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Modulation by D,L-Buthionine-S,R-Sulphoximine of Etoposide Cytotoxicity on Human Non-small Cell Lung, Ovarian and Breast Carcinoma Cell Lines

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Treatment with 25 μmol/l D,L-buthionine-S,R-sulphoximine (BSO) for at least 24 h depleted glutathione (GSH) in human non-small cell lung (SW-1573), ovarian (A2780) and breast carcinoma (MCF-7) cell lines to about 20% of control, and was accompanied by a 2-fold potentiation of the cytotoxicity of etoposide, doxorubicin and cisplatin. Cellular etoposide, but not doxorubicin or cisplatin, concentrations were increased 2-fold due to decreased efflux. This occurred independently of the presence of BSO during 1 h of etoposide exposure, but required prolonged exposure to BSO (at least 24 h). Energy depletion as well as cotreatment, but not pretreatment, of the cells with daunomycin, doxorubicin, vinblastine or vincristine increased cellular etoposide accumulation. Treatment of control cells with verapamil caused similar changes in etoposide cytotoxicity and cellular pharmacokinetics as GSH depletion, but did not further increase etoposide cytotoxicity and accumulation in GSH-depleted cells. Etoposide efflux may have been inhibited, not because of (competitive) inhibition by BSO or disturbance of the energy required for this process, but probably because of plasma membrane alterations.
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INTRODUCTION

THE TRIPEPTIDE glutathione (GSH) is the most abundant non-protein sulphydrylic compound in mammalian cells (cellular concentrations ranging from 0.1 to 10 mmol/l). This compound, either by itself or in reactions catalysed by GSH peroxidases and GSH S-transferases, plays an important role in the detoxification of various hazardous agents of endogenous as well as of exogenous origin. This process is based on elimination by reduction and conjugation reactions of these compounds themselves, their toxic metabolites or toxic oxygen-derived free radicals generated during drug metabolism [1–5].

Elevation of GSH content has been observed to protect cultured cells from damage by radiation and by a variety of cytostatic agents such as melphalan, platinum compounds and anthracyclines. It has further been associated with the development of (multi)drug resistance *in vitro*. On the other hand, cultured cells which are depleted of GSH, may exhibit an increased sensitivity to these cytotoxic challenges [1–5]. Thus, the efficacy of anticancer treatment could depend substantially on tumour GSH content. Based on this finding, phase I studies have been initiated, with the purpose of improving chemotherapeutic treatment with melphalan through depletion of tumour GSH levels [6]. GSH depletion can be achieved by treatment with BSO, a water-soluble, relatively non-toxic peptide which inhibits the activity of γ-glutamyl-cysteine synthetase, the rate-limiting enzyme for GSH biosynthesis [7, 8]. Since most normal cells contain an excess of GSH, whereas the GSH concentrations of malignant cells are close to those required for survival [1–5], such an approach could be of clinical benefit.

From previous results it might be anticipated that a decrease of cellular GSH content could also affect the cytotoxicity of etoposide. This anti-neoplastic agent has found widespread use in the treatment of several malignant tumours, either as a single agent or in combination therapy [9]. Its mechanism of action could be based on the formation of DNA strand-breaks, DNA protein cross-links and possibly also DNA adducts [10 and references therein, 11, 12]. These DNA lesions could be a result of interaction of etoposide with DNA topoisomerase II and/or of metabolic activation to DNA-binding intermediates such as the orthoquinone, the catechol and the semiquinone-free radical of etoposide. Initial indications for a possible involvement of GSH in etoposide cytotoxicity came from the observation that mice which were treated with etoposide exhibited decreased GSH and increased GSSG concentrations in the liver [13]. Since etoposide itself does not react with GSH, this observation could be due to a reaction of GSH with one or more etoposide metabolites [13]. A doxorubicin-resistant MCF-7 human breast carcinoma subline which contained increased concentrations of GSH peroxidases and GSH S-transferases, exhibited a high degree of cross-resistance to etoposide [14], giving additional support for a possible involvement of GSH in etoposide cytotoxicity.

