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TYROSINASE-INDUCED PHENOXYL RADICALS OF ETOPOSIDE (VP-16): INTERACTION WITH REDUCTANTS IN MODEL SYSTEMS, K562 LEUKEMIC CELL AND NUCLEAR HOMOGENATES

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Etoposide (VP-16) is an antitumor drug currently in use for the treatment of a number of human cancers. Mechanisms of VP-16 cytotoxicity involve DNA breakage secondary to inhibition of DNA topoisomerase II and/or direct drug-induced DNA strand cleavage. The VP-16 molecule contains a hindered phenolic group which is crucial for its antitumor activity because its oxidation yields reactive metabolites (quinones) capable of irreversible binding to macromoleculer targets. VP-16 phenoxyl radical is an essential intermediate in VP-16 oxidative activation and can be either converted to oxidation products or reduced by intracellular reductants to its initial phenolic form. In the present paper we demonstrate that the tyrosinase-induced VP-16 phenoxyl radical could be reduced by ascorbate, glutathione (GSH) and dihydrolipoic acid. These reductants caused a transient disappearance of a characteristic VP-16 phenoxyl radical ESR signal which reappeared after depletion of the reductant. The reductants completely prevented VP-16 oxidation by tyrosinase during the lag-period as measured by high performance liquid chromatography; after the lag-period VP-16 oxidation proceeded with the rate observed in the absence of reductants. In homogenates of human K562 leukemic cells, the tyrosinase-induced VP-16 phenoxyl radical ESR signal could be observed only after a lag-period whose duration was dependent on cell concentration; VP-16 oxidation proceeded in cell homogenates after this lag-period. In homogenates of isolated nuclei, the VP-16 phenoxyl radical and VP-16 oxidation were also detected after a lag-period, which was significantly shorter than that observed for an equivalent amount of cells. In both cell homogenates and in nuclear homogenates, the duration of the lag period could be increased by exogenously added reductants. The duration of the lag-period for the appearance of the VP-16 phenoxyl radical signal in the ESR spectrum can be used as a convenient measure of cellular reductive capacity. Interaction of the VP-16 phenoxyl radical with intracellular reductants may be critical for its metabolic activation and cytotoxic effects.

KEY WORDS: Etoposide, tyrosinase, phenoxyl radical, ascorbate, glutathione, dihydrolipoic acid, K562 leukemic cells

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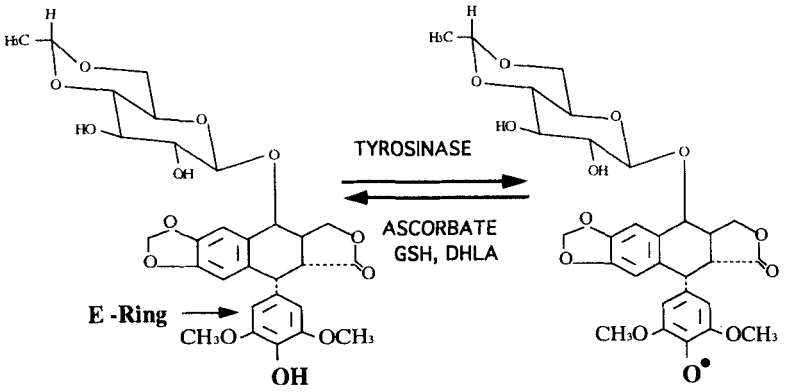

FIGURE 1 A scheme illustrating redox interconversions of VP-16 and its phenoxyl radical.

INTRODUCTION

Etoposide (VP-16) is an important antineoplastic agent used alone or in combination with other drugs in chemotherapy for several types of tumors [1]. The cytotoxicity of etoposide is considered to be dependent on a dual mechanism of DNA strand cleavage via inhibition of DNA topoisomerase II and/or direct DNA damage [2,3]. It has been suggested that metabolic activation of etoposide is essential for its cytotoxicity [4–8]. In particular, cytochrome P450-dependent monooxygenases, peroxidases, prostaglandin synthetase and tyrosinase may be involved in VP-16 metabolic activation [4–8].

Recently, Sinha *et al.* [9] demonstrated that VP-16 was significantly more cytotoxic to B-16/F-10 melanoma cells possessing high tyrosinase activity compared to MCF-7 breast tumor cells with low tyrosinase activity. Phenylthiocarbamide, an inhibitor of tyrosinase activity, selectively decreased VP-16 toxicity only in melanoma cells. Based on this data it was suggested that the VP-16 oxidation product formed by tyrosinase-catalyzed reaction (via intermediate formation of a VP-16 phenoxyl radical) possesses an enhanced cytotoxicity. However, direct ESR measurements failed to detect the tyrosinase-induced VP-16 radical intermediates in melanoma cells although the characteristic ESR signal of the VP-16 phenoxyl radical was observed in a model system containing purified tyrosinase [9].

VP-16 is a hindered phenol (Figure 1) and the presence of the phenolic 4′-hydroxy group in the benzene ring (E-ring) was found to be a structural prerequisite for its antitumor activity [3,10]. Phenoxyl radical is the first intermediate in peroxidative VP-16 activation which ultimately results in the formation of VP-16 quinone derivatives [6]. Interaction of phenoxyl radicals with reductants having appropriate redox-potential is known to result in the phenoxyl radical reduction, i.e. in the regeneration of the initial phenolic form of the compound [11,12]. This "regeneration" process has been found to be very efficient for relatively stable phenoxyl radicals generated from hindered phenols, e.g. vitamin E, butylated hydroxytoluene, and probucol [12–14].

We hypothesize that reduction of the VP-16 phenoxyl radical by intracellular reductants (in some intracellular compartments) may decrease the efficiency of its

metabolic activation and hence attenuate its cytotoxicity. In particular, this interaction of the VP-16 phenoxyl radical with reductants may be responsible for the lack of the ESR signal of the phenoxyl radical generated from VP-16 by its enzymatic activators (e.g. tyrosinase) in cell suspensions.

