High cellular accumulation of sulphoraphane, a dietary anticarcinogen, is followed by rapid transporter-mediated export as a glutathione conjugate

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Sulphoraphane (SF), a naturally occurring isothiocyanate, is a potent anticarcinogen in animal experiments. The mechanism of action of sulphoraphane includes induction of Phase 2 detoxification enzymes, inhibition of carcinogen-activating Phase 1 enzymes, induction of apoptosis and cell cycle arrest, and anti-inflammation. We have recently found that it was accumulated in mammalian cells by up to several hundred-fold over the extracellular concentration, primarily by conjugation with intracellular GSH. The intracellular accumulation levels of SF can reach millimolar concentrations. The anticarcinogenic activity of SF is at least partly dependent on its accumulation levels in cells. Here we show, however, that the accumulated SF was rapidly exported mainly in the form of GSH conjugate (GS-SF) in cultured human cells. It appeared that to sustain the intracellular accumulation levels required a continuous uptake of SF to offset

the rapid export of SF/GS-SF. These findings may have important implications for the development of an effective dosing regimen for SF. Moreover, the export was temperature-sensitive and was inhibited by known inhibitors of membrane pumps, suggesting the involvement of such a pump in exporting accumulated SF/GS-SF. Indeed, studies with human leukemia cells (HL60) with or without overexpression of multidrug resistance associated protein-1 (MRP-1) and human myeloma cells (8226) with or without overexpression of P-glycoprotein-1 (Pgp-1) indicated that both MRP-1 and Pgp-1 are involved in the export of intracellular SF/GS-SF.

Key words: anticarcinogen, isothiocyanate, MRP-1, P-glycoprotein-1, sulphoraphane transport.

INTRODUCTION

Sulphoraphane (SF) was originally isolated from broccoli as a potent inducer of Phase 2 detoxification enzymes [e.g., glutathione S-transferases (GSTs)] [1], and subsequently shown to block chemical carcinogenesis in a number of animal models [2,3]. It is a member of a large isothiocyanate family that is widely distributed in plants. In addition to inducing Phase 2 enzymes, SF is also known to induce apoptosis, arrest cell cycle, inhibit several Phase 1 enzymes that may activate chemical carcinogens, and inhibit inflammation [4–7]. These properties of SF have led to the view that it is not only an important cancer chemopreventive agent capable of inhibiting multiple steps of the carcinogenic process, but also may be a potentially useful cancer therapeutic drug. We recently have shown that SF, as well as some of its analogues, rapidly accumulated in all cell lines tested, and its intracellular concentrations can reach millimolar levels [8,9]. SF appeared to enter cells freely, but was almost entirely conjugated with GSH in cells [9,10]. Our results revealed that cellular GSH was the principal driving force for accumulation while cellular GST further enhances such accumulation [8,10]. This mechanism is consistent with the fact that SF undergoes spontaneous conjugation with GSH under mild conditions to give rise to the corresponding dithiocarbamate (GS-SF) and that the reaction is accelerated by GST [11]. Preliminary evidence suggests that the accumulated GS-SF may be further metabolized, such as by binding to cellular proteins and the formation of other dithiocarbamate metabolites [9,12]. Cellular accumu-

lation of SF appeared critical for its anticarcinogenic activity. We found that its potent activity in inducing many Phase 2 detoxification enzymes in cultured cells was related to its high intracellular accumulation levels [8,13]. As part of our goals to understand its anticarcinogenic mechanisms and to achieve the best cancer chemopreventive efficacy by modulating its intracellular accumulation levels, we have investigated the elimination of the accumulated SF/GS-SF. Intracellular SF/GS-SF refers to the total intracellular accumulation level of SF. SF is known to accumulate principally as the GS-SF conjugate in human breast cancer cells (MCF-7) [9] and in human prostate carcinoma cells (Y. Zhang and E. C. Callaway, unpublished work). However, there might be other minor metabolites of SF that were detected by the method used in this study, namely the cyclocondensation assay. Our studies indicated that cells rapidly exported the accumulated SF/GS-SF and that the intracellular accumulation level was maintained by a continuous uptake of SF to offset the rapid export. Moreover, intracellular SF/GS-SF was exported primarily as GS-SF and appeared to involve membrane drug transporters.

