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Manipulation of oxidative stress to induce cell death in Ewing's sarcoma family of tumours

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ABSTRACT

Ewing's sarcoma family of tumours (ESFT) are childhood cancers whose aggressive behaviour and propensity to relapse prompts the need for new treatment approaches. In this study, the role of cellular antioxidants in determining the sensitivity of ESFT cell lines to the cytotoxicity of the antineoplasic agent fenretinide was investigated with a view to identifying targets for the development of new treatment strategies. ESFT cell lines differentially express cellular antioxidants, although cellular glutathione (GSH) was identified as the major determinant of sensitivity to fenretinide. The importance of GSH in ESFT physiology was demonstrated by the depletion of intracellular GSH using L-buthionine (S,R) sulphoximine (BSO), which decreased cell viability. Furthermore, pre-treatment of ESFT cells with BSO sensitised them to fenretinide-induced death. Overall, these results demonstrate that ESFT cells are sensitive to changes in intracellular redox environment, and that targeting specific cellular antioxidants might be a viable strategy in treating ESFT.

1. Introduction

Ewing's sarcoma family of tumours (ESFT) comprise Ewing's sarcoma, peripheral primitive neuroectodermal tumour (pPNET), neuroepithelioma and Askin tumour, all of which are characterised by the non-random t(11;22)(q24;q12) chromosome rearrangement.[1–3] Ewing's sarcoma is the second most common malignant tumour of the bone found in children and young adults between the ages of 10 and 20 years.[4] Despite the intensification of treatment strategies involving surgery, radiation and chemotherapy, 10-year survival of those with metastatic disease is below 30% due to disease progression,[4] hence there is an urgent need for new treatment strategies in ESFT. We have previously reported that fenretinide (N-(4-hydroxyphenyl)-retinamide) induces cell death and delayed tumour growth in ESFT[5]; this is effected in part through the rapid induction of intracellular reactive oxygen species (ROS) and sustained activation of $p38^{MAPK}$.[5] Nevertheless, ESFT cell lines differentially respond to the induction of cell death by fenretinide *in vitro*,[5] and we propose that this may in part reflect the different levels of intracellular antioxidants and the capacity to neutralise ROS.

ROS are important effectors in signal transduction processes, especially in pathways leading to programmed cell death (apoptosis) in response to cytotoxic drugs, cytokines or other forms of stress.[6,7] Low levels of ROS are essential for

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normal physiological functions of the cell as they promote mitogenic proliferation and also act as crucial second messengers in many redox-sensitive signalling cascades[7–9]; however, intermediate to high levels of ROS promote cellular senescence, apoptosis or necrosis depending on cell type and microenvironment.[7,9] Under normal physiological conditions, intracellular ROS levels are tightly regulated by a battery of ROS-scavenging antioxidant enzymes in conjunction with non-enzymatic antioxidants such as glutathione, thioredoxin, ascorbate and vitamin E.[10,11] In cancer, however, changes in glucose metabolism that occur as part of the malignant transformation process create a pro-oxidant intracellular environment amenable to proliferation and immortalisation of cells.[12,13] The inherent oxidative stress in cancer cells may indeed provide a variety of therapeutic strategies that exclusively target malignant cells. Modulating the intracellular redox balance to achieve high levels of ROS (oxidative stress) through either depletion of cellular antioxidants,[14,15] or administering agents that trigger ROS production *in vivo*[16,17] are two of the strategies that have been exploited to achieve and enhance cell killing in some cancers. The aim of this therapeutic approach is to sustain a high intracellular level of ROS that can overwhelm the antioxidant defence machinery of chronically stressed malignant cells and trigger cell death pathways.[18]

Fenretinide is a synthetic retinoid with documented toxicity against several malignant cell types, and has been explored for the treatment of several cancers.[17,19,20] Unlike other retinoic acid derivatives, fenretinide induces overproduction of ROS in malignant cells[17,21] that is consistently associated with activation of the cell death cascade. The generation of ROS in malignant cell, but not in normal cells is thought to explain the tumour-specific effects of fenretinide,[22,23] and may explain its minimal toxicity in phase I studies in adults[24,25] and children.[26,27] Furthermore, compared to other retinoids that exert their anticancer activities through the retinoic acid receptor (RAR), fenretinide has been shown to exert its effects via both RAR-dependent[28] and RAR-independent[29] mechanisms. Fenretinide thus may act to synergistically or additively increase the efficacy of other agents in the treatment of cancer[30] its minimal toxicity making it an attractive therapeutic compared to more toxic retinoids.

In this study, the role of the cellular antioxidants: cytosolic and mitochondrial superoxide dismutases (SOD1 and SOD2, respectively), catalase (CAT), glutathione peroxidase-1 (GPX1), glutathione S-transferase (GST) and glutathione (GSH) in determining the sensitivity of ESFT cell lines to fenretinide was investigated. The specific objectives were (1) to investigate the relationship between production of ROS and induction of cell in ESFT cell lines *in vitro*, (2) to characterise the antioxidant defences of ESFT cells, (3) to assess any correlation between antioxidant defences and sensitivity to fenretinide-induced death and (4) to investigate whether depletion of specific cellular antioxidants could intensify the cytotoxicity of fenretinide in ESFT cell lines.

2. Materials and methods

Cell culture media (RPMI 1640, DMEM, MEM, DMEM/F-12 and McCoy's) were purchased from Sigma Chemical Company (Sigma). Bovine foetal calf serum (FCS) was obtained from Seralab (Sussex, UK) and stored frozen at –20 °C. Fenretinide (*N*-4-hydroxyphenyl)retinamide) was a kind gift from Janssen-Cilag (Basseldorf, Switzerland) and stored protected from light as a 10 mM stock solution in absolute ethanol at –20 °C. Actinomycin D, doxorubicin, etoposide and vincristine were purchased from Sigma; stock solutions in DMSO (actinomycin D and etoposide) or double-distilled water (vincristine and doxorubicin) were prepared immediately prior to use. The glutathione depleting drug, L-buthionine (*S*,*R*) sulphoximine (BSO), was obtained from Sigma and stored at 4 °C; 10 mM stock solutions in phosphate buffered saline were prepared fresh for use. The redox-sensitive dye, 5-(6-)-chloromethyl-2′-7′-dichlorofluorescein diacetate (CM-H_2DCFDA, Molecular Probes, Eugene, OR) used for ROS detection, was dissolved in DMSO (0.1 M concentration) immediately prior to use and shielded from direct light. Assays for the antioxidant enzymes CAT (FluoroCatalase™), GPX1 (Glutathione Peroxidase Assay kit™), GST (Glutathione S-transferase Assay Kit™) and SOD (Superoxide Dismutase Assay Kit II) were purchased from Bachem AG (Germany), Cayman Europe (Estonia) and Calbiochem® (Nottingham, United Kingdom), respectively. The rabbit polyclonal antibodies for CAT (ab16731), GPX1 (ab16798), SOD1 (ab16831), SOD2 (ab13534) and β-actin (ab8227) were purchased from Abcam® (Cambridge, UK). All antibodies were stored frozen at –20 °C in 10 μL aliquots until required. The Mitochondrial Isolation Kit™ was purchased from Pierce Biotechnology, Inc. (Rockford, IL, USA).

