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Interaction of 2-Deamino- and 2-Deamino-2-nitroactinomycin D with Calf-Thymus DNA and Formation of Ion-Radicals

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2-Deaminoactinomycin D (**3a**) and 2-deamino-2-nitroactinomycin D (**2a**) were prepared in one step from actinomycin D (**1a**, AMD) by reaction with nitrous acid. New DNA-binding (calf-thymus) data obtained by difference uv and CD spectra and ΔTm were presented. *In vitro* cell growth inhibitory activity of CCRF-CEM cells was also reported. The 2-deamino analog, **3a**, does not bind to DNA strongly nor by intercalation of its chromophore. However, some binding with DNA was indicated by CD which is attributed only to hydrogen bondings of the peptides with the DNA helix; the affinity for binding is in the order **1a** $\gg$ **2a** $>$ **3a**. The 2-nitro analog, **2a**, is a more potent agent against CCRF-CEM cells than the 2-deaminoactinomycin D, **3a**; the potencies are in the order **1a** $>$ **2a** $\gg$ **3a**. Furthermore, the microsomes activate the analogs to free radical states which catalyze the production of superoxide, as indicated by electron paramagnetic resonance studies and oxygen consumption experiments.

INTRODUCTION

The naturally occurring antibiotic actinomycin D (AMD, **1a**) (Figs. 1 and 2), whose potent activity in the treatment of Wilms' tumor (*1*) and gestational choriocarcinoma (*2*) is well known in the clinic, has been used extensively as a molecular probe in studies related to RNA metabolism due to its specific inhibition of RNA synthesis (*3–5*). The mechanism of action of AMD derives from its ability to intercalate with double-stranded DNA, and the overall result of this interaction is the inhibition of DNA-dependent RNA synthesis (*6, 7*). Previous work stressed the importance of the intact peptide lactones, and the 2-amino, 3-oxo, and 4- and 6-methyl groups of the phenoxazinone ring as being necessary for its biological activity (*8*). Work from our laboratories has shown that the actinomycin molecule can be altered at the N^2 site without adversely affecting its DNA-binding and antitumor properties (*9–11, 16, 17*), and that a free amino group at C-2 is not crucial for the antitumor activity of AMD. In some cases N^2-amine-modified analogs acted even as superior antitumor agents especially when they were activated *in vivo* (*12–15*). A similar trend has been observed in certain N^2 spin-labeled analogs of AMD which are prone to internal bioactivation to free radical species.

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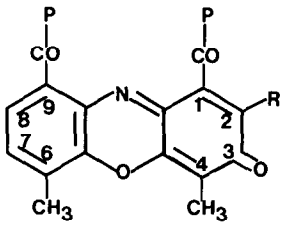

a, P= -Thr-D-Val-Pro-Sar-MeVal-
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b, P= $-N(C_2H_5)_2$

	R
1	$-NH_2$
2	$-NO_2$
3	-H

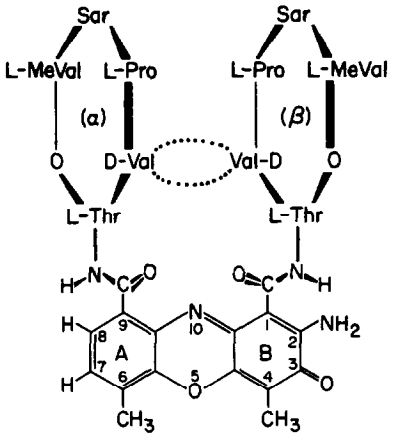
FIG. 1. Structures of actinomycin D and its C-2-substituted analogs.

These analogs have shown marked activity against P388 tumor in mice in spite of the reduction of their binding affinity for DNA (*12, 13*).

