Selective Activation of Mitomycin A by Thiols To Form DNA Cross-links and Monoadducts: Biochemical Basis for the Modulation of Mitomycin Cytotoxicity by the Quinone Redox Potential

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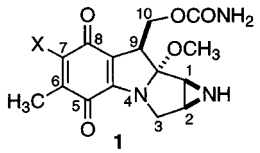
Mitomycin A (MA) but not mitomycin C (MC) cross-linked linearized ^{32}P-pBR322 DNA in the presence of dithiothreitol (DTT) or glutathione (GSH), as shown by a sensitive DNA cross-link assay. Incubation of calf-thymus DNA with MA and DTT or mercaptoethanol (MER) resulted in the formation of MA−DNA adducts, which were isolated from nuclease digests of the drug−DNA complexes by HPLC. The adducts were characterized by their UV absorption spectra, electrospray ionization mass spectrometry (ESIMS), and facile conversion from 7-methoxy- to 7-amino-substituted mitosene type adducts upon 10% NH_4OH treatment, which were identical with known adducts of MC. Both DNA interstrand and intrastrand cross-link adducts, linking two deoxyguanosine residues at N^2, as well as several deoxyguanosine-N^2 monoadducts of MA, were identified. No DNA adducts were formed with MC under the same conditions. A specificity of DNA cross-link formation for the CpG sequence was observed using 12-mer synthetic oligodeoxyribonucleotides as substrates and as DNA sequence models, in analogy to the known CpG sequence specificity of MC-induced DNA cross-links. MA is known to be more cytotoxic by 2−3 orders of magnitude than MC, and this property correlates with redox potentials of MA (−0.19 V) and MA analogues that are higher than those of MC (−0.40 V) and its analogues. It is suggested that the biochemical basis for the higher cytotoxic potency of MA is MA's propensity to be reductively activated by cellular thiols while MC is resistant to thiol activation. This distinction is probably derived from the large difference between the quinone redox potentials of the two drugs.

The mitomycin antitumor antibiotics are distinguished by their ability to cross-link the complementary strands of DNA. This property is generally regarded as the intrinsic cause of the potent cytotoxic activity of the mitomycins.[1] Two other natural antibiotics, carzinophilin (azinomycin)[2] and bioxalomycin,[3] were recently shown to cross-link DNA, although this property was demonstrated so far only in purified cell-free systems in both cases. Another distinct property of the mitomycins is that reductive activation is required to unmask their alkylating and cross-linking activities; hence, mitomycin C (MC;[4] **1a**, Chart 1) is regarded as the prototype of bioreductive antitumor drugs.[5] MC has been in use for clinical anticancer therapy in the U.S. since 1974. Mitomycin A (MA; **1b**), which differs from MC only in its 7-substituted group (7-methoxy instead of 7-amino), exhibited high toxicity in experimental animals, which prevented its clinical use. Furthermore, MA is several orders of magnitude more cytotoxic to tumor cells than MC.[6,7] Extensive SAR studies established that mitomycins with 7-alkoxy substituents had higher redox potentials than 7-nitrogen-substituted mitomycins (e.g., $E_{1/2}$ of MA and MC are −0.19 and −0.40 V, respectively) and that a strong correlation existed between $E_{1/2}$ and the activity of these mitomycins.[8,9]

Chart 1



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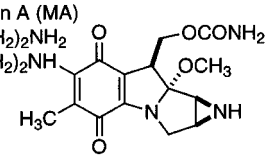
1a X = NH_2; mitomycin C (MC)
1b X = CH_3O; mitomycin A (MA)
1c X = $NH(CH_2)_2SS(CH_2)_2NH_2$
1d X = $NH(CH_2)_2SS(CH_2)_2NH$



1e X = $NH(CH_2)_2SS$–C$_6$H$_4$–NO_2

Reductive activation of MC as prodrug is catalyzed by several different flavoreductases in mammalian cells.[5] As a result of extensive studies utilizing in vitro chemical and enzymatic reduction systems, the chemical mechanism of the "mitomycin activation cascade" has been elucidated.[10a−d,11−13] The activation cascade (Scheme 1) is triggered by reduction of the quinone of the mitomycin (**1a**,**b**) to semiquinone or hydroquinone.[10b,c] The semiquinone is further reduced to the latter (**2**) by disproportionation.[11] Elimination of methanol to give the reduced mitosene[12] **3**[10b,c] followed by hydroquinone-assisted ring opening (**4**)[13,14] completes the activation process. The active form **4** can alkylate small-molecule nucleophiles or DNA, and it also tautomerizes to the

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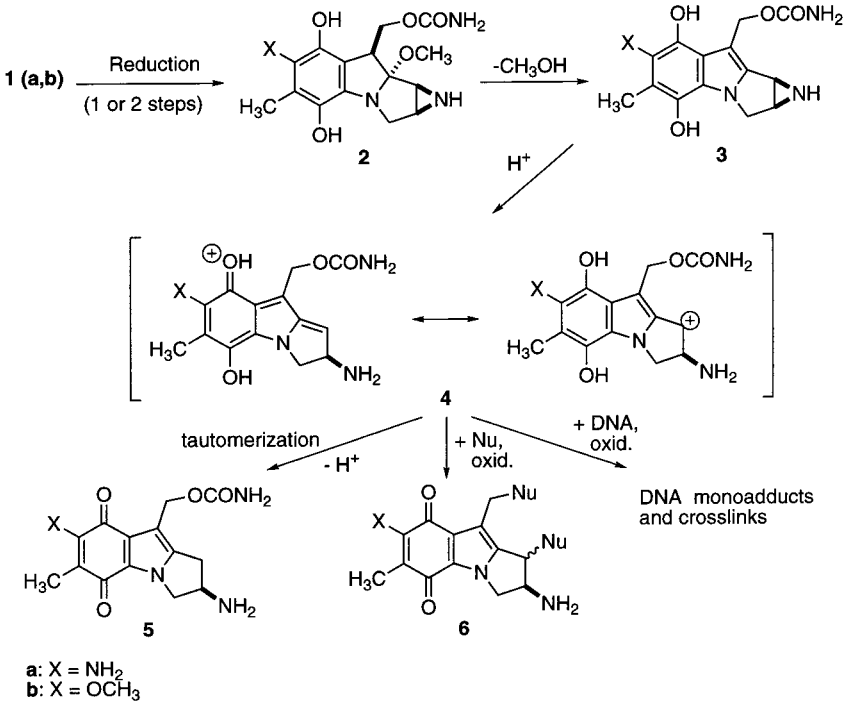
Scheme 1. Reductive Activation Cascade of Mitomycins