MATERIALS AND METHODS

Chemicals

SF was purchased from LKT Laboratories (St. Paul, MN, U.S.A.). Cyclosporin A, DL-buthionine (R,S)-sulphoximine (BSO), and glibenclamide were purchased from Sigma (St. Louis, MO, U.S.A.). 3-[(3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl)-(3-dimethyl amino-3-oxopropyl)-thio]methylthio]propanoic acid; MRP-1, multidrug resistance associated protein-1; Pgp-1, P-glycoprotein-1; SF, sulphoraphane [1-isothiocyanato-4-(methylsulphonyl)butane]; 8226/S, human myeloma cells;

Abbreviations used: BSO, DL-buthionine (R,S)-sulphoximine; DPBS, Dulbecco's phosphate buffered saline; GST, glutathione S-transferase; HL60/S, human leukemia cells; HL60/AR, adriamycin-resistant derivative of HL60; LNCaP, human prostate carcinoma cells; MCF-7, human breast cancer cells; MK571, 3-[(3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl)-(3-dimethyl amino-3-oxopropyl)-thio]methylthio]propanoic acid; MRP-1, multidrug resistance associated protein-1; Pgp-1, P-glycoprotein-1; SF, sulphoraphane [1-isothiocyanato-4-(methylsulphonyl)butane]; 8226/S, human myeloma cells; 8226/Dox40, adriamycin-resistant derivative of 8226/S cells.

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Cell culture

LNCaP cells (human prostate carcinoma cells), human leukemia HL60/S and HL60/AR cells (obtained from Dr A. F. List, Arizona Cancer Center, University of Arizona, Tucson, AZ, U.S.A.), and human myeloma 8226/S and 8226/DOX 40 cells (obtained from Dr S. P. Stratton and Dr R. T. Dorr, Arizona Cancer Center) were cultured in RPMI 1640 medium with 10% (v/v) fetal bovine serum, plus streptomycin sulphate (0.1 mg/ml) and penicillin G (100 units/ml). The medium and serum were purchased from Life Technologies (Rockville, MD, U.S.A.) and Omega Scientific (Tarzana, CA, U.S.A.) respectively. Cells were grown with 10 ml of medium in each 10-cm diameter plate with 5% CO₂ in a humidified incubator at 37 °C.

Measurement of cellular accumulation and export of SF

The procedures involving cell exposure to SF, cell harvest, preparation of cell lysates, and measurement of the lysates by the cyclocondensation assay for SF accumulation have been described previously [8]. Briefly, 5–10 ml cell suspensions were laid on top of a 2 ml layer of a mixture of an equal volume of dibutyl phthalate and di-isononyl phthalate in a 15-ml plastic centrifuge tube and centrifuged for 2 min at 1283 *g* (2500 rev./min) and 4 °C. The density of the phthalate oils allowed the cells to be centrifuged through the oil layer with the medium remaining on top of the oil. The cell pellet was retrieved, suspended in a small volume of water and sonicated. The lysates were subjected to the cyclocondensation assay (reaction with 1,2-benzenedithiol) [14].

To measure the export of intracellularly accumulated SF/GS-SF, cells were grown on 10-cm diameter plates for 3 days to reach 5×10^6 cells/plate. In each experiment, up to 15 plates of cells were pooled and suspended in fresh medium (approximately 0.5×10^6 cell/ml), and then incubated with a specified concentration of SF for 1 h in a 15-cm diameter plate. At the end of the incubation, two aliquots of the cell suspension (10 ml each) were removed and processed for determination of the total SF accumulation as described above. The remaining cells were separated from the medium by centrifugation [1283 *g* (2500 rev./min), 4 °C for 2 min]. The pellets were resuspended in an equal volume of fresh medium without SF and incubated at 37 °C. The above procedure of removing two aliquots of cells from the culture and replacing the original medium with an equal volume of fresh medium for the remaining cells was repeated at 0.5, 1, 2, 3, 4 and 4.5 h. The effect of temperature on the cellular export of SF/GS-SF was similarly assessed by incubating SF-loaded cells in medium at either 4 °C or 37 °C.

