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Iron-dependent hydroxyl radical formation and DNA damage from a novel metabolite of the clinically active antitumor drug VP-16

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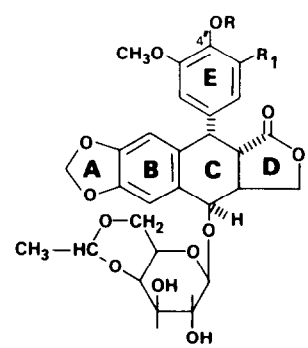
The dihydroxy derivative of VP-16 (DHVP), a novel metabolite of the clinically active antitumor drug VP-16, was cytotoxic to human breast tumor cells. It was found that DHVP chelates iron and catalyzes the formation of hydroxyl radicals from hydrogen peroxide and reduced glutathione. Ethanol, polyethylene glycol and *t*-butanol inhibited the formation of DMPO-OH, suggesting that perferryl iron was not involved in $OH^{\cdot}$ formation. Under conditions which formed hydroxyl radicals, DHVP also induced nicking of SV40 DNA, suggesting that the mechanism of tumor cell killing by DHVP may involve iron-dependent free radical formation.

VP-16; VP-16 catechol metabolite; Iron complex; Hydroxyl radical; Spin trapping

1. INTRODUCTION

VP-16 (fig.1) is a clinically active antitumor drug for the treatment of a number of human neoplasms. Studies to elucidate the mechanism of action have shown that VP-16 induces DNA damage in tumor cells and in isolated nuclei [1–3], and topoisomerase II has been identified as the intracellular enzyme responsible for these DNA strand breaks [4]. Since metabolic activation has been shown to be important in biological activities of a number of antitumor drugs, we have probed other biochemical factors responsible for VP-16-induced tumor cell killing. Recently, we have shown that VP-16 is activated by cytochrome P-450 and peroxidases (horseradish and prostaglandin synthetase) to reactive intermediates which bind irreversibly to cellular macromolecules [5–9]. We have identified two key metabolites of VP-16, *o*-dihydroxy (DHVP) and *o*-quinone (VP-16-Q) derivatives, as the potentially damaging and cytotoxic metabolites (fig.1).

Catechols are known to chelate metal ions, to form semiquinone radicals during oxidations, and to generate oxygen-dependent toxic free radicals. To identify the possible mechanism of tumor cell killing by DHVP and therefore of VP-16, we have



WHEN

R = H, R_1 = OCH_3 = VP-16

R = R_1 = OH = DHVP-16

OR = R_1 = = O = VP-16-Q

Fig.1. Structures of VP-16, and its metabolites DHVP and VP-16-Q.

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examined the iron-dependent hydroxyl radical formation from this novel metabolite of VP-16, and the DNA damage in vitro in the presence of DHVP, hydrogen peroxide and reduced glutathione (GSH).

2. MATERIALS AND METHODS

VP-16 was a gift from Bristol-Myers Co. (Syracuse, NY). The *o*-dihydroxy derivative of VP-16, prepared from VP-16 by oxidative *o*-demethylation followed by the reduction of the *o*-quinone, was provided by Dr Nemec of Frederick-NCI Research Facility, MD. Diethylenetriaminepentaacetic acid (DETAPAC), and 5,5-dimethyl-1-pyrroline oxide (DMPO) were obtained from Aldrich, WI. DMPO was purified by passing over activated charcoal before use. Highly polymerized calf thymus DNA (type I), RNA (purified from bakers yeast, type XI), and GSH were obtained from Sigma (St. Louis, MO). SV40 DNA was obtained from Bethesda Research Laboratories (Gaithersburg) and was used without further purification. DNA and RNA were freed of metal ions by extensive dialysis against 0.1% DETAPAC as in [10].

The ESR studies were performed using an IBM-Brucker (ER 200 D SRC) spectrometer operating at 9.5 GHz and equipped with an NMR gaussmeter (ER 035) and a TM cavity. The spin-trapping studies were carried out by reacting the pre-formed iron-DHVP complexes with 100 μM H_2O_2 in 1 ml phosphate-buffered saline (pH 7.4) containing 50 mM DMPO and recording the spectrum. The iron-DHVP complex was prepared by reacting 20 μM Fe^{3+} with increasing concentrations of DHVP (20–100 μM) at pH 3.0. When present, DNA or RNA (200 μg) was added to the pre-formed complex.

