Vol. 136, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1986 Pages 1-7

DEGRADATION OF DNA BY METALLOANTHRACYCLINES: REQUIREMENT FOR METAL IONS

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Received January 21, 1986

Metallodaunomycin has been shown to cleave DNA only in the presence of oxygen, a reducing agent and a metal ion under reaction conditions similar to those used for the cuprous-phenanthroline complex. The intermediacy of $O_2^{\cdot-}$ and H_2O_2 has been substantiated by experiments with superoxide dismutase and catalase, respectively. Only partial inhibition by OH$^{\cdot}$ scavengers was observed. An important feature of the reaction is that no specificity for Cu(II) was observed. This observation has led us to propose a reaction mechanism different from that proposed for the cuprous-phenanthroline complex. The mechanism proposed includes a catalytic role for metal ions other than Cu(II) as well as the direct participation of products of metal-catalyzed redox reactions such as semiquinone and/or hydroquinone of daunomycin.

The anthracyclines are structurally akin to streptonigrin, mitomycins B and C and certain 5,8-quinolinediones. Since the latter agents and bleomycin have been found to cleave DNA in a reaction that depends on molecular oxygen and metal ions(1), metalloanthracyclines may also mediate DNA strand scission and this mediation may actually be partially responsible for their therapeutic effects. Recently, the 1,10-phenanthroline-cuprous complex which cleaves DNA in the presence of oxygen(2,3), has been suggested to be a simple model for studying the mechanism of action of a variety of antibiotics(2). This suggestion, in part, but primarily the structural similarity of the anthracyclines to other antineoplastic agents whose quinone structure endows them with the ability to cleave DNA in the presence of metal ions, has prompted us to investigate metalloanthracyclines. In this report, new evidence on the daunomycin-induced DNA scission and the role of certain metal ions in the degradation is described. Unlike the 1,10-phenanthroline case, the evidence shows that the cleavage reaction is not specific to cuprous ion only(2). This has led us to propose a reaction mechanism, different from that proposed for the phenanthroline-cuprous, OP_2-Cu(II), complex(2).

0006-291X/86 $1.50

EXPERIMENTAL

[Methy-^{3}H] thymidine 5'-triphosphate, tetrasodium salt (0.0070 mg/ml, specific activity 81.5 Ci/mmole) was purchased from Schwarz/Mann and E. Coli DNA polymerase I was from Bethesda Research Laboratories, Inc., while Chelex 100, minus 400 mesh was from Bio-Rad Laboratories. All metal salts, reagent grade, were supplied by Fisher Chemical Co. Other materials, unless otherwise specified, were from Sigma Chemical Co. DNA and daunomycin concentrations were determined spectrophotometrically (using the respective extinction coefficients of 6600 and 11,500 M^{-1} CM^{-1} at 260 and 480 nm respectively) on a Varian 210 Spectrophometer.

Poly(dA-[^{3}H]T) was prepared by using a modified procedure of Modrich and Lehman(4). The degradation of poly(dA-[^{3}H]T) was determined by acid solubilization following the method of Downey et al(5). The reaction mixture contained, in a final volume of 0.1 ml, 50 μM daunomycin, 50 mM Hepes buffer, pH 7.4, 20 μM poly(dA-[^{3}H]T), 1 mM 2-Mercaptoethanol and 1 μM $CuSO_4$. After 30 min at 25°C, the reaction was stopped by the addition of 0.01 ml of (M EDTA, pH 8.0, 0.1 ml of Salmon sperm DNA, 2 mg/ml, and 0.5 ml of 1M perchloric acid. After 10 min at 0°C, the solution was centrifuged at 7,500 rpm for 10 min and an aliquot of the supernatant was counted in 10 ml Biofluor in a liquid scintillation counter to determine the percent acid soluble product.

Superoxide dismutase(6) and Catalase(7) were assayed for purity using standard procedures before use. Distilled water and all solutions other than those of metal salts were Chelex-treated to scavenge metal ion contaminants.