1-dihydromitosene **5** as an end-product.[13] The latter is formed as a major metabolite of MC in tumor cells and tissues.[15] As chemical reducing agents, usually in catalytic hydrogenation, $NaBH_4$ or $Na_2S_2O_4$ has been employed in the above model studies. Thiols do not reduce MC. However, they react as nucleophiles with *reduced* MC, generated by other agents.[16a,b]

In contrast to MC, several natural cytotoxins, notably the enediynes neocarzinostatin, calicheamycin,[17a] and dynemicin,[17b] as well as leinamycin,[17c] are known to be activated in their prodrug forms by thiols, and these processes are thought to be important in vivo. The antitumor antibiotics FR66979 and FR900480 are also activated reductively by thiols. The presence of a catalytic amount of ferrous salt is required for this activation.[17d] Thiols are also highly reactive with quinones. In the latter reactions in general, Michael addition generates a thioether-substituted hydroquinone.[18] However, thiols are inert toward the quinone in MC, and this has been attributed to the fact that tetrasubstituted quinones cannot tautomerize to the hydroquinone, following Michael addition.[19] Surprisingly, MA (**1b**), which differs from MC only in its 7-substituent (methoxy instead of amino), was recently found to react rapidly with thiols, resulting in *reduction* of the MA quinone system by a novel mechanism.[20]

We report here that the reaction of MA with thiols represents activation of the antibiotic to alkylate and cross-link DNA. This was evidenced by the isolation and characterization of inter- and intrastrand DNA cross-links and monofunctional minor groove adducts that were structurally identical with those generated under conventional reductive activation of MA or MC by flavoreductases, H_2/PtO_2, or $Na_2S_2O_4$. The dramatic difference between MC and MA in their susceptibility to activation by thiols to DNA-damaging species provides now a biochemical rationale for the greater toxicity and antitumor potency of MA relative to that of MC, shown by the earlier SAR studies.

Materials and Methods

Mitomycin C and mitomycin A were received from Kyowa Hokko Kogyo Co., Ltd., Tokyo, Japan. Other materials and their sources were as follows: EcoRI, pBR322, and Klenow fragment of DNA polymerase I (exo-), New England Biolabs; ^{32}P-labeled nucleotide triphosphate, NEN; agarose (DNA grade) and HPLC solvents, Fisher Scientific; P1 nuclease, calf thymus DNA, and NADPH−cytochrome *c* reductase, Sigma; DNase I (code D), phosphodiesterase I, and bacterial alkaline phosphatase, Worthington. UV spectra were recorded using a Beckman System Gold model 168 diode array UV−visible detector. Absorbance readings were performed in a Gilford 250 spectrophotometer. LC−ESIMS (electrospray ionization mass spectrometry, positive ion mode) was performed with a Hewlett-Packard series 1100 diode array HPLC system connected to a Hewlett-Packard series 1100 mass selective detector (MSD) mass spectrometer. Conditions for the HPLC were the following: Rainin C-18 Microsorb, 5 mm × 250 mm column; buffer system, solvent A was 10 mM TEAA, pH 6.8, solvent B was a mixture containing 60% CH_3CN and 40% buffer A; linear gradient from 10% B to 60% B in 25 min, flow rate of 1 mL/min. HPLC for product isolation was performed using a Beckman System Gold 125 instrument equipped with a diode array detector System Gold 168, fitted with a Rainin C-18, 5 mm × 250 mm column. Agarose gels were run on a DNA subcell apparatus (Bio-Rad). Agarose gels were prepared by dissolving 1 g of agarose in 100 mL of buffer containing 40 mM Tris-AcOH, 2 mM EDTA, pH 8.2. After electrophoresis, gels were transferred onto sequencing filter paper (Biorad), covered with Saran Wrap, and dried at 80 °C in a Biorad model 583 gel dryer. Phosphorimagery was performed with a storage phosphor screen (Applied Biosystems), using ImageQuant software (Applied Biosystems). Ethanol precipitations were performed by admixing the aqueous solution of DNA with 0.1 volumes of 3 M NaOAc, pH 5.0, and 7 volumes of cold ethanol followed by centrifugation (12 000 rpm, 4 °C, 15 min). The supernatant was removed, and the pellet was dried in a speed-vac. Calf thymus DNA was sonicated and dialyzed before use.

