Vol. 133, No. 1, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 27, 1985 Pages 15-22

HOW CAN IRON SALTS MEDIATE THE DEGRADATION OF NUCLEOS(T)IDES BY ELLIPTINIUM ACETATE VIA FREE-RADICALS ?

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Received September 26, 1985

Elliptinium acetate (NSC 264137) is an antineoplastic agent currently used in anticancer chemotherapy. We report here the first evidence that this drug is able to modify DNA models via a redox process with iron salts. In presence of iron (III) salts, EDTA and H_2O_2, 9-OH-NME degrades deoxyguanosine. The two main products are guanine and 8-hydroxydeoxyguanosine which result from the formation of hydroxyl radicals. The biological implications of this phenomenon are briefly discussed.

A brief survey of recent developments in the field of antitumoral drugs reveals that many of these drugs interact with transition metals and DNA to cause single-strand breaks by the formation of radical species. Some metal complexes in which the drug acts as ligand are known : adriamycin (1), bleomycin (2), streptonigrin (3). Some drugs may also undergo redox reactions with transition metals such as copper and iron : m-AMSA (4), mitomycin C (5), adriamycin (5a), streptonigrin (6). DNA breaks are also observed in vivo for these drugs (for a review, see ref. 7) and the generation of Reactive Oxygen Intermediates (ROI) by these drugs might play a role in the production of these breaks in vivo.

Ellipticium acetate (9-OH-NME) is antineoplastic agent currently used in anticancer chemotherapy (8). Besides its intercalative behaviour (9), it has been recently shown that this drug, after oxidation, can bind covalently to biological molecules such as ribonucleosides and RNA (10). Irreversible covalent binding to DNA of L1210 cells has been observed for 9-OH-NME (11) and DNA breaks are also

0006-291X/85 $1.50

observed (12). Whereas the covalent binding is related to the "bioxydative alkylation" process (13), DNA breaks are probably produced by an alternative route since they are reversible (12). We describe here the cleavage and hydroxylation of a simple nucleoside as model for the interaction with DNA at molecular level and discuss the possible role of transition metals in the interaction of elliptinium with DNA.

MATERIALS AND METHODS

Materials : 9-OH-NME is a gift of Sanofi Company.
PBN (α-phenyl-N-t-butyl nitrone) was obtained from Aldrich.
dG was obtained from Sigma Chemical Company.
Other reagents and solvents were purchased from Merck or Prolabo.

Reaction conditions : 1.3 mM dG was incubated in presence of 0.5 mM EDTA, 0.2 mM $FeCl_3$, 1.0 mM 9-OH-NME, 5 mM H_2O_2, 50 mM phosphate buffer PH 7.4 at 37°C for 1.5 hour. The mixture was centrifuged for 5 min at 2000 rpm to eliminate the precipitate and the supernatant was diluted 5 times with H_2O prior to analysis by HPLC on a reverse-phase column (μC_{18} Bondapack) using 10 mM ammonium acetate (NH_4OAc) pH 4.5 with 10 % (v/v) methanol as eluent, at a flow rate of 1.5 mL/min.

Identification of compound A an B

Compound A was identified by its HPLC retention time.

Compound B was identified by UV, NMR and mass spectrometry after purification under the following conditions : 50 mL of reaction solution were prepared as described in experimental conditions, but with 18 hours incubation at 37°C. The solution was centrifuged, the supernatant injected into a "C_{18} Sep-pak" cartridge and eluted with MeOH/H_2O (1/9), (v/v). The solvant of eluate was evaporated under vacuum and the residue dissolved in 2 mL H_2O. The 8-OH-dG was then purified on a semi-preparative (μC_{18} Bondapak) column (0.78 x 30 cm) using AcOH/MeOH/H_2O (0.1/15/84.9, v/v/v) as eluent at a flow rate of 1.5 mL/min.
MS data : m/z : 284 (MH^+), 168 (base H^+) and 134 (deoxyribose).
^1H NMR : (d_6 DMSO) expressed in δ (ppm) (reference TMS) : 6.50 (2p,5,NH_2), 6.04 (1p,t,1'-H), 5.02 (1p, b.s,3'-OH), 4.81 (1p,b.s,5'-OH), 4.34 (1p,m,S'-H), 3.75 (1p, m, 4'-H), 3.58 (2p, m, 5'-H), 2.97 (1p,m,2'-H), 1.94 (1p,m,2'-H).
U.V. visible (H_2O) λ max observed at 244 and 291 nm.
Peak C is the peak of deoxyguanosine which is obtained in all control reactions.

