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Reduction of Phenoxyl Radicals of the Antitumour Agent Etoposide (VP-16) by Glutathione and Protein Sulfhydryls in Human Leukaemia Cells: Implications for Cytotoxicity

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Abstract—Phenoxyl radicals are inevitable intermediates in the oxidative enzymatic metabolism of a phenolic antitumour drug, etoposide (VP-16), by peroxidases, cytochrome *P*-450, prostaglandin synthetase and tyrosinase, as well as in its interactions with oxygen and peroxyl radicals. It has been shown that one-electron reduction of the VP-16 phenoxyl radical by ascorbate and thiols prevents/delays its oxidative metabolism by tyrosinase both in model systems and in cell homogenates. To elucidate the role of endogenous thiols in the reduction of VP-16 phenoxyl radicals, K562 human leukaemia cells grown in Dulbecco's modified Eagle's medium which does not contain vitamin C (ascorbate) were used, thus excluding the ascorbate-dependent reduction of VP-16 phenoxyl radicals. VP-16 phenoxyl radicals were reduced by endogenous reductants in K562 cell homogenates, intracellular thiols mainly being responsible. Depletion of endogenous thiols by mersalyl acid resulted in almost complete inhibition of the ability of cell homogenates to reduce VP-16 phenoxyl radicals. Three systems were used to evaluate the contribution of thiol-dependent reduction of VP-16 phenoxyl radicals: (1) K562 cell homogenates depleted or supplemented with glutathione (GSH) *in vitro*; (2) homogenates derived from K562 cells with a decreased level of endogenous thiols and GSH (using a specific inhibitor of γ-glutamyl cysteine synthetase, buthionine-*S*,*R*-sulfoximine; BSO) and (3) homogenates derived from K562 cells with increased content of endogenous thiols as a result of treatment with cadmium chloride. Depletion of thiols in K562 cells or cell homogenates proportionally decreased the ability of homogenates to reduce VP-16 phenoxyl radicals. Similarly, depletion or supplementation of K562 cells or cell homogenates with GSH proportionally decreased or increased the ability to reduce VP-16 phenoxyl radicals. Reduction of VP-16 phenoxyl radicals by K562 cell homogenates was similar to that obtained from cell homogenates isolated from K/VP.5 cells, a VP-16 resistant cell line derived from K562 cells. Elevation of endogenous thiols by cadmium chloride increased the ability of homogenates to reduce VP-16 phenoxyl radicals but did not reveal any significant difference in the ability of the two types of cells to interact with VP-16 radicals. Finally, BSO treatment of K562 cells led to potentiation of VP-16-induced DNA damage and to an increase in VP-16-induced growth inhibition, suggesting that, in the absence of ascorbate, modulation of endogenous thiols may be an important factor determining the oxidative metabolism and cytotoxic activity of VP-16 in tumour cells.

INTRODUCTION

Many phenolic compounds exert their toxic effects through the formation of quinone metabolites (Guyton *et al.*, 1991 and 1994). Etoposide (VP-16), a semi-synthetic derivative of podophyllotoxin (4′-demethylepipodophyllotoxin-9-(4,6-*O*-ethylidene-β-D-glucopyranoside), is one of the most widely used clinical antitumour drugs (Slevin, 1991). It is frequently used as a first-line drug for treating small lung cancer, germ cell tumours, lymphomas and, more recently, Kaposi's sarcoma associated with AIDS. The drug is also used to treat a variety of leukaemias including acute non-lymphocytic leukaemia. VP-16 stabilizes the covalent binding of

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Abbreviations: AMVN = 2,2′-azobis(2,4-dimethyl-valeronitrile); BSO = buthionine-*S*,*R*-sulfoximine; DFO = deferoxamine mesylate; DTNB = 5,5-dithio-bis-(2-nitrobenzoic acid); ESR = electron spin resonance; GSH = reduced glutathione; GSH-Px = glutathione peroxidase; SSB = single-strand breaks; topo II = DNA topoisomerase II.

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DNA topoisomerase II (topo II) to DNA resulting in DNA strand breakage (Corbett and Osheroff, 1993). Although VP-16-induced DNA damage has been correlated with its cytotoxicity, the precise molecular mechanism for drug action is not known. VP-16 contains a hindered phenolic ring, the presence of which is a critical structural prerequisite for its antitumour activity (Loike and Horwitz, 1976; Long *et al.*, 1984). It has been suggested that metabolic activation of VP-16 proceeds by way of intermediate formation of a phenoxyl radical (Sinha *et al.*, 1990), ultimately resulting in the VP-16 quinone derivatives (Haim *et al.*, 1987). This metabolic activation may be essential for its cytotoxicity at the level of inhibition of DNA topo II or through direct interaction of a VP-16 intermediate with DNA (Van Maanen *et al.*, 1988). Cytochrome *P*-450-dependent monooxygenases, peroxidases, prostaglandin synthetase and tyrosinase may be involved in VP-16 metabolic activation (Haim *et al.*, 1987; Sinha *et al.*, 1990; Van Maanen *et al.*, 1985 and 1988). Recently, Usui and Sinha (1990) demonstrated that VP-16 was significantly more cytotoxic to B-16/F-10 melanoma cells possessing high tyrosinase activity than to MCF-7 breast tumour cells with low tyrosinase activity. Phenylthiocarbamide, an inhibitor of tyrosinase activity, selectively decreased VP-16 toxicity only in melanoma cells. On the basis of this data, it was suggested that the VP-16 oxidation product(s) formed by tyrosinase-catalysed reaction (through intermediate formation of a VP-16 phenoxyl radical) possesses enhanced cytotoxicity.