In the present work, we studied interactions of tyrosinase-induced VP-16 phenoxyl radicals with physiologically relevant reductants, such as ascorbate, GSH and dihydrolipoic acid in a model system. To further support our hypothesis, we tested whether oxidative depletion of endogenous reductants by a tyrosinase-catalyzed oxidative process was able to induce characteristic VP-16 phenoxyl radical ESR signals in K562 cell homogenates and nuclei suspensions. Finally, we compared the time-course of the VP-16 phenoxyl radical steady-state concentrations as measured by ESR with changes in oxidized VP-16 concentrations assayed by HPLC during tyrosinase-catalyzed oxidation in a model system or using K562 cell and nuclear homogenates.

MATERIALS AND METHODS

Preparation of K562 Cell Homogenates

Human leukemia K562 cells were grown in continuous culture in Dulbecco's modified Eagle's medium in the presence of 5% fetal bovine serum medium. Log-phase K562 cells (2.4×10^7) were centrifuged for 5 min at 1,000 *g* and the supernatant was discarded. Sedimented cells were washed four times by resuspending in phosphate buffered saline (PBS, 120 mL) and centrifuging under the same conditions. An aliquot of cell pellet (100 μL) was diluted using 200 μL of 0.1 M phosphate buffer (PH 7.4 at 25°C) and the suspension was sonicated (six 5-second pulses at ice-cold temperature) using a tip sonicator (Ultrasonic Homogenizer 4710 Series, Cole-Palmer Instrument Co., Chicago, IL).

Preparation of K562 Nuclear Homogenates

Nuclei from K562 cells were isolated by suspending cells in 1 mL of an ice-cold buffer A (pH 6.4) containing 1 mM KH_2PO_2, 5 mM $MgCl_2$, 150 mM NaCl, and 1 mM EGTA [15]. An additional 9 mL of the original buffer containing 0.3% Triton X-100 were added to lyse cells. After incubation on ice for 30 min, 40 mL of buffer A were added, and nuclei were pelletted by centrifugation at 1,000 rpm for 10 min, resuspended in 0.1 M phosphate buffer (pH 7.4 at 25°C) and sonicated (six 5-second pulses at ice-cold temperature) using a tip sonicator (Ultrasonic Homogenizer 4710 Series, Cole-Palmer Instrument Co., Chicago, IL).

HPLC Assay of VP-16 Oxidation in a Model System

VP-16 (0.5 mM) and tyrosinase (2.8 U/μL) were incubated in 0.1 M phosphate buffer (pH 7.4 at 25°C). Aliquots (10 μl) were taken at given time intervals and transferred from the reaction mixture into a glass tube containing 0.3 mL 50% CH_3OH. The dispersion thus formed was filtered through a C-18 cartridge (1 mL Sep-Pak Cartridge, Waters Division of MIILLIPORE, Millipore Co., Milford, MA) and the filtrate was used for the HPLC assay [16]. A C-18 reverse phase column (Ultrasphere ODS, 5μ, 4.6 mm × 25 cm, Beckman) was used for HPLC

determinations. Shimadzu LC-10A HPLC system was employed with LC-600 pump and SPD-10AV UV-detector (detection was by absorbance at 284 nm). Eluent was $CH_3OH:H_2O$ (55:46) adjusted to pH 3.1 by CH_3COOH. The flow rate was 1 ml/min. Under these conditions the retention time for VP-16 was 6.0 min. Control experiments have shown that the recovery of VP-16 using the above described filtration procedure was more than 98%.

Recording of the UV-absorbance difference spectra was made by stopping the eluent flow and scanning the spectrum. The data acquired were exported from the SPD-10UV detector using EZChrom software (Shimadzu), re-exported as ASCII files and plotted using SlideWrite Plus Software. The mobile phase spectrum was subtracted from the spectrum of the peak.

ESR Spectroscopy for Detection of the VP-16 Phenoxyl Radicals and Semidehydroascorbyl Radicals

ESR measurements were performed on a JEOL-RE1X spectrometer at 25°C in gas-permeable Teflon tubing (0.8 mm internal diameter, 0.013 mm thickness obtained from Zeus Industrial Products, Raritan N.J. USA) [13]. The permeable tube (approximately 8 cm in length) was filled with 60 μL of a mixed sample, folded into quarters and placed in an open 3.0 mm internal diameter ESR quartz tube in such a way that all of the sample is within the effective microwave irradiation area. Spectra were recorded at 335.5 mT center field, 10 mW power, 0.32 gauss modulation and 2.5 mt/minute scan time.

Recording of the kinetics of the VP-16 phenoxyl radicals was performed by field-lock at 336.3 mT using 0.32 × 1 mT field modulation, 5×10^2 gain, 1.0 sec time constant and scan time 64 min/360 mm.

Materials

Acetic acid, ascorbic acid, glutathione (reduced and oxidized), dihydrolipoic acid, sodium phosphates, tyrosinase were purchased from Sigma (St. Louis, MO). Methanol, CCl_4 (both HPLC grade) and dehydroascorbic acid were purchased from Aldrich (Milwaukee, WI). Deferoxamine mesylate was purchased from Ciba (NJ). Etoposide was a generous gift from Bristol-Myers Squibb Co.

RESULTS

Tyrosinase-Induced VP-16 Phenoxyl Radicals in a Model System

Addition of tyrosinase to a solution of VP-16 in a phosphate buffer results in an immediate appearance of an ESR signal (Figure 2) which had characteristic features of the previously reported VP-16 phenoxyl radical [9,17]. The ESR signal of the VP-16 phenoxyl radical was not observed in the absence of either tyrosinase or VP-16. Under conditions used, the magnitude of the VP-16 phenoxyl radical ESR signal was linearily dependent on the VP-16 concentrations within the range of 100-250 μM and was saturable at higher VP-16 concentrations (Figure 2). HPLC measurements showed that incubation with tyrosinase resulted in a time-dependent consumption of VP-16 and accumulation of its oxidation product(s) (Figure 3A). A characteristic absorbance maximum at 284 nm was detected in the UV-spectrum