RESULTS

The degradation of poly(dA-[^{3}H]T) by Dm requires a metal ion, a reducing agent and molecular oxygen (Table 1). The reaction can proceed effectively in a 50-fold excess of Dm over Cu(II). This and the obligatory presence of Cu(II) strongly suggests that only catalytic levels of Cu(II) are required. The re-

Table 1. Requirements for the Degradation of DNA

components	average[d] cpm	% acid soluble[e]
complete[a]	2631	51
minus Cu(II)[b]	209	4
minus 2-mercapto-ethanol[b]	102	2
minus daunomycin[b]	117	2
minus oxygen[b,c]	76	<1

[a]The complete reaction mixture contained in a final volume of 0.1 ml 50mM Hepes, pH 7.4, 20 μM poly(dA-[^{3}H]T), 1mM 2-mercaptoethanol, 1 μM $CuSO_4$, and 50 μM daunomycin. After 30 min at 24°C, acid soluble radioactivity was determined. [b]The reaction mixture contained all components as in a minus the component indicated. [c]This experiment was carried out by bubbling N_2 gas through the reaction mixture. [d]Counts indicate averages of at least three runs and are for a 10 μl-aliquot of the supernatant from the reaction mixture. [e]The control cpm used for the calculation (see Experimental) was 5107 cpm.

Table 2. Effect of Superoxide Dismutase and Catalase on the Degradation of DNA by Daunomycin-Cu(II) Complex[a]

conditions	average[d] cpm	% acid soluble[e]
complete[a]	2190	52
plus catalase[b] (10 µg/ml)	130	3
plus superoxide dismutase[b] (40 µg/ml)	2452	58
plus boiled catalase[b,c] (10 µg/ml)	1230	29

[a]Poly(dA-[^{3}H]T) (20 µM) was cleaved in 0.1 ml solution containing 50mM Hepes, pH 7.4, 1mM 2-mercaptoethanol, 50 µ M daunomycin, 1.0 µ M $CuSO_4$. [b]Indicates the complete system plus the component indicated. [c]The boiled catalase was prepared by heating the catalase for 10 min before addition. [d]As in Table 1. [e]Control cpm: 4206.

quirements for a reducing agent and molecular oxygen suggest that a reduced form of oxygen may be the reactive species responsible for the degradation.

The involvement of $O_2^{\cdot-}$ and H_2O_2 was established by experiments with superoxide dismutase (SOD) and catalase, respectively. The effects of SOD and catalase on the degradation are shown in Table 2. The experiment with SOD gave slightly increased acid soluble product (58%) compared to the control (52%). This indicates that $O_2^{\cdot-}$ is not a primary reactive species, since if it were one would expect inhibition. Catalase, on the other hand, provided with complete protection, indicating that the reaction is peroxide-dependent. Apparently, the reduction of oxygen to $O_2^{\cdot-}$ leads to H_2O_2 which may then react further to form OH. radicals. Production of OH. from H_2O_2 via a Fenton reaction or a metal-catalyzed Haber-Weiss reaction(8) is well documented. The implicit assumption that can be made here is then that the OH• radical is the primary reactive species(9).

The role of the OH• radical was then examined by several experiments. Table 3 shows the results of the effects of some hydroxyl free radical scavengers. Benzoate and acetate were only partially effective inhibitors when present at a concentration of 50 mM. Sodium chloride had virtually no effect

Table 3. Effect of Various Hydroxyl Radical Scavengers on the Thiol-Dependent Daunomycin-Cu(II) Complex

scavenger	concn (mM)[b]	average[b,d]	%inhibition[c,b]
acetate[a]	50(100)	821(1505)	18(33)
benzoate[a]	50(100)	912(2189)	20(48)
NaCl[a]	50(100)	91(91)	2(2)

[a]The complete reaction mixture contained, in a final volume of 0.1 ml, 50 μM daunomycin, 50mM Hepes, pH 7.4, 20 μM poly(dA-[^{3}H]T, plus the component indicated. [b]Values in parentheses are for 100mM concentrations. [c]The percent inhibition is calculated by substracting the percent acid soluble from the complete system represented as 100%. [d]As in Table 1.

even at 100 mM concentration. When the concentration of benzoate and acetate was doubled, the inhibition was rather modest(10). The results seem to suggest that the reaction may proceed only in part via a mechanism which involves a hydroxyl radical.