The phenoxyl radical is an inevitable intermediate in the peroxidative activation of VP-16 by different oxidizing enzymes (Haim *et al.*, 1986; Kalyanaraman *et al.*, 1989). Formation of phenoxyl radicals may also be induced by interacting phenols with peroxyl radicals, resulting ultimately in accumulation of quinoid end-products (Sinha and Trush, 1983). Thus, peroxyl radicals generated by endogenous or exogenous sources may be used for VP-16 activation to a potentially therapeutically more efficacious form. In recent experiments, we demonstrated that the combination of VP-16 with a lipophilic azo-initiator of peroxyl radicals, 2,2′-azobis(2,4-dimethylvaleronitrile), AMVN, caused both a time- and concentration-dependent enhancement of VP-16-induced topo II/DNA binding, and potentiated VP-16-induced DNA single-strand breaks (Yalowich *et al.*, 1993a, b). Also, AMVN-dependent formation of the VP-16 phenoxyl radical was demonstrated by electron spin resonance (ESR) (Gantchev *et al.*, 1994). In preliminary experiments, incubation of human leukaemia K562 cells for 1 hr with a non-toxic concentration of AMVN (250 μM) resulted in a two-fold potentiation of VP-16-induced growth inhibition.

Intracellular reductants may prevent the formation of the VP-16 free radical intermediate and reactive quinones by the reduction of the VP-16 phenoxyl radical, thus providing a critical protective mechanism against the toxic effects of VP-16. In particular, ascorbate, reduced glutathione (GSH) and protein thiols are known to be major intracellular reductants (Buettner and Jurkewiscz, 1993; Kosower and Kosower, 1978). Phenoxyl radicals of several natural and synthetic phenolic antioxidants such as vitamin E, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have been shown to be reduced by ascorbate, resulting in antioxidant recycling (Mukai *et al.*, 1991; Packer *et al.*, 1979; Scarpa *et al.*, 1984). The phenoxyl radicals of these phenolic compounds, however, are not efficient in their direct interactions with thiols (Kagan *et al.*, 1992). We have found that VP-16 phenoxyl radicals can be directly reduced by low molecular weight thiols and protein thiols, both in model systems and in homogenates prepared from different cell lines, whereas other biomolecules (e.g. lipids, DNA) were not involved in direct interactions with the VP-16 phenoxyl radicals (Tyurina *et al.*, 1995). Thus, the VP-16 phenoxyl radical-induced oxidation of intracellular thiols may be viewed as a special sulfhydryl-selective type of oxidative stress. Elucidation of the mechanisms and hierarchy of interaction of these reductants with the VP-16 phenoxyl radical and with each other may be crucial for elaborating strategies to promote or to suppress VP-16 oxidative metabolism, that is, to enhance or to attenuate its cytotoxic effects in cancer cells and surrounding tissues, respectively.

The goal of the work described here was to study the role of endogenous GSH and protein thiols in the reduction of VP-16 phenoxyl radicals by cell homogenates. In addition, we examined the consequences of decreasing the level of endogenous thiols on the DNA-damaging and cytotoxic effects of VP-16. Because commonly used cell culture media are devoid of ascorbate (Freshney, 1987), we were able to evaluate directly the interactions of GSH and protein thiols with the VP-16 phenoxyl radicals, without any interference by ascorbate.

MATERIALS AND METHODS

Reagents

Sodium phosphate, GSH, 5,5-dithio-bis(2-nitrobenzoic acid), (DTNB), cumene hydroperoxide, glutathione peroxidase (GSH-Px), *o*-[(3-hydroxymercuri-2-methoxy-propyl)carbamoyl]phenoxyacetic acid (mersalyl acid), bovine serum albumin (BSA), tyrosinase and etoposide (VP-16) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Deferoxamine mesylate (DFO) was purchased from Ciba (NJ, USA). Sodium chloride was purchased from Fisher Scientific Co. (Pittsburgh, PA, USA). The Bio Rad protein assay kit and Chelex-100 chelating resin were purchased from Bio Rad Laboratories (Hercules, CA, USA).

Cells, media and incubation conditions

Human leukaemia K562 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and the VP-16 resistant clone K/VP.5 was derived from K562 cells in one of our laboratories (Ritke *et al.*, 1994b). Cells were grown in continuous culture in Dulbecco's modified Eagle's medium (DMEM) in the presence of 5% foetal bovine serum (FBS) medium. Every 3 months, new frozen stocks of cells were thawed for use. In some experiments K562 cells were treated for 24 hr with 0.5, 1.0 or 2.0 mM buthionine-*S,R*-sulfoximine (BSO) or for 24, 48 and 72 hr with 2.0 mM BSO. In addition, in some experiments K562 or K/VP.5 cells were incubated for 24 hr in the presence of 5 μM $CdCl_2$.

Drug-induced growth inhibition

Log-phase K562 cells were adjusted to 1.2×10^5 cells/ml and incubated for 48 hr in the presence or absence of 1 mM BSO and various concentrations of VP-16, after which cells were counted on a model ZBF Coulter counter (Coulter Electronics, Hialeah, FL, USA). The extent of growth (beyond the starting concentration of 1.2×10^5 cells/ml) in VP-16-treated versus control cells was ultimately expressed as percentage inhibition of control growth. The 50% growth-inhibitory concentration for VP-16 in BSO treated and untreated cells was calculated from replicate concentration–response curves generated from separate experiments.