To determine the effect of a drug on the export of intracellular SF/GS-SF, the SF-loaded cells were incubated with fresh medium in the presence of the drug for 1 h. Cells were then harvested for determination of the remaining intracellular SF/GS-SF concentrations. To compare the intracellular accumulation levels of SF in cells expressing different levels of multidrug resistance associated protein-1 (MRP-1) or P-glycoprotein-1 (Pgp-1), the relevant cells were incubated with SF for 1 h and then measured for intracellular accumulation levels of SF/GS-SF.

Identification of the chemical form of SF released from cells

Cells were incubated with a specific concentration of SF for 30 min at 37 °C (5×10^6 cells/ml, up to 150 ml in a 15-cm

diameter plate). At the end of the exposure, two 5 ml aliquots of the culture were removed to determine the level of intracellular accumulation of SF as described above. The remaining SF-exposed cells were separated from the medium by centrifugation. The resultant cell pellets were washed once with ice-cold Dulbecco's phosphate buffered saline (DPBS) (approx. 2×10^6 cells/ml), resuspended in a small volume of DPBS (approx. 7.5×10^6 cells/ml), and incubated at 37 °C for 30 min. At the end of the incubation, the cells were separated from the DPBS supernatant by centrifugation through the phthalate oils as described above. The entire supernatant portion was collected and the cell pellets were suspended in a small volume of water, both of which were stored at –70 °C and quantified within one day by the cyclocondensation assay for SF/GS-SF content.

A large portion of the DPBS supernatant was fractionated by paired-ion HPLC that we had developed previously for the separation of SF and its dithiocarbamate metabolites, including GS-SF. Briefly, an analytical reverse phase column (Whatman Partisil 10 ODS-2, 4.5 mm $\times$ 250 mm) was eluted with a gradient of solvent A (6 mM tetraethylammonium bromide in water) and solvent B (acetonitrile) at 2 ml/min. GS-SF and free SF were eluted at 5.4 and 13.0 min respectively [9]. Potential metabolites of GS-SF, including cysteinylglycine-SF, cysteine-SF and *N*-acetylcysteine-SF, that may arise through further modification of the GSH structure were eluted at other time points [9]. The elution fractions (2 ml each) as well as the original sample were quantified by the cyclocondensation assay.

The fractions that were cyclocondensation reaction-positive were pooled from several HPLC runs and filtered through a Centricon filter (YM-30; pore size: 30000 Da molecular mass cut off). The filtrate was concentrated by Speed Vac and desalted by another reverse phase-HPLC run (same column, eluted with water at 2 ml/min). The cyclocondensation reaction-positive fractions were again concentrated by Speed Vac, followed by MS analysis (electrospray).

Measurement of cell volume

Cell volume was measured only in LNCaP cells because the majority of experiments were conducted in these cells. Thus, the total content of SF/GS-SF measured in lysates of LNCaP cells was converted to an intracellular concentration based on a calibration line relating intracellular volume to the protein content in cell lysates. The procedures for protein quantification and cell volume determination have been described previously [8]. Briefly, to determine total cell volume, the cells were suspended in a medium containing both ³H₂O and [¹⁴C]inulin for 5 min and were separated from the medium by centrifugation. The cell pellet was retrieved and lysed in small volume of detergent. The radioactivities of both ³H and ¹⁴C and the protein content in each lysate were determined. Inulin crosses cell membranes slowly and was used to determine the contamination of the radioactivities in the extracellular space. A linear correlation between cell volume and protein content of the lysate for LNCaP cells is shown in Figure 1.

Determination of cellular GSH level and GST activity

Cells which were grown in 10-cm diameter dishes ($0.5\text{--}1 \times 10^6$ cells/dish with 10 ml of medium) for 3 days were separated from the medium by centrifugation [1183 *g* (2400 rev./min) for 5 min at 4 °C]. Each cell pellet was washed with 10 ml of ice-cold DPBS, recentrifuged to remove the DPBS, and sonicated in 200 μ l of DPBS. In experiments where depletion of GSH was required, cells were incubated with a specific concentration of BSO for 24 h before harvest.