2.1. Cell culture

Five substrate-adherent ESFT cell lines A673, RD-ES, SKES-1, TC-32 and TTC-466 were studied. All cell lines were cultured as previously described.[5] Incubations and treatments of cells were carried out in humidified incubators (Sanyo Gallenkamp, Loughborough, UK) containing 5% CO_2 and 95% air at 37 °C.

2.2. ROS production and induction of cell death by fenretinide in cultured cells

To investigate the relationship between ROS production and induction of cell death by fenretinide and other anticancer agents, ESFT cells (2×10^5 cells in 10 cm^2 dish; 60–70% confluent) were treated with the anticancer agent or vehicle (ethanol or DMSO), and ROS were then detected using the redox-sensitive dye, CM-H_2DCFDA, at time intervals ranging from 15 min to 16 h. Flow cytometry and Cell Quest Pro™ were used to assess fluorescence of the dye; 10,000 events were counted for each sample. The concentrations of actinomycin D, doxorubicin, etoposide, fenretinide and vincristine used to treat ESFT cells are shown in Table 1.

To assess the effect of fenretinide on cell viability, cells were seeded into 24-well plates, allowed to adhere overnight, and then treated with 0–10 μM fenretinide. Viable cell number was determined for each cell line using the automated Vi-cell trypan blue exclusion assay (Beckman Coulter).

Table 1 – Concentrations of some cancer chemotherapeutic agents used to test for induction of reactive oxygen species (ROS) in Ewing's sarcoma family of tumours (ESFT) cells

Drug[a]	Cell line[a]				
	A673	RD-ES	SKES-1	TC-32	TTC-466
Actinomycin D	6	30	18	3.22	6.44
Doxorubicin	8	13	11.4	3.48	13.3
Etoposide	1074	247	596	62	87.3
Fenretinide	1250	2500	4000	1700	1460
Vincristine	2026	11.2	4.2	8.64	7.18

a Drug concentrations are IC-50 values for each agent in nanomoles/litre.

2.3. Antioxidant enzyme activities

ESFT cell lines at 60–70% confluence were harvested into ice-cold PBS using a sterile rubber scraper and were centrifuged at 2000*g* for 10 min at 4 °C.

CAT activity was determined using the FluoroCatalase™ (Peninsula Laboratories) kit, which utilises a non-fluorescent detection reagent to detect H_2O_2 substrate remaining following the catalase reaction. Fluorescence was measured at excitation 530 nm and emission was detected at 590 nm.

GPX1 activity was measured indirectly by a coupled reaction with glutathione reductase (Glutathione Peroxidase Assay kit™, Cayman Europe). Briefly, the oxidised glutathione produced by the GPX1 reaction utilising cumene hydroperoxide substrate is recycled to its reduced state by reductase and NADPH. The decrease in absorbance of NADPH when oxidised to $NADP^+$ was measured spectrophotometrically at 340 nm.

The Glutathione S-Transferase Assay Kit™ (Cayman Europe) was used to determine total GST activity (cytosolic and microsomal) by spectrophotometrically measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) to reduced glutathione, which is accompanied by an increase in absorbance at 340 nm.

SOD1 and SOD2 enzymatic activities were measured using an optimised and validated Superoxide Dismutase Assay Kit II™ (Calbiochem®). SOD2 activity was measured in the mitochondrial pellets. The assay utilises a tetrazolium salt to detect superoxide anions generated by the conversion of hypoxanthine to uric acid by xanthine oxidase. One unit of SOD activity is described as the amount of enzyme needed to inhibit 50% of the oxidation of the tetrazolium salt monitored by absorbance at 405 nm.

2.4. Western blotting

ESFT cells at 60–70% confluence were harvested by scraping into lysis buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10 mM $MgCl_2$, 1% Triton X-100, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM sodium orthovanadate, 25 mM NaF) and incubating on ice for 30 min. The cell lysates were then centrifuged at 11,600*g* (4 °C) to pellet the insoluble material. The protein concentration of each cell extract was determined using the Bio-Rad DC Protein Assay (Bio-Rad, Herts., UK). The remaining supernatants were analysed by Western blot for the expression of the antioxidant enzymes.[5] The primary antibodies for CAT, SOD1 and SOD2 were used at a dilution of 1:2000, GPX1 antibody was used at a dilution of 1:1000. The β-actin antibody (loading control) was used at a 1:5000 dilution. Protein bands were detected and quantified using the Li-cor™ imaging system (Li-cor Biosciences, Lincoln, NE).

2.5. Measurement of GSH levels

ESFT cells at 60–70% confluence were harvested as described above. The cells were immediately centrifuged at 1500*g* for 5 min and the pellets were homogenised in 100–500 μl MES buffer (400 mM 2-(*N*-morpholino)-ethanesulphonic acid, 100 mM phosphate and 2 mM EDTA, pH 6.0) on ice. The homogenates were further centrifuged at 10,000*g* for 15 min (4 °C) and de-proteinated by adding an equal volume of 10% metaphosphoric acid. After centrifugation, the de-proteinated samples were neutralised with freshly prepared 0.2 M (final concentration) triethanolamine (TEAM) solution (Sigma) before measuring GSH.

Concentrations of reduced (GSH) and oxidised (GSSG) glutathione were determined using a glutathione reductase-based enzymatic recycling method (Glutathione Assay Kit™, Cayman Europe). To enable the measurement of GSSG from the cell extracts, reduced GSH was first removed from the TEAM-treated samples by adding 10 mM 2-vinylpyridine (Aldrich) and incubating at room temperature for 1 h before carrying out the assay.

2.6. Effects of fenretinide treatment on GSH levels

Whether treatment of ESFT cells with fenretinide resulted in depletion of intracellular GSH was investigated to establish if this was important for the induction of cell death by fenretinide. ESFT cell lines ($n = 5$) were treated with fenretinide (3 μM; a dose in excess of the IC-50 value for each cell line). Control groups were treated with ethanol alone or untreated; untreated controls were included to check whether ethanol alone had an effect on cellular GSH. Following treatment with fenretinide, cells were harvested at 0, 1, 2, 4, 6 and 24 h and rapidly snap-frozen in liquid N_2 until analysed for GSH as described above.

2.7. The effect of depleting intracellular GSH on sensitivity to fenretinide

Whether depletion of intracellular GSH using BSO increased the sensitivity of the ESFT cell lines to fenretinide-induced death was investigated. BSO is a specific inhibitor of γ-glutamylcysteine

synthetase (γ-GCS, a rate-limiting enzyme in GSH biosynthesis) and blocks cellular resistance to chemotherapy by inhibiting this enzyme.[31] ESFT cells were pre-treated with 5 μM BSO or PBS (vehicle for BSO) for 24 h (a period that ensured maximum depletion of GSH in the BSO-treated group), and then both groups were challenged with fenretinide (0–5 μM). After a further 24 h, cells were harvested by trysinization and the viable cell number was counted using the Vi-Cell trypan blue exclusion assay as before.