Assay of Cross-Linking ^{32}P-pBR322 DNA with DTT- or GSH-Activated Mitomycins. The standard assay of DNA cross-linking, developed by Hartley et al.,[21] was applied. A series of test mixtures were prepared containing 10 ng of ^{32}P-pBR322 DNA, 1 mg of calf thymus DNA, 80 μM of DTT, and 0−80 μM MA or MC in 25 μL of 20 mM Tris-HCl, 14 mM

EDTA, pH 7.4 buffer. The mixtures were incubated for 1 h at room temperature under aerobic conditions. An analogous series, using 4 mM GSH instead of 80 μM DTT, was also prepared. The DNA was precipitated by ethanol, dissolved in strand separation buffer (30% DMSO, 1 mM EDTA, pH 9, 0.25% xylene cyanol, 0.25% bromophenol blue), heated at 90 °C for 5 min, then immediately ice-cooled. Samples were loaded on a 1% agarose gel and run at 40 V for 16 h. The gels were dried, and the bands were visualized and quantified by phosphorimagery.

Reaction of MA with Calf Thymus DNA in the Presence of MER as Activator. A solution of sonicated calf thymus DNA, 1 mM MA, and 100 mM MER in 0.1 M Tris-HCl, pH 7.5, containing 5% methanol (total volume 1 mL) was incubated at room temperature for 2 h under aerobic conditions. The DNA was precipitated with cold ethanol.

Reaction of MA with Calf Thymus DNA, Using DTT as Activator. A solution of 3 mM DNA, 1 mM MA, and 1 mM DTT in 0.1 M Tris-HCl, pH 5.0, was incubated for 2 h at room temperature under aerobic conditions. The same reaction was also conducted at pH 7, in 0.1 M Tris-HCl, pH 7.4, as reaction medium.

Isolation of MA−DNA Adducts from the above Reaction Mixtures after Enzymatic Digestion of MA−DNA Complex. The covalent complex formed in the above reactions complex was precipitated with cold ethanol. The pellet was dissolved in 2 mL of 10 mM NH_4OAc, pH 5.0, and 0.05 mL of this solution was subjected to enzymatic digestion with P1 nuclease (1 unit) for 3 h at 37 °C. After this period the pH of the solution was increased by the addition of 0.06 mL of 0.5 M Tris-HCl and 5 mM $MgCl_2$, pH 8.3, then 3 units of phosphodiesterase I and 5 units of bacterial alkaline phophatase were added, and the mixture was incubated for 5 h at 37 °C. The digest containing the MA−DNA adducts was chromatographed by HPLC, using a Rainin C-18 reversed phase column (5 mm × 250 mm). For elution, solvent A was 10 mM TEAA, pH 6.8, and solvent B was a mixture containing 60% CH_3CN and 40% solvent A. Linear gradient from 10% B to 60% B in 25 min was employed at a flow rate of 10 mL/min. The eluate was monitored for absorbance at 254 and 340 nm; appropriate fractions were collected for further characterization.

Cross-linking of 12-mer Self-Complementary Oligodeoxyribonucleotides 13 and 14 by MA, Activated by MER. A total of 5 OD_{260} units of **13** or **14** (0.04 μmol of guanine nucleotide) was annealed in 0.1 M Tris-HCl, pH 7.4, by cooling from 55 to 4 °C in 2 h. MA (0.25 μmol) and MER (2.5 μmol) were added, and the mixture was incubated overnight under aerobic conditions. Control reactions containing MC instead of MA were also incubated. The mixtures were chromatographed over a Sephadex G-50 column (2.5 cm × 56 cm) heated at 40 °C, using 0.02 M NH_4HCO_3 as eluant and collecting approximately 126 mL fractions.

Cross-linking of 13 and 14 by MC and MA, Activated by $Na_2S_2O_4$ under Anaerobic Conditions. The cross-linking was carried out using previously published procedures.[22,23]

Conversion of MA−DNA Adducts to MC−DNA Adducts. This was accomplished by incubation of MA−DNA adducts as isolated from HPLC in 10% aqueous NH_4OH for 3 h at room temperature. The resulting product was directly analyzed by HPLC.

Results

Cross-linking of Linear ^{32}P-pBR322 DNA. Figure 1 illustrates that thiols promote cross-linking of DNA by MA but not by MC. This was shown by employing a sensitive assay developed by Hartley et al.[21] Both DTT and GSH were effective in the case of MA. The gel of the DTT reactions illustrates that MC was completely inert to activation by DTT. The quantitative plots (Figure 1b) show that DTT was more effective than GSH in activating MA to cross-link. Neither DTT nor GSH activated MC to cross-link DNA.

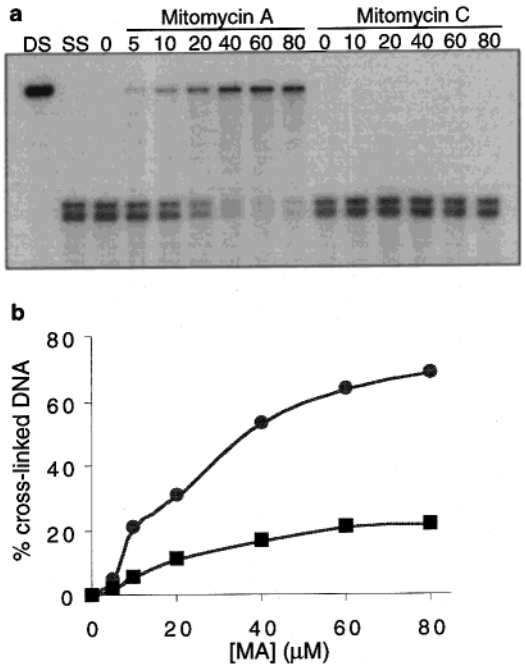
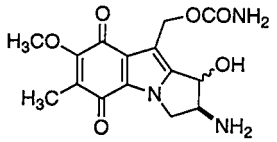


Figure 1. Assay of ^{32}P-pBR322 DNA cross-linking by MC and MA under activation by thiols. (a) Autoradiogram of agarose gels: activator, 80 μM DTT; lane 1, control linear ^{32}P-pBR322 DNA; lane 2, control linear ^{32}P-pBR322 DNA, heat denatured; lanes 3−8, heat-denatured reaction mixtures containing 0.5, 10, 20, 40, 60, and 80 μM MA, respectively; lanes 9−15, same as lanes 3−8 except the drug is MC. (b) Plot of % cross-linking of ^{32}P-pBR322 DNA as a function of MA concentration, measured by densitometry of the corresponding gel autoradiogram (●, MA/DTT; ■, MA/GSH).