Figure 1 Standard curve relating the protein content in cell lysates to the intracellular fluid volume of LNCaP cells

The protein was quantified using the BCA assay [32]. The intracellular volume was measured by $^3\text{H}_2\text{O}$ uptake which was corrected for extracellular contamination by [^{14}C]inulin. A linear correlation was established: cell volume (μl) = $3.29 \times \text{protein quantity (mg)} - 0.007$, $r = 0.99$. Each value is the mean for triplicate determinations (standard errors were generally less than 4% of the means).

Cellular GSH levels were determined by the GSH reductase-coupled 5,5'-dithiobis-2-nitrobenzoic acid ('DTNB') assay in 96-well microtitre plates [13]. Cytosolic GST activity was assayed with 1-chloro-2,4-dinitrobenzene ('CDNB') as substrate in 96-well microtitre plates [13].

Western blot analysis

Cells were cultured, harvested and washed as described above. Each cell pellet was suspended in a small volume of lysis buffer (10 mM Tris/HCl, 10 mM NaCl and 1 mM MgCl_2 , pH 7.4), incubated on ice for 20 min, and homogenized with a Dounce glass homogenizer. The homogenates were then centrifuged at 994 g (2200 rev./min) and 4 °C for 10 min to remove cell debris and nuclei. The supernatant portion was then centrifuged at 100000 g at 4 °C for 1 h. The pellet was suspended in a small volume of DPBS, sonicated and stored at -70 °C. Each sample (25 μg of protein) was resolved by SDS/PAGE [7.5% (w/v) gel] and blotted on to a PVDF sheet. The sheet was then probed with monoclonal antibody against either MRP-1 or Pgp-1 (Kamiya Biochemical, Seattle, WA, U.S.A.), and the MRP-1 and Pgp-1 bands were visualized by an amplified alkaline phosphatase and chemiluminescence system (VECTOR, Burlingame, CA, U.S.A.).

RESULTS AND DISCUSSION

Rapid cellular uptake and export of SF

The experiments described in this paper were mainly conducted in LNCaP cells. Selection of these cells was based on the following: 1) exposure of LNCaP cells to SF led to a rapid, high and dose-dependent accumulation of the compound. Intracellular SF/GS-SF concentrations reached 4.4, 9.5 and 13.3 mM when the cells were incubated with 0.028, 0.084 and 0.28 mM SF respectively for 30 min at 37 °C, a 47–155-fold accumulation; 2) the cells grow in monolayers in culture, but can be readily rinsed off the plates and separated from each other without trypsin treatment; and 3) LNCaP cells appeared to behave similarly to other cell types in response to SF as described below.

The maximal accumulation level of SF was achieved in less than 2 h in LNCaP cells, but the intracellular SF/GS-SF levels

Figure 2 Time course of SF accumulation levels in LNCaP cells

(A) LNCaP cells were incubated with 5 μM SF at 37 °C. At indicated times a portion of cells was removed and total intracellular SF accumulation levels were determined by the cyclocondensation assay. (B) LNCaP cells were incubated with 50 μM SF at 37 °C for 1 h. At the end of the incubation, cells were separated from the medium by centrifugation, washed once with DPBS, and resuspended in fresh medium without SF. At each indicated time point, a portion of cells was removed for determination of intracellular SF accumulation levels by the cyclocondensation assay, while the remaining cells were resuspended in an equal volume of fresh medium and incubated again. The total SF/GS-SF content detected in cell lysates was converted to intracellular SF/GS-SF concentration based on the standard curve shown in Figure 1. Each value is the mean for four determinations (standard errors were less than 4% of the means).