The effect of BSO on mitochondrial GSH levels was also investigated. ESFT cell lines were treated with BSO as described above (controls were treated with PBS), and harvested by scraping into ice-cold PBS after 24 h. Mitochondria were isolated from 2×10^7 cells for each group using the reagent-based protocol described in the Mitochondrial Isolation Kit™ (Pierce Biotechnology Inc., Rockford, IL, USA). Isolated mitochondria were re-suspended in 50–100 μL MES buffer, and GSH levels were assayed as described above. The purity of the mitochondrial fraction was confirmed by Western blot probed for cytochrome *c* (a mitochondrial-specific protein).

2.8. Statistical analyses

One-way ANOVA and post-hoc analyses were used to test for statistical significance between the activities of antioxidants in ESFT cell lines, whereas correlations between antioxidant activities or ROS production and sensitivity to fenretinide were evaluated using the parametric Pearson's correlation. Values of $p \leqslant 0.05$ were considered statistically significant.

3. Results

3.1. Fenretinide triggers ROS production in ESFT cell lines

Treatment of the ESFT cell lines A673, RD-ES, SKES-1, TC-32 and TTC-466 with fenretinide (0–10 μM) led to a significant dose-dependent increase in the intracellular ROS levels relative to vehicle controls (Fig. 1). Whilst the levels of ROS observed 1 h following exposure to fenretinide correlated with the degree of cell death observed within each cell line, similar levels of ROS resulted in different levels of cell death across the different ESFT cell lines (data not shown).

3.2. ROS production is specific to fenretinide-induced cell death in ESFT

In ESFT cell lines treated with five different anticancer drugs, induction of ROS (within 30 min of treatment) was consistently associated with fenretinide-induced death but rarely detected in cells treated with actinomycin D, doxorubicin, etoposide or vincristine (Fig. 2). Furthermore, the levels of ROS induced by fenretinide were several-fold higher than those induced by any of the other drugs in all the five cell lines. ROS levels peaked within 2 h of fenretinide treatment and remained elevated for up to 16 h in RD-ES and SKES-1 cell lines. However, the maximum levels of ROS achieved following fenretinide treatment differed significantly between cell lines ranging from 1.6- to 12-fold above the level of control untreated cells.

3.3. ESFT cells differentially express the key antioxidant enzymes

Antioxidant enzyme protein levels were detected by Western blotting in five ESFT cell lines (Fig. 3). All cell lines except A673 expressed high levels of both SOD1 and SOD2 proteins. A673 expressed high levels of SOD2, but lower levels of SOD1. Expression of GPX1 and CAT was heterogeneous. GPX1 protein levels were very low in A673 and RD-ES cell lines.

3.4. Antioxidant activities in ESFT cell lines are heterogeneous

The activities of the antioxidant enzymes and the concentration of intracellular GSH are shown in Table 2. A673 cells had the lowest activity of GPX1, which mirrored the low protein levels observed on Western blots, as well as the lowest GSH levels and GSH/GSSG ratio. There was no significant correlation ($p > 0.05$) between protein levels and antioxidant activity for any of the other four cell lines indicating that protein levels do not always reflect the biological activity. SOD1 activity was always at least 2-fold higher than SOD2 activity in all the cell lines. SOD2 activity was very low in all ESFT cell lines and was very close to the detection limit for the assay, hence there were poor reproducibility and large standard errors on the values obtained. The redox status of all the cell lines (assessed by the GSH/GSSG redox couple) was below 20 (Table 2), suggesting that ESFT cells were oxidatively stressed in routine culture conditions.

3.5. Sensitivity of ESFT cells to fenretinide is determined by GSH levels

The fenretinide IC-50 values (drug concentrations that caused 50% death of ESFT cells in culture) were plotted against activities of the enzymes in five ESFT cell lines to determine whether there was any relationship between antioxidant activity and sensitivity to fenretinide-induced death. There were strong positive correlations between total cellular GSH ($p < 0.02$) and mitochondrial GSH ($p < 0.009$) levels and fenretinide IC-50 value for each cell line, indicating that cells with higher GSH levels were less susceptible to fenretinide-induced death compared to those with lower levels (Fig. 4). A strong but non-significant negative correlation ($p < 0.1$) was observed between total GST activity and IC-50 values for fenretinide, whereas the activities of the other antioxidant enzymes did not correlate with the sensitivity to fenretinide (Fig. 4). However, the activities of GPX1 and SOD1 correlated strongly with the GSH/GSSG ratio of all the five ESFT cell lines (Table 3, $p < 0.02$ and $p < 0.05$, respectively), suggesting that they may be involved in regulation/maintenance of cellular redox status. Taken together, these results suggest that GSH is the major determinant of ESFT sensitivity to fenretinide, whereas SOD1 and GPX1 may have a role in the regulation of intracellular redox balance under normal conditions.

3.6. Fenretinide depletes intracellular GSH levels in some, but not all, cell lines

Glutathione is the most abundant intracellular antioxidant in cells, and its levels within cells constitute a redox buffer that is

