British Journal of Cancer (1996) 74, (Suppl. XXVII) S109-S113

The interaction of buthionine sulphoximide (BSO) and the topoisomerase I inhibitor CPT-11

TE Sawyer and JA Bonner

Department of Oncology, Mayo Clinic, Rochester, MN 55905, USA.

Summary Buthionine sulphoximide (BSO)-induced depletion of glutathione (GSH) was found to be associated with an increased sensitivity to CPT-11 (topoisomerase I-reactive agent) in V79 hamster lung fibroblast cells. When V79 cells were exposed to 2.5 mM BSO for 28 h beginning 4 h prior to a 24 h coincubation with CPT-11, cytotoxicity was increased compared with CPT-11 alone. It was determined that BSO resulted in a G_1 cell cycle arrest and a decrease in the percentage of cells in S-phase. Since CPT-11 is known to be S-phase-specific, this BSO-induced cell cycle redistribution did not appear to account for the chemosensitisation of CPT-11. Additionally, BSO did not alter intracellular accumulation of CPT-11, conversion of CPT-11 to its active metabolite SN-38, or efflux of either CPT-11 or SN-38 from the cell. Finally, BSO resulted in a slight reduction, rather than an increase, in the number of stabilised DNA-topoisomerase I complexes induced by CPT-11. Therefore, these results suggest that BSO-induced sensitisation of V79 cells to the cytotoxic effects of CPT-11 occurs by a mechanism independent of the stabilisation of DNA-topoisomerase I complexes.

Keywords: glutathione depletion; V79 cells; cell cycle distribution; camptothecin

CPT-11 is a derivative of camptothecin and like the parent drug, CPT-11 is known to stabilise the formation of DNA-topoisomerase I complexes (cleavable complexes). Additionally, CPT-11 undergoes *in vivo* hydrolysis to the metabolite SN-38 (Pommier and Tanizawa, 1993). It has been shown that SN-38 has greater cytotoxic potency and results in increased stabilisation of cleavable complexes compared with CPT-11 in human colon carcinoma cells (Tanizawa *et al.*, 1994). Although the stabilisation of cleavable complexes is believed to be a critical event in CPT-11-induced cytotoxicity, other CPT-11 associated events suggest that the overall mechanism is very complex. For instance, studies of the parent compound, camptothecin, have suggested that the topoisomerase I-reactive compounds may decrease RNA synthesis (Zhang *et al.*, 1988; Schaak *et al.*, 1990). Additionally, camptothecin has been shown to induce gene expression associated with cellular differentiation of a monocytic cell line (Aller *et al.*, 1992).

The reducing capacity of naturally occurring intracellular thiols such as glutathione has been shown to be involved in the mechanism of action or activity of many chemotherapeutic agents (Chasseaud, 1979). Therefore, this investigation was initiated to determine the interaction of glutathione (a scavenger of free radicals) depletion and the topoisomerase I-reactive compound CPT-11. In fact, it was determined that BSO-induced glutathione depletion was associated with marked sensitisation to the cytotoxic effects of CPT-11 in V79 cells. Studies were performed to characterise this interaction.

Materials and methods

Cell culture and cell survival

V79 hamster lung fibroblast cells were cultured as described previously (Bonner *et al.*, 1992). Cell survival was assessed by a standard colony formation assay as described previously (Bonner *et al.*, 1992). Briefly, following appropriate exposure to CPT-11 or SN-38 in the presence or absence of BSO, cells were washed twice with phosphate-buffered saline (PBS) and plated to yield between 50 and 100 colonies per dish. After fixation and staining, colonies with greater than 50 cells were counted.

Drugs

CPT-11 and SN-38 were provided as generous gifts from Yakult, Japan and Dr Scott Kaufmann (Mayo Clinic, Rochester, MN), respectively. CPT-11, SN-38 and BSO were made fresh for each experiment.

Glutathione (GSH) content

Intracellular GSH was assessed by a previously published method (Rice *et al.*, 1986) with minor modifications. Following treatment of cells with the desired BSO exposure, the cells were washed and monochlorobimane (MCIB) was added as a fluorescent probe which specifically labels reduced GSH. The reaction was stopped with trichloroacetic acid and the degree of fluorescence (excitation 398 nm, emission 488 nm) correlated with the quantity of GSH. A standard curve of GSH was made for each experiment and data were expressed as the percentage control of GSH in untreated cells.