In order to study this possibility in more detail, the effects of decreased GSH contents on etoposide cytotoxicity were investigated in cultured tumour cells. Comparison of the results using etoposide with those from parallel experiments using doxorubicin and cisplatin, gave insight into a possible biochemical mechanism, since GSH depletion is presumed to sensitise cells to these anticancer agents by reduced scavenging of toxic

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free radicals or by diminished conjugation, respectively [15–21]. Our results demonstrated a potentiating effect of GSH depletion on the cytotoxicity of etoposide which was probably mediated by a mechanism different from that presumed for doxorubicin and cisplatin.

MATERIALS AND METHODS

Drugs and chemicals

Non-labelled etoposide and cisplatin were obtained from Bristol-Myers. Non-labelled daunomycin and doxorubicin were purchased from Farmitalia Carlo Erba. ^{3}H-Labelled etoposide (1000 mCi/mmol) was purchased from Moravek Biochemicals, Brea, California. ^{14}C-Labelled doxorubicin (59 mCi/mmol) was from Amersham, UK. Verapamil, vinblastine, vincristine and BSO were obtained from Sigma. GSH was from Boehringer Mannheim. All other chemicals were from the laboratory stock and were well qualified for analytical use.

Cell lines

Human non-small cell lung carcinoma cells (SW-1573, cell doubling time about 23 h) were originally isolated by Dr A. Leibovitz, Scott and White Clinic, Temple, Texas and were obtained from Dr H. Joenje, Department of Human Genetics, Free University, Amsterdam. Human ovarian carcinoma cells (A2780, cell doubling time about 20 h) were supplied by Dr R.F. Ozols, National Cancer Institute, Bethesda. Human breast carcinoma cells (MCF-7, cell doubling time about 25 h) were obtained from Dr K.H. Cowan, National Cancer Institute, Bethesda. Cells were cultured in Dulbecco's modified essential medium (DMEM) from Gibco, supplemented with 10% fetal calf serum (FCS) from Flow at 37°C in a humidified atmosphere of 5% CO_2 in air. Only cells in exponential growth were used for the experiments.

Determination of total cellular GSH content

Depletion of cellular GSH content was achieved by culturing cells in the presence of 25 μmol/l BSO for 48 h. Preliminary studies had indicated that such BSO treatments themselves cause only a relatively low level of cell growth inhibition (less than 15%). The course of GSH depletion during this period of time was determined by measuring total cellular GSH levels (reduced and oxidised GSH) at several time points [22, 23]. Total cellular GSH concentrations (nmol per mg cellular protein) were derived from a calibration line of GSH and were expressed relatively to the amounts of total GSH determined in untreated controls. Protein was measured as described [24, 25].

Growth inhibition studies

Cell growth inhibition by etoposide, doxorubicin and cisplatin was examined upon exposure of untreated or 24-h BSO-treated samples of 6×10^4 cells, cultured in 6 well culture dishes (Costar), to different concentrations of the drugs in the absence or presence of either 25 μmol/l BSO, 16 μmol/l verapamil or both agents. After 1 h, the drug-containing medium was exchanged for drug-free medium without or with 25 μmol/l BSO, in which the cells were grown for an additional 23 h prior to receiving fresh, drug-free medium.

After 4 days, cells were counted with a Sysmex CC-110 Microcell-counter (TOA Medical Electronics, Kobe, Japan). Drug cytotoxicity was expressed as IC_{50} value, i.e. the drug concentration which resulted in 50% cell growth inhibition. The modulating effects of BSO and/or verapamil treatment on drug cytotoxicity were expressed as dose modifying factors (DMF), i.e. the IC_{50} value found upon drug treatment alone divided by that upon combination with BSO and/or verapamil. The latter was corrected for background effects caused by BSO and/or verapamil alone.

Cellular accumulation of etoposide, doxorubicin and cisplatin

Cellular accumulation of etoposide, doxorubicin and cisplatin was studied using cell samples ($1.5–4 \times 10^6$) either in monolayer or in suspension, since similar results were found under both conditions. Drug accumulation was examined by incubating cell samples which were pretreated for several periods of time with 25 μmol/l BSO, 5 μmol/l ^{3}H-labelled etoposide, 0.5 μmol/l ^{14}C-labelled doxorubicin or 10 μmol/l non-labelled cDDP, either in the absence or in the presence of 25 μmol/l BSO. In addition, cellular accumulation of ^{3}H-labelled etoposide (5 μmol/l) was measured after energy depletion and upon cotreatment or 1-h pretreatment with verapamil (16 μmol/l), daunomycin (50 μmol/l), doxorubicin (50 μmol/l), vinblastine (20 μmol/l) or vincristine (20 μmol/l). Energy depletion was achieved by pre-incubating cell samples for 30 min in glucose-free medium which contained 10 mmol/l sodium azide.