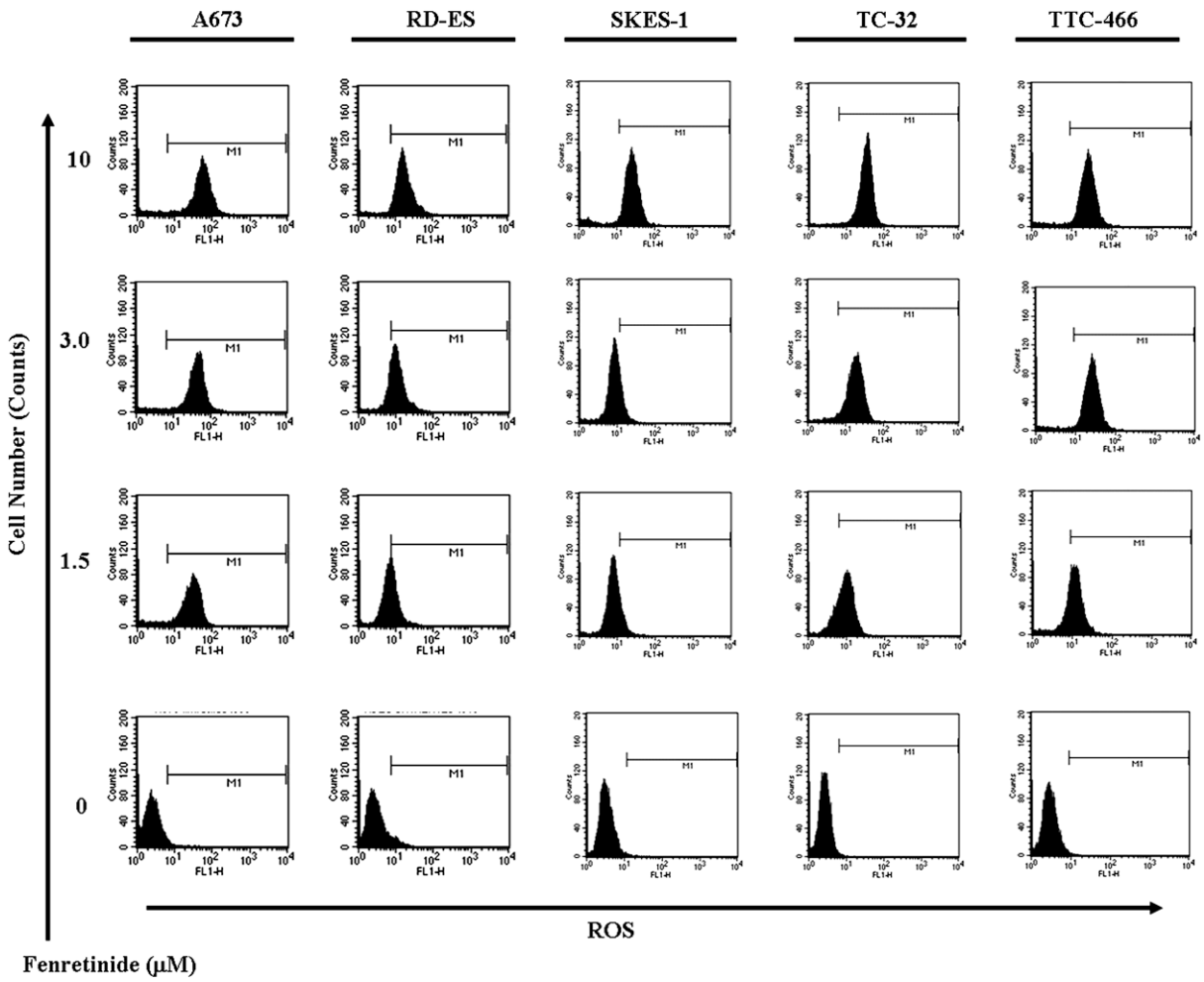

Fig. 1 – Accumulation of reactive oxygen species after treatment of Ewing's sarcoma family of tumours (ESFT) cell lines with fenretinide. Cells were treated with fenretinide (0–10 μM; 1 h) or vehicle (0.20% v/v ethanol) and reactive oxygen species (ROS) were determined using CM-H_2DCFDA and FACS analysis, where fluorescence is proportional to ROS. Fluorescence of cells in the M1 region indicates cells with ROS levels higher than those in vehicle-treated control. An increase in median value of the histogram, *i.e.* a shift of the peak to the right, indicates an increase in ROS. The vertical axis on all panels indicates the number of cells counted whereas the horizontal axis indicates ROS levels.

routinely used as a measure of intracellular redox status.[32] In this study, when ESFT cells were treated with 3 μM fenretinide, the effects on GSH levels were cell line-specific (Fig. 5). In RD-ES, TC-32 and TTC-466 cells, fenretinide (3 μM) decreased GSH levels 24 h after treatment ($p < 0.01$), but no similar decreases were observed in A673 and SK-ES1 cells. The magnitude of the effect of fenretinide on GSH levels was also independent of absolute basal levels of GSH in each cell line. Fenretinide had no effect on GSH levels at 1–6 h in any cell line (results not shown). These results demonstrate that fenretinide does not always decrease cellular GSH in ESFT, and that depletion of intracellular GSH is not a universal mechanism of or prerequisite for fenretinide-induced death in ESFT.

3.7. *Inhibition of GSH biosynthesis increases sensitivity of ESFT cells to fenretinide*

Treatment of ESFT cells with fenretinide resulted in depletion of intracellular GSH in the three of the five cell lines within 24 h (Fig. 5), suggesting a defensive role for GSH against fenretinide-induced ROS. To establish whether depletion of intracellular GSH increased the cytotoxicity of fenretinide to ESFT cells, GSH was depleted by treating with BSO prior to treatment with fenretinide.

Treatment of ESFT cell lines with BSO alone (10–200 μM) induced both a dose- and time-dependent decrease in the viable cell number in all ESFT cells studied (Fig. 6a), consistent with a survival role for GSH in ESFT cell lines. However, treatment with 5 μM BSO had no significant effect on the viable cell number up to 48 h (Fig. 6a), but lowered GSH levels in ESFT cell lines by between 78% and 98% in 24 h (Table 4). Furthermore, BSO (5 μM) treatment did not increase ROS production in ESFT cell lines (results not shown), although much higher doses (1 mM) have been shown to increase levels of ROS in neuroblastoma cells *in vitro*.[33] Fenretinide toxicity was enhanced by BSO-induced GSH depletion in ESFT cell lines (Table 4). However, there was no significant correlation between the extent of GSH depletion and increased sensitivity to fenretinide-induced