Growth inhibition

Growth inhibition was assessed as previously described (Bonner *et al.*, 1994). Briefly, cells were allowed to enter exponential growth, and control and treated cells were subsequently counted (haemocytometer and Coulter counter) at various time points.

Flow cytometry

Cell cycle distributions were determined as described previously (Crissman and Steinkamp, 1982) with minor modifications (Minehan and Bonner, 1993).

CPT-11/SN-38 intracellular accumulation and efflux

Intracellular CPT-11 and SN-38 accumulation were determined by fluorescence spectrophotometry as described by Niimi *et al.*, 1992. (CPT-11: excitation 370 nm, fluorescence

Correspondence: JA Bonner, Department of Oncology, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA.

430 nm; SN-38: excitation 380 nm, fluorescence 550 nm). Data were expressed as ng of drug per million cells.

CPT-11/SN-38 stabilised DNA–topoisomerase I complexes

Complexes were detected as single-stranded DNA breaks using alkaline elution under conditions with and without proteolysis with proteinase K as described previously (Tanizawa *et al.*, 1994; Bonner *et al.*, 1992). Data were presented as the cGy equivalent of DNA single strand breaks as determined from a calibration curve of elution rate *vs* radiation dose in V79 cells.

Statistical analysis

All experiments were performed at least three times but representative experiments are shown. Error bars represent the standard error of the mean of triplicate samples within a given experiment.

Results

Since glutathione (GSH) plays a significant role in the metabolic pathways of multiple chemotherapeutic agents, experiments were performed to determine the interaction of BSO-induced GSH depletion on CPT-11-induced cytotoxicity utilising V79 hamster lung fibroblast cells. Firstly, studies were designed to determine the time course of BSO-induced GSH depletion for various concentrations of BSO. It was determined that exposure of V79 cells to 4 h of either 2.5 mM or 5.0 mM BSO [5 mM was utilised in previous studies by our laboratory (Bonner *et al.*, 1992)] resulted in depletion of GSH to less than 15% of control values. The time course of depletion for cells exposed to 2.5 mM or 5 mM BSO was essentially identical (data not shown).

Experiments were performed to determine the effect of these BSO treatments on CPT-11 sensitivity. When cells were exposed to 5 mM BSO for 7 h beginning 4 h prior to a 3 h coincubation with various concentrations of CPT-11, no chemosensitisation occurred (Figure 1). Exposure to 5 mM BSO alone for 7 h did not result in cytotoxicity. Next, studies were undertaken with more protracted exposures to CPT-11, as it is difficult to achieve significant cytotoxicity with short exposures to CPT-11 exposures (<5 h) due to the fact that CPT-11 is S-phase-specific and that CPT-11-induced cytotoxicity is believed to occur as the cells traverse the S-phase (Pommier and Tanizawa, 1993). When longer exposures to 5 mM BSO were used (>7 h), minimal cytotoxicity occurred (approximately a 25% decrease in surviving fraction). Therefore, 2.5 mM BSO was used for the experiments involving longer BSO exposures and this concentration of BSO resulted in no cytotoxicity even after 28 h. When V79 cells were pretreated with 2.5 mM BSO for 4 h followed by a 24 h coincubation of BSO and various concentrations of CPT-11, chemosensitisation occurred, as demonstrated by an approximate 3-fold reduction in the IC_{50} (Figure 1).

It was then of interest to determine the effect of BSO pretreatments on SN-38-induced cytotoxicity, as SN-38 is the active metabolite of CPT-11. Marked chemosensitization occurred when the V79 cells were exposed to either 5 mM BSO for 7 h beginning 4 h prior to a 3 h coincubation with various concentrations of SN-38, or 2.5 mM BSO for 28 h beginning 4 h prior to a 24 h coincubation with various concentrations of SN-38 (Figure 2). It was not clear why BSO pretreatments resulted in chemosensitisation for the 4 h SN-38 exposures but not for the 4 h CPT-11 exposures.