After incubation at 37°C in a shaking waterbath, cells were chilled on ice, washed three times with 5 ml ice-cold 0.9% NaCl and lysed with distilled water. Cellular concentrations of etoposide and doxorubicin were determined from the amounts of radioactivity counted in a Betamatic Liquid Scintillation Counter (Kontron, Zürich) with 23 ml Insta-gel II (Packard Instruments). Cellular concentrations of cisplatin were determined by atomic absorption spectrometric measurement of cellular platinum, using a 5000 GF atomic absorption spectrometer, an A450 autosampler and a HGA 500 graphite furnace (Perkin Elmer). Cellular digests were prepared as described [26] and dry-ashed at 1400°C.

Cellular drug accumulation was expressed as pmol per 10^6 cells. Data were corrected for zero-time values, obtained by adding ice-cold drug solution to cells on ice, followed by immediate removal of the drug-containing medium and washing of the cells as described above.

Cellular etoposide concentrations retained after drug efflux

To determine cellular etoposide concentrations retained after drug efflux, untreated, 47-h BSO-pretreated or 1-h verapamil-cotreated cells were loaded with ^{3}H-labelled etoposide (5 μmol/l) for 1 h, thoroughly washed (4°C) and immediately postincubated in prewarmed medium (37°C) alone or in prewarmed medium containing either 25 μmol/l BSO or 16 μmol/l verapamil. Cellular etoposide concentrations after 5 min drug efflux were determined as described in the previous paragraph.

Statistics

All experiments were performed at least three times in duplicate or triplicate. Means (S.D.) are presented in Figures and Tables. $P < 0.05$ was taken to indicate statistical significance (Student's t test).

RESULTS

Depletion of cellular GSH contents by treatment with BSO

The effect of BSO treatment (25 μmol/l) for several periods of time on total GSH levels in wild-type SW-1573, A2780 and MCF-7 cells was examined. As shown in Fig. 1, cellular GSH concentrations decreased with time, until after BSO treatment for 24 h minimum levels of about 20% of control values were reached.

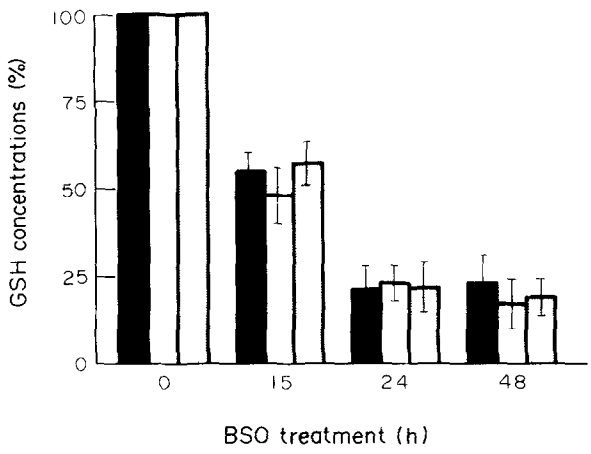

Fig. 1. Course of GSH depletion in SW-1573 (■), A2780 (□) and MCF-7 cells (▨) upon treatment with 25 μmol/l BSO for different periods of time. Data are expressed as % of basal total GSH concentrations as determined in untreated cells. Basal total GSH concentrations were 28.3 (3.5), 25.0 (3.2) and 48.3(3.6)nmol per mg cellular protein, for SW-1573, A2780 and MCF-7 cells, respectively.