The requirement for a metal ion was examined further to establish the specificity for metal ions (Table 4). The percent acid soluble product observed for the metal ions tested was about a third of that of Cu(II). Furthermore, a 100-fold increase in the concentration of the metal ions resulted in only a

Table 4. Effect of Metal Ions on the Degradation of DNA[a]

metal ion	conc(μM)[b]	average[b,d] cpm	%acid soluble[b,c]
Zn(II)	1.0(100)	927(1264)	18(33)
Mg(II)	1.0(100)	931(1337)	18(35)
Fe(II)	1.0(100)	910(1075)	17(28)
Fe(III)	1.0(100)	906(1081)	17(28)
Ca(II)	1.0(100)	912(1416)	17(27)
Yb(III)[e]	1.0(100)	1967(2810)	38(54)
Cu(II)	1.0	2633	51

[a]Poly(dA-[^{3}H]T) (20 μM) was cleaved in a reaction mixture containing 50mM Hepes, pH 7.4, 1mM 2-mercaptoethanol, 50 μM daunomycin, and a given concentration of the metal ion. The acid soluble radioactivity was then determined after 30 min at 24°C. [b]Values in parentheses are for 100 μM concentrations. [c]The control cpm for the 1 μM concentrations (metal) was 5107 and that for the 100 μM, 3728. [d]As in Table 1. [e]Control cpm: 5205.

two-fold increase in the percent acid soluble product. The observed two-fold increase in the cleavage reaction is not consistent with the almost 100-fold increase in the concentration of the complex that is expected to form(11). Since the extent of degradation appears to be about the same for all the metal ions, except Cu(II), this observation does not reflect the differences in the stability constants of the metal ions. Moreover, simple binding of the metal ions to the polymer does not explain the data because such binding should lead to different stability constants and different degradation effects.

DISCUSSION

The several lines of evidence presented strongly indicate that $O_2^{\cdot-}$ and H_2O_2 participate in the degradation reaction. These observations are consistent with those reported for the OP_2-Cu(II) systems(2,3), except that the orthophenanthroline system showed specificity for Cu(II). The novel features of the Dm system are (1) the effectiveness of a number of metal ions (Table 4), (2) that there were no marked differences in the extent of degradation by the other metal ions examined, (3) Cu(II) is more effective compared to the other metal ions. These findings coupled with the observation that the presence of a metal ion is a requirement suggest several implications: (1) the metal ions may play only a catalytic role, (2) that they probably do not participate in a rate-determining step, and (3) that two pathways may be operative in the Cu(II) system.

The partial inhibition by OH$^{\cdot}$ scavengers can be interpreted in two ways: (1) OH$^{\cdot}$ may be only partially responsible for the degradation, (2) OH$^{\cdot}$ may be produced in the proximity of the degradation site and may react soon after it is generated; hence, OH$^{\cdot}$ scavengers will only be partially effective. The latter is probably true for the Cu(II) case. The former possibility, on the other hand, calls for several considerations: (1) in systems in which Cu(II) is present, OH$^{\cdot}$, derived from H_2O_2, is probably the primary reactive species and the reaction proceeds predominantly via the mechanism outlined by Que et. al.(2); (2) in systems in which no Cu(II) is present, redox reactions involving the divalent metal ions may actually occur to a significant extent leading to

degradation; (3) Dm itself may undergo metal-catalyzed, redox reactions generating reactive Dm intermediates which lead to degradation.

Since the data in Table 4 indicated no differential effects, it is reasonable to rule out case 2(12). Case 1 involves the known redox reaction of Cu(II)(8). Further credence for this mechanism comes from the observation that SOD had no significant effect on the reaction as well as from the complete protection provided by catalase. Case 3, if operative, should also be applicable in the Cu(II) system probably only as a minor reaction pathway.