changed very slowly thereafter, decreasing by 8% in 4 h (Figure 2A). Similar accumulation kinetics of SF were observed in murine hepatoma Hepa 1c1c7 cells [8] and human colon cancer Colo 205 cells (results not shown). The experiments typically were performed with a low extracellular SF concentration to avoid inducing cytotoxicity during long incubation times. In order to understand whether the slowly changing intracellular SF accumulation levels were the result of slow export of the accumulated SF/GS-SF, cells were first loaded with SF and then incubated in medium without the compound. Thus, LNCaP cells were pretreated with 50 μM SF for 1 h at 37 °C and then incubated in medium without the compound. A portion of the cells was collected for determination of intracellular SF/GS-SF levels at 0, 0.5, 1, 2, 3, 4 and 4.5 h, while the remaining cells were transferred to new medium for further incubation in SF-free medium at each time point. As shown in Figure 2(B), there was a first-order decrease of intracellular SF/GS-SF, with a half-life of approximately 1 h. A very similar result was obtained in Colo 205 cells (results not shown). These results were in direct contrast to the finding shown in Figure 2(A) and indicated that the elimination of intracellularly accumulated SF/GS-SF was extremely fast and was not saturated. It is unlikely that the rapid decrease of intracellular SF/GS-SF resulted from simple leaks in the cell membrane because those cells appeared morphologically normal and, as described below, lowering the temperature almost completely blocked the release. Because extracellular SF content in these experiments was kept negligible by frequent medium replacement, whereas SF was continuously present in the medium

Figure 3 Chemical structure of the GS-SF conjugate

SG, glutathione.

in the experiment shown in Figure 2(A), it seems highly likely that the intracellular accumulation pattern of SF seen in Figure 2(A) was achieved by a continuous uptake of SF to offset the rapid export of the accumulated SF/GS-SF. However, whether or not SF in the medium may inhibit the elimination process is unknown at the present time.

Intracellularly accumulated SF/GS-SF was mainly exported as the GSH conjugate

From our previous studies, it was known that nearly all SF accumulated in MCF-7 cells as the GSH conjugate [9]. Our preliminary results showed that SF also accumulated mainly as the GSH conjugate in LNCaP cells (results not shown). Thus, it was intriguing to know whether intracellular SF/GS-SF in LNCaP cells was exported as the GSH conjugate. Cells were preloaded with SF and the accumulation was released to DPBS for determination of the chemical nature of the released SF. Total intracellular SF/GS-SF concentration reached 8.6 mM when the cells were incubated with 0.065 mM SF for 30 min at 37 °C. Intracellular SF concentration dropped to 5.6 mM when these cells were washed and then incubated in a small volume of DPBS for 30 min at 37 °C. More than 90 % of the decrease was recovered in the DPBS supernatant. When the DPBS portion was analysed by paired-ion HPLC as described previously, all of the released SF was recovered in two locations: 85 % co-eluted with synthetic GS-SF and 15 % co-eluted with SF. Subsequent analysis of the fractions co-eluted with GS-SF by electrospray MS confirmed that it was indeed GS-SF (both the calculated molecular ion and the detected molecular ion are 485.1; see Figure 3 for the structure). Moreover, the UV spectrum of the isolated compound was identical to that of the synthetic GS-SF. Similar results were obtained when the same cells were re-incubated in DPBS for another 30 min (results not shown). Since it is known that GS-SF can dissociate into SF at a substantial rate in physiological conditions [9], it is likely that the majority of the free SF detected in the DPBS supernatant might have dissociated from the conjugate exported from the cells. Hence, we conclude that accumulated SF/GS-SF in LNCaP cells is principally exported in the form of the GS-SF conjugate.