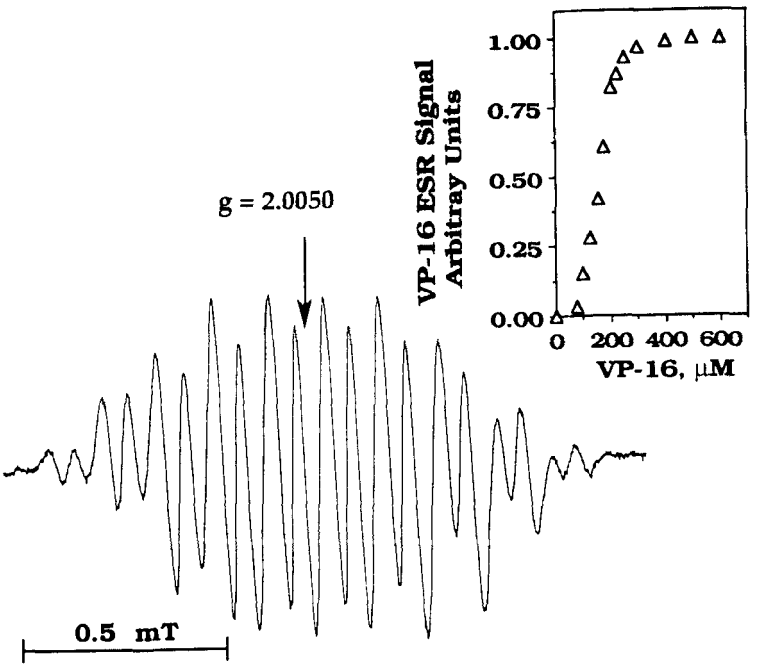

FIGURE 2 ESR spectrum of the tyrosinase-induced VP-16 phenoxyl radical in phosphate buffer. Incubation conditions: VP-16 (0.5 mM), tyrosinase (2.8 U/μL) and deferoxamine (250 μM) in 0.1 M phosphate buffer (pH 7.4 at 25°C). ESR conditions: center field 335.5 mT, sweep width 1 mT, gain 5×10^2, field modulation 0.32 × 0.1 mT. Insert: Dependence of the magnitude of the tyrosinase-induced VP-16 phenoxyl radical ESR signal on the concentration of VP-16.

of the VP-16 peak in the HPLC tracing (Figure 3B), while two new peaks with the maxima at 250 and 320 nm were observed in the spectrum of VP-16 oxidation product (Figure 3B). Based on the UV-absorbance spectrum of the product and its higher lipophilicity compared to VP-16 (an increased retention time in HPLC tracing), we suggest that the VP-16 oxidation product is a quinone. NMR and mass spectral identification of this VP-16 oxidation product is underway.

When ascorbate was added to the incubation medium, the VP-16 phenoxyl radical signal was not detected in the ESR spectrum; instead the doublet ESR signal of semidehydroascorbyl radical was observed (Figure 4A). This signal was transient and was substituted by the VP-16 phenoxyl radical ESR signal which appeared only after the semidehydroascorbyl radical ESR signal disappeared from the spectrum (Figures 5A,B). The duration of semidehydroascorbyl radical ESR signal was dependent on the concentration of ascorbate added (Figure 4B).

In the absence of tyrosinase and VP-16, slow autooxidation of ascorbate catalyzed by adventitious transition metals resulted in weak ESR signals of semidehydroascorbyl radicals in the ESR spectra. To avoid possible interference due to ascorbate autooxidation by adventitious iron, the same measurements were performed in the presence of deferoxamine (250 μM). In the presence of deferoxamine no semidehydroascorbyl radicals were detected in the ESR spectra (not shown). The time-course of semidehydroascorbyl and VP-16 phenoxyl radicals were not significantly different in the presence and in the absence of deferoxamine.

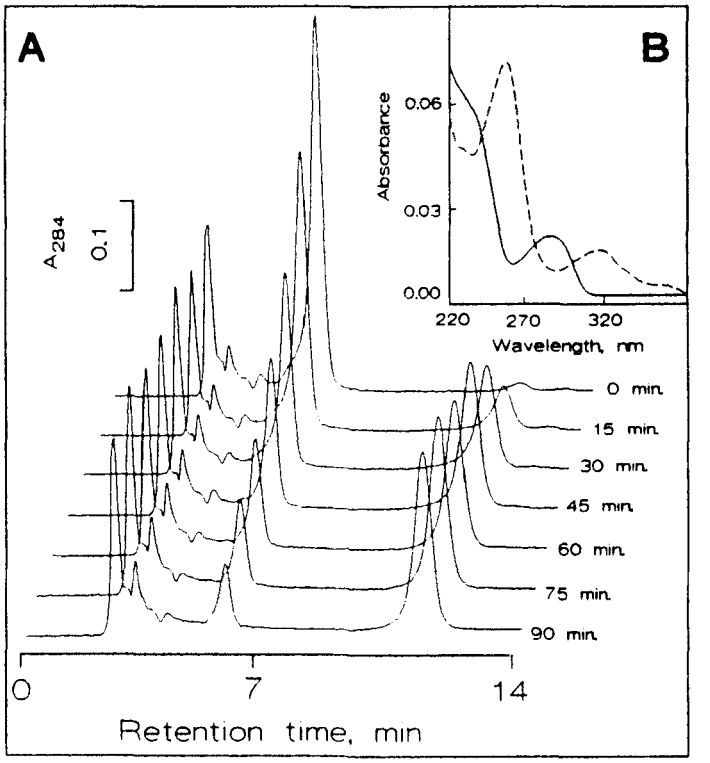

FIGURE 3 HPLC tracings of VP-16 and its oxidation product formed in the course of incubation with tyrosinase in phosphate buffer. Incubation conditions: VP-16 (0.7 mM) and tyrosinase (2.8 U/μL) in 0.1 M phosphate buffer (pH 7.4 at 25°C). Insert: UV-absorbance spectra of VP-16 (solid line) and its oxidation product (dashed line) for the two HPLC peaks with the retention times 5.5 and 11.2 min, respectively. Recording of the spectra was performed by stopping the eluent flow. Reference-eluent.

Signals of deferoxamine radicals were observed in the ESR spectra together with the VP-16 phenoxyl radical signals (Figure 4A). These signals of deferoxamine radical were reported previously [18] and were quenched by ascorbate.