Formation of MA−DNA Adducts in the Reaction of MA and Calf Thymus DNA under Activation with MER. HPLC of the digested MA−DNA complex showed, in addition to the four unmodified nucleosides, the presence of four major UV-absorbing products marked as **10b**, **9b**, **12**, and **11** with increasing elution times (Figure 2a).

Structural Identification of DNA Adducts from MER Activation Reaction. UV Spectra. The 7-alkoxy mitosene chromophore, as in *apo*-mitomycin A, is characterized by absorption maxima at 232, 285, and 340−360 nm,[24] as shown in Figure 3. Each of the four UV peaks marked **10b**, **9b**, **12**, and **11** in Figure 2a displayed UV spectra that indicated the presence of this chromophore; in addition, the strong absorbance in the 250 nm region was suggestive of the deoxyguanosine chromophore(s). The UV spectra of **10b** and **9b** are shown in Figure 3. LC−ESIMS (positive ion mode) (Figure 4) indicated that all four products are 1:1 or 2:1 adducts of deoxyguanosine and MA. Each displayed clearly the mass of a protonated molecule (MH^+), and the presence of the additional $[M]Na^+$, $[M]K^+$, and $[M]Et_3NH^+$ species in each spectrum unambiguously



apo-mitomycin A

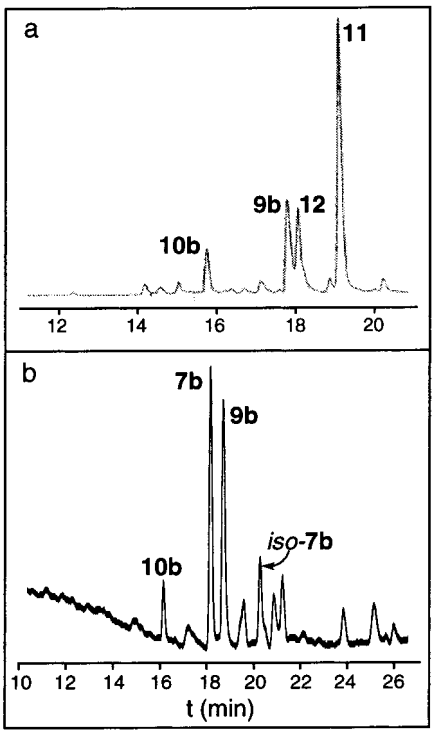


Figure 2. HPLC of digests of calf thymus DNA−MA complexes: (a) complex formed under activation by MER; (b) complex formed under activation by DTT. For HPLC conditions, see Materials and Methods.

defined MH^+. The molecular mass of peaks in the order of increasing elution time corresponded to the calculated mass for adducts **10b** ($M = 851$; DNA intrastrand cross-link), **9b** ($M = 790$; DNA interstrand cross-link), **11** ($M = 601$), and **12** ($M = 646$), respectively (Chart 2). It is to be noted that in the formulas of **11** and **12** the parentheses around the MER moieties signify that either the nucleophile S or O may be linked to the mitomycin. The UV and MS data are insufficient to specify the particular isomer.

Unambiguous Structural Identification of the Interstrand (9b) and Intrastrand (10b) DNA Cross-link Adducts of MA by Their Conversion to Authentic Cross-link Adducts of MC (9a and 10a, Respectively) by 10% NH_4OH. Figure 5 illustrates that the putative MA adducts **10b** and **9b** in the HPLC in Figure 2a were quantitatively converted to authentic MC adducts **10a**[25] and **9a**,[26] respectively, after 3 h of incubation in 10% NH_4OH. This is shown by HPLC elution time comparisons (Figure 5a−d). Furthermore, the UV spectra of the authentic MC adducts and the converted MC adducts were also identical. Parts b and c of Figure 3 show the UV spectra of the interstrand cross-link adduct **9b** before and after its conversion to **9a**, and intrastrand cross-link adduct **10b** before and after its conversion to **10a**, respectively, in 10% ammonia.

Formation of MA−DNA Adducts in the Reaction of MA with Calf Thymus DNA under Activation by DTT. HPLC of digested MA−DNA complex showed, in addition to four unmodified nucleosides, the presence of 10 sharp peaks (Figure 2b). Among them, the intrastrand and interstrand cross-links **10b** and **9b** were identified by comparison of their elution times with **10b**

