MOLECULAR PHARMACOLOGY, 17, 432–434

Stimulation of Superoxide Formation by Actinomycin D and Its N^2-Substituted Spin-Labeled Derivatives

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Received September 17, 1979; Accepted November 17, 1979

SUMMARY

SINHA, B. K., AND M. G. COX. Stimulation of superoxide formation by actinomycin D and its N^2-substituted spin-labeled derivatives. *Mol. Pharmacol.* **17:** 432–434 (1980).

The mechanism of action of actinomycin D (AMD) is related to its interactions with DNA and inhibition of RNA synthesis. Recently, it has been shown that AMD stimulates the formation of superoxide when incubated with microsomal proteins. We have prepared N^2-[4-(2,2,6,6-tetramethyl-1-piperidinyloxyl)]actinomycin D and the related 1,3-diaminopropane analogs, which were found to be more active than AMD *in vivo* against P-388 leukemia cells in spite of their poor DNA binding properties. We have investigated the stimulation of superoxide formation by these compounds as a possible mechanism of action. These analogs are more effective in stimulating O_2 uptake and the formation of $\dot{O}_2^-$ than the parent AMD. The better antitumor activities of these analogs may be related to the increased $\dot{O}_2^-$ formation *in vivo*.

INTRODUCTION

Actinomycin D (NSC,3503,AMD,1), a cyclic pentapeptide antibiotic, has been used clinically as a potent antitumor agent in the treatment of Wilm's tumor (1) and gestational choriocarcinoma (2). The mechanism of action of AMD is believed to result from its ability to interact with double-stranded DNA and the consequent inhibition of RNA synthesis (3, 4). Müller and Crothers (5) and Krugh (7) have shown that deoxyguanosine residues are essential for the binding of AMD to DNA. Sobell (6), using X-ray crystallographic techniques, has further shown that the phenoxazone ring system of AMD is preferentially intercalated between base-paired nucleotide sequences of pdG–pdC, and that the peptide subunits lie in the minor groove of the DNA helix and interact with the deoxyguanosine residues on the opposite chains through hydrogen bonds. In addition, AMD is carcinogenic and induces chromosomal damage in cultured human leukocytes and in HeLa cells (8).

While the binding of AMD to DNA may in part be responsible for its biological actions, recently we have shown that AMD also binds to the membranes of human red blood cells and to mastocytoma cells (9). Studies by Bachur *et al.* (10, 11) and others (12, 13) have shown that anticancer agents containing quinone moieties, such as adriamycin and daunoribicin, are metabolized to free radicals which augment O_2 uptake with the formation of $\dot{O}_2^-$. When incubated with microsomal proteins, AMD has been shown to generate a free radical intermediate, which stimulates O_2 uptake with the formation of $\dot{O}_2^-$ (14). These observations have suggested that the formation of $\dot{O}_2^-/H_2O_2$ may play an important role in the antitumor effectiveness of these drugs.

In an earlier paper, we have described the synthesis and biological properties of N^2-substituted spin-labeled AMD analogs **2** and **3** (Fig. 1) (15). These analogs were prepared as probes for nucleic acids, since spin-labeled drugs have been used extensively to study drug–macromolecule interactions (16, 17). Our previous studies have shown that analogs **2** and **3** bind weakly to DNA as compared to AMD and are poor inhibitors of DNA-dependent RNA polymerase. However, they showed better antitumor activities ($\%T/C = 276$) than AMD ($\%T/C = 181$) against P-388 leukemia cells *in vivo* (15). In view of the recent implication of $\dot{O}_2^-$ derived from AMD as a cytotoxic species, we have investigated the production of $\dot{O}_2^-$ by AMD, **2**, and **3** to gain some insight into the mode of action of these compounds.

MATERIALS AND METHODS

Materials. Actinomycin D (NSC 3503) was the gift of the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute. The spin-labeled analogs **2** and **3** were prepared from AMD according to the published method (15).