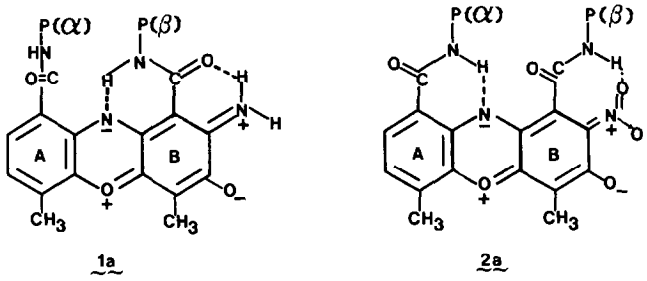
In addition to the antibiotic's action of binding to DNA and inhibiting biochemical reactions involving DNA, it is known to cause chromosomal damage and appears to require active cell processes for this to occur (*14, 15*). In light of the similarity of quinone-containing antibiotics (anthracyclines, mitomycin C, streptonigrin, etc.) and the quinoneimine structure of AMD, Bachur and his co-workers proposed a mechanism involving enzymatic reduction of AMD to a free radical intermediate with subsequent transfer of the electron to oxygen to yield superoxide. These free radicals may be the critical activated form of the antibiotic to cause intracellular DNA damage (*14, 15*).

In our continuing efforts to synthesize improved antitumor actinomycin D derivatives capable of bioactivation to free radical species, we examined the possibility of introducing a nitro group into actinomycin D at the 2-position to enhance its radical-forming ability in the presence of red-ox enzymes in cells, and also in the proliferating tumor cells. A possible route to achieve this was suggested by the work of Jones and Robins (*18*), who were able to convert a series of 8-aminopurines by the action of nitrous acid via diazotization to 8-nitropurines. In this manner a nitro group was introduced into the 2-position of the imidazole moiety in the purine ring system. This approach was then followed by the synthesis of yet another antibiotic, azomycin, by Beaman *et al.* (*19*), who succeeded in making the naturally occurring 2-nitroimidazole in appreciable yields from 2-aminoimidazole after diazotization in the presence of cupric sulfate.

Initially we attempted this reaction with chromophore model compound **1b** (R = NH_2), in which the pentapeptide lactone amide moieties at positions 1 and 9 in AMD (**1a**) were replaced by diethylamino groups (*20*). Reaction of the diazonium



Actinomycin D (1a)



1a 2a

FIG. 2. Proposed hydrogen-bonded structures and conformations of AMD **1a** and its analog, **2a**.

salt with nitrous acid in the presence of cupric sulfate by a modification of the procedure of Beaman *et al.* (*19*) indeed afforded the desired 2-nitro chromophore, **2b** (R = NO_2), and also the 2-H chromophore, **3b** (R = H), by elimination of diazo groups as products (*20*). Encouraged by these findings, we then examined this reaction with AMD (**1a**) under identical conditions. The resulting 2-deamino-2-nitroactinomycin D (**2a**) and 2-deaminoactinomycin D (**3a**) were isolated by preparative layer chromatography and were characterized by ir and NMR (*21*). The identity of **2a** was also established by its catalytic hydrogenation to **1a**, confirming that a nitro group had been introduced at the desired 2-position of the phenoxazinone moiety. The NMR assignments for the 2-deamino derivative, **3a**, and the 2-deamino-2-nitro derivative, **2a**, were consistent with the unambiguous assignments of the C_2-H, C_7-H, and C_8-H protons of model systems (**1b–3b**) made by PMR, CMR, and gated nondecoupling with NOE CMR experiments (*20*) and with the values assigned by Mosher for **3a** (*22*). In the infrared, a band at 1585 cm^{-1}, which is ascribed to planarity of the chromophore in AMD (**1a**) (*23*), is absent both in the spectra of **2a** and **3a**, implying the loss of planarity in the chromophore. Detailed procedures for the synthesis of **2a** and **3a** have been reported (*21*).

