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TYROSINASE-INDUCED FREE RADICAL FORMATION FROM VP-16,213: RELATIONSHIP TO CYTOTOXICITY

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Tyrosinase-dependent activation of hydroxybenzenes forms reactive compounds, including catechols and o-quinones, and some of which show antitumor activity against pigmented melanomas. Since VP-16 is a phenoxy-containing antitumor drug, forms free radicals and reactive o-quinones during peroxidative activation, we evaluated the cytotoxicity of VP-16 to both tyrosinase-containing and non-tyrosinase-containing tumor cells. Our results show that VP-16 is significantly more cytotoxic to B-16/F-10 melanoma cells than human MCF-7 breast tumor cells. Phenylthiocarbamide, an inhibitor of tyrosinase activity, selectively decreased VP-16 toxicity only in melanoma cells. Furthermore, VP-16 was readily activated to its phenoxy free radical intermediate by purified tyrosinase, indicating tyrosinase may play a role in VP-16 toxicity in pigminted melanomas.

KEY WORDS: Free radicals, VP-16, tyrosinase, pigmented melanomas, o-quinones, cytotoxicity.

INTRODUCTION

Etoposide (VP-16-213), a semi-synthetic derivative of podophyllotoxin, is one of the most active antitumor agents in the treatment of various malignancies.[1] While VP-16 has been shown to induce topoisomerase II-dependent DNA strand breaks in tumor cells, and good correlation has been found between the drug cytotoxicity and DNA damage, the precise cellular and molecular mechanism for tumor cell kill is not clear. Metabolic alteration, catalyzed by either cytochrome P-450 or peroxidases (horse radish and prostaglandin synthetase) in the dimethoxyphenol ring of etoposide has been implicated in its cytotoxicity.[2,3] This enzymatic activation results in the formation of both the dihydroxy- and the o-quinone-derivative of VP-16. The intermediate for the formation of both derivatives is the VP-16 phenoxy radical, formed as a result of the one-electron oxidation of the 4′-OH of VP-16.[4] Recently, evidence has been presented to indicate that both of these metabolites cause DNA damage in a process that is independent of topoisomerase II, and leads to the inactivation of øX174 DNA.[5,6]

Tyrosinase is a copper-containing enzyme widely distributed in nature and is mainly involved in melanin biosynthesis. Tyrosinase hydroxylates phenols to form catechols and generates o-quinones. Thus activation of ∂-L-glutaminyl-hydroxybenzene and other phenols by tyrosinase is believed to result in the formation of active

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intermediates which show selective cytotoxicity towards malignant melanomas.[7-11] Since VP-16 is also a phenoxy compound, it is possible that tyrosinase may activate VP-16 to intracellulary to free radical intermadiates and other toxic species that kill melanotic melanoma cells. We have evaluated this possibility by comparing the cytotoxicity of VP-16 in B-16/F-10 melanoma and non-melanotic MCF-7 breast cancer cells. Our results show that VP-16 is selectively more toxic to F-10 melanoma cells possibly as the result of activation by tyrosinase to free radical intermediate and other species.

MATERIALS AND METHODS

Drugs and Reagent

VP-16 was a gift from Bristol Myers Co. (Syracuse, NY). Phenylthiocarbamide (PTC), tyrosinase, dimethyl sulfoxide (DMSO) and gentamicin were purchased from Sigma Chemical Company (ST. Louis, MO). Dulbecco Modified Eagle Medium (DMEM), Improved Modified Eagle Medium (IMEM), trypsin-EDTA, fetal bovine serum (FBS), and phosphate buffered saline (PBS) were purchased from Gibco Laboratories (Grand Island, NY). L-[3,5-^{3}H] Tyrosine (specific activity 49 Ci/mmol) and [methyl-^{3}H]-thymidine (specific activity 20 Ci/mmol) were purchased from Amersham (Arlingtn, IL). [^{3}H]-VP-16, labeled in the aromatic rings (900 mCi/mmol), was obtained from Moravek Biochemical, Inc. (Brea, CA).

ESR Study

The ESR studies for the detection of VP-16-derived free radicals following tyrosinase activation were performed by incubating VP-16 (100–250 μ) with tyrosinase (100–2000 U/ml) or F-10 cells (1×10^7 cells/ml; 70–80% confluent) in PBS at room temperature. The ESR spectrum was recorded on a ER 220 D IBM-Brucker spectrometer (9.5 GHz), equipped with a TM cavity.