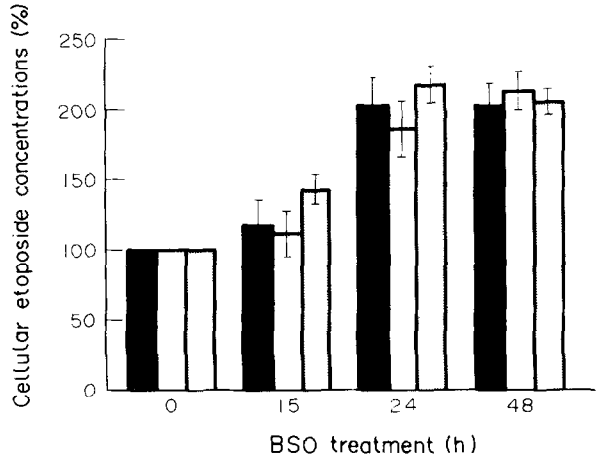

Fig. 2. Accumulation of etoposide in SW-1573 (■), A2780 (□) and MCF-7 cells (▨), after treatment for 0, 15, 24 and 48 h with 25 μmol/l BSO. Cells were exposed for 1 h to 5 μmol/l etoposide, 0.5 μmol/l doxorubicin or 10 μmol/l cisplatin. Data are expressed as % of etoposide concentrations found in untreated cells. Cellular etoposide concentrations after exposure of the cells for 1 h at 37°C, were 5.4(0.7), 7.1(1.8) and 9.3(1.7)pmol per 10^6 cells in SW-1573, A2780 and MCF-7, respectively; for doxorubicin these values were 26.0(1.0), 26.1(1.8) and 45.0(3.5)pmol per 10^6 cells, respectively, and for cisplatin 30.3(1.6), 42.2(4.5) and 24.2(4.7)pmol per 10^6 cells, respectively.

Potentiation of etoposide, doxorubicin and cisplatin cytotoxicity by GSH depletion

As shown in Table 1, GSH depletion resulted in a 2-fold potentiation of etoposide cytotoxicity. Comparison with doxorubicin and cisplatin showed a similarly increased toxicity of these agents in GSH-depleted cells (Table 1).

For etoposide, the potentiating effects of GSH depletion were compared with those of the chemomodulating agent verapamil [17, 27–29] in both control and GSH-depleted cells. Etoposide cytotoxicity in control cells was increased 2-fold upon coincubation with verapamil (Table 1), while preincubation with this agent was without effect. On the other hand, etoposide cytotoxicity in GSH-depleted cells was not further increased in the presence of verapamil (Table 1).

No differences in IC_{50} values of etoposide were found in GSH-depleted cells, but also not in control cells, whether or not BSO was present in the incubation mixtures during the time of exposure to etoposide.

Table 1. Potentiation of drug cytotoxicity in human carcinoma cell lines upon GSH depletion by treatment for 48 h with 25 μmol/l BSO and upon cotreatment with 16 μmol/l verapamil*

	DMF*		
	SW-1573	A2780	MCF-7
Etoposide + BSO†	2.1(0.7)	2.4(0.4)	2.2(0.5)
+ verapamil‡	2.2(0.5)	1.9(0.4)	2.3(0.5)
+ BSO + verapamil	2.1(0.3)	2.2(0.3)	2.3(0.5)
Doxorubicin + BSO	2.3(0.5)	2.0(0.1)	1.9(0.3)
Cisplatin + BSO	2.1(0.4)	2.0(0.3)	2.0(0.4)

*Potentiation of drug cytotoxicity was expressed as DMF (dose modifying factor; see Materials and Methods). IC_{50} values for etoposide after a 1-h drug exposure period at 37°C were 4.4(1.1), 2.5(0.5) and 7.7(1.1) μmol/l for SW-1573, A2780 and MCF-7 cells, respectively; for doxorubicin these values were 0.9(0.2), 0.8(0.1) and 1.4(0.2) μmol/l, respectively, and for cisplatin 6.9(1.3), 7.2(1.6) and 8.4(2.3) μmol/l, respectively.

†The presence of BSO in the incubation mixture during the time of exposure to etoposide was without effect on its cytotoxicity in either untreated or GSH-depleted cells (not shown).

‡Results were obtained upon cotreatment with verapamil (16 μmol/l); pretreatment with this agent did not affect etoposide cytotoxicity significantly (not shown).