EXPERIMENTAL PROCEDURES

IR spectra were taken with a Perkin–Elmer Model 457A grating spectrophotometer in KBr micropellets. UV–visible spectra were obtained on a Gilford 250 spectrophotometer, which, with the addition of a baseline reference compensator (Analog Multiplexer 5053) and thermoprogrammer, auto four-cell programmer, and thermoelectric cell holder 2577, were used to obtain thermal denaturation curves; experiments were performed as reported (*9, 16*). NMR spectra were obtained in a JEOL FX90Q MHz spectrometer equipped with Fourier transform. EPR spectra were recorded on a Varian E-9 EPR spectrometer. Actinomycin D (NSC 3053, lot L554651-0-10) was generously provided by Dr. John Douros, Natural Products Branch, National Cancer Institute (Silver Spring, Md.). Cd spectra were measured according to the previously described methods (*17*) in a Cary 61 spectrophotometer. NADPH and calf thymus DNA, type 1, were purchased from Sigma Chemical Company (St. Louis, Mo.); the latter was used without further processing (*9*). Rat liver microsomes were prepared following the reported procedure (*13*).

RESULTS AND DISCUSSION

DNA Binding; UV–Visible and CD Spectra

The difference spectra (*24*) of the analog–DNA in Fig. 3 clearly demonstrate that both **2a** and **3a** do not have any appreciable binding affinity for DNA; this is in contrast with AMD–DNA binding. The result is in agreement with the result reported by Moore *et al.* (25) on the 2-deamino analog, **3a**, binding. These results are also in accord with the thermal denaturation of DNA experiments (Table 1). It is clear that both analogs **2a** and **3a** fail to stabilize the DNA against strand separation; AMD is found to stabilize the DNA double helix (**1a**, $\Delta Tm = 8 \pm 1$°C) against thermal denaturation under identical condition. 2-Deaminoactinomycin D shows very negligible binding (**3a**, $\Delta Tm = 1.8 \pm 0.46$°C) and **2a** shows no binding to DNA. We have further observed that **2a** itself is not very stable at high temperatures, i.e., at or near 90°C. In short, the analogs **2a** and **3a** do not appear to bind with DNA via interactions of their chromophores.

The CD spectra of AMD and the analogs emanate directly from the peptide groups, since the nearly planar chromophore lacks a chiral center. It is known that the conformation of the peptide rings in AMD has some steric relationship with its chromophore and also with respect to each other (*10*). In the structure of free AMD (Fig. 2), the 2-amino function of the planar chromophore is hydrogen-bonded to the amide carbonyl residue of the adjacent β-pentapeptide lactone. Elimination of this C-2 amino function, or its replacement by the strongly electron-withdrawing (-I) nitro group, changes this stereochemistry. 2-Deaminoactinomycin D (**3a**), lacks this peptide-binding function at the 2-position. In addition, replacement of the amino group by a nitro group at C-2 may change the nature of bonding interactions between the chromophore and the pentapeptide moieties.

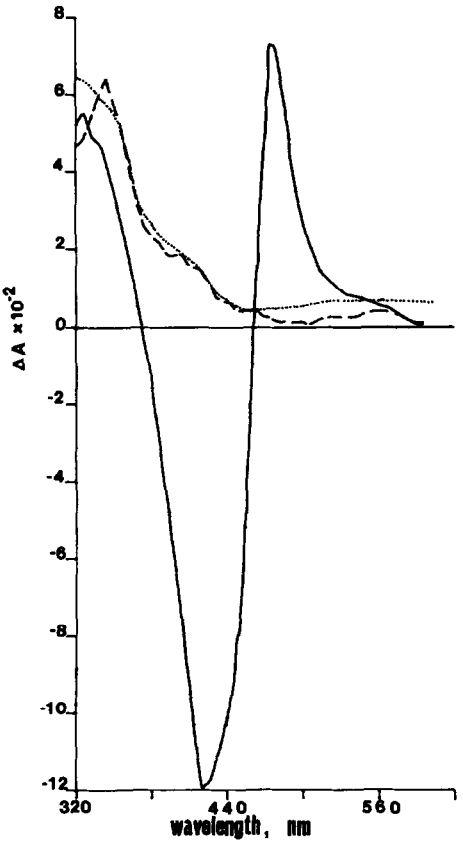


FIG. 3. The difference visible absorption spectra of free and DNA-bound actinomycin D or its analogs in 0.01 M phosphate (pH 7) containing EDTA (10^{-5} M). [AMD], 0.0213 mM; [**2a**], 0.0210 mM; [**3a**], 0.0432 mM; [DNA], 4.92 mM. Spectra were recorded after 1 h equilibration for complex formation: **1a** (—), **2a** (---), **3a** (···).