Tumor cell culture and cytotoxicity assays

Mouse B-16/F-10 melanoma cells were maintained in DMEM medium supplemented with 10% FBS and 30 mg/500 ml of gentamicin. Human breast (MCF-7) cancer cells were grown in monolayer in IMEM medium with 5% FBS under standard culture conditions at 37° C in a humidified 5% CO_2 atmosphere. Ctyotoxicity assays were carried out by seeding 10,000 cells/ well in 6-chambered Limbro dishes (Coster, Cambridge, MA) in complete medium, and 24 hrs later fresh medium containing the appropriate concentrations of the drugs were added. The cells were incubated for 72 hrs, trypsinized, and counted with a model ZM Coulter counter (Hialeah, FL). Data were collected as triplicates.

^{3}H-TdR incorporation assay

The inhibition of DNA synthesis was evaluated by the ^{3}H-TdR incorporation assay. The cells were harvested from exponential-phase-maintained cultures by trypsin-

EDTA, suspended in medium and adjusted to 1×10^4 cells/ml. Cell suspensions were distributed into 96-well culture plates (Coster) at 1000 cells/well. Following 24 hrs of incubation, VP-16 was added in a broad range of concentrations in a volume of 0.1 or 0.5 ml/well, and the plates were incubated for another 72 hrs. Cells were distributed into 96-well plates and were treated with drugs. The cells were then pulsed for 3 hrs with 0.25 μCi of ^{3}H-TdR, cells were trypsinized for 20 min, harvested onto glass fiber filters (LKB, Finland) with a multichannel microharvester (Skarton, Sterling VA0), and then radioactivity was counted on a Beta Plate counter (cpm). Data were collected as replicates of 6 wells.

Tyrosinase Assay

The method used was a modification of the procedure of Pomerantz[12] by Kern *et al.*[11] The medium was removed from 25-cm^2 culture flasks (Coster) containing cells at 70% confluency and replaced with 2 ml of fresh culture medium containing 10 μCi of L-[3,4-3H] tyrosine. Following 24 hrs incubation, 100 μl aliquots were added to AG 50 W $\times$ 8 poly-prep prefilled chromatography columns (Bio-Rad laboratories, Richmond, CA) previously equilibrated with 0.1 mM citrate buffer (pH 7.2). Tritiated water was washed off the column with 4 ml buffer, aliquots of 100 μl were added to 10 ml Hydroflor, and counted by liquid scintillation counter (Packard Instrument Co., Downers Grove, IL). The limit of detection under these assay conditions was 0.2 unit of tyrosinase/ml.

RESULTS

Electron Spin Resonance Study

The incubation of VP-16 with purified tyrsinase resulted in the formation of the VP-16 free radical. The radical spectrum consisted of multi-lines (Figure 1) and the hyperfine coupling constants were identical to those formed by peroxidase systems from VP-16[13], indicating that radical formed during tyrosinase activation was the phenoxy radical. However, when VP-16 was incubated with either F-10 or MCF-7 cells, no VP-16 free radical was detected (data not shown), possibly due to a rapid reduction by cellular reductant.

Cytotoxicity of VP-16 to F-10 and MCF-7

The ctyotoxicity of VP-16 to the two cell lines was evaluated following the drug exposure for 72 hrs using DNA synthesis inhibition and cell proliferation assay. Data (IC_{50}) presented in Table-I show that significatly (5–10–fold) higher concentrations of VP-16 were required either to inhibit DNA synthesis or to kill the MCF-7 cells compared to F-10 melanoma cells. Because the differential toxicity of VP-16 to F-10 melanoma cells may have resulted from tyrosinase activation of VP-16 to a reactive species e.g., 0-quinone, we compared the relative activity of this enzyme in the two cell lines. As shown in Table-I, the tryosinase activity in F-10 melanoma cells was 76.7 ± 4.27 units/10^7 cells compared to 0.1 units/10^7 cells for MCF-7 cells.