Since BSO-induced depletion of GSH was associated with chemosensitisation of both CPT-11 and SN-38 under conditions in which cells were exposed to CPT-11 or SN-38 for 24 h, studies were undertaken to determine whether these protracted exposures to BSO altered various proliferative parameters of the V79 cells which may have made

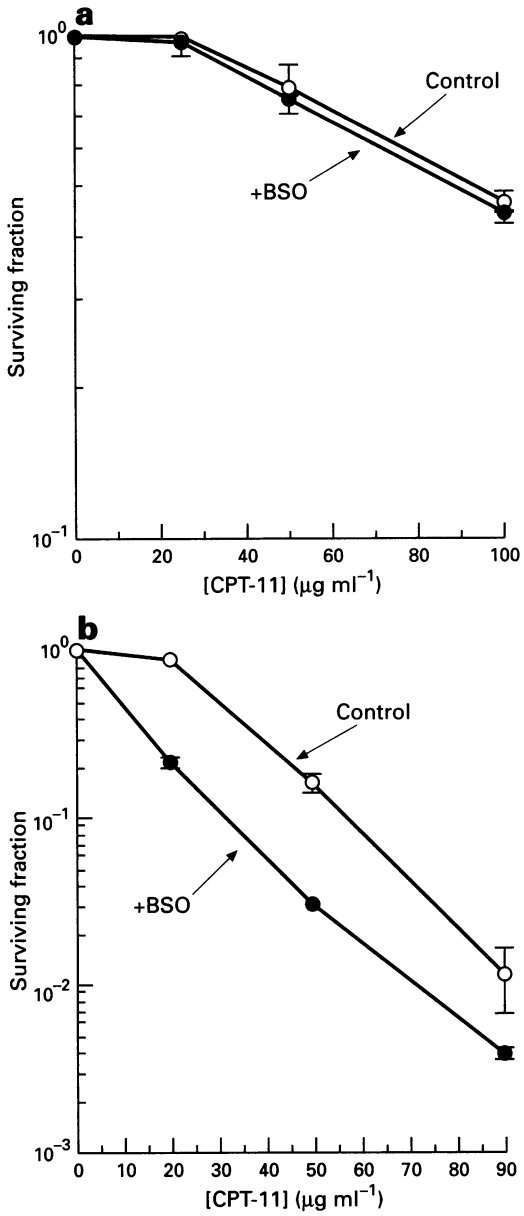

Figure 1 Preincubation of V79 cells with 5 mM BSO for 4 h followed by a 3 h coincubation with BSO and various concentrations of CPT-11 did not lead to chemosensitisation (**a**). However, marked chemosensitisation occurred when the cells were preincubated with 2.5 mM BSO for 4 h followed by a 24 h coincubation with BSO and various concentrations of CPT-11 (**b**).

them more sensitive to CPT-11 or SN-38. (As mentioned above, exposure to 2.5 mM BSO for 28 h did not cause cytotoxicity as measured by colony formation.) Two types of experiments were performed to assess the effect of BSO on cellular proliferation.

First, growth kinetics were assessed by a standard daily growth assessment (Bonner *et al.*, 1994). It was determined that a 28 h exposure to 2.5 mM BSO did slow exponential growth of V79 cells compared with control cells. However, following the removal of BSO, the cells resumed exponential growth consistent with that of the control cells (Figure 3). Second, flow cytometry experiments were designed to determine whether this BSO-induced growth arrest caused the cells to enter a more CPT-11/SN-38-sensitive cell cycle phase. When exponentially growing V79 cells were exposed to 2.5 mM BSO for various time periods and subsequently assessed for cell cycle distributions, it was determined that BSO caused a G_1 arrest and that fewer cells were in S-phase throughout the 28 h incubation with BSO (Figure 3).

Therefore, it did not appear that the BSO-induced chemosensitisation was due to a cell cycle redistribution, as it is known that CPT-11 and SN-38 are S-phase-specific (Slichenmyer *et al.*, 1993).