RESULTS AND DISCUSSION

When 9-OH-NME was incubated with deoxyguanosine, in presence of $FeCl_3$, EDTA and H_2O_2, the HPLC analysis of the solution indicated the presence of two main compounds derived from dG (Fig. 1). Compounds A and B were identified by chromatographic behaviour as guanine and 8-hydroxydeoxyguanosine (8-OH-dG), respectively. To confirm the

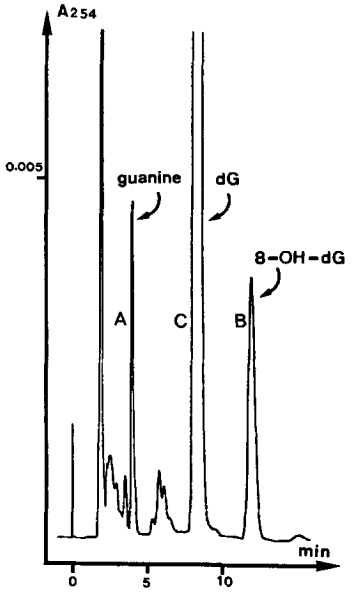

Fig. 1 : HPLC spectrum of the reaction after 1.5 hour incubation (see reaction conditions under materials and methods). The chromatogram was obtained from a 10 µl injection on a μC_{18} Bondapak colum.

structure of B, the compound was isolated and its UV-visible, ^1H NMR and mass data are consistent with the reported values for 8-OH-dG (14). For an initial concentration of dG equal to 1.3 mM, we obtained, after a 1.5 hours incubation at 37°C, 54 µM guanine (yield 4 %) and 94 µM 8-OH-dG (yield 7 %). When one of the reagents was omitted, neither guanine nor 8-OH-dG was observed. If we consider that 9-OH-NME, may reduces Fe^{III} salts, to give Fe^{II} species, then we might expect that dG is degraded by radicals generated by the Fenton reaction

$$Fe^{II} + H_2O_2 \longrightarrow Fe^{III} + HO^{\cdot} + HO^{-}$$

which is known to be activated by EDTA (15). Indeed, the disappearance of 9-OH-NME in the reaction mixture observed by HPLC suggest that this molecule has been oxidized while Fe^{III} is reduced. In fact, formation of both guanine and 8-OH-dG can be explained by the reactivity of $HO^{\cdot}$: guanine can be liberated after hydroxylation of a ribose ring and $HO^{\cdot}$ is known to be a strong hydroxylating agent for aromatic molecules. As confirmation, marked inhibition was observed when the reaction was

Table 1 : Inhibition (%) of guanine and 8-OH-dG formation by various components

inhibitor	guanine	8-OH-dG
10 mM mannitol	47	40
10 mM ethanol	52	46
10 mM benzoate (sodium)	80	72
10 mM thioura	94	90
10 mM DTPA	78	69
reaction under argon	27	10

Experimental conditions : a solution of 1.3 mM dG, 0.5 mM EDTA, 0.2 mM $FeCl_3$, 5.0 mM H_2O_2, 1.0 mM 9-OH-NME in 50 mM phosphate buffer PH 7.4 was incubated for 1.5 hour at 37°C, in presence of various components. Then reaction products were analyzed by HPLC as described under fig. 1. % inhibition was calculated for the different components.

performed in the presence of hydroxyl radical scavengers (Table 1). Inhibition of the reaction was also observed when we added diethylene triaminepentaacetic acid (DTPA), a chelating ligand known to inhibit the Fenton reaction (15). When the reaction was performed under argon, a slight inhibition was obtained, indicating that oxygen participates in the reaction but to a lesser extent.

To confirm the participation of HO$^{\bullet}$, we tried to detect the formation of radical-spin trap adducts by electron spin resonance (ESR) using the spin trap α-phenyl-N-t-butyl nitrone (PBN) (16). In the presence of 0.1 M PBN, we observed the signal shown in fig. 2A. (g = 2.0063, a_N = 16.0 G, a_β^H = 3.2 G), which strongly indicates that the hydroxyl radical adduct PBN/OH was obtained. This spectrum and that recorded in the presence of ethanol (10 %) (Fig. 2D) differ slighthy in the hyperfine coupling constants from the spectrum reported for hydroxyl adducts generated from bleomycin/ Fe(II) (2a) in the presence of ethanol. However, our spectrum recorded in the presence of ethanol was identical to the spectrum of Fenton reagent in the presence of ethanol (Fig. 2E), thus removing all doubt as to the nature of our adduct.

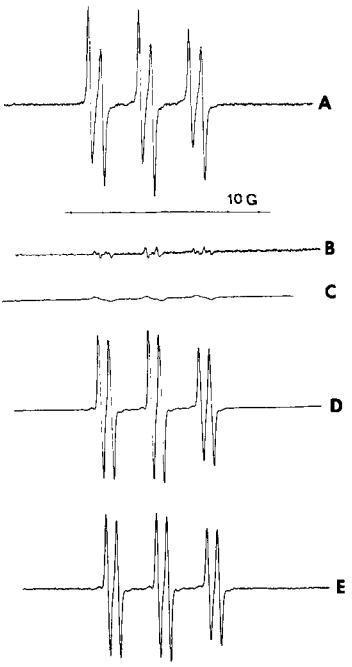

Fig. 2 : EPR experiments

Spectra were recorded on a Bruker ER 200 recorder equipped with an aqueous sample cell. Time constant : 0.2s, modulation amplitude : 0.5 Gpp, scan rate : 20 Gauss/min.