Preparation of cell homogenates

Cells were centrifuged for 5 min at 1000 ***g*** and the supernatant was discarded. Sedimented cells were washed four times by resuspending in 0.15 M phosphate buffer containing 0.15 M NaCl, pH 7.4, and centrifuged under the same conditions. The cell pellet was suspended in 0.05 M phosphate buffer containing 0.1 M NaCl, pH 7.4 at 25°C (1.6×10^7 cells/ml). Then, the cell suspension was sonicated (six 5-sec pulses [10 W, 20 kHz] at ice-cold temperature) using a tip sonicator (Ultrasonic Homogenizer 4710 Series, Cole-Palmer-Instrument Co., Chicago, IL, USA).

Tyrosinase-catalysed oxidation of VP-16 in the presence and in the absence of cell homogenates

For measurements of VP-16 phenoxyl radical formation, VP-16 (0.6 mM) and tyrosinase (2.8 U/μl) were incubated in 0.05 M phosphate buffer containing 0.1 M NaCl and 100 μM DFO (pH 7.4, at 25°C) in the presence or absence of cell homogenates, as described earlier (Tyurina *et al.*, 1995). The phosphate buffer was pretreated with Chelex-100 to remove possible transition metal ion contaminants.

ESR spectroscopy for detection of the VP-16 phenoxyl radicals

ESR measurements were performed on a JEOL-RE1X spectrometer at 25°C in gas-permeable teflon tubing (0.8 mm i.d., 0.013 mm thickness) obtained from Alpha Wire Corp., (Elizabeth, NJ, USA). The tube (approx. 8 cm in length) was filled with 60 μl of a mixed sample, folded into quarters and placed in an open 3.0 mm i.d. ESR quartz tube in such a way that all of the sample was within the effective microwave irradiation area. Spectra of VP-16 phenoxyl radicals were recorded at 335.5 mT centre field, 20 mW power, 0.04 mT field modulation, 5 mT sweep width, 500 receiver gain, and 0.03 sec time constant.

Measurements of the lag period for the appearance of the VP-16 phenoxyl radical signal in ESR spectra

Cell homogenates (25 μl—4×10^5 cells) were added to the incubation mixture containing VP-16, tyrosinase and DFO (time = 0) and the final volume was adjusted to 60 μl with 0.05 M phosphate buffer (pH 7.4 at 25°C). The lag period was then determined as the time prior to an appearance of the VP-16 phenoxyl radical signal (arbitrarily chosen as $\times 2$ of background noise).

Assay of thiols in cell homogenates

Total thiol concentration in sonicated cell homogenates was determined by Ellman's assay (Ellman, 1959) using 200 μM DTNB. A standard curve was established by addition of GSH (10–100 μM) to 200 μM DTNB solution in phosphate buffer (10 mM, pH 7.4) and the thiol content was calculated. A UV 160U UV-visible recording spectrophotometer (Shimadzu) was used for determination (detection was by absorbance at 412 nm) using Shimadzu PC 160 PLS software.

Determination of GSH concentration in cell homogenates

GSH concentrations in cell homogenates were determined as the difference in DTNB-titrable thiols in the presence and in the absence of GSH peroxidase and cumene hydroperoxide. The sonicated cell homogenates were incubated with GSH-peroxidase (1.94 U/μl), cumene hydroperoxide (333 μM) and DFO (100 μM) for 30 min at 25°C. An aliquot of cell homogenate was then added to 200 μM DTNB, and the protein was precipitated by centrifugation for 5 min, 10,000 ***g*** at 4°C. The absorbance of the supernatant at 412 nm was determined.

In some experiments, aliquots of 1, 2, 3, 5, 6 and 8 μl exogenous GSH (1.5 mM) in 0.05 M phosphate buffer containing 0.1 M NaCl and 100 μM DFO (pH 7.4 at 25°C) were added to K562 cell homogenates and the final volume was adjusted to 60 μl with phosphate buffer (pH 7.4 at 25°C).

Depletion of thiols in cell homogenates by titration with mersalyl acid

K562 cell homogenates were incubated with an excess of mersalyl acid (400 μM) for 40 min at 25°C. Since mersalyl acid inhibits tyrosinase, the excess of mersalyl acid was eliminated by reacting it with GSH and the excess of GSH was removed with GSH-Px plus cumene hydroperoxide. The treated cell homogenates were then used to determine the lag period in the VP-16-tyrosinase system.

DNA damage assays

For DNA damage experiments, K562 cells were labelled for 48 hr with [2-^{14}C]thymidine (0.02 Ci/ml) in the presence or absence of 0.5 mM BSO. A separate aliquot of K562 cells were labelled for 48 hr with [*methyl*-^{3}H]thymidine (0.1 μCi/ml). Unlabelled thymidine was added to allow for a final thymidine concentration of 1 μM in the culture medium. VP-16-mediated DNA damage in BSO-treated or untreated cells was assessed using the alkaline elution technique for high-frequency single-strand breaks (SSB) (Kohn *et al.*, 1976). One million VP-16-treated K562 cells (previously labelled with [2-^{14}C]thymidine) were combined with 7.5 × 10^{5} internal standard K562 cells (containing [^{3}H]DNA) which had received 1500 rad irradiation. Cells were layered onto a polyvinyl chloride filter (pore size 0.8 μm; Gelman Sciences, Inc., Ann Arbor, MI, USA) in a 20 ml solution of ice-cold phosphate buffered saline and lysed with a solution of 2% sodium dodecyl sulfate, 10 mM disodium EDTA and 0.5 mg proteinase K/ml. DNA was eluted from the filter with tetrapropylammonium hydroxide, pH 12.1. The elution flow rate was 0.16 ml/min, with a fractional interval of 5 min. Cells containing [^{3}H]DNA were irradiated on ice with a ^{137}Cs source irradiator (J. L. Sheppard and Associates, Glendale, CA, USA). The frequency of VP-16-induced DNA SSB was quantified as the fraction of [^{14}C]DNA remaining on the filter when 75% of the ^{3}H-labelled internal standard DNA remains. A calibration curve for relating the frequency of VP-16-induced DNA SSB to a corresponding effect of radiation (radiation equivalent DNA damage) using ^{14}C-labelled cells was obtained by plotting rads *v.* [^{14}C]DNA retention at 75% retention of the [^{3}H]DNA internal standard.