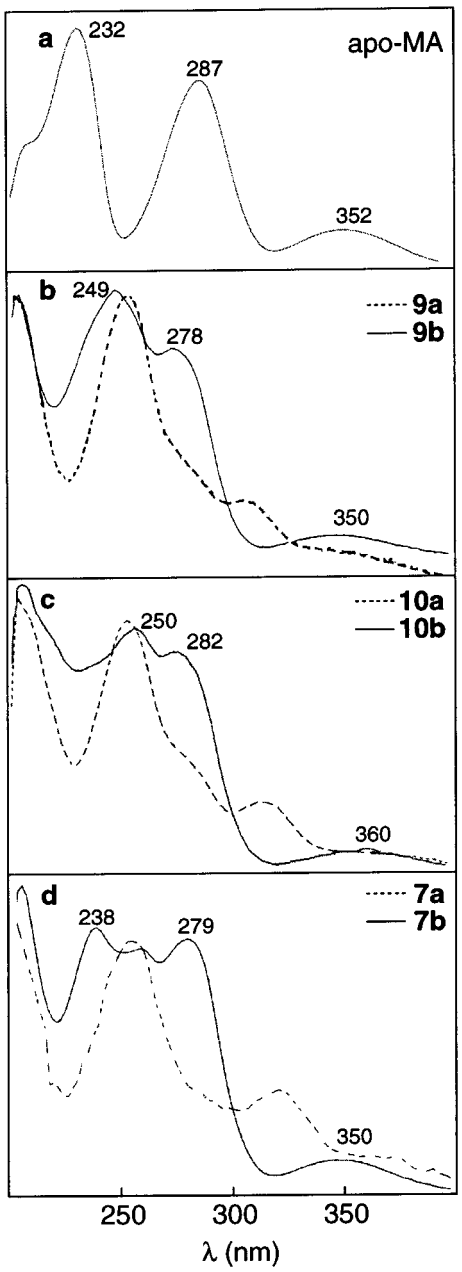


Figure 3. UV spectra of DNA adducts of MA and MC. For conditions, see Materials and Methods.

and **9b** isolated and elucidated from the MER-activated MA−DNA reaction above (Figure 2a). A major adduct peak at 18 min, not observed in the MER-activated reaction, was rigorously characterized as the MA−dG monoadduct **7b** by its UV spectrum and its conversion by 10% NH_4OH to authentic **7a**, a major DNA adduct[27] formed with MC under conventional reductive activation conditions. This conversion is illustrated by a shift of the elution time of the 18 min peak to 13 min elution time, coeluting with authentic **7a**, (parts e and f of Figure 5), and by identity of the UV spectra of the converted product and authentic **7a** (Figure 3d). The peak marked iso-**7b** is probably the 1″-β isomer of **7b**, since its UV spectrum is identical to that of **7b**. The formation of the minor 1″-β isomer of **7a** occurs also with MC.[27] Additional peaks were not identified, although the UV spectra of most of them indicated the presence of the guanine chromophore (see above). Thus, they may

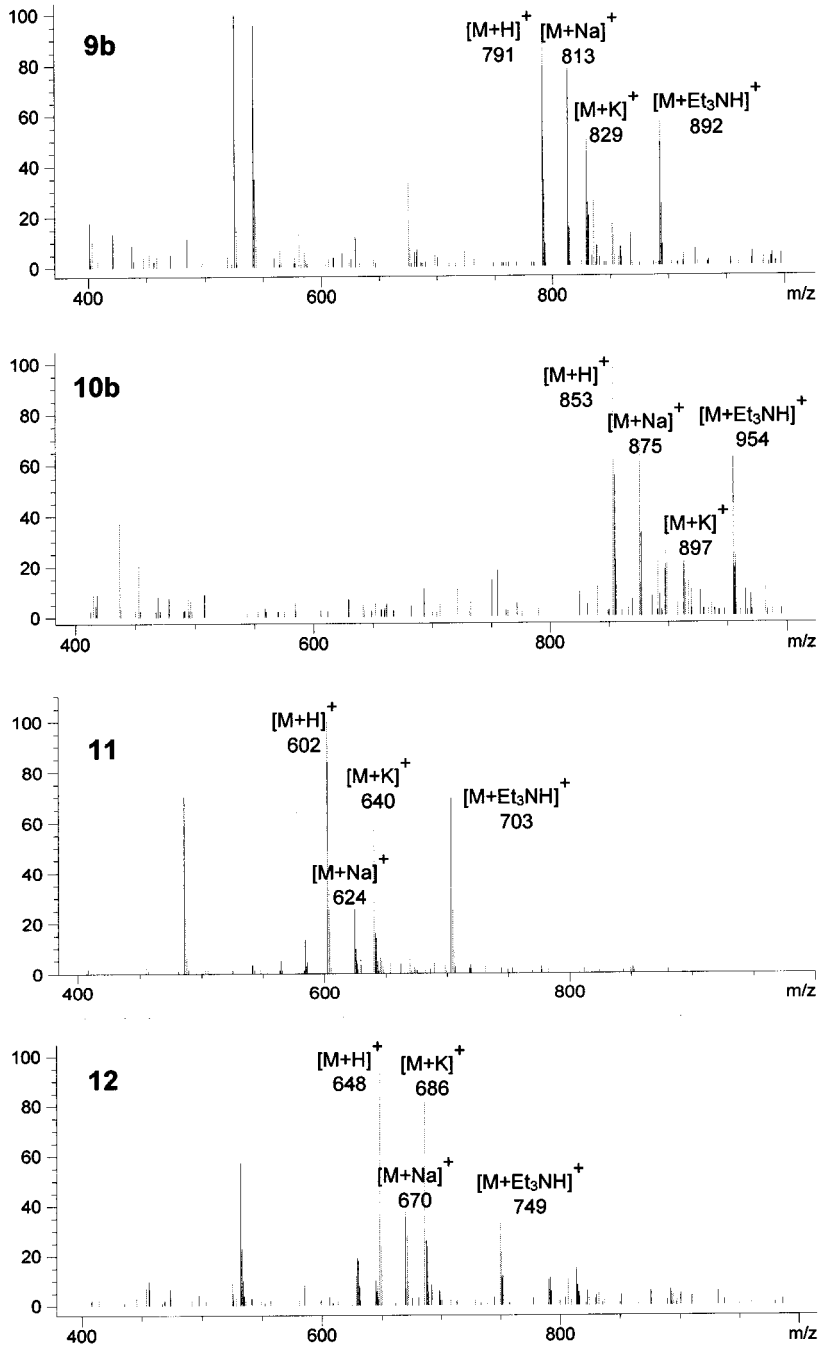


Figure 4. LC−ESIMS spectra of DNA adducts of MA. Adducts are, from top panel down, **9b**, **10b**, **11**, and **12**.

be dG adducts substituted by DTT in electrophilic mitosene positions, in analogy to the MER adducts **11** and **12** (Chart 2).