Since BSO-induced alterations of cellular proliferation did not appear to account for the chemosensitisation, it was hypothesised that BSO may alter CPT-11 or SN-38 intracellular accumulation, as BSO treatments have been shown to increase intracellular accumulation of the topoisomerase II-reactive agent, etoposide (Schneider *et al.*, 1995). In order to test this hypothesis, V79 cells were exposed to 20 $\mu g\ ml^{-1}$ of CPT-11 and assessed for intracellular drug accumulation after various times of exposure (maximum 24 h). Efflux of CPT-11/SN-38 was assessed at various times following a 24 h exposure to CPT-11 and was found to be extremely rapid, as previously described by others (Niimi *et al.*, 1992). Fluorescence spectrophotometry was used to assess the intracellular accumulation of CPT-11. Additionally, conversion of CPT-11 to SN-38 was assessed as described in the Materials and methods. It was determined that intracellular CPT-11 accumulation, conversion of CPT-11 to SN-38, and the subsequent efflux of both CPT-11 and SN-38 were similar in cells pretreated with BSO (as in the cell survival experiments) compared with cells not treated with BSO (Figure 4).

Since BSO did not affect intracellular CPT-11 accumulation, it was important to determine whether BSO affected the number of CPT-11-induced stabilised DNA–topoisomerase I complexes (cleavable complexes), as these complexes are believed to be critically involved in CPT-11-induced cytotoxicity (Pommier and Tanizawa, 1993). When cells were exposed to 2.5 mM BSO for 28 h beginning 4 h prior to a 24 h coincubation with various concentrations of CPT-11, a slight reduction in the number of stabilised cleavable complexes was measured as compared with cells treated with CPT-11 alone (Figure 4). It is of note that the combination of BSO and CPT-11 resulted in a similar number of non-protein-associated DNA single strand breaks compared with CPT-11 alone when alkaline elution was performed without proteinase K (data not shown).

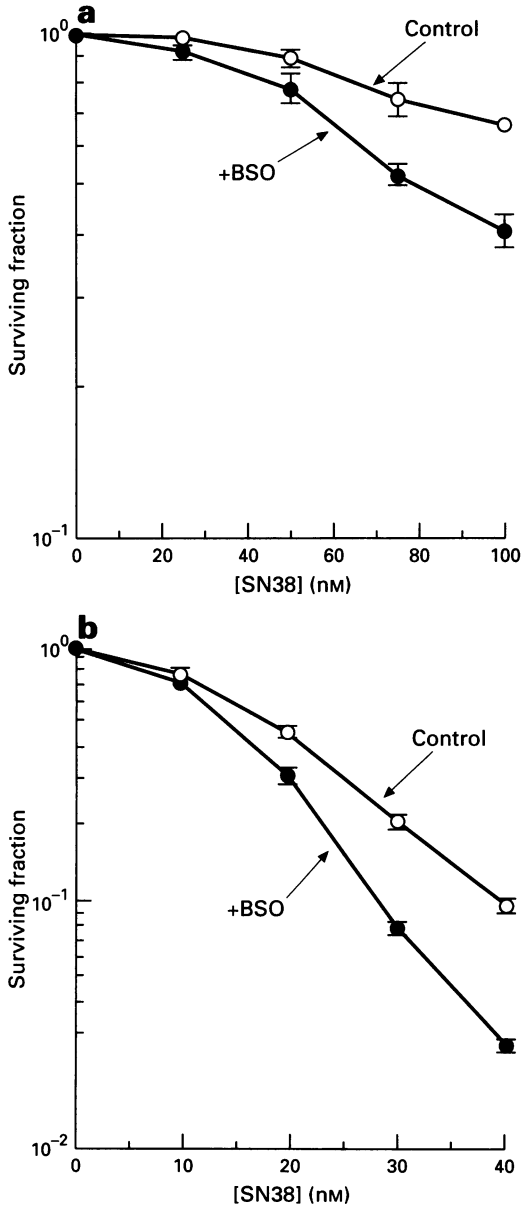

Figure 2 Preincubation of V79 cells with 5 mM BSO for 4 h followed by a 3 h coincubation with BSO and various concentrations of SN-38 lead to chemosensitisation (**a**). Additionally, chemosensitisation occurred when the V79 cells were preincubated with 2.5 mM BSO for 4 h followed by a 24 h coincubation with BSO and various concentrations of SN-38 (**b**).