A. Spectrum obtained after a 1.5 hour incubation at 37°C of 1.3 mM dG, 1.0 mM 9-OH-NME, 5 mM H_2O_2, 0.2 mM $FeCl_3$, 0.5 mM EDTA, 50 mM phosphate buffer PH 7.4, 0.1 M PBN. Power : 16 mW, gain : 3.2×10^5 ($g = 2.0063$, $a_N = 16.0$, $a_\beta^H = 3.2$)

B. H_2O_2 was omitted, power : 16 mW, gain : 3.2×10^5

C. 9-OH-NME was omitted, power : 63 mW, gain : 10^5

D. Spectrum obtained as A, in presence of 10 % ethanol (v/v). power : 16 mW, gain 4×10^4 ($g = 2.0064$, $a_N = 16.0$, $a_\beta^H = 3.2$)

E. Spectrum obtained after 10 min incubation of 0.5 mM EDTA, 0.2 mM Fe^{II}, 5 mM H_2O_2 (Fenton reagents), 50 mM phosphate buffer PH 7.4, 0.1 M PBN, 10 % ethanol (v/v). Power : 16 mW, gain : 2×10^5, ($g = 2.0064$, $a_N = 16.0$, $a_\beta^H = 3.2$).

When Fe^{III}-EDTA was omitted, no signal was recorded.

These facts bear out the proposed mechanism in fig. 3 : (i) reduction of Fe^{III} by 9-OH-NME, (ii) generation of HO$^\cdot$ by Fe^{II}/H_2O_2, (iii) formation of guanine and 8-OH-dG by reaction of HO$^\cdot$ with dG. 8-OH-dG results from the hydroxylation at C_8, whereas guanine might be released from dG after an attack of HO$^\cdot$at the C_1 position of the sugar

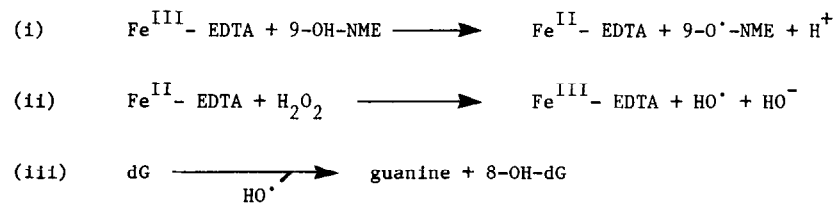

Fig. 3 : Proposed mechanism for the formation of guanine and 8-OH-dG from dG by interaction with 9-OH-NME and iron complexes.

ring as previously observed in the radiolysis of aqueous solutions of nucleosides (17).

When the system was extended to other nucleosides (A,U,G,C), deoxynucleosides (dA,dC,dG,dT) or deoxynucleotides (dAMP, dCMP, dGMP, dTMP), the liberation of the corresponding base was observed. For dinucleotides (ApG, GpA), both bases were released. Cu^{II} salts were less active in similar conditions than Fe^{III} complexes. Moreover, it is interesting to note that when we replaced 9-OH-NME by N^2-methyllellipti-nium (the corresponding non hydroxylated derivative of 9-OH-NME which is not antineoplastic) we detected neither guanine nor 8-OH-dG.

Biological implications : Generally, drugs which are able to generate radical species in the presence of transition metals induce DNA scission in cells (7). Many questions remain about the mechanism of the generation of the DNA breaks. In vitro, DNA breaks are produced from metal-drug-DNA interactions, and also by topoisomerase-drug-DNA interactions in the case of some drugs mentioned above : m-AMSA (18), adriamycin (19), 9-OH-NME (20) and Liu has proposed topoisomerase to be a common target for such antitumor drugs. Comparison of results obtained from m-AMSA and its inactive isomer o-AMSA, reveals a correlation between the abilities of these drugs (i) to produce DNA break on cells (21) (ii) to produce in vitro DNA breaks associated with topoisomerase (18) (iii) to interact with copper and thus generate DNA break in vitro (4). In the present study, we observed that 9-OH-NME reacts with $iron^{III}$, and degrades deoxynucleosides by generation of radical species,

and having in mind the results observed for m-AMSA, o-AMSA and other drugs, we propose that transition metals are the common feature in the cytotoxicity of the following drugs : elliptinium, anthracyclines, streptonigrin, bleomycin, mitomycin C, m-AMSA.

Thus, if we remember that iron is associated with the proliferation of neoplastic cells (22,23), and that antitumor drugs specifically kill neoplastic cells we ask ourselves if transition metals could be one of the possible keys in the understanding the selectivity of anticancer drugs toward neoplastic cells. Moreover, such an hydroxylation of dG has been observed with mutagenic substances (24), and in the case of 9-OH-NME, this reaction could be responsible of mutagenicity observed for this drug (25).

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