Specificity of Cross-linking by MA to the CpG Sequence in 12-mer Oligodeoxyribonucleotides. Oligonucleotide **13** (Chart 3), containing a single duplex CpG sequence, was cross-linked by MA under both anaerobic $Na_2S_2O_4$ and aerobic MER activation, yielding 28% and 6% cross-linked 12-mer duplex, respectively, as seen by a Sephadex G-50 gel chromatography assay[22] (upper curves of parts a and b of Figure 6). In contrast, oligonucleotide **14** (Chart 3), containing a single duplex GpC sequence, was not cross-linked (lower curves of parts a and b of Figure 6). MC showed analogous specificity of cross-linking of **13** and **14** under $Na_2S_2O_4$ activation,[28] but it did not cross-link either **13** or **14** when MER was used for activation (data not shown). The cross-linked oligonucleotide fraction from the MER-activated reaction (shaded peak; Figure 6b, upper curve) was digested to nucleosides, and the digest was chromatographed by HPLC. A single adduct was detected, eluting at 49 min (Figure 7a). When the digest was treated with 10% NH_4OH, this adduct was converted to **9a**, indicating that the original single adduct of the cross-linked oligonucleotide was **9b** (Figure 7b).

Discussion

Over 1000 analogues of MC have been made in the past in efforts to prepare analogues with improved therapeutic properties.[29] Most of this work centered on

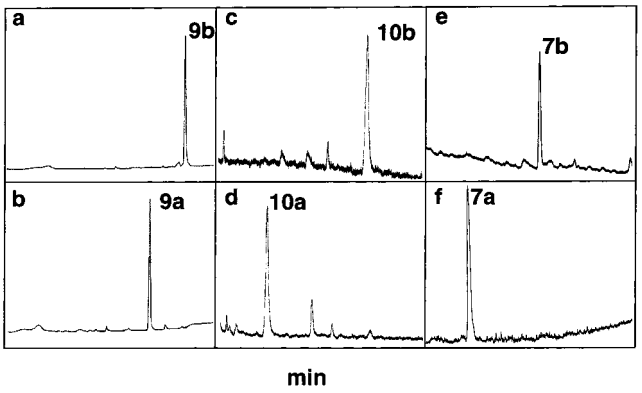
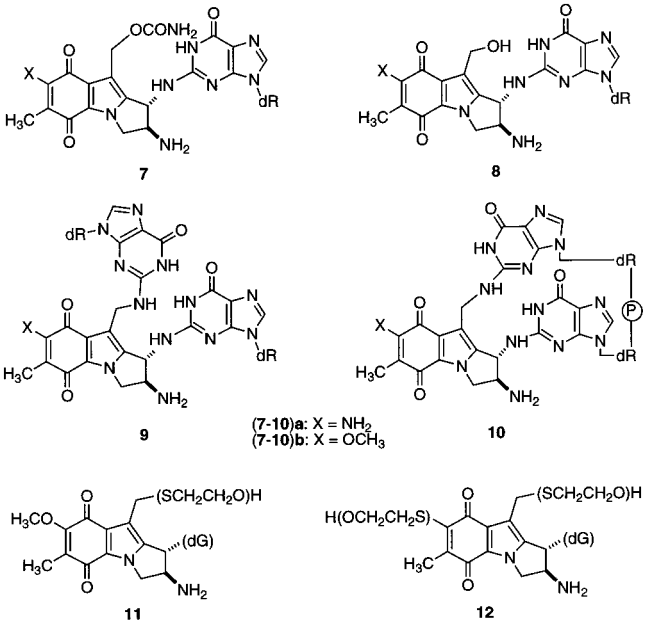


Figure 5. Conversion of MA−deoxyguanosine adducts to MC−deoxyguanosine adducts by treatment with 10% aqueous NH_4OH (demonstration by HPLC). MA adducts (a) **9b**, (c) **10b**, and (e) **7b** were collected from HPLC of digests of MA−DNA complexes. The above MA adducts were treated with 10% NH_4-OH and admixed with standard MC adducts (b) **9a**, (d) **10a**, and (e) **7a**.

Chart 2



varying the mitosane[12] 7-substituent. This could be achieved by facile displacement of the 7-methoxy group of MA by nitrogen nucleophiles, yielding MC analogues, or by alcohols and phenols under base catalysis, yielding MA derivatives. A major conclusion from the SAR studies was that any MA derivative had a highly significant probability of having greater antitumor potency than its MC equivalent.[6] Typically, MA derivatives were 2 orders of magnitude more potent than the corresponding MC derivatives.[8] Furthermore, a significant correlation was observed between in vitro cardiotoxicity and redox potential, MA being 3 orders of magnitude more cardiotoxic than MC to rat heart cells.[7] From their incisive QSAR studies Remers and coworkers concluded that the limiting factors in the antitumor potency of the mitomycins appear to be tumor cell uptake as measured by the lipophilicity factor log *P* and the "ease" of intracellular bioreductive activation as measured by the redox potential $E_{1/2}$. All MA analogues had higher redox potential and thus greater reducibility than their corresponding MC derivatives.[8]