Methods. Hepatic microsomes were prepared from 160- to 180-g male C–D rats by homogenizing livers in 3 vol of 150 mM KCl–50 mM Tris buffer at pH 7.4. The

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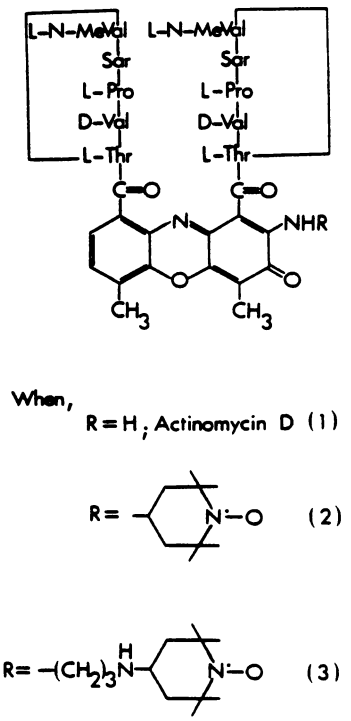

FIG. 1. *Structure of actinomycin D and its N^2-substituted spin-labeled analogs*

homogenates were centrifuged at 9000*g* for 15 min. The pellet was discarded and the supernatant was centrifuged at 165,000*g* for 40 min. The microsomal pellets were resuspended in KCl–Tris buffer and resedimented at 165,000*g*. The microsomal protein was determined by the method of Sutherland *et al.* (18) using bovine serum albumin as a standard. Oxygen uptake was determined with a Clark electrode in a water-jacketed glass vassel at 37°C and filled with 150 mM KCl–50 mM Tris–5 mM $MgCl_2$ (pH 7.4). The drugs (100 μM) were incubated with microsomes (1.5 mg protein/ml) for 2.0 min. The reaction was started by adding NADPH (0.4 mM) and the rate was taken as the initial slope.

The adrenochrome assay for $\dot{O}_2^-$ was performed at 37°C in an Aminco DW2A spectrophotometer in the split-beam mode. A solution of epinephrine (30 μl, 0.02 M in 0.02 N HCl) and the microsomes were added to both cuvettes. One minute later, NADPH (0.4 mM) was added to the sample side only and the absorbance at 480 nm was monitored. The rate of adrenochrome formation was determined using an extinction coefficient of 4.02 mM^{-1} cm^{-1} (19). Where indicated 10 μg/ml SOD[1] was added with the microsomes. Drug solutions were freshly prepared in 150 mM KCl–50 mM Tris at pH 7.4.

RESULTS AND DISCUSSION

Bachur *et al.* (14) have shown that the incubation of AMD with purified microsomes stimulates O_2 uptake. However, the addition of AMD (100 μM) to microsomes containing NADPH stimulated O_2 uptake by only 13%. This effect may be dramatized by solubilizing the microsomes with Triton N-101 (2%, v/v), which lowers endogenous O_2 consumption without affecting NADPH-dependent O_2 uptake. In the presence of the solubilized microsomes, AMD stimulated O_2 uptake by 110% over the basal level (Table 1). In contrast, the addition of spin-labeled AMD analogs **2** and **3** (100 μM) in the absence of detergent drastically stimulated O_2 uptake, by 154 and 1273%, respectively. The observed differences in the stimulation of O_2 uptake by **2** and **3** must not be due to the presence of the nitroxide moieties, per se, since addition of the nitroxide compound Tempo[1] alone under similar conditions resulted in a very small stimulation (12%).

The formation of adrenochrome from epinephrine has been used as an indicator of the formation of $\dot{O}_2^-$ (20, 21). The addition of AMD, **2**, and **3** stimulated the rate of oxidation of epinephrine to adrenochrome by 90, 260, and 982%, respectively. The addition of 10 μg/ml SOD inhibited the formation of adrenochrome, suggesting that $\dot{O}_2^-$ was responsible for adrenochrome formation.

Sato *et al.* (13) have shown that the DNA-bound anthracycline antitumor drugs, adriamycin and daunorubicin, are not substrates for microsomal activation. In order to ascertain whether AMD, **2**, and **3** will generate $\dot{O}_2^-$ when bound to DNA, excess calf thymus DNA (nucleotide:drug = 15:1) was preincubated with the drugs for 30–45 min and assays were performed as described. While the preincubation with DNA abolished the stimulation of O_2 consumption by AMD, it reduced this stimulation by **2** and **3** by 71 and 50%, respectively (Table 2). The presence of DNA also abolished the stimulation of epinephrine oxidation by AMD and **2** (Table 2) and reduced the stimulation by **3** by 50%. These results suggest that, when bound to DNA, these analogs are poor substrates for activation to their reactive intermediates for the reduction of molecular O_2 to superoxide.