The antitumor activity of DHVP was determined by colony formation assay following a 1 h incubation with human breast tumor (MCF-7) cells grown in improved modified essential medium containing 5% fetal bovine serum and 50 μg/ml gentamycin. Briefly, the MCF-7 cells were trypsinized and 500 cells were seeded in triplicate in six-well Linbro dishes in 2 ml of the medium and allowed to attach for 18 h at 37°C. The medium was removed and DHVP dissolved in dimethyl sulfoxide (DMSO), diluted with serum-free medium, was added. Following 1 h DHVP exposure, the drug was removed and the cells were washed with medium. The cells were then supplemented with complete medium and allowed to grow for 12–14 days, stained with 0.5% methylene blue in 50% methanol, and the colonies were counted.

The DNA degradation induced by DHVP-iron complexes in the presence of either H_2O_2 or GSH was carried out as described [11]. Briefly, this consisted of reacting 100 μM SV40 DNA (>95% form I) with DHVP (dissolved in polyethylene glycol and diluted with 20 mM phosphate buffer, pH 7.4, in a final volume of 200 μl) in the presence or absence of ferric acetohydroxamic acid (100 μM). In the controls without Fe^{3+}, 1 mM desferal was added to DNA 5 min before the drug addition. The reaction was initiated by adding either H_2O_2 (100 μM) or GSH (0.25 mM) and incubation carried out for 2 h at 37°C. The reactions were then terminated by adding 1 mM desferal followed by two extractions with buffer-saturated phenol. The reacted DNA (20 μl) was then loaded onto 1% agarose gels and a constant current (80 V, 2.5 h) was applied in Tris-acetate buffer. The gels were stained with ethidium bromide and pictures were taken under ultraviolet light.

3. RESULTS AND DISCUSSION

The relative cytotoxicity of VP-16 and DHVP is shown in fig.2. While VP-16 is fairly active in the MCF-7 cell line with an IC_{50} (concentration required to inhibit 50% of colony formation) of 5 μM, DHVP was less active (25%) with an IC_{50} of 20 μM under the same conditions. The decreased activity of DHVP is probably due to the fact that it is more polar than the parent drug. When added exogenously to cells under tissue culture conditions, polar drugs are not taken up, and thus the intracellular concentrations are not comparable.

Since DHVP is a catechol, and the mechanism of this antitumor activity of DHVP could involve formation of free radicals, we next examined iron-dependent $OH^{\cdot}$ formation using DMPO as a spin-trapping agent. Addition of H_2O_2 to DHVP or Fe^{3+} in the presence of DMPO formed very little DMPO-OH spin adduct. However, when H_2O_2 was added to a preformed DHVP-Fe^{3+} complex, a large increase in spin adduct formation was detected (fig.3), and the maximum DMPO-OH was formed at a drug/Fe^{3+} ratio of 2 (table 1). Addition of nucleic acids also stimulated the formation of $OH^{\cdot}$ (table 1). The presence of low-M_r polyethylene glycol or ethanol inhibited the formation of DMPO-OH adducts with concomitant formation of carbon-centered radical adducts with DMPO (fig.3) indicating the formation of free $OH^{\cdot}$. Since perferryl species may be involved in the iron-dependent free radical chemistry and have been shown to react with most of the quenchers of $OH^{\cdot}$ at an appreciable rate [12], we performed spin-trapping experiments in the presence of *t*-butanol. Perferryl species react with *t*-butanol at a much slower rate than the hydroxyl radical.

t-Butanol inhibited DMPO-OH formation in a dose-dependent fashion with the formation of a DMPO-carbon-centered radical adduct. These observations indicate that the perferryl species is not involved in the formation of OH radical from DHVP-iron complex and thus appears to be different from adriamycin, where formation of perferryl species has been implicated during the

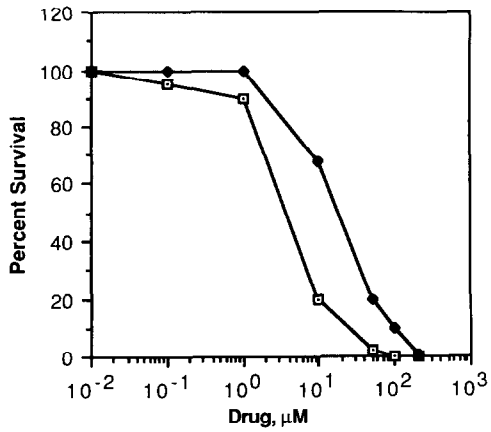

Fig.2. Relative cytotoxicity of VP-16 and DHVP in human breast tumor (MCF-7) cell line following 1 h drug treatment in colony formation assay. VP-16 (□); DHVP (■).

reductive activation of the drug in the absence of metal ion chelators [13].