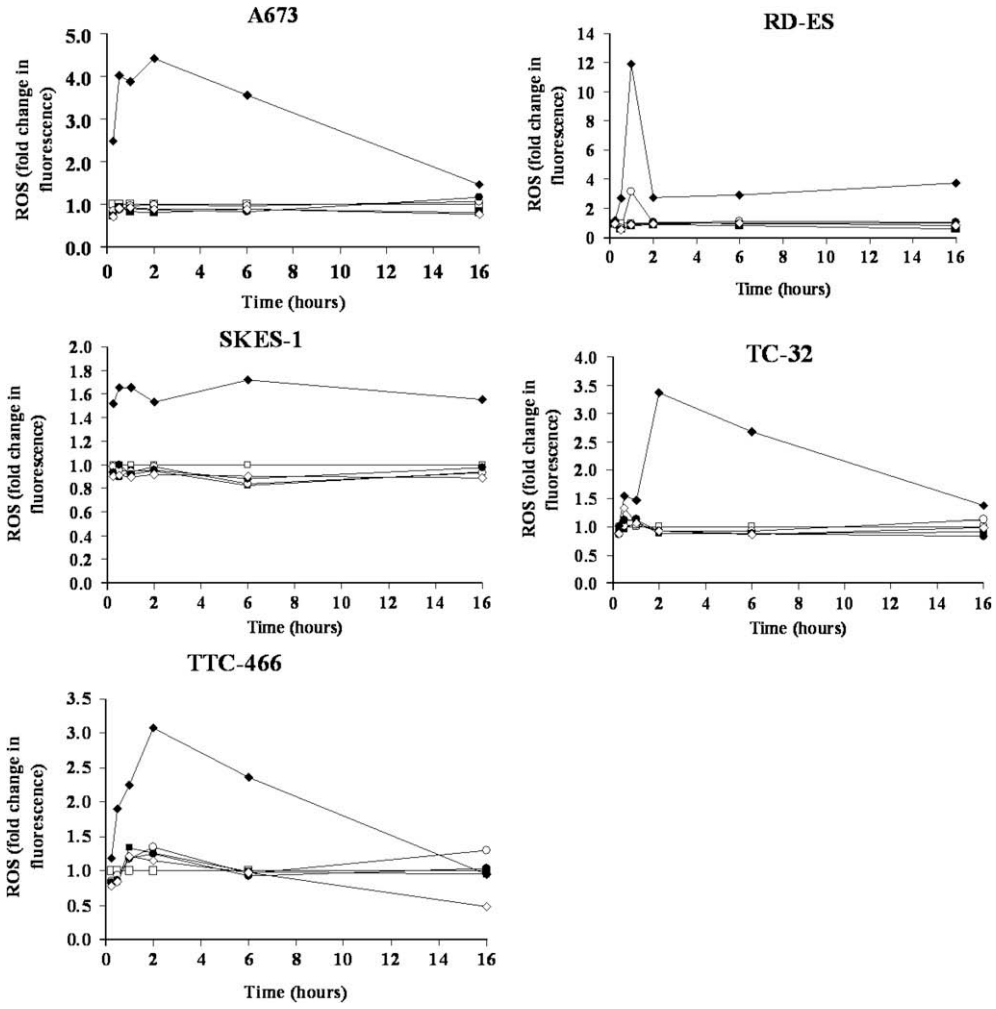

Fig. 2 – Induction of ROS is an early initiating event in fenretinide-induced cell death and is less prominent in cell death induced by other anticancer drugs. Mean fold increase in ROS level relative to vehicle-treated controls in ESFT cell lines treated with 5 different anticancer drugs is shown as means ± SEM ($n = 6$). Cells were seeded into 6-well plates at a density of 2×10^5 cells per well and allowed to adhere overnight before treatment with the IC-50 concentrations of fenretinide and other chemotherapeutic agents. ROS production was assessed using the CM-H_2DCFDA (Molecular Probes, Eugene, OR) assay and FACS analysis at 15 min, 30 min, 1 h, 2 h, 6 h and 16 h post treatment. Symbols: □, vehicle control; ■, actinomycin D; ○, doxorubicin; ●, etoposide; ♦, fenretinide; ◇, vincristine.

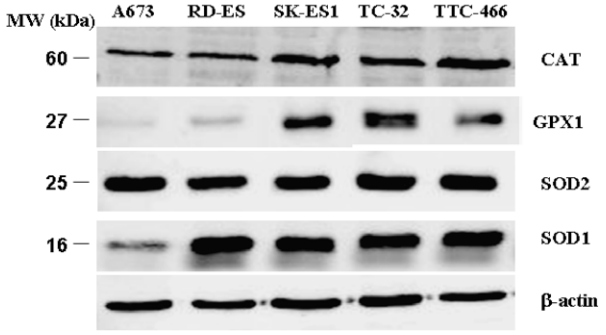

Fig. 3 – Expression of antioxidant enzymes in ESFT cells. SOD2 is abundantly expressed in all ESFT cell lines whereas there is variability in the expression pattern for SOD1, GPX1, and CAT. β-Actin was used as a control to check for equal loading of protein.

death ($p > 0.05$), suggestive of a cell line-specific GSH threshold level for each cell line beyond which further depletion would exacerbate fenretinide toxicity.

3.8. *BSO depletes mitochondrial GSH levels in ESFT cells*

The lack of correlation between the degree of GSH depletion by BSO and enhanced sensitivity to fenretinide suggests that total GSH is not an absolute determinant of cellular response to agents that induce death through the production of ROS, like fenretinide. We therefore hypothesised that depletion of GSH in the mitochondria (critical compartment effecting an intrinsic death cascade) might be a useful strategy to induce cell death and sensitise ESFT cells to agents that activate a death cascade through ROS-dependent mechanisms. Consistent with this hypothesis BSO (which induces cell death in ESFT cells, Fig. 6a) causes depletion of mitochondrial GSH levels (70–99%) in 24 h (Fig. 6b).

Table 2 – Antioxidant enzyme activities and glutathione levels in ESFT cell lines

Cell line	CAT	GPX1	GST	Total GSH	GSH/GSSG ratio	SOD1	SOD2
A673	2.21 ± 0.05	2.53 ± 0.02	56.64 ± 5.92	56.70 ± 2.4	0.86 ± 0.06	3.43 ± 1.02	1.18 ± 0.43
RD-ES	2.10 ± 0.02	7.72 ± 1.11	59.61 ± 1.73	317.8 ± 13.2	7.18 ± 1.20	7.92 ± 1.36	3.23 ± 0.40
SK-ES1	10.1 ± 0.14	6.68 ± 0.50	24.94 ± 1.14	374.1 ± 22.2	5.70 ± 0.36	5.09 ± 1.07	1.31 ± 0.68
TC-32	11.9 ± 0.13	8.43 ± 0.84	47.70 ± 1.31	135.5 ± 6.9	14.9 ± 2.88	7.42 ± 1.99	1.72 ± 0.74
TTC-466	4.73 ± 0.02	13.9 ± 1.00	63.09 ± 2.18	113.2 ± 3.7	20.6 ± 2.68	10.09 ± 1.17	3.81 ± 1.70

Enzymatic activities are given as CAT: U/mg protein; GPX1: μmol/min/mg protein; GST: μmol/min/mg protein; SOD1 and SOD2: U/mg protein. Units for GSH concentration: nmol/mg protein. All abbreviations are explained in the text. Values are means ± SEM of $n = 6$ determinations.

4. Discussion

The generation of ROS is a common feature in the initiation of fenretinide-induced death in ESFT and other cell lines[5,17,21,22,28,34] We have hypothesised that the differential response of ESFT cells to fenretinide *in vitro* could reflect the activities of intracellular antioxidants and their capacity to neutralise ROS. Characterisation of the key antioxidant defences in ESFT was therefore crucial in understanding the differential response of ESFT to fenretinide-induced death. We observed that ESFT cell lines differentially express the key antioxidant enzymes CAT, GPX1, SOD1 and SOD2 as well as the non-enzymatic antioxidant GSH. However, only cellular GSH appeared to be directly involved in defence against fenretinide-induced death in ESFT; this finding was supported by experiments showing that depletion of GSH using BSO could sensitise ESFT cells to fenretinide-induced death.

In this paper, we have shown for the first time that the early induction of ROS (within 30 min) as a trigger for cell death is associated with the activity of fenretinide in ESFT cells, whereas initiation of the death cascade by chemotherapeutic agents such as actinomycin D, doxorubicin, etoposide or vincristine is rarely associated with the production of ROS. These results support the hypothesis that ROS production is an initiating event in fenretinide-induced death of ESFT and that this may be specific for the induction of the death cascade by fenretinide, but not by conventional chemotherapeutics. Overall, there appears to be no common threshold of ROS required to trigger cell death in ESFT, as the maximum levels achieved differed between cell lines by 1.6–12-fold. Thus, the levels of ROS generated after fenretinide treatment, as well as the amount of ROS required to trigger cell death, are cell line-specific; both seem to depend on the antioxidant capacity of individual ESFT cell lines.