Cellular steady-state concentrations of etoposide, doxorubicin and cisplatin after GSH depletion

To examine a possible effect of GSH depletion on cellular accumulation of etoposide, doxorubicin and cisplatin, drug concentrations in cells which were pretreated with BSO for increasing periods of time were compared to those in untreated cells after a 1-h drug exposure period. After such a period of time, cellular etoposide concentrations had reached a plateau level (results not shown), which increased with increasing GSH depletion to reach a maximum of 200% of control values after 24 h GSH depletion (Fig. 2). In contrast, (1 h) cellular concentrations of doxorubicin and cisplatin after GSH depletion were not significantly altered.

Cellular pharmacokinetics of etoposide upon GSH depletion, energy depletion and treatment with verapamil, daunomycin, doxorubicin, vinblastine or vincristine

To investigate whether the increased accumulation of ^{3}H-labelled etoposide in GSH-depleted cells after 1 h incubation could be the result of alterations in cellular pharmacokinetics, cellular steady-state etoposide concentrations and cellular etoposide concentrations retained after 5 min efflux in drug-free medium were determined in GSH-depleted and in untreated cells. ^{14}C-labelled doxorubicin and cisplatin were excluded from this set of experiments, since GSH depletion was found to be without significant effect on their cellular concentration (see above).

The results with etoposide, presented in Table 2, showed 2-

Table 2. Effects of treatment with BSO (25 μmol/l, 48 h pre-incubation) and verapamil (16 μmol/l, 1 h coincubation) on steady-state concentrations and retention of etoposide in human carcinoma cell lines

	Cellular steady-state concentrations*	Cellular etoposide concentrations after 5 min efflux†
SW-1573, control	100	9(2)
+ BSO	205(13)	16(4)
+ verapamil	209(15)	14(4)
A2780, control	100	10(3)
+ BSO	198(29)	18(2)
+ verapamil	179(21)	23(5)
MCF-7, control	100	16(5)
+ BSO	207(13)	35(3)
+ verapamil	209(15)	34(4)

*Cellular steady-state concentrations of etoposide were determined at 60 min incubation time and are expressed as % of control values as presented in legend to Fig. 2. Cells were exposed for 1 h at 37°C to 5 μmol/l ^{3}H-labelled etoposide.

†Cellular concentrations of etoposide retained after 5 min drug efflux were determined by postincubating cells which were loaded for 60 min with 5 μmol/l ^{3}H-labelled etoposide, for 5 min in drug-free, prewarmed medium and are expressed as % of cellular etoposide steady-state concentrations measured at 60 min incubation time.

fold higher cellular steady-state concentrations in both GSH-depleted and verapamil-cotreated cells compared to untreated control cells. After 5 min efflux in drug-free medium, significantly more etoposide was retained in GSH-depleted and in verapamil-cotreated cells compared with control cells (Table 2). As found for etoposide cytotoxicity, etoposide concentrations in both control and GSH-depleted cells were independent of the presence of BSO in the incubation mixtures during the time of drug exposure (Table 3). Cotreatment of GSH-depleted cells with 16 μmol/l verapamil was without significant effect on 1-h steady-state concentrations of etoposide observed upon GSH depletion alone (Table 3).

Cellular etoposide concentrations found upon GSH depletion were also determined upon energy depletion and upon cotreatment with verapamil (16 μmol/l), daunomycin (50 μmol/l), doxorubicin (50 μmol/l), vinblastine (20 μmol/l) or vincristine (20 μmol/l), since cellular etoposide concentrations were reported to be elevated under all these conditions [17, 27–32]. Energy depletion by pre- and co-incubation in glucose-free medium containing 10 mmol/l sodium azide, as well as co-incubation only, but not pretreatment alone with verapamil, daunomycin, doxorubicin, vinblastine or vincristine, also resulted in 2-fold increased cellular steady-state etoposide concentrations (Table 3).

In Table 3, only data found with SW-1573 cells are presented; however, comparable results were obtained with A2780 and MCF-7 cells.