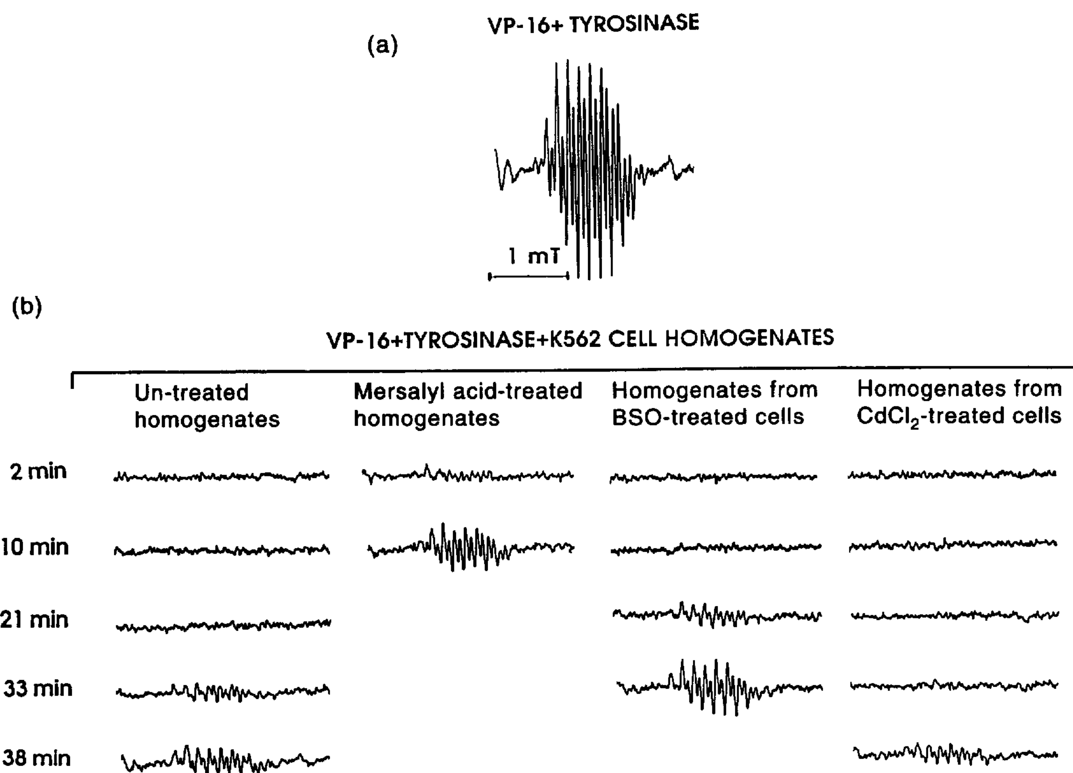

Fig. 1. ESR spectra of VP-16 phenoxyl radicals generated by tyrosinase alone (a) or when K562 cell homogenates were added together with tyrosinase (b). VP-16 (0.7 mM) was incubated with tyrosinase (2.8 U/μl) at 25°C in 0.05 M phosphate buffer (pH 7.4) containing 0.1 M NaCl and 100 μM deferoxamine. The ESR spectrum in (a) was recorded after 6 min incubation. In separate experiments, K562 cell homogenates (from an equivalent of 4 × 10^{5} cells) were added together with VP-16 and tyrosinase and ESR spectra were observed at the various times indicated in (b). Cell homogenates were depleted of thiols by treatment with 400 μM mersalyl acid, as described in Materials and Methods. In addition, K562 cells were depleted of thiols by treatment with BSO (2 mM) for 48 hr, following which homogenates were prepared. K562 cells were also treated with 5 μM CdCl$_2$ for 24 hr to increase total thiols, following which homogenates were prepared.

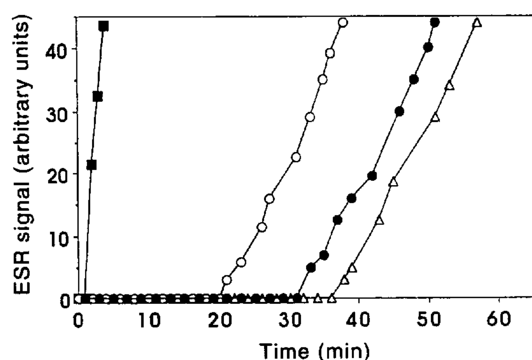

Fig. 2. Time course of formation of VP-16 phenoxyl radicals generated by tyrosinase in the absence (■) and in the presence of K562 cell homogenates. K562 cell homogenates were made from cells incubated in the absence (●) or presence of BSO (○) or $CdCl_2$ (△), as indicated in the legend to Fig. 1. Incubation conditions were identical to those described in Fig. 1.