The nitro group in **2a** is likely to form hydrogen bonds, perhaps with the N–H hydrogen adjacent to the carbonyl at C-1 (Fig. 2). This is supported by a large downfield shift in the NMR of the β-threonine-NH proton resonance in the case of the 2-deamino-2-nitro derivative, **2a**, compared to **1a** and **3a** (Table 1). The charge distribution predicted by this structure (Fig. 2) would favor hydrogen bond formation between the ring nitrogen (N_{10}) and α-threonine-NH, as well as between the 2-nitroxyl oxygen and the β-threonine-NH. These interactions would be expected to result in a downfield shift of the threonine-NH protons with a larger shift for those in the ring adjacent to $-NO_2$ on the chromophore (*21*).

The CD spectra of the free drugs are shown in Fig. 4. AMD shows a negative band at 250–270 nm. 2-Deamino-2-nitro-substituted **2a** shows very strong negative bands at 230 nm like AMD and a minor band in the region 300–330 nm. AMD has a negative band at 350–370 nm. The CD spectrum of 2-deaminoactinomycin D (**3a**) shows a general lack of features all throughout the spectrum. With regard to conformation, the CD spectra of the free drugs confirm that the analogs **2a** and **3a**

TABLE 1

NMR PROPERTY (δ), ELEVATION OF T_m VALUES OF DNA (ΔT_m), AND *in Vitro* TUMOR GROWTH-INHIBITORY ACTIVITY (ID_{50})

Compound	δ values[a] (β-Thr-NH proton)	ΔT_m[b] (°C)	*In vitro* CCRF-CEM[c] (ID_{50}, μM)
1a	7.82 (6.2)	8.0 ± 1.0	0.043
2a	8.51 (7.2)	−0.7 ± 0.68[d]	0.276
3a	7.78 (7.2)	1.8 ± 0.46	>7.0

[a] Chemical shift in ppm; the data in parentheses are *J* values; NMR in $CDCl_3$ solution with Me_4Si as internal standard.

[b] $\Delta T_m = T_m$ of DNA–drug complex minus *Tm* of DNA. Concentration of drug, 1.0×10^{-5} M; of DNA (P), 5×10^{-5} M; in 0.01 M phosphate buffer (pH 7.0), 5×10^{-3} M EDTA.

[c] Using human lymphoblastic leukemia cells in log-phase growth (*9–11*). Compounds were dissolved in Me_2SO–saline; the final growth medium contained less than 1% Me_2SO. Data show the concentration (μM) required for 50% inhibition of cell growth in 44 h.

[d] Negative ΔT_m, if real, indicates complexation-destabilized DNA.

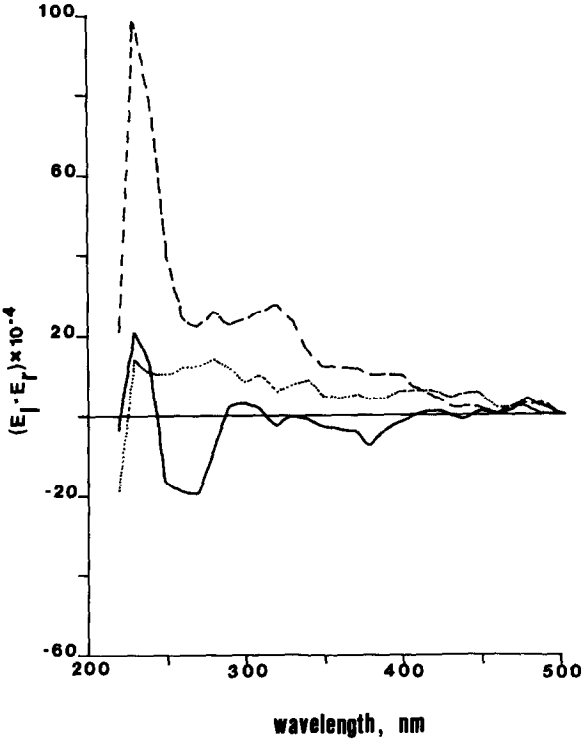


FIG. 4. CD spectra of uncomplexed **1a** (—), **2a** (---), and **3a** (···) in 0.01 M phosphate (10^{-5} M)–EDTA buffer; [drug], 1.0×10^{-5} M.