The semidehydroascorbyl ESR radical signal could be observed upon incubation of ascorbate with tyrosinase (but without VP-16). In the absence of VP-16, the magnitude of the semidehydroascorbyl ESR signal was about two-fold smaller than in its presence and the signal declined very slowly (by 25% over 90 min incubation) indicating that a slow tyrosinase-dependent oxidation of ascorbate occurs (Figure 5B). In the presence of both tyrosinase, VP-16 and the same concentration of ascorbate, the semidehydroascorbyl radical ESR signal could be only observed for 30 min, after which it disappeared abruptly and was replaced by the VP-16 phenoxyl radical signal (Figures 4A,5A,B). The oxidized form of ascorbate, dehydroascorbate, did not change the time-course of the tyrosinase-induced VP-16 phenoxyl radical (data not shown).

HPLC tracings demonstrated that ascorbate was consumed in the course of incubation with tyrosinase and VP-16 (Figure 5D). No decay of VP-16 was observed until ascorbate was completely consumed (Figures 5C,D). After depletion of ascorbate the rate of VP-16 oxidation was the same as in the absence of ascorbate.

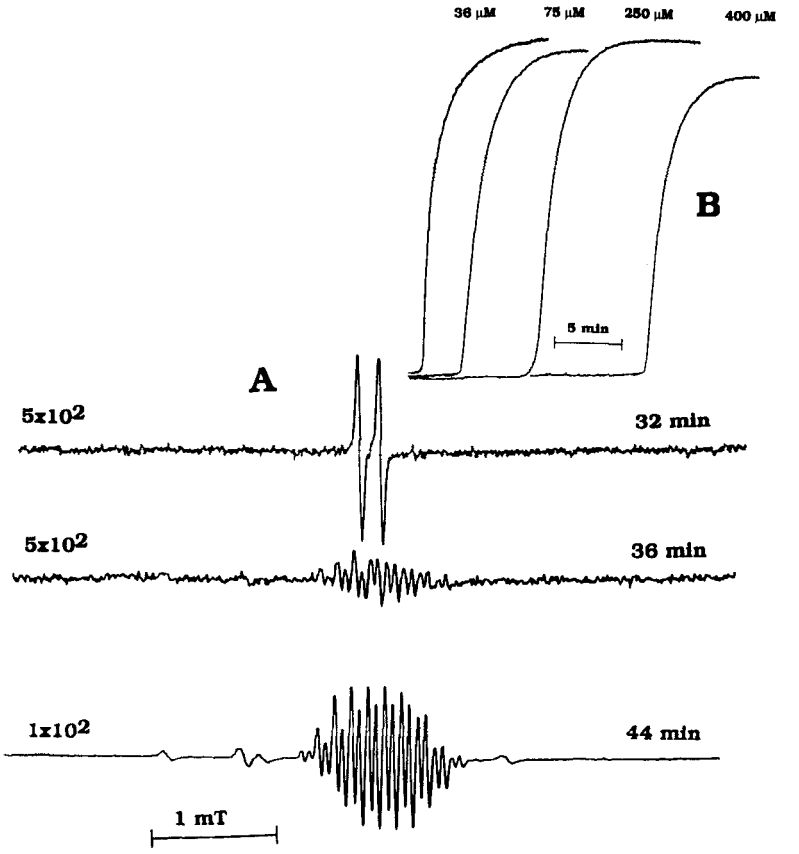

FIGURE 4 A. ESR spectra of the tyrosinase-induced semidehydroascorbyl radicals and VP-16 phenoxyl radical in phosphate buffer in the presence of ascorbate. Ascorbate concentration was 0.8 mM. Other conditions were as given in the legend of Figure 2. B. ESR-kinetics of the tyrosinase-induced VP-16 phenoxyl radicals in the presence of different concentrations of ascorbate. Ascorbate concentration were as indicated. Other conditions were as given in the legend of Figure 2.

In the absence of VP-16, a slow tyrosinase-catalyzed oxidation of ascorbate occurred. Ascorbate concentration decreased from 0.83 mM to 0.44 mM during a 30 min incubation with tyrosinase alone. In the presence of VP-16, ascorbate concentration was reduced to 0.04 mM at the end of the 30 min incubation period.

When reduced glutathione (GSH) or dihydrolipoic acid (DHLA) was added to the VP-16 solution in the presence of tyrosinase, the VP-16 phenoxyl radical signal could not be detected in the ESR spectrum; no other additional signals were observed (Figure 6). After a lag-period, the VP-16 phenoxyl radical ESR signal appeared in the spectrum and was persistent over time. The duration of the lag-period during which the VP-16 phenoxyl radical ESR signal was absent in the spectrum depended on the concentration of the reduced thiol added (Figures 7A,B). Oxidized glutathione (GSSG) and the oxidized form of dihydrolipoic acid (lipoic acid) did not prevent the immediate appearance of the characteristic VP-16 phenoxyl radical signal in the presence of tyrosinase (data not shown).