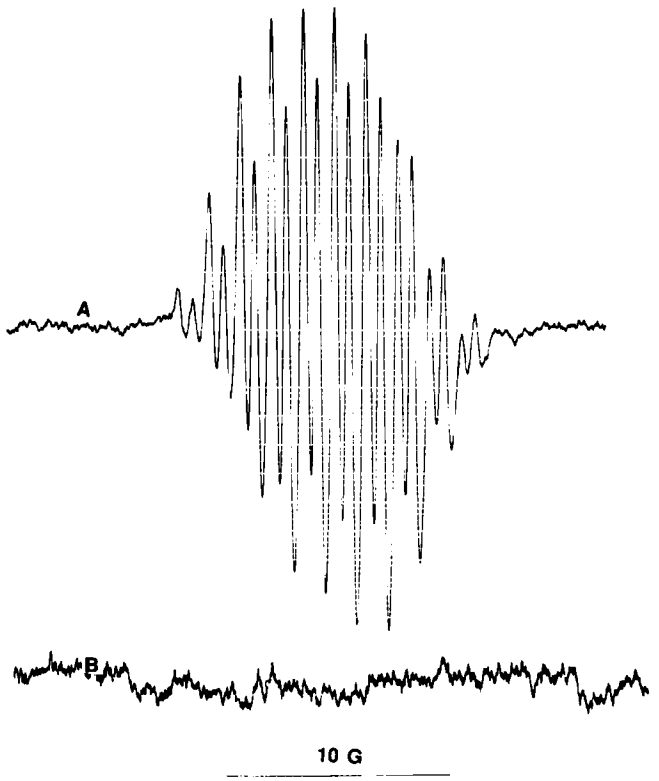

FIGURE 1. The electron spin resonance spectrum obtained during incubation of VP-16 (250 μM) with (A) purified tyrosinase (220 U/ml) and (B) F-10 cells (1×10^7/ml) in PBS buffer at room temperature. The ESR settings were: Field = 3480 G; modulation amplitue = 0.1 G; scan range = 50 G and the receiver gain 1.25×10^5 for (A) and 1×10^6 for (B).

TABLE I
Relative cytotoxicity of VP-16 and tyrosinase activity in F-10 melanoma and MCF-7 breast tumor cells

Cells	Median Doses (nM) ^{3}H-TdR*	Cell Proliferation†	Tyrosinase (units/10^7 cells)
F-10	5.3 ± 1.2	31.5 ± 2.2	76.7 ± 4.3
MCF-7	57.1 ± 4.4	146.2 ± 10.7	<0.1

*The results from ^{3}H-thymidine incorporation assay.
†The results from growth inhibition assay.

Inhibition of tyrosinase activity by phenylthiocarbamide (PTC)

Because PTC inhibits tyrosinase activity[11], we examined its effect on VP-16 toxicity in both DNA synthesis and cell survival assays. As shown in Figure 2 the cytotoxicity of VP-16 to F-10 cells was inhibited by PTC in both the DNA inhibition and the tumor cell survival assays. In contrast, PTC did not alter the toxicity VP-16 to MCF-7 cells (Figure 3). Under these experimental conditions, PTC alone (up to 100 μM) was not toxic to cells.

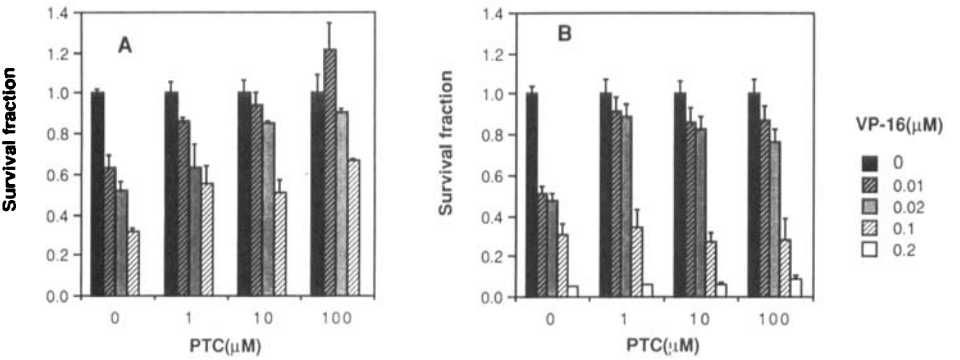

FIGURE 2. Effects of phenylthiocarbamide (PTC) on VP-16 – induced inhibition of cell survival (A) and DNA synthesis (B) in F-10 cells.