differ from each other and also from AMD; this is in agreement with NMR data (*21*).

The chromophore in AMD intercalates between adjacent base pairs of double-stranded DNA, thereby disturbing its helical structure (*17*). Figure 5 shows the difference CD spectrum, i.e., the spectrum of AMD–DNA complex minus the spectrum of AMD. A comparison of these difference spectra with CD of unbound DNA confirms that there are some definite perturbations of the DNA helix upon AMD binding (Fig. 5) (*17*).

The CD spectrum of DNA shows strong negative and positive ellipticities at 243 and 275 nm, respectively. In the presence of AMD, the peak at 275 nm shifts to 280 nm (*17*). At the lower wavelength, the negative DNA maximum appears at 252 nm. Both shifted peaks show a decrease in intensity, indicating that the intercalation of the chromophore has introduced a perturbation into the DNA helix. Neither the 2-deamino-2-nitro (**2a**) nor the 2-deamino derivative (**3a**) shows any shift at 275 nm. The 2-deamino-2-nitro derivative (**2a**) shifts the ellipticity slightly from 243 to 245 nm, but the maximum at 243 nm is unchanged in the presence of 2-

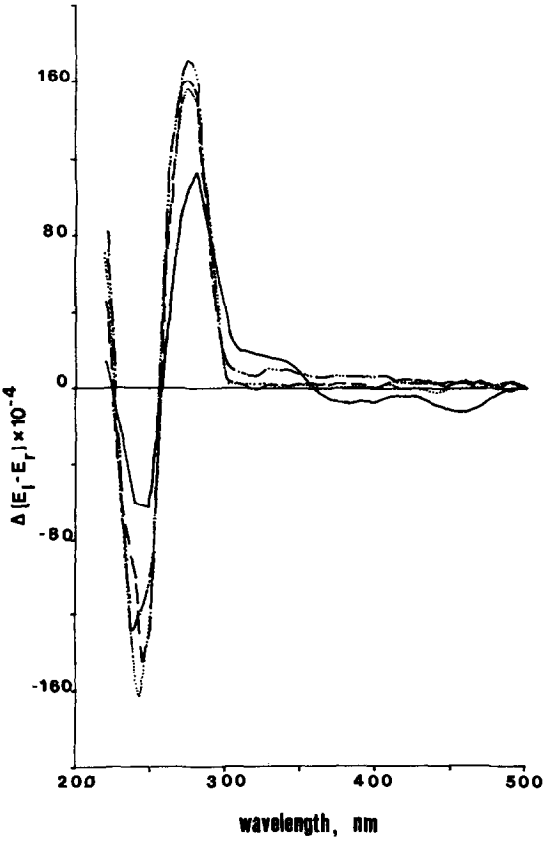


FIG. 5. Difference CD spectra of the drug–DNA complexes and the free drugs; buffer and symbols are as in Figs. 3 and 4. [Drug], 1.0×10^{-5} M; [DNA], 1.5×10^{-4} M. CD spectrum of free DNA (—···—); [DNA], 1.5×10^{-4} M.

deamino derivative (**3a**). The 2-substituted analogs therefore show no evidence of CD spectral shifts and leave the DNA helix relatively undisturbed.

The CD difference spectrum, obtained by subtracting the spectra of both the free drug and DNA from that of the drug–DNA spectrum, further elucidates conformational changes in the peptides of DNA-bound drugs. The CD difference spectrum of AMD shows a negative trough in the region 350–480 nm. A positive band is present at 300 nm. Additional strong negative and positive bands appear at 260 and 247 nm, respectively (Fig. 6).