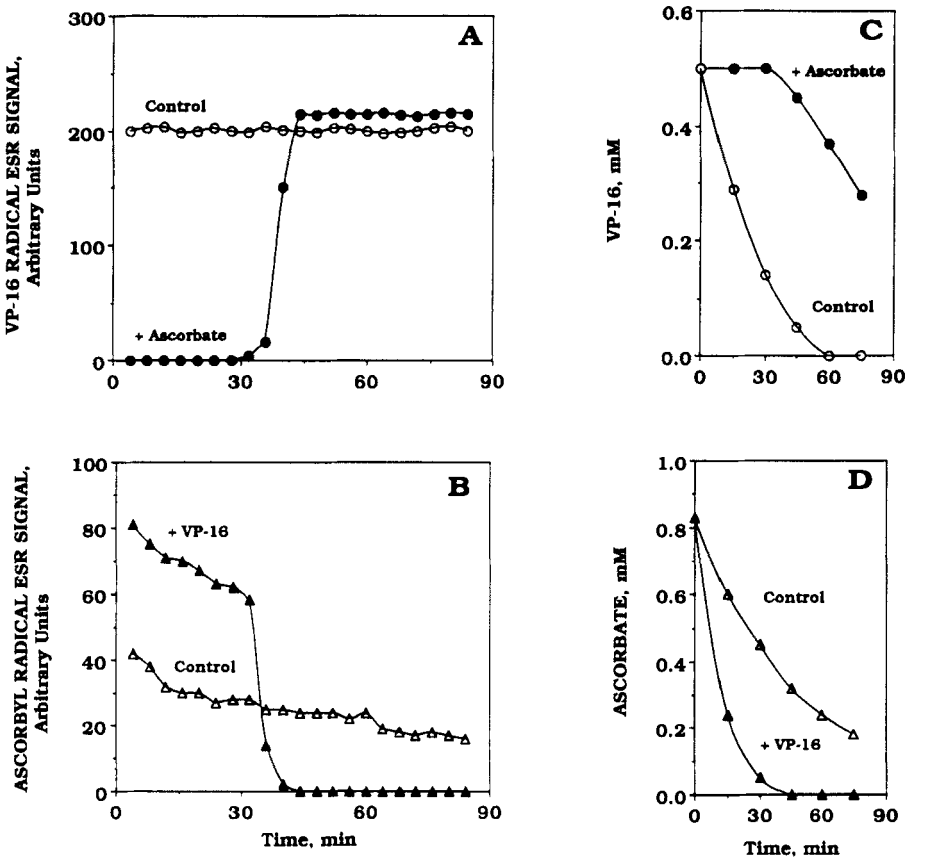

FIGURE 5 Time-course of VP-16 phenoxyl radicals (A) and of semidehydroascorbyl radicals (C) as measured by ESR and of VP-16 (B) and ascorbate (D) oxidation as measured by HPLC during tyrosinase-catalyzed VP-16 oxidation in the presence or absence of ascorbate. Ascorbate concentration was 0.83 mM. Other conditions were as given in the legend of Figure 2.

In accord with these ESR data, our HPLC measurements confirmed that VP-16 oxidation proceeded with a lag period during which the concentration of VP-16 did not change (Figure 7C). The duration of the lag-periods for the appearance of the VP-16 phenoxyl radical signal in the ESR spectra in the presence of GSH and DHLA (Figures 7A,B) were exactly the same as the lag periods observed for the onset of VP-16 oxidation in HPLC tracings (Figure 7C). Similar to results obtained in the presence of ascorbate (Figures 4-6), it is likely that only after GSH or dihydrolipoic acid were oxidized by the tyrosinase-induced VP-16 radical, did consumption of VP-16 begin. Neither GSSG nor lipoic acid affected the time course of VP-16 oxidation by tyrosinase (data not shown).

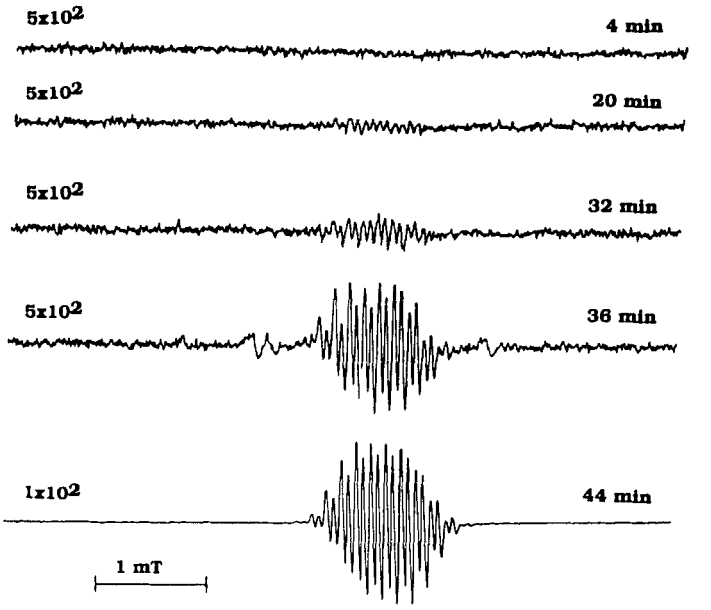

FIGURE 6 ESR spectra of VP-16 phenoxyl radicals generated by tyrosinase-catalyzed VP-16 oxidation in the presence of reduced glutathione (GSH). GSH concentration was 140 μM. Other conditions were as given in the legend of Figure 2.

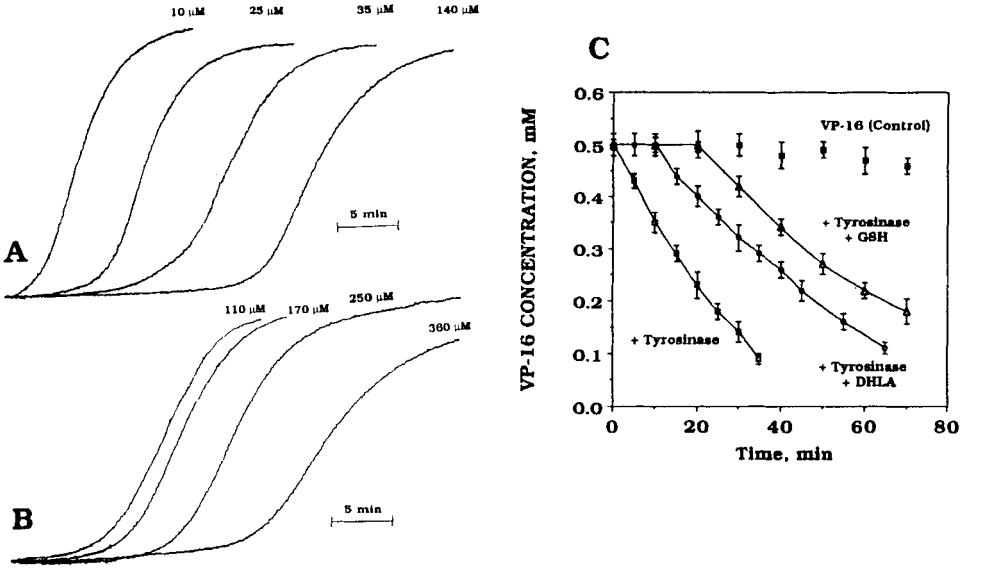

FIGURE 7 Effect of different concentrations of GSH (A) and DHLA (B) on the kinetics of the tyrosinase-induced VP-16 phenoxyl radical in the ESR spectra and on the kinetics of VP-16 oxidation as measured by HPLC (C). Incubation conditions were as given in the legend of Figure 2. A,B - concentrations of GSH and DHLA as indicated, C - concentrations of GSH and DHLA were 140 μM and 170 μM, respectively. Data are means ± SD. N = 4.