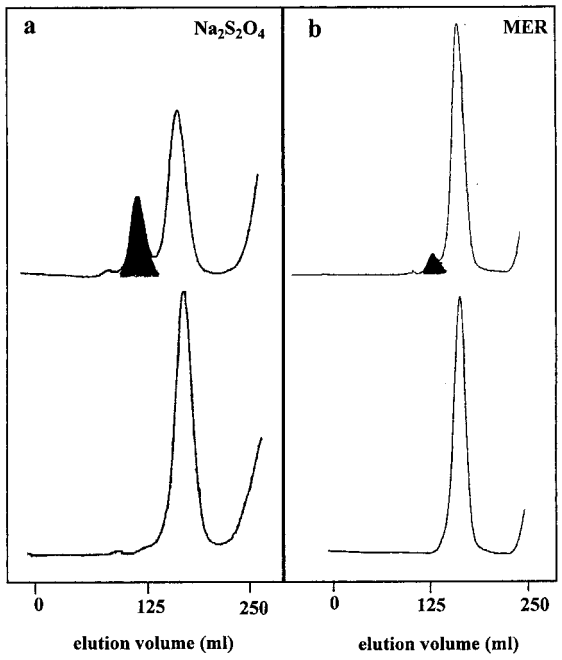


Figure 6. Assay of CpG specificity of cross-linking of 12-mer oligonucleotides by MA using Sephadex G-50 gel chromatography: (a) reaction mixture of MA and **13** (upper curve) and MA and **14** (lower curve), both activated by $Na_2S_2O_4$; (b) reaction mixture of MA and **13** (upper curve) and MA and **14** (lower curve), both activated by MER. The shaded peaks indicate the elution of cross-linked oligonucleotides. For conditions, see Materials and Methods.

Chart 3

5'-ATATACGTATAT	5'-ATATAGCTATAT
13	**14**

Similar relationships were observed by Pan and Gonzalez.[9] The biochemical basis for these variations in activity between the two closely related antibiotics and their analogues is unknown. In vitro, enzymatically or chemically reduced MA alkylates cross-link DNA similarly to MC, forming analogous adducts in similar yields.[23] However, the presence of air in such in vitro model systems inhibited MA adduct formation to a lesser extent than formation of MC adducts, and it was speculated that in vivo O_2 is less inhibitory to the activation of MA than to that of MC, which may explain the more potent cytotoxicity of MA.[23] As another potential factor, enzymatic reduction of MA was found to be faster than that of MC.[9] Although both in vitro phenomena appear to be related to the higher $E_{1/2}$ of MA, their relevance to the biological activities of MA and MC in vivo remains speculative.

A more compelling mechanism is uncovered by the present study, which is based on an *absolute* difference between the two drugs: MA is activated by thiols and MC is not. The origin of this work lies in an earlier report by Kono et al.[30] of the complex reaction of MA with cysteamine. Two products were isolated (**1c** and **1d**), each of which was substituted by the amino group of *oxidized* cysteamine (i.e., cystamine). The rest of the original MA was converted to a mixture of mitosenes. An unstable intermediate had the UV properties of leucomitomycin A (**3b**). It was proposed that the thiol function of cysteamine reduced the quinone **1b**, generating the disulfide cystamine, which then displaced the

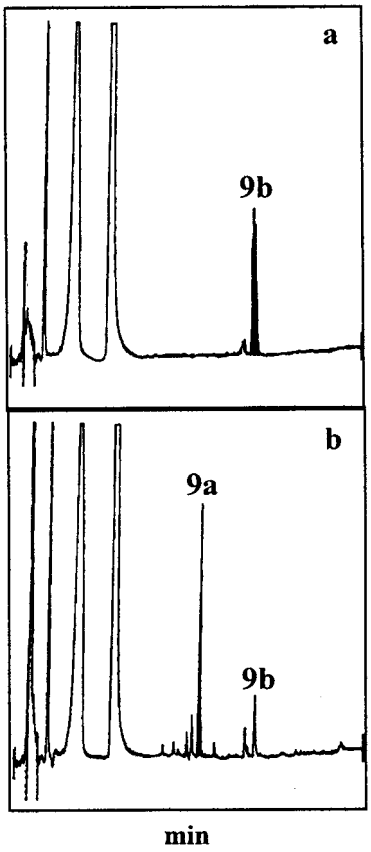
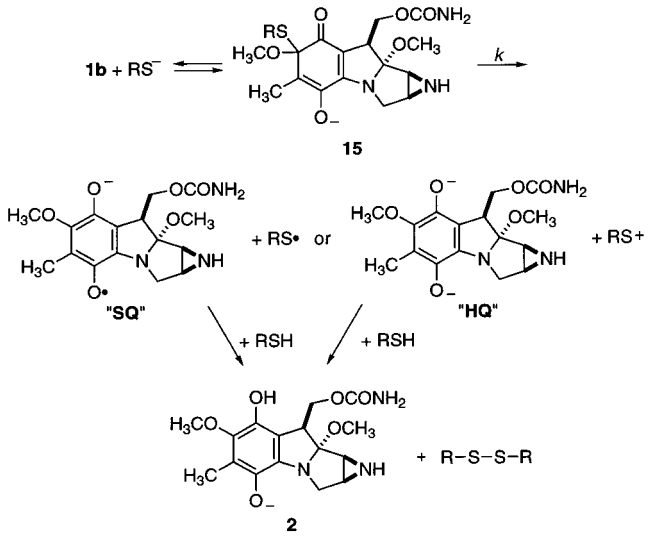


Figure 7. HPLC results demonstrating conversion of the MA adduct (**9b**) of the oligonucleotide **13** to MC adduct (**9a**) by treating the nuclease digest of the cross-linked oligonucleotide with 10% NH_4OH. For conditions, see Materials and Methods.