Recent studies by Thayer (22) have revealed that the cardiotoxicity of adriamycin may be related to its ability to stimulate the formation of $\dot{O}_2^-/H_2O_2$ in heart tissue. Bachur *et al.* (10) have suggested that the antitumor

TABLE 1

Stimulation of oxygen uptake and adrenochrome formation by actinomycin D and its analogs by rat hepatic microsomal incubations

Drug	Triton N-101	O_2 uptake[a]	SOD	Adrenochrome formation[a]
		nmol/min/mg protein	*10 μg/ml*	*nmol/min/mg protein*
—	−	7.23 ± 0.27	−	1.29 ± 0.10
	+	1.22 ± 0.04	−	−
AMD	−	8.17 ± 0.81 (13)	−	2.45 ± 0.18 (90)
	+	2.57 ± 0.36 (110)	+	−
2	−	18.37 ± 0.71 (154)	−	4.66 ± 0.17 (260)
			+	−
3	−	99.28 ± 3.55 (1273)	−	13.96 ± 1.16 (982)
			+	−
Tempo	−	8.10 ± 0.3 (12)	−	1.48 ± 0.15 (15)

[a] The percentage stimulation = [(drug-stimulated rate − basal rate)/basal rate] × 100 and is given in parentheses. Values for O_2 uptake and adrenochrome formation are averages ± standard errors of triplicate incubations.

[1] Abbreviations used: SOD, superoxide dismutase; Tempo, 2,2,6,6-tetramethyl-1-piperidinyloxyl.

TABLE 2

Effect of DNA (nucleotide:drug = 15:1) on the oxygen uptake and the adrenochrome formation by actinomycin D and its analogs by rat hepatic microsomal incubations

Drug	O_2 uptake[a]	Adrenochrome formation[a]
	nmol/min/mg protein	*nmol/min/mg protein*
—	10.86 ± 0.37	2.51 ± 0.23
AMD	11.63 ± 0.33	3.05 ± 0.11
2	15.8 ± 1.2 (45)	3.07 ± 0.23
3	79.78 + 2.33 (635)	13.19 ± 1.69 (425)

[a] The percentage stimulation = [(drug-stimulated rate-basal rate)/basal rate] × 100 and is given in parentheses. Values for O_2 uptake and adrenochrome formation are averages ± standard errors of triplicate incubations.

properties of the anthracycline antitumor drugs may also be related to their biochemical activation to free radical metabolites. These workers have further suggested that these "site specific free radicals" either may bind to cellular components such as DNA, RNA, and proteins or may generate $\dot{O}_2^-/H_2O_2$ for cytotoxicity. Tomasz (23) has shown that mitomycin C also produces H_2O_2 when activated, suggesting that the formation of $\dot{O}_2^-/H_2O_2$ may be involved in the antitumor activity of this drug. H_2O_2 is known to inactivate transforming DNA (24, 25) and cause chromatic aberration in ascites tumor cells (26). Furthermore, H_2O_2 has been shown to cause base liberation and backbone breakage of DNA (27). The toxic effects of $\dot{O}_2^-$ and/or species derived from $\dot{O}_2^-$ in biological systems are well established (21).

Our findings indicate that AMD and its spin-labeled derivatives **2** and **3** stimulate the formation of superoxide by microsomal proteins. The increased stimulation of $\dot{O}_2^-$ by **2** and **3** is quite surprising in light of the fact that Tempo has little or no effect. Therefore, it seems that covalently attached nitroxide moieties in the N^2 position of AMD (as in **2** and **3**) increase the rate and ease of formation of the free radical metabolite of AMD, which then stimulates the reduction of oxygen to superoxide. However, it is also possible that the free radical metabolites of **2** and **3** may react more rapidly with O_2 to form $\dot{O}_2^-$. $\dot{O}_2^-$ is converted to H_2O_2 either spontaneously or by SOD which is also present in cancer cells (28). Either $\dot{O}_2^-$, H_2O_2, or reactive oxygen species may lead to lipid peroxidation or induce DNA damage. Khandwala and Kasper (29) and, recently, Bornstein *et al.* (30) have shown that the nucleus contains a cytochrome *P*-450 system that is capable of metabolizing benz[*a*]pyrenes. Thus, it is possible that analogs **2** and **3** may be metabolized more effectively than AMD in the nucleus due to their poor DNA binding properties. The active metabolites of **2** and **3** then may lead to the formation of $\dot{O}_2^-$. Increased $\dot{O}_2^-$ formation in the nuclei may, in part, be related to their increased antitumor effectiveness. However, other mechanism of actions such as membrane bindings (9) cannot be ruled out at this time.

ACKNOWLEDGMENTS

The authors wish to thank Drs. C. F. Chignell and R. P. Mason for their helpful discussions during the preparation of the manuscript.

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