Since free radicals have been shown to induce DNA damage from anthracycline-iron complexes [14,15], we next examined metal-dependent destruction of SV40 DNA. In the presence of H_2O_2, DHVP-Fe^{3+} complex was an effective catalyst for the nicking of SV40 DNA (fig.4A). DHVP or iron alone in the presence of hydrogen peroxide was without any effects. VP-16 in the presence of Fe^{3+} caused some (<20%) DNA cleavage indicating a slow formation of a reactive metabolite, most likely DHVP. Both 1:1 or 2:1 DHVP-Fe^{3+} complexes were equally effective in inducing DNA damage in the viral DNA. GSH was also effective when substituted for H_2O_2 in inducing this DNA damage (fig.4B) from DHVP-Fe^{3+} complex, however, hydrogen peroxide was a better reactant for the DHVP-Fe complex to induce DNA nicking.

Our previous studies have shown that VP-16 undergoes metabolic activation, catalyzed by either cytochrome P-450 or peroxidases, to form DHVP and VP-16-Q. We have postulated that these bioactivations of VP-16 result in the formation of potentially cytotoxic species [5–9]. Studies reported here show that the dihydroxy derivative of VP-16 is cytotoxic to MCF-7 tumor cells although it is less active than the parent drug. However, the lower antitumor activity of DHVP may be due to poor bioavailability to the cells in

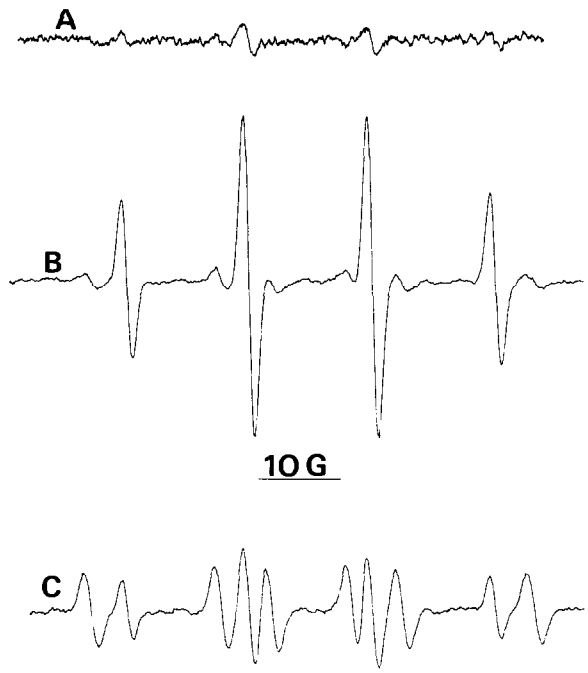

Fig.3. ESR spectrum of the DMPO-OH formed in the presence of 100 μM H_2O_2 from (A) Fe^{3+} (20 μM), (B) DHVP (40 μM)-Fe^{3+} (20 μM) and (C) identical to (B) except the reaction mixture contained 2% polyethylene glycol. ESR settings: field, 3480 G; modulation frequency, 100 kHz; modulation amplitude, 2.0 G; microwave power, 20 G; receiver gain, 5×10^4.

tissue culture system as a result of increased polarity of DHVP. We speculate that the production of hydroxyl radical is a mechanism for the antitumor effects. We have shown that both peroxide and

Table 1

Hydroxyl radical formation from DHVP-Fe^{3+} as a function of drug/iron ratio and effects of nucleic acids

Reactant	Relative DMPO-OH
Iron, alone	1
DHVP-Fe (1:1)	16
DHVP-Fe (2:1)	32.5
DHVP-Fe (3:1)	30
DHVP-Fe (5:1)	32.5
DHVP-Fe (2:1) + DNA (200 μg)	45
DHVP-Fe (2:1) + RNA (200 μg)	52

The reaction mixtures contained in 1 ml: 20 μM Fe^{3+}, DHVP, and DMPO with or without nucleic acids. The reaction was initiated by adding 100 μM H_2O_2. The relative OH formation was obtained by monitoring the peak heights of the DMPO-OH signal and is given in arbitrary units