The maximum that is present at 260 nm for all three drugs may reflect interactions of the peptide lactones with the DNA. Intercalation of the chromophore and hydrogen and hydrophobic interactions by the peptide groups of actinomycin D during complexation with DNA are known to act cooperatively (*16*). The peptide lactones of derivatives **2a** or **3a** associate with DNA despite the lack of the intercalative binding by their chromophores (and unlike AMD). In the bound state the P groups might be expected to attain the conformation of the P groups of actinomycin D in a AMD–DNA complex (*26*). This may be the source of the

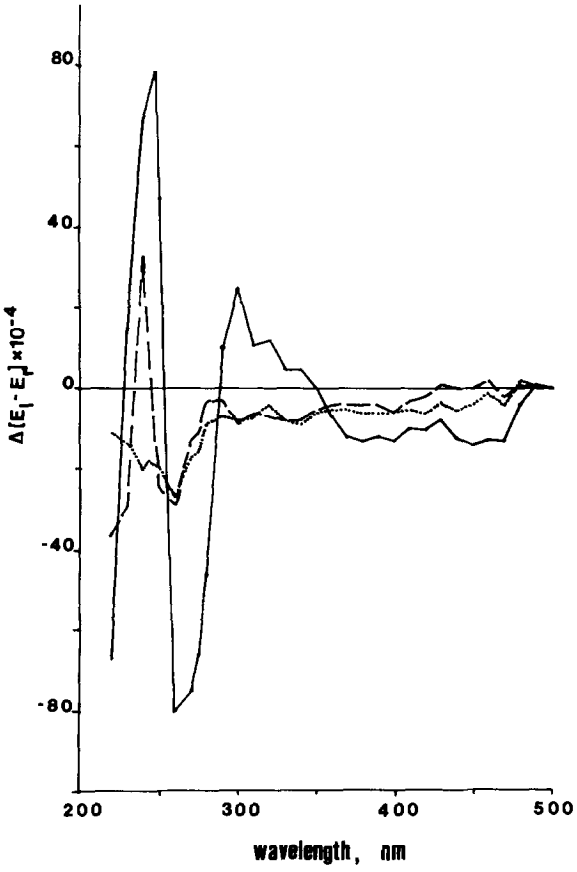


FIG. 6. Difference CD spectra of the drug–DNA complexes and the free drug + free DNA; buffer, concentrations and symbols are as in Fig. 5.

observed change in ellipticities at 260 and 247 nm of both AMD and the 2-deamino-2-nitro derivative (**2a**) (see also Fig. 2). We have already mentioned that the analogs' association with DNA does not disturb the DNA helix, and no change in DNA ellipticity at 275 nm is observed. In the region 240–250 nm, both AMD and **2a** show a maximum which is conspicuously absent in the 2-deamino analog (**3a**). The 2-deamino-2-nitro analog, **2a**, shows a strong change at 240 nm, and AMD shows a stronger change at 247 nm.

AMD is known to bind to DNA by intercalation and shows a CD spectrum markedly different from those of **2a** and **3a** in the visible (>380 nm) region. Neither uv (Fig. 3) nor CD difference spectra of the drug–DNA complexes (Figs. 4–6) show evidence of intercalation of chromophores of these analogs into DNA. DNA denaturation studies with AMD also support this inference. In conclusion, the analogs' interactions with DNA may be restricted to specific or nonspecific binding of their peptide functions only.