DISCUSSION

From previous results it could be suggested, that cellular GSH concentrations are a determinant for etoposide cytotoxicity [13, 14, 33]. The results from the present study confirm this suggestion, by demonstrating that etoposide cytotoxicity was increased with a factor 2 (Table 1) upon depletion of cellular GSH concentrations (Fig. 1). This was accompanied by a similar increase in cellular etoposide concentrations, probably due to inhibition of cellular etoposide efflux (Table 2).

GSH depletion was found to potentiate the cytotoxicity of doxorubicin and cisplatin with a factor 2 as well (Table 1). In contrast to that of etoposide, concentrations of doxorubicin and cisplatin in GSH-depleted cells were not significantly altered compared to control cells. The mechanisms involved in the potentiation of etoposide cytotoxicity upon GSH depletion thus seem to be different from those presumed for doxorubicin and cisplatin, i.e. reduced elimination of toxic free radicals or diminished conjugation of the drug, respectively [15–21].

A diminished (GSH-mediated) elimination of cytotoxic metabolites like the orthoquinone and the semiquinone free radical intermediates of etoposide (reviewed in [10]) is probably not responsible for the observations with etoposide. This can be inferred from the results of high-performance liquid chromatography analyses of cellular extracts, which showed no significant differences in the distribution of radioactivity between control and GSH-depleted cells treated with ^{3}H-labelled etoposide (not shown). On the other hand, cellular etoposide concentrations in GSH-depleted cells (Fig. 2) exhibited comparable kinetics as the course of GSH depletion (Fig. 1). It can thus be strongly suggested, that GSH depletion sensitised cells to etoposide, by affecting biochemical mechanisms associated with the net cellular accumulation of the drug.

To characterise these mechanisms in more detail, the effects of GSH depletion were compared to those of treatments which also increase cellular etoposide concentrations, i.e. energy depletion and cotreatment with verapamil, daunomycin, doxorubicin, vinblastine or vincristine [17, 27–32]. In line with these previous data, all these treatments were found to induce a 2-fold increase of cellular steady-state etoposide concentrations or of etoposide cytotoxicity (Tables 1–3). These results indicate, that also for the cell lines used in the present study, cellular etoposide concentrations are determined by energy-dependent drug efflux mechanisms which can be competitively inhibited by structurally unrelated agents.

BSO-mediated GSH depletion induced similar changes in etoposide cytotoxicity and in cellular steady-state etoposide concentrations as the above mentioned treatments (Tables 1–3). In contrast to the latter treatments, however, these effects were independent of the presence of BSO in the incubation medium during exposure to etoposide (legend to Table 1, Table 3). Thus, the increased etoposide accumulation observed for GSH-depleted cells was probably not the result of competitive inhibition of energy-dependent etoposide efflux by BSO. Support for this conclusion is provided by the observation that GSH-depleted cells were not further sensitized to etoposide by verapamil, which is supposed to modulate etoposide cytotoxicity by inhibiting its cellular efflux [27–29], and did not accumulate more etoposide in the presence of this agent (Tables 1–3).

An explanation for the similarly increased cellular accumulation and cytotoxicity of etoposide under all the above-mentioned experimental conditions is not readily apparent from the present data. P-glycoprotein-mediated cellular efflux of etoposide was most probably not involved in this phenomenon. This is indicated by the fact that the A2780 and MCF-7 cell lines used in the present study did not contain messenger RNA encoding for P-glycoprotein (unpublished observations), whereas the SW-1573 cell line contained only a relatively low amount of messenger RNA encoding for P-glycoprotein [35]. Modifications in plasma membrane circumference also did not

*Table 3. Effects of GSH depletion, energy depletion or treatment with doxorubicin, daunomycin, vinblastine and vincristine on the steady-state accumulation of etoposide in SW-1573 cells**

Control		100
BSO†	1 h cotreatment	112(17)
	47 h pretreatment	211(13)
	47 h pre-, 1 h cotreatment with BSO	205(13)
	47 h pre-, 1 h cotreatment with BSO + verapamil	218(20)
verapamil‡	pretreatment	105(8)
	cotreatment	209(15)
DN‡	pretreatment	123(30)
	cotreatment	217(26)
DX‡	pretreatment	113(35)
	cotreatment	195(19)
VBL‡	pretreatment	112(16)
	cotreatment	206(9)
VCR‡	pretreatment	96(15)
	cotreatment	192(23)
Energy depletion		217(19)