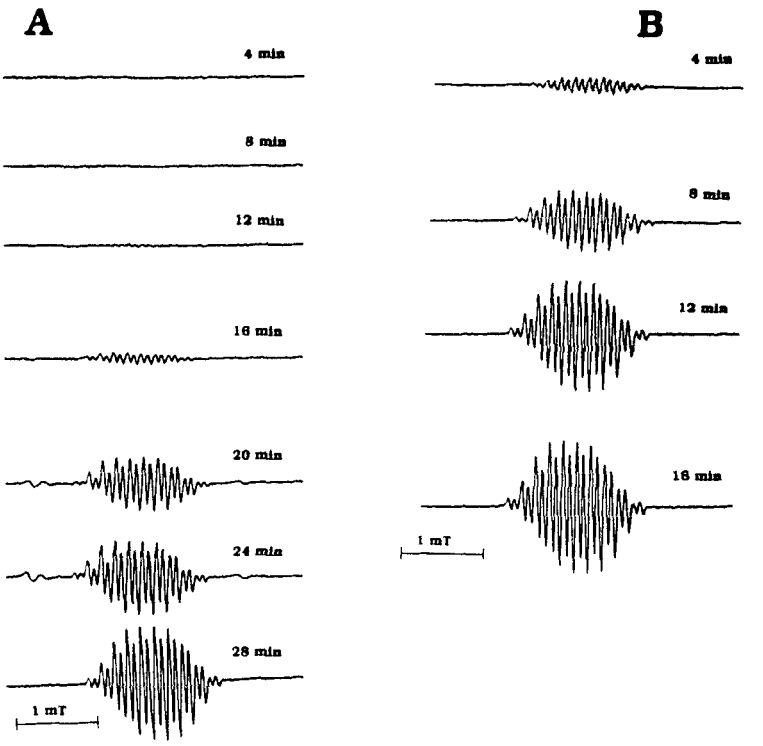

FIGURE 8 ESR spectra of VP-16 phenoxyl radicals generated by tyrosinase-catalyzed VP-16 oxidation in the presence of K562 cell homogenate (A) or nuclear homogenate prepared from K562 cells (B). A – homogenates prepared from 0.9×10^6 K562 cells were added the incubation medium. Other conditions were as given in the legend of Figure 2. B – number of nuclei was 1.2×10^6. Other conditions were as given in the legend of Figure 2.

Tyrosinase-Induced VP-16 Phenoxyl Radicals in K562 Leukemic Cell Homogenates

No ESR signals were detected upon addition of tyrosinase and VP-16 to K562 cell homogenates (Figure 8A). However, a characteristic ESR signal of the VP-16 phenoxyl radical appeared after a lag period and increased over time until it reached a maximum. The duration of the lag period before the VP-16 phenoxyl radical signal was observable in the ESR spectrum depended on the concentration of cells used to make the homogenate (Figure 9A). After obtaining the maximum VP-16 phenoxyl radical ESR signal (by incubating cell homogenates with VP-16 in the presence of tyrosinase) the signal could be quenched again by exogenous reductants (ascorbate, GSH, dihydrolipoic acid) added to the suspension (data not shown).

HPLC measurements revealed two phases in a tyrosinase-catalyzed VP-16 oxidation in the presence of cell homogenates: a lag period which corresponded to the lag-period for appearance of the VP-16 phenoxyl radical ESR signal, and a second phase during which VP-16 oxidation occurred (Figure 9C). The onset of the VP-16 oxidation coincided in time with the appearance of the VP-16 radical ESR signal (Figure 9A).

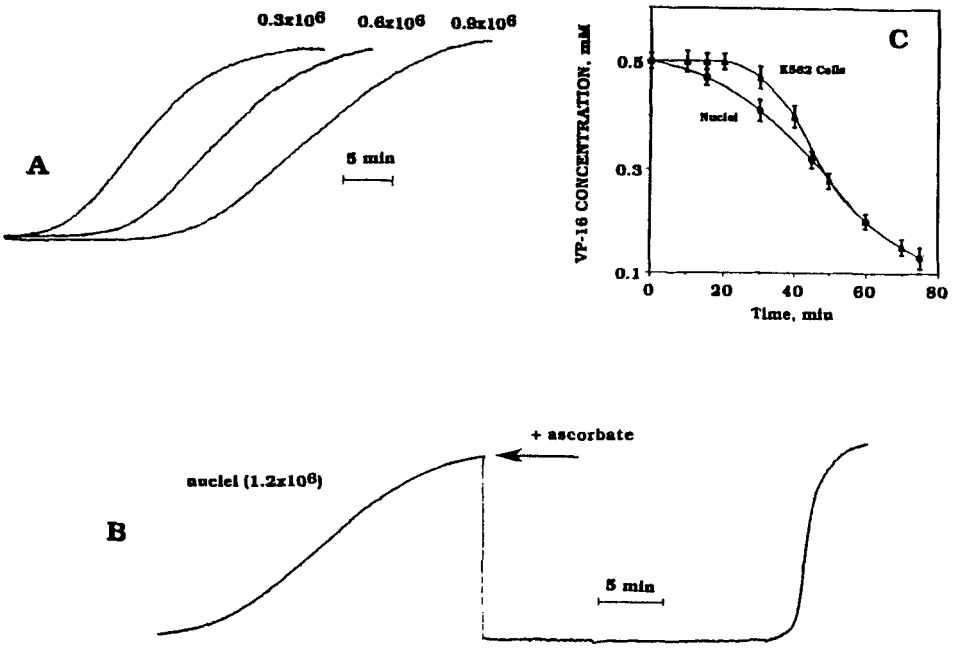

FIGURE 9 Effect of K562 cell homogenates (A) or nuclear homogenates (B) on the kinetics of the tyrosinase-induced VP-16 phenoxyl radical in the ESR spectra and on VP-16 oxidation as measured by HPLC (C). A - number of cells as indicated. B - number of nuclei as indicated, concentration of ascorbate 0.5 mM. C - number of cells and nuclei were 0.9×10^6 and 1.2×10^6, respectively. Other conditions were as given in the legend of Figure 2. Data are means ± SD. N = 4.