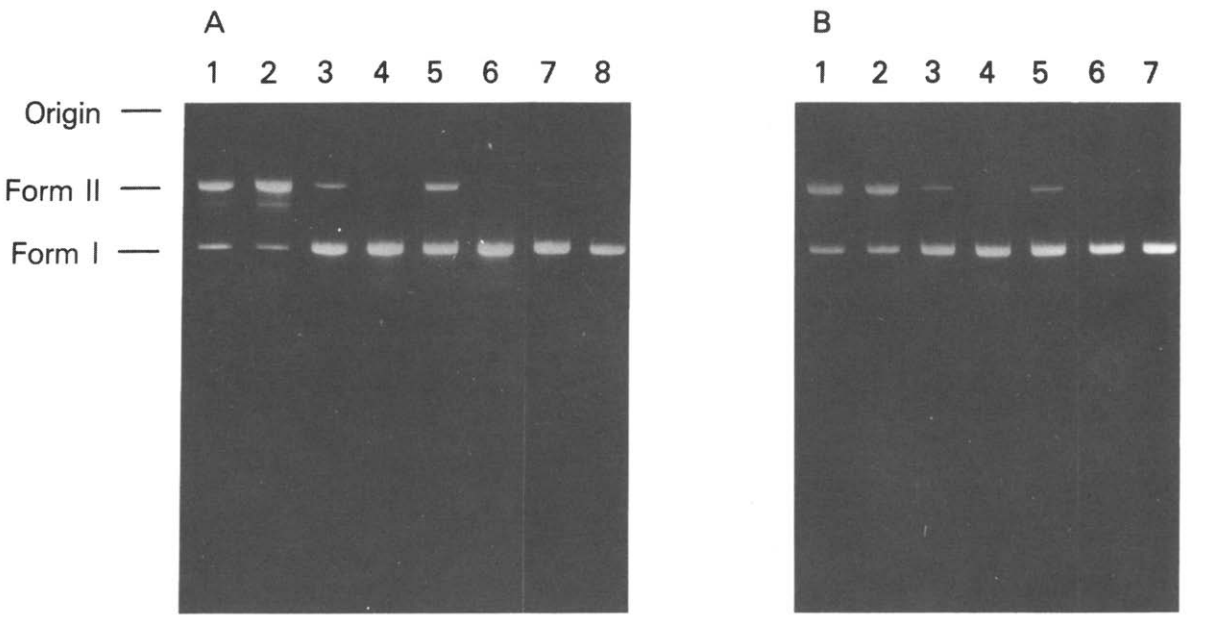

Fig.4. Hydrogen peroxide (A, 100 μM) and GSH (B, 250 μM) dependent DNA cleavage of SV40 by DHVP-Fe^{3+}. (A) (1) DHVP (200 μM)-Fe (100 μM); (2) DHVP (100 μM)-Fe^{3+} (100 μM); (3) Fe^{3+}; (4) DHVP (100 μM) desferal was added to DNA before DHVP addition; (5) VP-16 (100 μM)-Fe^{3+} (100 μM); (6) VP-16 (100 μM); (7) H_2O_2, and (8) control DNA. (B) (1) DHVP (200 μM)-Fe^{3+} (100 μM); (2) DHVP (100 μM)-Fe^{3+} (100 μM); (3) Fe^{3+} (100 μM); (4) DHVP (100 μM), desferal was added before DHVP addition; (5) VP-16 (100 μM)-Fe^{3+} (100 μM); (6) VP-16 (100 μM), and (7) control DNA. See section 2 for details.

GSH act as the reductants of oxygen to $OH^{\cdot}$ with the drug-iron complex. Since neither DHVP nor Fe^{3+} alone produced any significant amounts of $OH^{\cdot}$, it appears that DHVP chelation or binding to Fe^{3+} results in the reduction of Fe^{3+} to Fe^{2+} since peroxide-dependent $OH^{\cdot}$ formation requires Fe^{2+} in the Fenton system. A significant stimulation of $OH^{\cdot}$ formation in the presence of DNA or RNA would suggest that Fe^{2+} formed is stabilized from polymerization, and that this stabilized form is redox active as previously observed by Floyd and Lewis [16]. In the present study, we have identified DNA as one of the targets for the DHVP-dependent iron-mediated damage for the cytotoxicity of DHVP. The damage is catalyzed by the formation of hydroxyl radicals. It is of interest to note that in this mechanism of antitumor activity of DHVP and possibly VP-16, there is a direct radical-dependent DNA damage which does not require participation of topoisomerase II for its activity. It is interesting to speculate, however, that $OH^{\cdot}$ formed from DHVP-iron complex may oxidize SH groups of topoisomerase II causing its inactivation. It is not known at this time how iron would become available for this free radical-dependent DNA damage to occur in vivo. However, it must be pointed out that the formation of DHVP-iron-dependent free radical would be important in certain proliferating tumor cells which contain large iron pools [17,18]. Alternatively, iron may be mobilized from ferritin in reactions involving radicals [19,20].

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