RESULTS

Formation and reduction of VP-16 phenoxyl radicals: the role of total thiols

Tyrosinase-catalysed oxidation of VP-16 in phosphate buffer results in the formation of phenoxyl radicals with typical ESR spectral characteristics (Fig. 1a). The tyrosinase-induced VP-16 phenoxyl radical ESR signal reached its maximum within 5–7 min of incubation (Fig. 1a), after which time the signal was persistent and its magnitude did not decline over a 40–60-min period (data not shown). Immediately after addition of K562 cell homogenates to this oxidative system, no VP-16 phenoxyl radical ESR signal was observed (Fig. 1b). The signal appeared in the ESR spectrum only after a lag period (Figs 1b, 2) the duration of which was linearly dependent on the concentration of cells in homogenates (data not shown) and the thiol content in cell homogenates (Figs 2 and 3).

No ascorbate-dependent reduction of VP-16 phenoxyl radicals is expected to occur in K562 cell homogenates because the cell culture medium that was used for growing K562 cells does not contain vitamin C (ascorbate). Indeed, no characteristic ESR

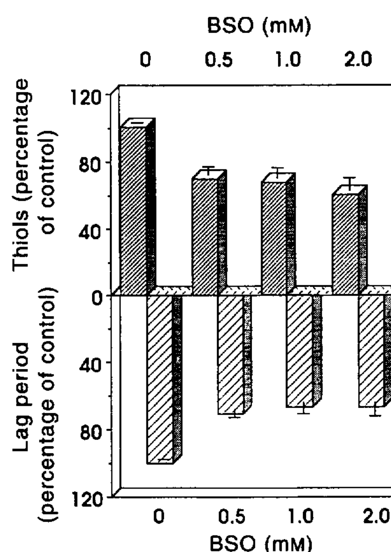

Fig. 4. Effect of 24 hr treatment of K562 cells with different concentrations of BSO on the cell thiol content and the lag period for appearance of the VP-16 phenoxyl radical ESR signal in the presence of K562 cell homogenates. Incubation conditions were identical to those described in Fig. 1. Columns represent the mean values from at least four determinations ±SD.

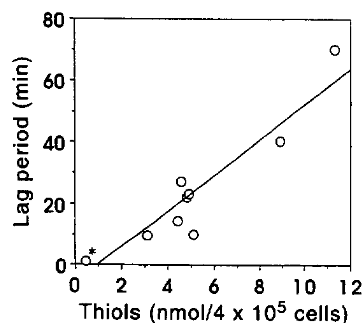

Fig. 3. Relationship between thiol content of K562 cell homogenates and the lag period for appearance of VP-16 phenoxyl radicals generated by tyrosinase. Incubation conditions were identical to those described in Fig. 1. Cell homogenates were made from different K562 cell cultures on different days. All homogenates were prepared after adjusting cell concentrations to 1.6×10^7 cells/ml, as described in Materials and Methods. The data point marked with an asterisk represents a K562 cell homogenate treated with 400 μM mersalyl acid to decrease thiol content. Based on linear regression analysis, the thiol content of cell homogenates was highly correlated ($\rho^2 = 0.89$) with the lag period for appearance of the VP-16 phenoxyl radical ESR signal.

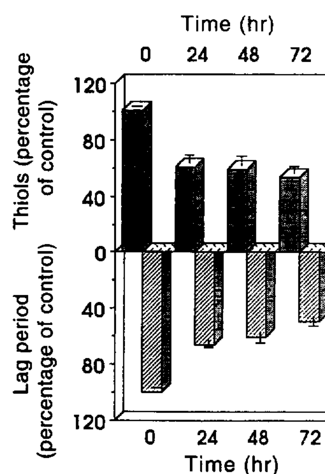

Fig. 5. Effect of 24–72 hr treatment of K562 cells with 2 mM BSO on the cell thiol content and the lag period for appearance of the VP-16 phenoxyl radical ESR signal in the presence of K562 cell homogenates. Incubation conditions were identical to those described in Fig. 1. Columns represent the mean values from at least four determinations ±SD.

Table 1. Effect of cadmium on thiol and GSH content in K562 and K/VP.5 cells and on the duration of lag period for appearance of VP-16 phenoxyl radical to ESR signal in the presence of cell homogenates prepared from these cell lines

Cell line	Thiols (nmol/4 × 10^5 cells) Total	GSH	Protein	Lag period (min) Total	GSH* dependent	Protein thiol* dependent
K562	9.3 ± 0.4	4.6 ± 0.1	4.7 ± 0.2	33.0 ± 1.0	17.0 ± 1.0	16.0 ± 0.5
K562 + $CdCl_2$	10.9 ± 0.6	5.6 ± 0.1	5.3 ± 0.2	38.0 ± 1.5	20.0 ± 1.5	18.5 ± 0.5
K/VP.5	10.1 ± 0.5	5.6 ± 0.4	4.5 ± 0.2	35.0 ± 1.0	20.0 ± 1.5	15.0 ± 0.5
K/VP.5 + $CdCl_2$	11.8 ± 0.6	7.0 ± 0.5	4.8 ± 0.2	41.2 ± 1.5	24.5 ± 2.0	16.5 ± 0.5

*Concentrations of GSH in cell homogenates were determined as the difference in DTNB-titrable total thiols in the presence and in the absence of GSH peroxidase and cumene hydroperoxide. Concentrations of protein thiols were determined as DTNB-titrable total thiols in the presence of GSH peroxidase and cumene hydroperoxide (for details see Materials and Methods).
Value are means ± SD (n = 4).

signal of the semidehydroascorbyl radical was detected when K562 cell homogenates were incubated with VP-16 and tyrosinase (data not shown). In contrast, in tissue homogenates and primary cell cultures (e.g. hepatocytes), we previously demonstrated that ascorbate provided for efficient reduction of VP-16 phenoxyl radicals (Kagan *et al.*, 1994). Thus, the role of intracellular thiols in reducing VP-16 phenoxyl radicals can be directly studied in cell culture by depleting and/or enriching K562 cells and/or homogenates with endogenous thiols.