Tumour cells are reported to have low SOD2 activity,[35] low activities of SOD1, CAT and GPX1 have also been reported in many transformed cell lines,[36,37] consistent with the hypothesis that low antioxidant levels may be a reflection of low levels of oxidative stress (ROS) in these cell lines.[38] However, results from this study are at variance with this hypothesis. We observed that whilst ESFT cell lines have low SOD2 and GPX1 activities, they also generally have very low GSH/GSSG ratios, indicating mild to severe oxidative stress under routine culture conditions. Our results are consistent with the hypothesis that tumour cells in culture may select for higher levels of oxidative stress essential for their rapid proliferation and maintenance of malignant phenotype through suppression of the activities of key antioxidants.[39] The prevalence of an oxidative stress environment in ESFT cells offers a potential target for anticancer strategies employing agents that generate ROS *in situ*, the objective being to further increase oxidative damage to a level that triggers cell death. In this study, we demonstrated that ROS-producing agents such as fenretinide effectively kill ESFT cells in culture, and that depletion of a key cellular antioxidant enhances fenretinide cytotoxicity.

The treatment of ESFT cells with fenretinide alone (and also in combination with BSO) demonstrated that depletion of cellular GSH is not a pre-requisite for the initiation and/or execution of cell death by fenretinide. BSO depleted GSH levels in both cytosolic and mitochondrial compartments of ESFT cells, but the effect of this depletion on sensitivity to fenretinide was cell line-specific. Sensitivity to fenretinide was not dependent on absolute level of GSH *per se*, but each cell line had a particular GSH threshold level below which further depletion increased fenretinide toxicity.

BSO has been used to sensitise oesophageal cancer cell lines to agents such as cisplatin[40] ovarian cancer cells to cyanomorpholino-doxorubicin,[41] and a combination of BSO-melphalan was observed to be more effective in treating neuroblastoma compared to melphalan alone.[42] Our results have shown BSO to be very toxic to ESFT cells at doses much lower than those used to deplete GSH in other cell lines[43] and well-tolerated in phase I studies *in vivo*,[44] consistent with the hypothesis that ESFT cells are particularly sensitive to oxidative stress. The depletion of mitochondrial GSH observed in this study is at variance with the notion that mitochondrial GSH is resistant to depletion by BSO (see review [31]). Whether this phenomenon is ESFT-specific and could be exploited for the development of novel treatment strategies remains to be seen.

The net redox status of a cell is determined by various factors amongst which are the enzymatic antioxidants (free-radical scavengers), non-enzymatic antioxidants (e.g. GSH), as well as auxiliary antioxidant enzymes such as GST. The lack of correlation between CAT, GPX1 and SOD1 and 2 enzyme activities and sensitivity of ESFT cells to fenretinide-induced death suggests that these antioxidants may not be directly involved in defence against fenretinide-induced ROS. Although cellular enzymatic antioxidants can be induced by ROS,[45] fenretinide causes elevation of ROS levels in ESFT cells within 5–15 min of treatment,[5] hence induction of these antioxidant

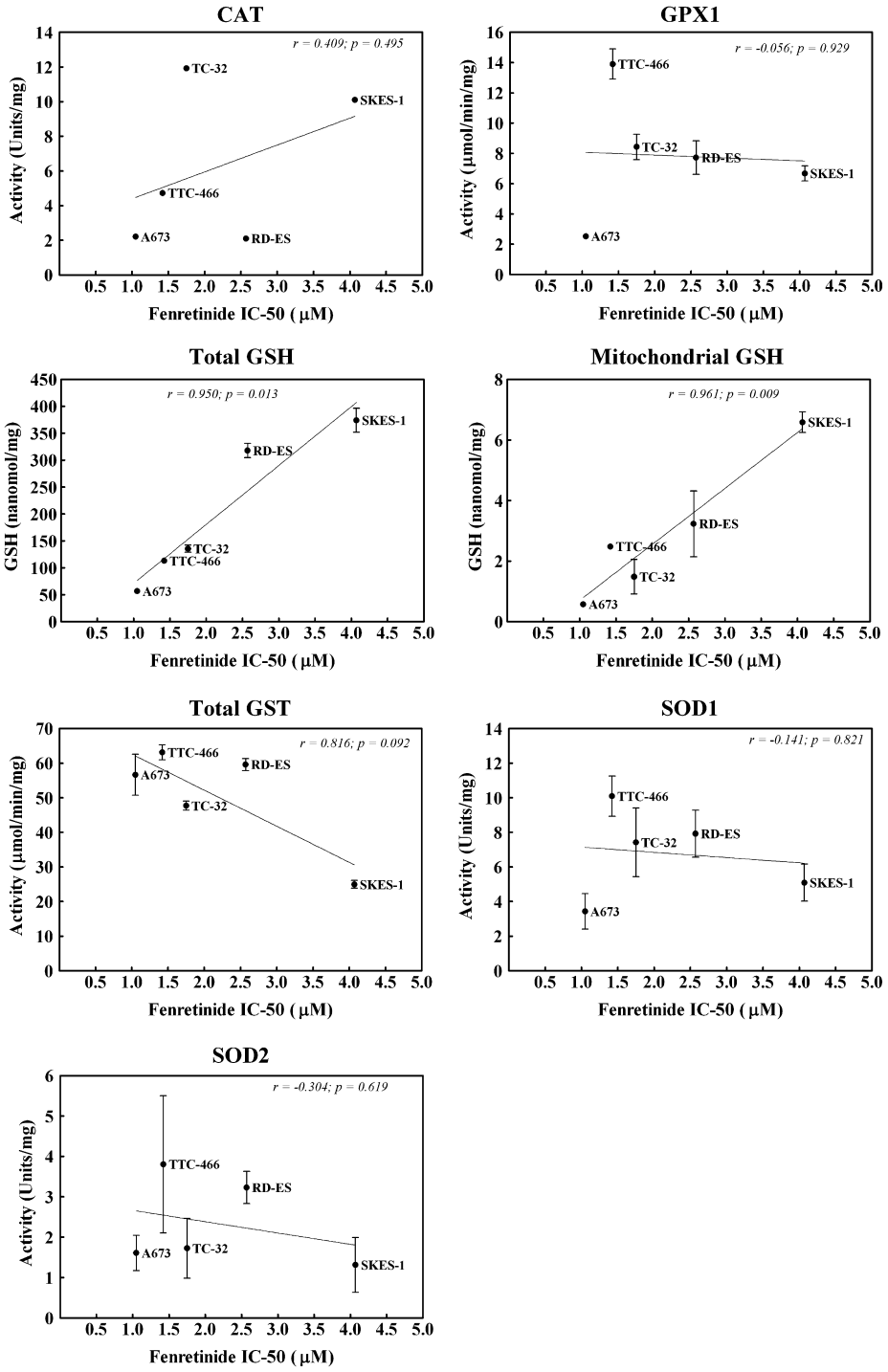

Fig. 4 – The relationships between antioxidant activity (or level) in ESFT cells and the effect of fenretinide (given as IC-50 values). The Pearson correlation coefficient (*r*) and the degree of statistical significance (*p*-value) are shown above each plot. Each point on the graphs is means ± SEM for *n* = 6 determinations.