In the absence of Cu(II) but in the presence of other metal ions, case 3 is an attractive reaction mechanism. Consequently, we propose the following:

$$Dm + 2HORSH \longrightarrow DmH_2 + 2HORS^{\bullet} \quad (1)$$

$$Dm + 2HORS^{\bullet} \longrightarrow DmH^{\bullet} + HORS\text{-}S\text{-}H \quad (2)$$

$$DmH^{\bullet} \longrightarrow Dm^{\bullet} \quad (3)$$

$$DmH^{\bullet} + 2HORSH \longrightarrow DmH_2 + HORS\text{-}S\text{-}ROH \quad (4)$$

$$2HORS^{\bullet} + O_2 \longrightarrow O_2^{\bullet -} + HORS\text{-}S\text{-}ROH \quad (5)$$

$$2O_2^{\bullet -} + 2H^{+} \longrightarrow H_2O_2 + O_2 \quad (6)$$

$$H_2O_2 + O_2^{\bullet -} \longrightarrow OH^{\bullet} + OH^{-} + O_2 \quad (7)$$

Step 1 represents reduction of Dm to the hydroquinone (DmH_2) in the presence of an excess of a reducing agent ($HOCH_2CH_2SH$, in this work). Step 2 shows the formation of a semiquinone ($DmH^{\bullet}$) from Dm by a one-electron reduction, probably involving a free radical, which can then be protonated by a solvent. $DmH^{\bullet}$, in turn, can be converted to a semiquinone methide ($Dm^{\bullet}$) which has been alluded to as a reactive intermediate. Finally, $DmH^{\bullet}$ can also be converted to DmH_2. Steps 5-7 are self-explanatory. The products of the reductive processes in steps 1-4 can be oxidized back to Dm. The mechanism as proposed is consistent with the requirements for oxygen and a reducing agent. A novel feature of the reactions that we are proposing here is that both the reductive and oxidative processes require a metal ion as a catalyst, since the degradation requires a metal ion. The proposed direct participation of the semiquinone and/or hydroquinone of Dm in the degradation reaction and the redox processes themselves are consistent with the work of Lown and Sim on similar systems(13).

Both anaerobic and aerobic reduction of Dm have been reported. Moreover, the aerobic reduction of Dm leads to reactive oxygen species(14). It has been proposed that these reductive processes are responsible at least in part for

the biological activity including the acute cardiotoxicity of the anthracyclines(15). The results reported here seem to strongly suggest that metalloanthracyclines mediate this proposed biological activity(16). Work in this area is therefore pertinent to the understanding of the mechanism of the mode of action of the anthracyclines. Results of further work currently in progress in our laboratory will be reported elsewhere.

ACKNOWLEDGMENTS: This research was supported by grants from the NIH/MBRS Program, Grant No. RR-08006.

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10. The second order rate constants for the reaction of a hydroxyl radical with acetate, benzoate, and sodium chloride were determined to be 4.2×10^7, 3.3×10^9, and 10^3 M^{-1} S^{-1}, respectively (J. Biol. Chem. 249, 2151 (1974); Int. J. Appl. Radiat. Isot. 18, 493 (1970)). Therefore, at least acetate and benzoate should be effective scanvengers of the hydroxyl radical.
11. A sample calculation (using a log stability constant of 1.1 (Glover, P. M. S. Thesis, Atlanta University, 1985) at pH 7.4) of the concentrations of the complexes that are expected to form at the two concentrations employed, 1 and 100 M, gives 2.5×10^{-4} M and 2.5×10^{-2} M, respectively.
12. For case 2 to occur, metal ions such as Mg(II), Zn(II), and Ca(II) would have to be reduced and oxidized quite readily. Such reactions are not however common. Furthermore, if the degradation reaction were dependent on the redox capabilities of the different metal ions, one would have then observed differential effects in the extent of the degradation.
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16. We have considered the possibility that the nonspecificity observed in this investigation might be due to Cu(II) impurity in the different metal ion concentrations. However, since the concentrations of the metal ions used were exceedingly low, any Cu(II) impurity is unlikely to give the results observed.