Tyrosinase-Induced VP-16 Phenoxyl Radicals in K562 Cell Nuclear Homogenates

In the nuclear homogenates prepared from K562 cells and incubated in the presence of VP-16 and tyrosinase, the maximum signal of the VP-16 phenoxyl radical appeared in the ESR spectrum only after a lag period (Figure 8B), similar to results obtained with K562 cell homogenates. The duration of the lag period was also dependent on the concentration of the nuclei in homogenate (not shown). However, in nuclear homogenates the durations of the lag periods were significantly shorter than those obtained for an equivalent number of K562 cells. After achieving a maximal level, the VP-16 phenoxyl radical signal could be quenched by addition of ascorbate (Figure 9B), GSH, or DHLA (not shown). The subsequent lag period before reappearance of the VP-16 phenoxyl radical ESR signal was prolonged by addition of these reductants, ascorbate (Figure 9B), GSH or DHLA (not shown).

In accord with the ESR data, our HPLC results demonstrated that the duration of the lag period in the VP-16 oxidation in the presence of nuclear homogenate was significantly shorter than in the presence of K562 cell homogenate (Figure 9C).

DISCUSSION

Intracellular oxidation of VP-16 is considered to be important for its mechanism of action either at the level of DNA topoisomerase II inhibition or direct DNA damage [2,3]. Formation of VP-16 quinone(s) may result in its efficient interaction with a criticial SH-group in topoisomerase II molecule [19]. Alternatively, the quinone form of VP-16 may be involved in the redox-cycling reactions producing oxygen radicals which then can directly oxidize DNA [20]. Both mechanisms may lead to VP-16-induced apoptotic cell death.

Recently we found that an enhanced inhibition of topoisomerase II and increased DNA strand breakage in K562 cells could be accomplished by using an exogenous source of free radicals (a lipophyllic azo-initiator of peroxyl radicals) which were able to react with the VP-16 phenolic group to initiate its oxidation [21]. In this case, cellular VP-16 oxidative activation was accomplished independently of intracellular metabolism. However, several enzymatic systems (cytochrome P450-supported oxygenases, peroxidases, prostaglandine synthetase, and tyrosinase) may be involved in VP-16 metabolic activation [4–9]. This may be particularly important for obtaining cytotoxic effects in the tumor cells which express high activity of VP-16 metabolizing enzymes. Consistent with this concept, Sinha *et al.* [9] demonstrated that cytotoxicity of VP-16 in B-16/F-10 melanoma cells was associated with its tyrosinase-catalyzed metabolism.

Reduction of the VP-16 Phenoxyl Radical in Model Systems

Phenoxyl radical is the first intermediate in VP-16 oxidation by tyrosinase or by peroxyl radicals (Figure 1) [8]. At the stage when the VP-16 phenoxyl radical is formed the reaction can be reversed if an appropriate reductant is present (Figure 1). The question is whether physiologically important reductants may be efficient in reducing the VP-16 phenoxyl radical, and if so, which of them are involved. To answer this question we studied the effects of three physiologically relevant reductants (ascorbate, GSH, DHLA) on tyrosinase-induced VP-16 phenoxyl radicals in a simple model system as well as in K562 cell homogenates and nuclei suspensions.

Tyrosinase-catalyzed oxidation of VP-16 was accompanied by the appearance of a characteristic ESR spectrum of the VP-16 phenoxyl radical in accord with previously published data [8–10,17]. The VP-16 phenoxyl radicals are fairly stable; their steady-state concentration was maintained by tyrosinase under the experimental conditions used, thus providing for persistence of the ESR signal over tens of minutes. All three reductants used – ascorbate, GSH and DHLA – were able to completely and transiently quench the ESR signal of the VP-16 phenoxyl radical producing a lag-period in VP-16 oxidation. Quenching of the VP-16 phenoxyl radical ESR signal by GSH in organic solvents has been demonstrated previously [22]. During the lag-period, the reductants completely prevented VP-16 oxidation measured by HPLC. The duration of lag-periods measured as an appearance of the VP-16 radical ESR signal or VP-oxidation was linearily dependent on the concentration of the reductant added. There was a very good correspondence between the lag-periods in tyrosinase-catalyzed VP-16-oxidation assayed by ESR- and HPLC-measurements.

The protective effects of reductants against VP-16 oxidation were not due to a tyrosinase inhibition; rather the reductants were able to regenerate VP-16 by

reducing the VP-16 phenoxyl radical formed in the course of tyrosinase-catalyzed VP-16 oxidation. This is evidenced by a complete reconstitution of both the magnitude of the VP-16 radical ESR signal and VP-16 oxidation rate after the lag-period was over. The protection afforded by the reductants against tyrosinase-catalyzed VP-16 oxidation was extremely efficient since no loss of VP-16 occurred during the lag period.

A slow tyrosinase-dependent oxidation of ascorbate took place in the absence of VP-16 as assayed by both ESR signals of semidehydroascorbyl radicals and HPLC-tracings of ascorbate. However, addition of VP-16 resulted in a several-fold stimulation of ascorbate oxidation. This suggests that reduction of the VP-16 phenoxyl radical by ascorbate was the major mechanism of ascorbate oxidation in the presence of tyrosinase and VP-16.

In contrast to ascorbate, which caused a very abrupt substitution of the semidehydroascorbyl signals in the ESR spectra for the VP-16 phenoxyl radical signals upon oxidation of ascorbate, both GSH and DHLA caused a slow and continual reappearance of the VP-16 phenoxyl radical ESR signal after its initial quenching. This can be explained by tyrosinase-catalyzed consumption of ascorbate both in the absence of VP-16 and in the reaction with the VP-16 phenoxyl radical or by a different reactivities of ascorbate and reduced thiols with VP-16 radicals. In fact, a much lower reactivity of reduced thiols with phenoxyl radicals as compared to that of ascorbate has been reported for the phenoxyl radical of acetaminophen [23] and tocopherol [24].