defences by fenretinide-induced ROS (which may take place after a few to several hours) may not be sufficient to protect against the oxidative stress. However, the strong positive correlations between GPX1 and SOD1 enzymatic activities and cellular GSH/GSSG ratios suggest that cellular redox status may also be largely influenced by the activities of these two enzymes. Further experiments with targeted knock-downs or over-expression of these antioxidants in ESFT cell lines are needed in order to delineate their physiological role. The negative correlation between GST activity and fenretinide IC-50 s of ESFT cell lines supports the hypothesis that depletion of intracellular GSH by this detoxifying enzyme may

Table 3 – Correlation between antioxidant enzyme activities and cellular redox status[a]

Antioxidant activity *versus* GSH/GSSH ratio	Pearson's correlation coefficient (*r* value)	*p*-Value[b]
CAT	0.30896	NS
GPX1	0.94345	<0.02
GST	0.30611	NS
SOD1	0.89716	<0.05
SOD2	0.60427	NS

a Cellular redox status is the GSH/GSSG ratio calculated from the GSH and GSSG concentrations for each cell line. Pearson's correlation coefficients (*r*) were calculated from the enzyme activities and GSH/GSSG ratios of $n = 5$ ESFT cell lines.

b Correlation statistically significant at confidence level (*p*-value) indicated; NS means *p*-value is not statistically significant.

leave ESFT cell lines susceptible to death induced through oxidative stress. At present, it is not known whether GSTs are involved in fenretinide metabolism, and there is insufficient data to support the notion that GST activity in ESFT could be a useful marker of sensitivity to fenretinide. Further work is required to ascertain whether fenretinide is a substrate for GSTs and to identify the isoforms involved.

In conclusion, results from this study have identified cellular GSH as a major determinant of sensitivity to fenretinide-induced cell death in ESFT. However, the differential expression of enzymatic antioxidants in ESFT cell lines does not predict sensitivity to fenretinide-induced cell death; sensitivity to fenretinide-induced death may be dependent on the abundance of non-enzymatic antioxidants, such as GSH, and a reduction in GSH-dependent detoxification of reactive electrophiles. The sensitivity of ESFT cells to modulation of

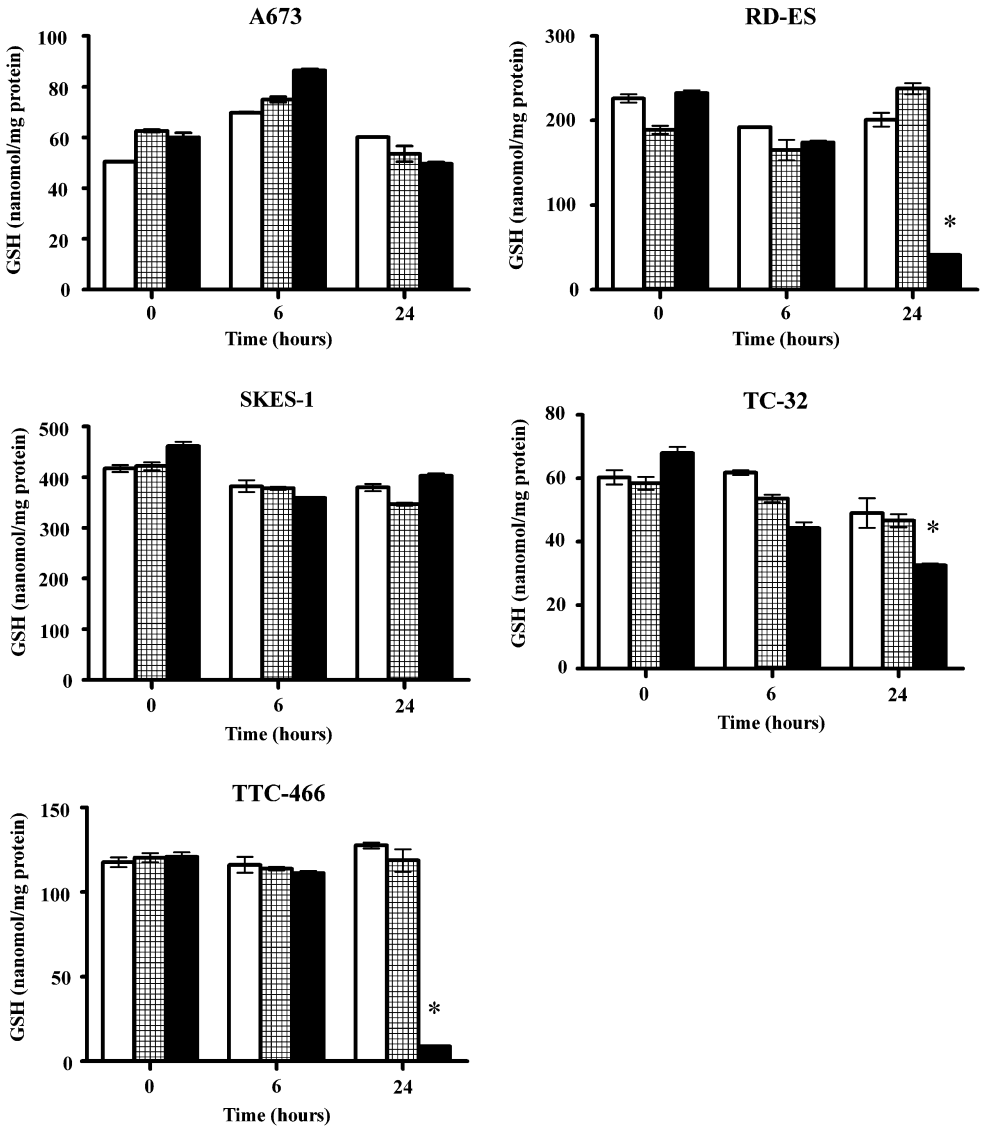

Fig. 5 – The effect of fenretinide (3 μM) on GSH levels in ESFT cell lines at 6 and 24 h. Bars represent means ± SEM for $n = 6$ experiments. *Value significantly different from both controls at maximum value of $p < 0.01$. Symbols: ▦, media control; □, ethanol control; and ■, fenretinide.