Reductive Activation to Free Radicals

A free radical intermediate with broad resonance peaks was detected by EPR

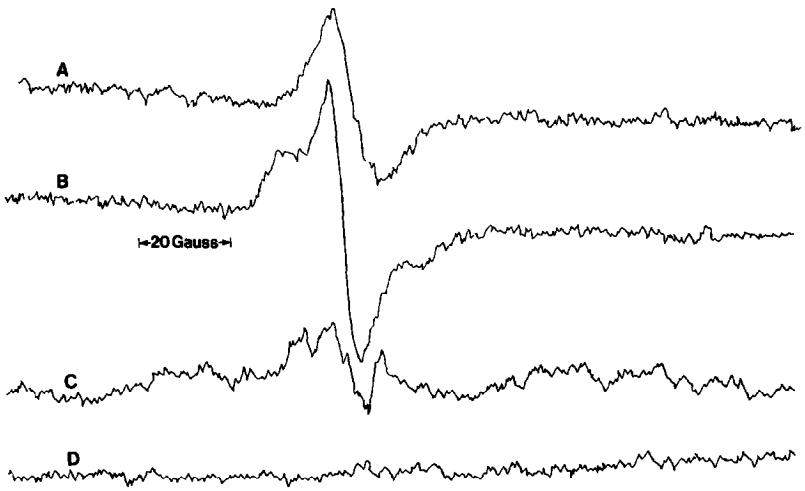


FIG. 7. (A) EPR spectrum of actinomycin D (**1a**) free radical anion formed in anaerobic 1-ml incubations of 5 mM NADPH, 1 mM AMD, 0.2 M potassium phosphate buffer (pH 7.4), and liver microsomes (0.5 mg). EPR conditions at room temperature were microwave power, 5 mW; microwave frequency, 9.51 GHz; modulation amplitude, 8 G; time constant, 0.5 s; scan time, 8 min; receiver gain, 6.3×10^4; magnetic field, 3380 G. (B) EPR spectrum of 2-deamino-2-nitroactinomycin D (**2a**) free radical anion formed in anaerobic 1-ml incubations of 5 mM NADPH, 1 mM **2a**, 0.2 M potassium phosphate buffer (pH 7.4), and liver microsomes (0.5 mg). EPR conditions at room temperature were microwave power, 5 mW; microwave frequency, 9.51 GHz; modulation amplitude, 8 G; time constant, 0.5 s; scan time, 8 min; receiver gain. 6.3×10^4; magnetic field, 3380 G. (C) EPR spectrum of 2-deaminoactinomycin D (**3a**) free radical anion formed in anaerobic 1-ml incubations of 5 mM NADPH, 1 mM **3a**, 0.2 M potassium phosphate buffer (pH 7.4), and liver microsomes (0.5 mg). EPR conditions at room temperature were microwave power, 5 mW; microwave frequency, 9.51 GHz; modulation amplitude, 8 G; time constant, 1.0 s; scan time, 8 min; receiver gain. 1.6×10^5; magnetic field, 3380 G. (D) Control and the model derivatives **1b–3b** under identical conditions.

when anaerobic solutions of NADPH, actinomycin D (**1a**), and potassium phosphate buffer were reacted with liver microsomes (Fig. 7A). Similarly, free radical intermediates were detected when anaerobic solutions of NADPH, 2-deamino-2-nitro derivative (**2a**), and 2-deamino derivative (**3a**) were reacted with liver microsomes (Figs. 7B and C, respectively). No free radicals were detected in the absence of enzyme or substrates. However, no free radical intermediate was detectable from the peptide lactone-free model phenoxazinone derivatives, **1b–3b**, under similar conditions. In short, the results of these experiments affirm the 2-deamino-2-nitro derivative **2a** to be a better substrate for radical formation, that AMD is significantly inferior to **2a** but superior to the 2-deamino derivative, **3a**, and that the peptide groups play an important role in determining their specificity for enzymes.

CONCLUSION

Actinomycin D is known to express its proximal biological activity via intercalation into DNA and inhibition of DNA-primed RNA synthesis; this might account for its cytotoxicity in biological systems. The antibiotic is also known to form radicals and cause DNA and chromosomal damage in cells; this mechanism may be related to its inhibition of DNA synthesis in proliferating cells. The 2-deamino-2-nitroactinomycin D, which does not intercalate and generates ion radicals enzymatically, might be able to distinguish between these proximal and distal mechanisms of action and help evaluate the role of these effects in the inhibition of certain tumor cells which depend primarily upon rapid DNA synthesis.

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