7-methoxy group of the remaining **1b** by its amino group(s) to give **1c** and **1d**. Recently, we showed conclusively that MA is reduced by thiols from the quinone to the hydroquinone level, as evidenced by the isolation of 1-dihydromitosene derivatives (**5b**) and a number of 1,10-disubstituted mitosene derivatives (**6**)[31] formed upon incubation of MA with simple thiols. The mechanism of the reduction of the MA quinone by thiols was proposed[20] to involve reversible addition of RS− to the quinone (**15**), which then undergoes a rate-determining intramolecular redox reaction (*k*) to give ultimately **2** and R−S−S−R (Scheme 2). The thiol-mediated reduction of the MA quinone represents an unusual reaction mechanism.[20] Typically, benzoquinone and mono-, di-, and trisubstituted quinones undergo irreversible Michael addition by thiols, resulting in thioether-substituted hydroquinones, which, however, do not constitute reduction products of the quinone.[18] Tetrasubstituted quinones, which cannot give stable (hydroquinone) Michael adducts, have been reported to be unreactive toward thiols.[32] Complex possible precedents for the reduction mechanism of MA (a tetrasubstituted quinone) are the reduction of adriamycin by GSH[33] and the reductive conversion of gonyautoxins into saxotoxins by thiols.[34]

The reaction of MA is also in sharp contrast to the facile displacement of the 7-methoxy substituent of MA by nitrogen nucleophiles or alkoxide ions.[35a,b] Thiols, however, do not appear to displace the 7-OCH_3. No 7-sulfur-substituted mitomycin derivatives have ever

Scheme 2. Proposed Mechanism of Reduction of MA by RSH[18]



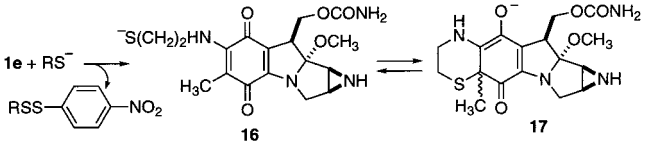
been isolated from reactions of MA. The failure to observe such reaction products with the bifunctional nucleophile cysteamine and MA, described above, is an example.[30] Lack of displacement of the 7-methoxy group by sulfhydryl is, indeed, as expected, considering that the 5-carbonyl of MA has properties of a vinylogous methylester:[35c] Formation of thioesters from esters and thiols is thermodynamically unfavorable.[36] In contrast, reactions of MA with amines to form vinylogous amides, with alcohols in transesterification, or with water in hydrolysis occur readily.[35c]

Conclusions

The present work relates the thiol-mediated reduction mechanism (Scheme 2)[20] to the biologically relevant endpoints of mitomycin transformation: formation of DNA adducts and cross-linking of DNA. We isolated the major monofunctional and bifunctional deoxyguanosine−MA adducts **7b**, **9b**, and **10b** (Chart 2) and demonstrated sequence-specific cross-linking of 12-mer oligodeoxyribonucleotides between guanines at the 5′-CG sequence, all of which are identical to the DNA lesions, observed under activation of mitomycin A by flavoreductases and chemical reducing agents.[23] They are also analogous to the MC−DNA adducts and are formed with identical sequence specificity.[28] This proves that in the case of MA, thiols induce the same mitomycin activation cascade, leading to the same lethal DNA lesions as those occurring in the case of MC. A significant feature of these findings is that the 7-amino-substituted mitomycin, MC, is not activated by thiols.[16a,20,19] This virtually absolute chemical discrimination appears to be due to the lower electrophilicity of the C_5 and C_8 quinone carbonyls of MC, both being vinylogous amides associated with a lower reduction potential, than that of MA ($E_{1/2} = -0.40$ and -0.19 V, respectively).[37]

Significance. The contrasting, efficient activation of MA by thiols offers a compelling biochemical basis for the previously observed structure−activity relationship between MC and MA in particular and between MC analogues and MA analogues in general.[8] Thus, we propose that the observed high potency and toxicity of

Scheme 3. Formation of Intramolecular Thiol Adducts from **1e**



MA relative to MC is related to its activation by intracellular thiols. GSH, present in up to 5 mM concentration in mammalian cells,[38] could generate activated MA that, together with enzymatically activated MA, would exceed the concentration of activated MC, which arises only from enzymatic activation. The higher level of activated MA formed in the cell under equivalent drug dose conditions may lead to additional toxic lesions. Whether these are manifested by enhanced cross-linking and alkylation of DNA as shown here or of other cellular nucleophiles, or some other reactions of reduced MA, e.g., enhanced redox cycling, is not defined by the present work. A striking precedent already exists for correlation of thiol-mediated drug activation in vitro and antitumor activity in a mitomycin analogue series.[30,19] The series features the structural unit $-S-S-(CH_2)_n-NH$ ($n \geq 2$) as the 7-substituent of mitosanes, as in **1d** and **1e**, for example. Only the $n = 2$ analogues were activated by thiols to cross-link DNA in vitro,[19] and these $n = 2$ analogues were 2−3 orders of magnitude more cytotoxic than any of the $n > 2$ analogues or MC itself.[30] This selectivity is attributed to the formation of a six-membered cyclic intermediate, which facilitates the intramolecular reduction of the quinone **17** (Scheme 3).[19,39]

On the basis of our results, it is predicted that tumor cells with elevated GSH concentration are more sensitive to MA than those with normal levels of GSH. Since tumor resistance to chemotherapeutic drugs is often associated with elevated GSH, it would be of interest to test this prediction in tumor cell cultures derived from such tumors. Thiol-activated antitumor agents in general may overcome tumor resistance to chemotherapeutic drugs that are detoxified by glutathione conjugation in the cell.

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(4) Abbreviations: DTT, dithiothreitol; GSH, glutathione; MC, mitomycin C; MA, mitomycin A; MER, mercaptoethanol; TEAA, triethylammonium acetate.

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