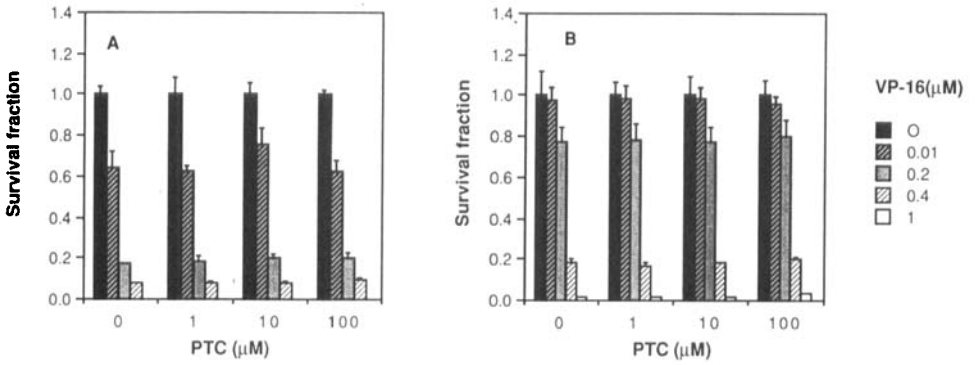

FIGURE 3. Effects of phenylthiocarbamide (PTC) on VP-16-induced inhibition of cell survival (A) and DNA synthesis (B) in MCF-7 cells.

Discussion

The selective toxicity of l-dopa for melanoma cells has been reported by Wick *et al.*[7-9] Additionally, Kern *et al.*[11] have also reported that a number of synthetic catechol analogs, including β-[(p-hydroxyphenyl) amino] alanine and N^{δ}-(p-hydroxy-phenyl) ornithine, were selectively more cytotoxic to human melanomas than to non-melanoma cells. It was further shown that the cytotoxicity of these compounds required intracellular activation by tyrosinase to quinones as phenylthiocarbamide (PTC), a specific inhibitor of tyrosinase, inhibited both the cytotoxicity and the oxidation of l-dopa derivatives to the quinones.

Our studies show that tyrosinase catalyzed the oxidation of VP-16 to its phenoxy radical. The phenoxy radical has been shown to form o-quinone derivative in a unimolecular pathway. Although we were unable to demonstrate VP-16 radical formation during incubation with F-10 cells, it is possible that the VP-16 radical was formed and was rapidly reduced by glutathione or other reducing agents present in cells, as previously reported.[13] This conclusion is further supported by the observation

that preformed tyrosinase-generated VP-16 radical was reduced upon the addition of F-10 or MCF-7 cells lysates (data not shown).

This study also showed that F-10 cells were significantly more sensitive to VP-16 than MCF-7 cells in both DNA synthesis inhibition and cell proliferation assays. The level of tyrosinase activity is much higher in F-10 cells than in MCF-7. Furhtermore, PTC in a dose-dependent manner reversed VP-16 induced inhibition of DNA synthesis and cell killing in F-10 cell line. In contrast, PTC was without any significant on the cytotoxicity of VP-16 to MCF-7 cells. These observations indicate that tyrosinase plays a role in VP-16 activation toxicity in F-10 cells.

The principal cellular target of VP-16 is thought to be DNA.[14–17] Loike and Horwitz[14] reported that the introduction of DNA strand breaks is an important mechanism for VP-16 cytotoxicity and a free hydroxyl group in the C-4′ is required for activity. In recent studies, it has become clear that the nuclear enzyme DNA topoisomerase II plays an important role in VP-16 induced DNA strand breaks. VP-16 induces topoisomerase II-dependent DNA strand breaks in MCF-7 cells which is, in part, related to the drug cytotoxicity.[17] PTC did not inhibit VP-16 toxicity in MCF-7 cell line, indicating that PTC does not inhibit cleavagable DNA-Topo II complex formatin in MCF-7 cells. Therefore, it is likely that the PTC-induced modulation of VP-16 toxicity in tyrosinase-containing F-10 melanoma cells is, in part, independent of topoisomerase II mediated DNA damage and results from the activation of the drug by tyrosinase to free radicals and reactive intermediates which kill cells.

It has been suggested that the OH group in the C4′-position of the pendulum ring of VP-16 is involved in free radical formation, metabolism, DNA cleavage and tumor cell kill.[2–4,18,19] Peroxidative oxidation of VP-16 produce an number of metabolites including o-quinone drivatives through oxidative o-demethyllation.[3,4] Although the detailed mechanism of VP-16 activation by tyrosinase is still unknown, this enzyme catalyzes the formation of the phenoxy radical *in vitro* and may be one of the reasons for the increased sensitivity of F-10 melanoma cells to VP-16 relative to the non-tyrosinase-containing MCF-7 cells. Work is in progress to further evaluate the role and mechanisms of this novel tyrosinase-dependent activation pathway for VP-16 toxicity in pigmented melanomas.

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