*Data are expressed as % of control values as presented in legend to Fig. 2. Cells were exposed for 1 h at 37°C to 5 μmol/l etoposide.
†Control cells or 47-h BSO pretreated cells were incubated for 1 h with etoposide in the absence or in the presence of 25 μmol/l BSO, or 25 μmol/l BSO + 16 μmol/l verapamil.
‡Energy depletion and pre- and cotreatment with verapamil, daunomycin, doxorubicin, vinblastine and vincristine were performed as described in Materials and Methods.

contribute to these observations, since diameters of 48-h BSO-treated cells were not altered when compared with untreated cells (not shown). It can be speculated that complete inhibition of the putative etoposide efflux causes a maximum increase in cellular drug concentrations to 200% of control values.

Summarising, the present results show that depletion of cellular GSH concentrations by BSO treatment renders tumour cells more sensitive to etoposide by inhibiting its cellular efflux and thus increasing its cellular steady-state concentrations. This phenomenon, which might be due to plasma membrane damage such as lipid peroxidation induced by the loss of protection by GSH [1–5], needs further investigation, in view of the clinical importance of etoposide.

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MTT Assays Allow Quick and Reliable Measurement of the Response of Human Tumour Cells to Photodynamic Therapy

Jean-Louis Merlin, Samir Azzi, Dominique Lignon, Carole Ramacci, Nadia Zeghari and François Guillemin

MCF-7 and HT-29 cell lines were selected as a reliable model to examine the possible parameters affecting the sensitivity of tumour cells to photodynamic therapy (PDT) using a dye-laser at 630 nm. The chemical composition of haematoporphyrin derivative (HPD) was determined by high-performance liquid chromatography (HPLC) analysis and was in agreement with reported values. MTT assays were performed to assess the time-dependency of PDT and the influence of the output power and light fluence. The results showed a maximal cytotoxicity 48 h after photoirradiation. The output power (1 or 2 W) did not significantly affect the cytoxicity when the fluence was constant (20 J/cm²). However, an increase in fluence (10–40 J/cm²) led to a significant enhancement of cytotoxicity until maximal values were reached (30–40 J/cm²). A further increase in fluence (50 J/cm²) proved to induce a fall-off in cytotoxicity related to the intense photobleaching of HPD.
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INTRODUCTION

PHOTODYNAMIC THERAPY (PDT) is based upon the specific properties of a compound such as haematoporphyrin derivative (HPD) to be retained in tumour tissue longer than in normal tissues such as skin, lung, brain, muscle [1]. When the sensitised tissue is exposed to an argon-dye laser generated red light (630 nm), the sensitiser is activated photochemically, thus permitting a selective destruction of the tumour. The resulting cytotoxicity has been reported to be induced by the generation of oxygen singlets [2], damaging mainly plasma membranes [3], mitochondria [4], cytoplasmic or nuclear organelles and enzymes [5, 6], in addition to the surrounding vessels [7]. Although it is well stated that the transport of HPD involves binding to serum proteins, the nature of the actual mechanism responsible for the selective tumoral biodistribution of HPD remains unclear [8] but has been demonstrated to be related to the pH-mediated generation of ionic species in tumoral sites. However, at the cellular level, it has been demonstrated that HPD accumulation involves passive diffusion facilitated by the low tumour pH [9].

Since PDT was first introduced for the treatment of malignant tumours, more than 4000 patients have undergone this therapy [10, 11]. HPD has dominated the biomedical studies since its discovery in 1961 [12] and is now the only photosensitiser permitted for use in clinical trials mainly for the treatment of tumours of the upper aerodigestive tract [13, 14], gastrointestinal [15] or disseminated intraperitoneal [16] neoplasms. New developments are being investigated for the treatment of brain tumours [17] and attempts to use topic administration of HPD [18] have been reported. The main advantage of HPD lies in the lack of severe toxicity, especially on nonirradiated areas, while its major drawback is its chemical composition, comprising a mixture of several porphyrin-derivated compounds including oligomeric species [19]. For this reason and despite numerous studies, the relationship between the chemical composition and the photodynamic activity of HPD remains unclear, the activity

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