We next studied the effect of the thiol reagent, mersalyl acid, on the time course of appearance of VP-16 phenoxyl radicals after addition of K562 cell homogenates (Fig. 1b). A 40-min incubation of K562 cell homogenates with 400 μM mersalyl acid resulted in a 10-fold decrease of endogenous DTNB-titrable thiols (data not shown) and almost a complete elimination of the lag period for appearance of the VP-16 phenoxyl radical ESR signal (Fig. 1b). The rate of the VP-16 phenoxyl radical generation by tyrosinase in the presence of mersalyl acid-treated homogenates was the same as in the control untreated cells (data not shown).

When K562 cells were treated with 0.5, 1.0 and 2.0 mM BSO for 24 hr, the total thiol content was reduced to 70, 68 and 61% of untreated K562 cell thiol content, respectively (Fig. 4). There was a corresponding decrease in the lag period for appearance of VP-16 phenoxyl radicals after addition of cell homogenates prepared from these BSO treated cells (Fig. 4). When K562 cells were treated for up to 72 hr

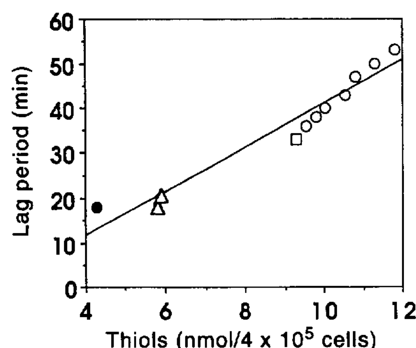

Fig. 6. Relationship between the changes in total thiols produced by GSH depletion or GSH supplementation of K562 cell homogenates and the lag period for appearance of VP-16 phenoxyl radicals generated by tyrosinase. In these experiments the K562 cell homogenates used contained 9.3 nmol thiol/4 × 10^5 cells, of which 4.6 nmol/4 × 10^5 cells was GSH (□). These K562 cell homogenates were either supplemented with exogenous GSH to increase total thiol levels (○) or were treated with GSH-Px and cumene hydroperoxide to deplete specifically homogenates of GSH (●). In addition, K562 cells were treated with 2 mM BSO for 48 hr prior to making homogenates in order to decrease GSH levels and hence total thiol levels (△). Incubation conditions were identical to those described in Fig. 1. Aliquots of 1, 2, 3, 5, 6 and 8 μl exogenous GSH (1.5 mM) in 0.05 M phosphate buffer containing 0.1 M NaCl and 100 μM DFO (pH 7.4 at 25°C) were added to K562 cell homogenates and the final volume was adjusted to 60 μl with phosphate buffer (pH 7.4 at 25°C). Based on linear regression analysis, the glutathione content of cell homogenates was highly correlated ($r^2 = 0.95$) with the lag period for appearance of the VP-16 phenoxyl radical ESR signal.

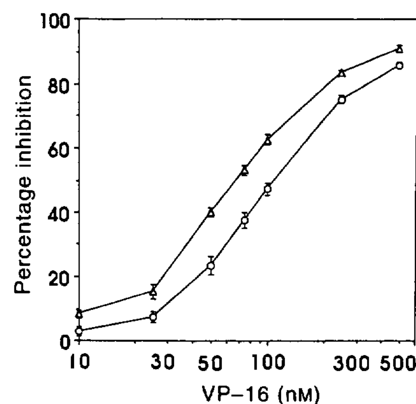

Fig. 7. Effect of BSO on VP-16-induced growth inhibition in K562 cells. Log-phase K562 cells were incubated for 48 hr in the presence (△) or absence (○) of 1 mM BSO and various concentrations of VP-16, after which growth inhibition was assessed as described in Materials and Methods. Under these experimental conditions BSO (1 mM) did not inhibit cell growth. Data points at each VP-16 concentration represent the mean from six separate experiments performed on separate days; bars indicate SE. Averaging results from six experiments, the 50% inhibitory concentrations were 108.5 ± 4.5 nM and 68.0 ± 1.5 nM for VP-16 alone and for VP-16 plus BSO, respectively ($P = 0.006$, Student's paired *t*-test).