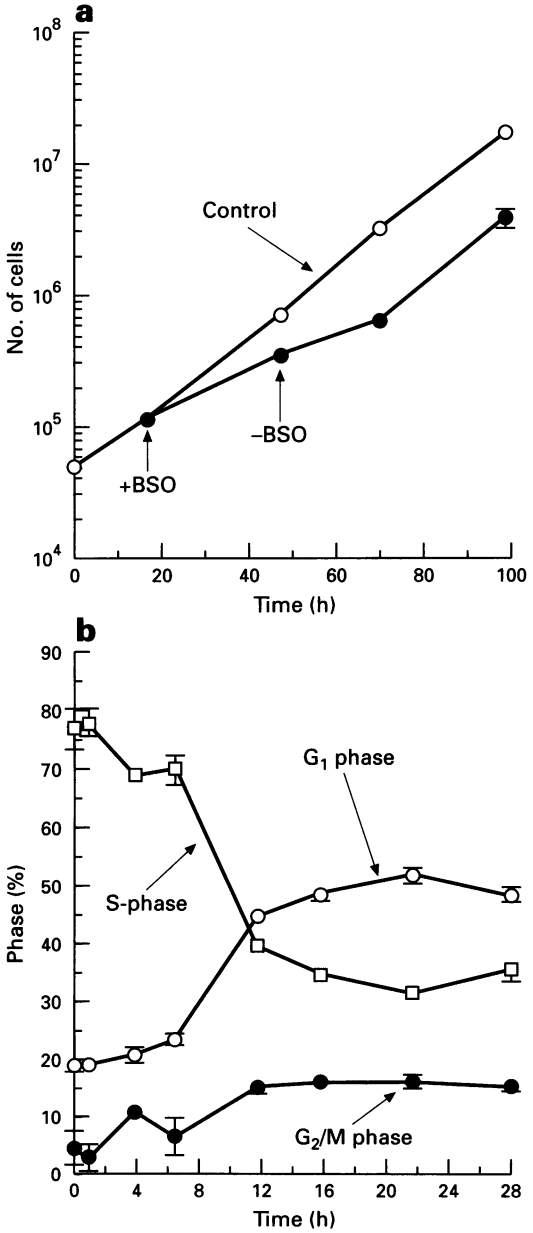

Figure 3 Exposure of V79 cells to 2.5 mM BSO for 28 h resulted in a growth delay followed by resumption of normal growth approximately 24 h after removal of BSO (**a**). Additionally, exposure of V79 cells to 2.5 mM BSO for 28 h resulted in a greater proportion of G_1 phase cells and fewer S-phase cells compared with the control cell-cycle distribution (**b**).

