitro DNA breakage studies to other acridine analogues (unpublished observations). Acridine and 9-aminoacridine, which are not cytotoxic, were tested. Results showed that both drugs, either in the presence or in the absence of Cu(II), were ineffective in inducing DNA breakage in the pDPT275 DNA system. Thus, it is conceivable that this difference in the DNA cutting potencies is at least partially responsible for the lack of antitumor activities of oAMSA, acridine, and 9-aminoacridine.

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Registry No. mAMSA, 51264-14-3; oAMSA, 51264-17-6; copper, 7440-50-8.

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