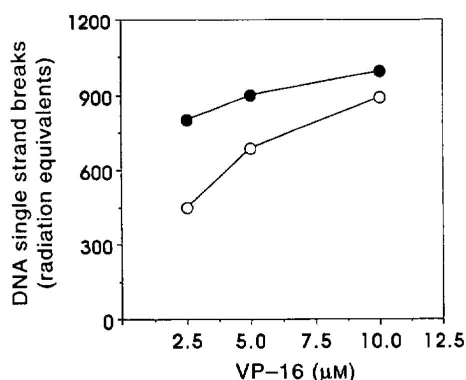

Fig. 8. Effect of BSO on VP-16-induced DNA SSB in K562 cells. K562 cells were preincubated for 48 hr with [2-^{14}C]thymidine (0.02 Ci/ml) in the presence (●) or absence (○) of 0.5 mM BSO. Cells were then treated for 1 hr with the indicated concentrations of VP-16 and assessed for DNA damage using alkaline elution of DNA fragments, as described in Materials and Methods. A calibration curve for relating the frequency of VP-16-induced SSB to a corresponding effect of radiation (radiation equivalent DNA damage) was obtained by plotting rads versus [^{14}C]DNA retention at 75% retention of the [^{3}H]DNA internal standard.

with 2 mM BSO, there was a time-dependent decrease in thiol content to 53% of the 0 hr level and a similar time-dependent decrease in the lag period for appearance of VP-16 phenoxyl radicals to 50% of that seen at 0 hr (Fig. 5).

K562 and K/VP.5 cells were next treated with 5 μM $CdCl_2$ for 24 hr to elevate endogenous thiol levels, as demonstrated previously with other cell lines (Li *et al.*, 1993 and 1994). $CdCl_2$ increased both the total thiol content and the total lag period for the appearance of the VP-16 phenoxyl radical ESR signal when cell homogenates from K562 and K/VP.5 cells were added to the tyrosinase plus VP-16 oxidation system (Table 1).

Effect of glutathione content on reduction of VP-16 phenoxyl radicals

GSH peroxidase and cumene hydroperoxide added to K562 cell homogenates depleted GSH, thus revealing that 50% of thiol content was GSH and that 48% of the lag period for appearance of the VP-16 phenoxyl radical ESR signal was due to the GSH content of cell homogenates (see Table 1 data). GSH-Px- and cumene hydroperoxide-mediated depletion of GSH in cell homogenates also resulted in a decreased lag period (shown in Fig. 6). Treatment of K562 cell homogenates with cumene hydroperoxide alone or GSH-Px alone did not significantly decrease the thiol content in the homogenates and did not affect the lag period (data not shown). When cells were incubated with 2 mM BSO for 48 hr to deplete cells of GSH, homogenates exhibited decreased lag periods compared with homogenates derived from untreated cells (Fig. 6). Conversely, exogenous GSH added to the tyrosinase plus VP-16 oxidation system in the presence of K562 cell homogenates produced a concentration-dependent increase in the lag period for the appearance of the VP-16 phenoxyl radical ESR signal (Fig. 6).

When K562 and K/VP.5 cells were incubated for 24 hr with 5 μM $CdCl_2$, elevation of GSH accounted for about two-thirds of the $CdCl_2$-induced increment in total thiols (Table 1). In addition, increased GSH accounted for two-thirds of the increase in the lag period for the appearance of VP-16 phenoxyl radicals in the presence of cell homogenates in both cell lines (Table 1). These results suggest that both GSH and protein thiols are involved in the enhanced ability of homogenates from $CdCl_2$-treated cells to reduce VP-16 phenoxyl radicals.

Potentiation of VP-16 activity by modulation of thiol levels

The 48-hr growth-inhibitory effects of VP-16 in K562 cells were potentiated 1.6-fold by addition of 1 mM BSO (Fig. 7). In addition, VP-16-induced DNA SSB were increased in K562 cells preincubated for 24 hr in the presence of 0.5 mM BSO (Fig. 8). Together, these results further suggest that BSO-mediated reduction in thiol content leads to greater VP-16 cytotoxicity through mechanisms linked to the oxidative metabolism of VP-16 to its phenoxyl radical.

DISCUSSION

In this work we have demonstrated that VP-16 phenoxyl radicals were reduced by endogenous reductants in K562 cell homogenates and that intracellular thiols were mainly responsible for this reduction. The present results extent our previous report indicating that the lag period for appearance of VP-16-phenoxyl radicals produced by cell homogenates from a number of cell lines was strongly correlated with their thiol content (Tyurina *et al.*, 1995). Whereas the formation of VP-16 *ortho*-quinone/sulfhydryl adducts is well documented and the formation of VP-16-semiquinone radical/sulfhydryl adducts is suggested (Mans *et al.*, 1992a), the role of VP-16 phenoxyl radicals in oxidative modification of protein thiols remains to be elucidated. Our previous studies demonstrated that the ratio of oxidized thiols to oxidized VP-16 during tyrosinase-catalysed reactions was 5:1, which significantly exceeded the 2:1 ratio maximally expected for the quinone-mediated oxidation of thiols (Tyurina *et al.*, 1995). This suggests that phenoxyl radical-driven oxidation of thiols is the major pathway resulting in a large-scale modification of the intracellular thiol pool.

Our results showed that, in untreated K562 cells, endogenous GSH is responsible for about 50% of the cell's ability to reduce VP-16 phenoxyl radicals in thiol-dependent reactions (see Table 1). Depletion or supplementation of K562 cells or cell homogenates