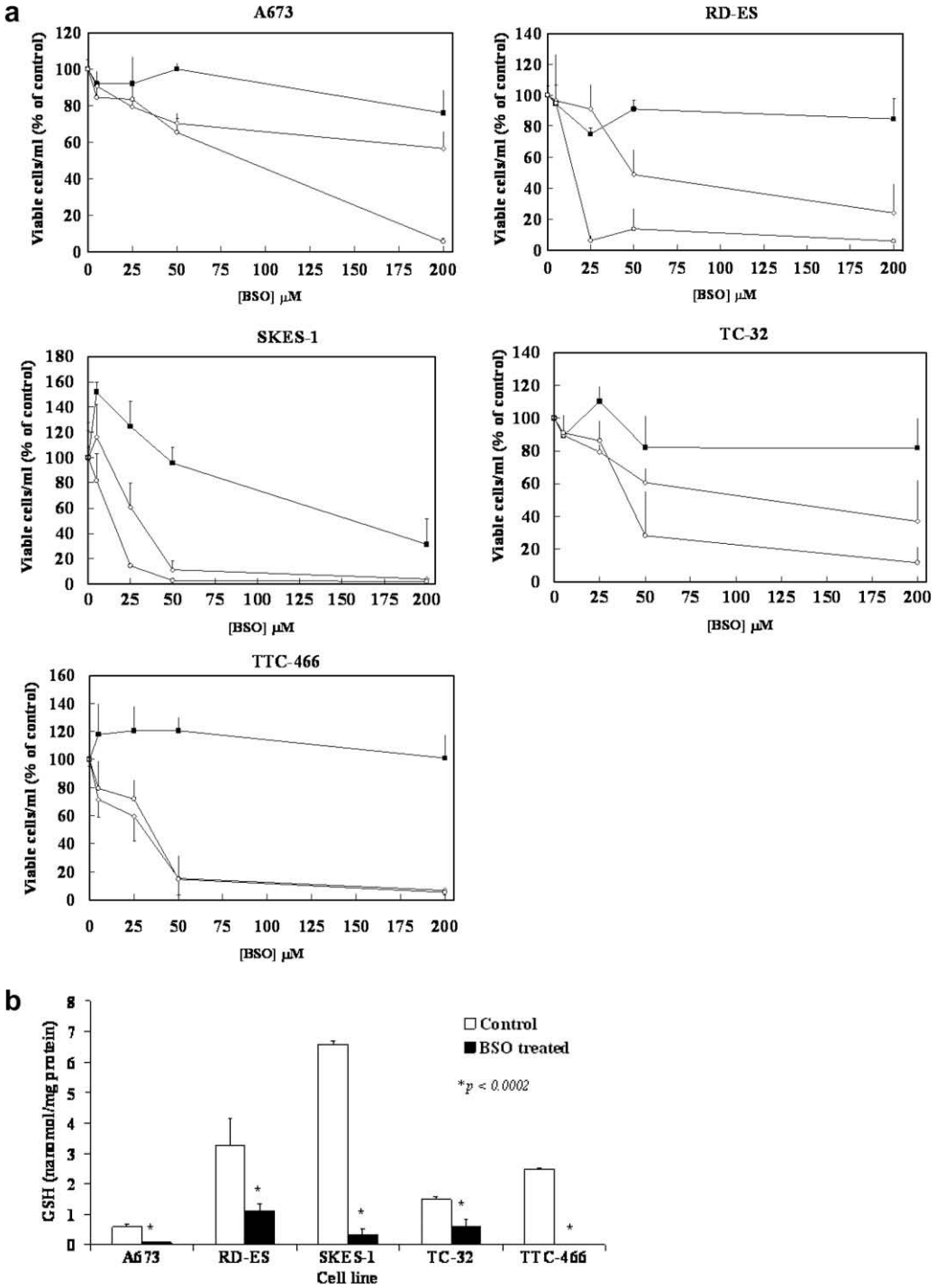

Fig. 6 – (a) Dose- and time-dependent effects of BSO (0–200 μM) on viable ESFT cell number in culture. Results are shown as means ± SEM (n = 6). Symbols: ■, 24 h; ◇, 48 h; ○, 72 h. (b) The effects of BSO on mitochondrial GSH levels in ESFT cells; results are shown as means ± SEM for n = 6 determinations. *Values significantly different from controls at maximum value of $p < 0.01$.

antioxidant status by agents such as BSO offers a potential therapeutic target that could be exploited in the treatment of ESFT by ROS-producing agents (Fig. 7). However, there is the possibility that the extreme sensitivity of ESFT cells to the effects of GSH depletion we observed in this study could be an artefact of culture conditions since these cell lines are already oxidatively stressed. Currently, there are no adequate procedures for accurately determining the levels of oxidative stress in tumour cells *in vivo*, hence future challenges should focus on determining the antioxidant status/redox balance in primary ESFT to ascertain whether manipulation of cellular oxidative stress is a viable strategy in the treatment of ESFT.

Table 4 – Effects of BSO on GSH levels and sensitivity of ESFT cells to fenretinide[a]

Cell line	ΔGSH (%)	IC-50 (control)	IC-50 (+5 μM BSO)	*p*-Value *versus* control[b]	Fold increase in sensitivity to fenretinide
A673	98.7 ± 1.5	1.099 ± 0.212	0.345 ± 0.116	<0.008	3.2
RD-ES	78.0 ± 12.1	2.778 ± 0.820	0.020 ± 0.014	<0.005	139
SK-ES1	88.7 ± 6.8	3.725 ± 1.398	1.195 ± 0.190	<0.036	3.1
TC-32	79.3 ± 5.1	1.739 ± 0.177	0.719 ± 0.013	<0.001	2.4
TTC-466	79.7 ± 11.1	1.258 ± 0.152	0.446 ± 0.023	<0.001	2.8

a Values are means ± SEM of triplicate determinations for $n = 3$ separate experiments. ΔGSH (%) is the average percentage decrease in GSH concentration achieved after treatment of ESFT cell lines with 5 μM BSO for 24 h.
b Values significantly different from control at p level indicated.

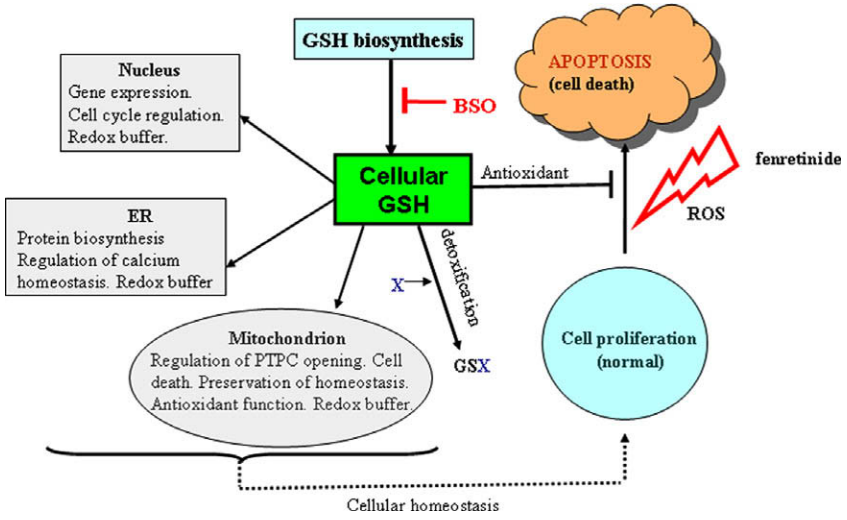

Fig. 7 – A hypothetical schema depicting the central role of glutathione in preserving cellular homeostasis and redox balance. Inhibition of GSH biosynthesis by BSO disrupts cellular homeostasis thereby sensitising the cell to ROS-induced damage and apoptosis and might be a viable strategy in the treatment of ESFT. ER, endoplasmic reticulum; GSX, glutathione conjugate; PTPC, mitochondrial permeability transition pore complex; X, foreign compound (xenobiotic). Other abbreviations are as explained in the text.

Conflict of interest statement

None declared.

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