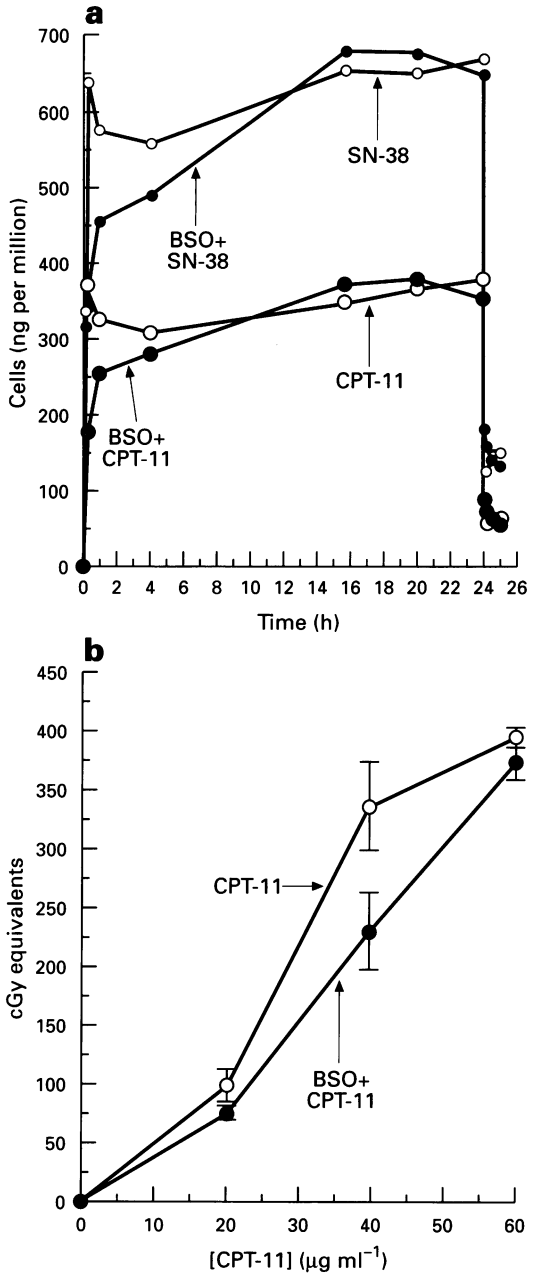

Figure 4 (**a**) Intracellular CPT-11 accumulation, conversion of CPT-11 to SN-38 and efflux of CPT-11/SN-38 from V79 cells were not affected by pre-exposure to BSO and subsequent coincubation with BSO and 20 $\mu g/ml^{-1}$ CPT-11 compared with CPT-11 treatment alone. Efflux was assessed after 24 h of exposure to CPT-11. (**b**) Preincubation of V79 cells with 2.5 mM BSO for 4 h followed by a 24 h coincubation with BSO and various concentrations of CPT-11 resulted in a slight reduction in the number of stabilised DNA–topoisomerase I complexes compared to CPT-11 treatment alone. Following appropriate drug exposures, cells were analysed by alkaline elution as described in the Materials and methods section.

Discussion

The current investigation was performed to assess the interaction of BSO-induced GSH depletion and CPT-11-induced cytotoxicity in V79 cells (hamster lung fibroblasts). When cells were exposed to 2.5 mM BSO for 4 h (resulting in GSH depletion to <15% control values) followed by a coincubation with BSO and CPT-11 or SN-38 for 24 h, chemosensitisation occurred as exemplified by an approximate 1.5–3.0-fold decrease in the IC_{50} values for either CPT-11 or SN-38 (Figures 1 and 2). Additionally, pretreatment of V79 cells with 5 mM BSO for 4 h followed by a 3 h coincubation with BSO and CPT-11 or SN-38, resulted in sensitisation of cells to SN-38 but not CPT-11. The mechanism of this difference is not clear.

The mechanism of BSO-induced chemosensitisation varies greatly depending on the chemotherapeutic agent involved. For instance, chemosensitisation in association with BSO-induced GSH depletion has been related to greater free radical production in the case of doxorubicin (Dusre *et al.*, 1989), greater intracellular drug accumulation in the case of etoposide (Schneider *et al.*, 1995) and partial inhibition of DNA repair in the case of cisplatin (Lai *et al.*, 1989).

The current investigation revealed that BSO pretreatments did not sensitise cells to CPT-11 or SN-38 by altering intracellular accumulation of either drug or by causing a redistribution of the V79 cells into a more CPT-11/SN-38-sensitive cell cycle phase. (Although, the overall reduction in S-phase cells following BSO exposure was dramatic, it should be noted that this reduction may have been accompanied by a change in the ratio of early S-phase cells/late S-phase cells as cells accumulate at the G1/S border. Further work would be necessary to fully characterize the BSO-induced G1 arrest.) Additionally, BSO pretreatments resulted in a slight reduction, rather than an increase, in the number of CPT-11-induced stabilised DNA-topoisomerase I complexes (Figure 4). It is possible that this reduction was due to the above noted cell cycle redistribution that occurred during the BSO exposure. Therefore, BSO pretreatments appeared to sensitise V79 cells to CPT-11 or SN-38 by a mechanism independent of the stabilisation of DNA–topoisomerase I complexes.

It is possible that GSH depletion is associated with an enhancement of the cytotoxic events which follow the stabilisation of DNA–topoisomerase I complexes by CPT-11. Further work will be necessary to study this hypothesis and fully decipher the mechanism of chemosensitisation.

Acknowledgements
This work was supported by an American Cancer Society Fellowship (TES), Moreton Onoclogy Fellowship (TES) and an American Cancer Society Career Development Award (JAB).