with GSH proportionally decreased or increased the subsequent ability of cell homogenates to reduce VP-16 phenoxyl radicals. GSH, which is present in cells in relatively high concentrations (up to 10 mM), is known to be involved in numerous cellular defences against toxicants, drugs, ionizing radiation and carcinogens (Kosower and Kosower, 1978; Meister, 1994; Orrenius and Moldeus, 1984). The major function of GSH is thought to be protection of cells against reactive oxygen species and chemical electrophiles produced by bioactivation of therapeutic and environmental chemicals (Ketterer *et al.*, 1983; Reed, 1985). The antioxidant function of GSH is mainly associated with its ability to scavenge oxygen radicals as well as peroxyl and alkoxyl radicals (Asmus, 1990). Since GSH is abundant in K562 leukaemic cells (Lin *et al.*, 1993), the demonstrated ability of GSH to interact with VP-16 phenoxyl radicals reflects its antioxidant and protective activity in this cell system. Incubation of K562 cells with concentrations of BSO demonstrated to decrease GSH levels resulted in an enhancement of both VP-16-induced DNA damage and growth inhibition (Figs 7 and 8). These experiments provide the critical link between modulation of thiol levels, reduction of VP-16 phenoxyl radicals and the cytotoxic effects of this clinically utilized anticancer agent. Recently, the treatment of human non-small cell lung (SW-1573), ovarian (A2780) and breast carcinoma (MCF-7) cells with BSO for 24 hr was shown to deplete endogenous GSH to about 20% of control and to be accompanied by a twofold potentiation of the cytotoxicity of VP-16 (Mans *et al.*, 1992b). Therefore, BSO depletion of GSH has been correlated with potentiation of VP-16 cytotoxicity in several cancer cell lines.

There is evidence that metallothioneins, thiol-containing proteins found in the cytoplasm and nucleus, exert radical-scavenging capability towards superoxide, hydroxyl and organic radicals in cells (Chibatsu and Meneghini, 1993; Sato and Bremner, 1993). Exposure of cells to $CdCl_2$ is known to result in an elevated level of both GSH and metallothioneins (Chibatsu and Meneghini, 1993). In our experiments, pretreatment of K562 and K/VP.5 cells with $CdCl_2$ resulted in an elevation of the DTNB-titrable thiols by 17% (Table 1) and an increased ability to reduce the VP-16 radical by 15–20%, as quantified by increased lag time for appearance of VP-16 phenoxyl radical (Table 1). Only two-thirds of the thiol increment and the increased reduction of the VP-16 radical was due to increased GSH levels. Therefore, protein thiols induced by $CdCl_2$ also participated in the reduction of VP-16 phenoxyl radicals. Our recent results demonstrated directly that exogenous metallothionein can reduce the VP-16 phenoxyl radical (Kagan *et al.*, 1994). In addition, over-expression of metallothioneins in NIH 3T3 cells resulted in an increased ability of cell homogenates to reduce the VP-16 phenoxyl radicals (Schwarz *et al.*, 1994). Thus, interaction of metallothioneins with phenoxyl radicals may contribute to their protection against oxidative stress. Quantitatively, however, metallothioneins represent a minor fraction of protein thiols, suggesting that other proteins may be oxidatively modified by VP-16 phenoxyl radicals.

The identification of protein targets whose sulfhydryl groups may be involved in interactions with VP-16 phenoxyl radicals is a major goal of our future investigations. We intend to identify the critical biomolecules responsible for both cytotoxic effects of VP-16 and for potential protection against VP-16 cytotoxicity. It is a general notion that oxidation of protein sulfhydryls may affect the catalytic activity of target enzymes. We have recently demonstrated that the VP-16 phenoxyl radical-mediated oxidation of sulfhydryl groups caused inactivation of Ca^{2+}-ATPase and Ca^{2+}-release channels of sarcoplasmic reticulum membranes from skeletal muscles, Na^+, K^+-ATPase and rhodopsin (Ritov *et al.*, 1995). Likewise, interaction of the VP-16 phenoxyl radical with critical thiols in DNA topo II may be related to this compound's inhibition of the enzyme (Kaufmann and Shaper, 1991).

The K/VP.5 cell line is a VP-16 resistant subline derived from K562 cells (Ritke *et al.*, 1994b) that contains decreased levels of DNA topo II and qualitative changes in topo II resulting in the reduced ability of VP-16 to stabilize topo II-DNA cleavable covalent complexes (Ritke *et al.*, 1994a,b). We examined whether elevated levels of intracellular reductants preventing VP-16 oxidation might contribute to cell resistance to VP-16. Elevation of endogenous thiols by $CdCl_2$ did not reveal any significant difference in the ability of the two types of cells to interact with VP-16 phenoxyl radicals (Table 1). Thus, while prevention of oxidative activation of VP-16 is not likely to be a determinant of VP-16 resistance in K/VP.5 cells, we have demonstrated that the ability of GSH and protein thiols to reduce phenoxyl radicals may be a very important protective mechanism against cytotoxicity of VP-16.

In conclusion, in ascorbate-free cell culture conditions, we have demonstrated thiol-dependent delays in the production of VP-16 phenoxyl radicals and related the modulation of thiol levels to the DNA damaging and cytotoxic effects of VP-16 in the human leukaemia K562 cell line. Supplementation with thiols and other antioxidants that prevent oxidative activation of VP-16 (e.g. ascorbate) may be critical for protection of normal cells from VP-16 cytotoxicity. Conversely, depletion of thiols in malignant tissue may be related to the clinical efficacy of VP-16 as an anticancer agent. Although in a clinical situation ascorbate is present and may contribute significantly to reduction of the VP-16 phenoxyl radical, ascorbate is a vitamin in humans and its reserves are limited and can easily be depleted. In contrast, GSH can be continuously regenerated by oxidized glutathione (GSSG) reductase and thus protect cells from VP-16 phenoxyl radical-mediated

toxicity. Hence, information from this study may help to design clinical strategies to sensitive cancer cells further to VP-16 and/or to protect normal tissues from toxicity, thereby leading to improved clinical application of VP-16.

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