Reduction of the VP-16 Phenoxyl Radicals in K562 Cell an Nuclear Homogenates

The time course of the tyrosinase-induced VP-16 phenoxyl radical in K562 cell homogenates as measured by ESR or by VP-16 consumption assayed by HPLC was quite similar to that obtained in a model system in the presence of reductants. Oxidation of VP-16 could be observed by both ESR and HPLC only after an initial lag-period. The duration of the lag period in K562 cell homogenates measured by ESR or HPLC was similar and was dependent on cell concentration in the sample. The magnitude of the VP-16 phenoxyl radical ESR signals appearing in cell homogenates and the rate of VP-16 consumption after the lag-period were identical to those obtained in a model system with equivalent concentrations of VP-16 and tyrosinase. No consumption of VP-16 was detected during the lag period. This data suggests that VP-16 regeneration via reduction of its phenoxyl radical by intracellular reductants rather than inhibition of tyrosinase occurred in cell homogenates.

In K562 cell homogenates no semidehydroascorbyl radical ESR signals were detected during the lag-period (preceeding the appearance of the VP-16 phenoxyl radical ESR signal) as was observed in a model system upon addition of ascorbate; hence ascorbate is not likely to be the major intracellular reductant responsible for the VP-16 regeneration. The slow kinetics of the VP-16 phenoxyl radical ESR signal after a lag period were similar to those observed in a model system in the presence of reduced thiols (GSH, DHLA). Addition of ascorbate to K562 cell or nuclear homogenates after completion of the lag-period resulted in the same kinetics of quenching and abrupt reappearance of the VP-16 phenoxyl radical ESR signal after ascorbate oxidation (as was observed in a model system). Exogenously added GSH (or DHLA) caused a rapid quenching and a slow reappearance of the VP-16

phenoxyl radical ESR signal if added to K562 cell homogenates after the initial lag-period was over. Thus it seems likely that reduced thiols may primarily contribute to the VP-16 regeneration in K562 cell homogenates.

Comparison of the lag-periods measured by ESR and HPLC in K562 cell homogenates with those obtained for nuclear homogenates prepared from a equivalent amount of K562 cells shows that the former were much greater than the latter. This suggests that low molecular weight cytosolic reducing factors (e.g. reduced thiols) dominate in the VP-16 regeneration in K562 cell homogenates, while nuclear reductants (probably protein thiols) may be of minor importance. GSH concentration in K562 cells was reported to be as high as 5.4 nmol/10^7 cells [25]. In our experiments K562 cell homogenates prepared from 10^6 cells were diluted to 60 μL and caused a lag period of 20 min. Simple calculations show that GSH concentration in our K562 cell homogenates was in the range of 8.0–8.5 μM. Comparison with our measurements in a model system shows that these concentrations of GSH can produce a lag period of about 3 min. When 10 μM of GSH were added after completion of the lag period to the K562 cell homogenate incubated with tyrosinase and VP-16, it produced a new lag period of about 3–4 min (data not shown). Thus GSH-dependent reduction of the VP-16 phenoxyl radical may be only a fraction of the intracellular mechanism(s) responsible for VP-16 regeneration which may in turn prevent its oxidation to a cytotoxic quinoid form. Obviously, other components contributing to an overall intracellular reductive capacity should be identified. In this regard, clarification of the role of metallothioneins may be of great importance [26].

It is known that reduced thiols protect cells against injury by various antitumor drugs, carcinogens, radiation and oxygen-derived radicals [27]. In particular, GSH has been shown to protect DNA against the VP-16-induced damage by interacting with VP-16 quinones or semiquinones [28]. However, conjugates of quinones with GSH are known to have a higher redox-cycling activity as compared to their non-conjugated counterparts [29]. In our experiments, the oxidation of VP-16 in the presence of tyrosinase was completely prevented by thiols (GSH, DHLA) indicating that the formation of the conjugates did not happen during the lag period and that the thiol-dependent disappearance of the VP-16 phenoxyl radical ESR signal was due to the reduction of the phenoxyl radical. Thus, the results of this study demonstrate that interaction of the VP-16 phenoxyl radical with reduced thiols (GSH, DHLA) which efficiently prevent VP-16 oxidation to form VP-16 quinone(s) may be crucial in preventing VP-16 metabolic activation.

It is worth mentioning that some other antineoplastic drugs which may act as topoisomerase II inhibitors (such as hydroxylated ellipticines), also express their cytotoxic effects after oxidative activation through electron transfer reactions [30]. The therapeutic efficiency of these drugs may be dependent on the above described reactions of their phenoxyl radicals with intracellular reductants.

In conclusion, our results demonstrate that intracellular reductants may eliminate activation of VP-16 by oxidative metabolism by a mechanism involving reduction of the VP-16 phenoxyl radical. This may give a reasonable explanation for the lack of the characteristic tyrosinase-induced VP-16 phenoxyl radical signal in ESR experiments with cells as was previously reported by Sinha *et al.* [9]. A delicate balance between oxidants (including oxidative enzymes) and reductants may predetermine prevalence of oxidative over reductive metabolism of VP-16 in specific intracellular compartments. Shifts in this balance may be partially responsible for the drug resistance of tumor cells via mechanism(s) involving reduction of the

VP-16 phenoxyl radical. Deliberate depletion of intracellular reductants (e.g. by photosensitizers generating reactive oxygen species, azo-initiators of peroxyl radicals, overexpression of oxidative enzymes) may be used in the future to enhance cytotoxic effects of VP-16.

The procedure we have developed for measuring the duration of the lag periods for the tyrosinase-induced VP-16 phenoxyl radical appearance in cell homogenates can be used for a convenient evaluation of cell reductive capacity. Experiments are now underway to compare quantitatively the reductive properties of sensitive and VP-16 resistant K562 cell lines.

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