References

ALLER P, RIUS C, MATA F, ZORRILLA A, CABANAS C, BELLON T AND BERNABEU C. (1992). Camptothecin induces differentiation and stimulates the expression of differentiation-related genes in U-937 human promonocytic leukemia cells. *Cancer Res.*, **52**, 1245–1251.

BONNER JA, CHRISTIANSON TJH AND LAWRENCE TS. (1992). Interaction of buthionine sulfoximide and the stabilization of DNA-topoisomerase II complexes by doxorubicin. *Int. J. Radiat. Oncol. Biol. Phys.*, **22**, 519–523.

BONNER JA, MAIHLE NJ, FOLVEN BR, CHRISTIANSON TJH AND SPAIN K. (1994). The interaction of epidermal growth factor and radiation in human head and neck squamous cell carcinoma cell lines with vastly different radiosensitivities. *Int. J. Radiat. Oncol. Biol. Phys.*, **29**, 243–247.

CHASSEAUD LF. (1979). The role of glutathione and glutathione s-transferases in the metabolism of chemical carcinogens and other electrophilic agents. *Adv. Cancer Res.*, **29**, 175–275.

CRISSMAN HA AND STEINKAMP JA. (1982). Rapid, one step staining procedures for analysis of cellular DNA and protein by single and dual laser flow cytometry. *Cytometry*, **3**, 84–90.

DUSRE L, MIMNAUGH EG, MYERS CE AND SINHA BK. (1989). Potentiation of doxorubicin cytotoxicity by buthionine sulfoximine in multidrug-resistant human breast tumor cells. *Cancer Res.*, **49**, 511–515.

LAI GM, OZOLS RF, YOUNG RC AND HAMILTON TC. (1989). Effect of glutathione on DNA repair in cisplatin-resistant human ovarian cancer cell lines. *J. Natl Cancer Inst.*, **81**, 535–539.

MINEHAN KJ AND BONNER JA. (1993). The interaction of etoposide with radiation: variation in cytotoxicity with the sequence of treatment. *Life Sciences*, **53**, 237–242.

NIIMI S, NAKAGAWA K, SUGIMOTO Y, NISHIO K, FUJIWARA Y, YOKOYAMA S, TERASHIMA Y AND SAIJO N. (1992). Mechanism of cross-resistance to a camptothecin analogue (CPT-11) in a human ovarian cancer cell line selected by cisplatin. *Cancer Res.*, **52**, 328–333.

POMMIER Y AND TANIZAWA A. (1993) Mammalian DNA topoisomerase I and its inhibitors. In *Cancer Chemotherapy* Hickman J, Tritton T. (eds). pp. 214–250. Blackwell: Oxford.

RICE GC, BUMP EA, SHRIEVE DC, LEE W AND KOVACS M. (1986). Quantitative analysis of cellular glutathione by flow cytometry utilizing monochlorobimane: Some applications to radiation and drug resistance in vitro and in vivo. *Cancer Res.*, **46**, 6105–6110.

SCHAAK J, SCHEDL P AND SHENK T. (1990). Transcription of adenovirus and HeLa cell genes in the presence of drugs that inhibit topoisomerase I and II function. *Nucleic Acids Res.*, **18**, 1499–1508.

SCHNEIDER E, YAMAZAKI H, SINHA BK AND COWAN KH. (1995). Buthionine sulphoximine-mediated sensitisation of etoposide-resistant human breast cancer MCF7 cells overexpression the multidrug resistance-associated protein involves increased drug accumulation. *Br. J. Cancer*, **71**, 738–743.

SLICHENMYER WJ, ROWINSKY EK, DONEHOWER RC AND KAUFMANN SH. (1993). The current status of camptothecin analogues as antitumor agents. *J. Natl Cancer Inst.*, **85**, 271–291.

TANIZAWA A, FUJIMORI A, FUJIMORI Y AND POMMIER Y. (1994). Comparison of topisomerase I inhibition, DNA damage, and cytotoxicity of camptothecin derivatives presently in clinical trials. *J. Natl Cancer Inst.*, **86**, 836–842.

ZHANG H, WANG JC AND LIU L. (1988). Involvement of DNA topoisomerase I in transcription of human ribosomal RNA genes. *Proc. Natl Acad. Sci.* USA, **85**, 1060–1064.