The export of SF/GS-SF was temperature-sensitive and inhibited by known inhibitors of membrane drug transporters

Since it had been widely reported that cell membranes contain pumps that transport a variety of GSH conjugates, it was important to know if GS-SF was also transported by such pumps. Indeed, our experiments appear to have implicated such a pump(s). First, lowering the temperature inhibited the export of intracellular SF/GS-SF. LNCaP cells were preloaded with SF by incubation with 50 μM SF for 1 h and then transferred to fresh medium without SF and incubated at either 4 °C and 37 °C. A portion of the cells was harvested at 0.5, 1, 2 and 3 h for determination of intracellular SF/GS-SF accumulation levels, whereas the remaining cells were transferred to an equal volume

Figure 4 Effect of temperature on the export of intracellular SF/GS-SF

LNCaP cells were incubated with 60 μM SF for 1 h at 37 °C. Cells were then separated from the medium by centrifugation, resuspended in an equal volume of fresh medium, and incubated at either 4 °C (■) or 37 °C (□). At each indicated time point, a portion of cells was harvested for determination of intracellular SF/GS-SF concentration, and the remaining cells were resuspended in an equal volume of fresh medium. The total SF/GS-SF content detected in cell lysates was converted to intracellular concentrations as described in Figure 2. Each value is the mean for four determinations (standard errors were less than 5 % of the means). Each value in the 4 °C treated cells was significantly different from that in control cells ($P < 0.05$; Student's *t* test).

Figure 5 Effect of MRP and Pgp inhibitors on the export of intracellular SF/GS-SF

LNCaP cells were incubated with 50 μM SF for 1 h at 37 °C. Cells were then separated from the medium by centrifugation, resuspended in an equal volume of fresh medium, and incubated at 37 °C for 1 h with or without an inhibitor. The cells were then harvested for the determination of intracellular SF/GS-SF concentrations. Each value in the drug-treated cells was significantly different from that in control cells (mean $\pm$ S.D., $n = 4$, $P < 0.05$; Student's *t* test). GB, glibenclamide; CS, cyclosporin A.

of fresh medium at each time point to prevent potential cellular reaccumulation of released GS-SF. As shown in Figure 4, while intracellular SF accumulation levels rapidly decreased at 37 °C (16 % remaining at 3 h), the decrease was much slower at 4 °C (85 % remaining at 3 h). A similar temperature effect on the intracellular SF accumulation levels was also observed in both Hepa 1c1c7 cells and MCF-7 cells (results not shown). Such a dramatic temperature effect on the export of GS-SF is consistent with a transporter-mediated mechanism [15,16] and has been observed with another GSH conjugate previously [17].

Figure 6 Western blot analysis of membrane proteins

Cell membranes were prepared from each cell line and 25 μ g of membrane proteins were resolved on 7.5% polyacrylamide gels and transferred on to PVDF sheets. After successive incubation of the PVDF sheets with monoclonal antibodies against MRP-1 or Pgp-1 and secondary antibodies, the bands of interest were visualized with an amplified alkaline phosphatase and chemiluminescence detection system.

Export of SF/GS-SF in LNCaP cells was also inhibited by known inhibitors of membrane pumps. Incubation of SF-loaded cells with MK571 and glybenclamide, known inhibitors of MRP proteins [15,16,18], resulted in a dose-dependent inhibition of the export of SF/GS-SF. For example, SF-loaded cells, in the presence of 0.5 mM MK571 or 1 mM glybenclamide retained 73 % or 54 % of intracellular SF/GS-SF at 1 h incubation respectively, whereas control cells retained only 30 % (Figure 5). However, cyclosporin A, a potent inhibitor of Pgp-1 [19,20], had only weak a inhibitory effect on the export, only 38–39 % of the accumulated SF/GS-SF was retained at 1 h incubation in cyclosporin A-treated cells (10 and 100 μ M) compared with 30 % in the control cells. These results suggested that MRP(s) may be primarily involved in the export of intracellular SF/GS-SF in LNCaP cells. Subsequent studies showed that whereas MRP-1, the ubiquitous MRP family member [21], was detected in LNCaP cells by Western blot analysis, no Pgp-1 was found in these cells (Figure 6). The Western blot result is consistent with published results [22,23]. The small inhibitory effect by cyclosporin A on GS-SF export in LNCaP cells probably resulted from inhibition of MRP-1 since cyclosporin A has been shown to inhibit MRP function previously [16].

Reduced SF accumulation in MRP-1 or Pgp-1 overexpressing cells

Because the export of SF/GS-SF in LNCaP cells is partially blocked by chemicals that are known to inhibit MRPs, and MRP-1 is present in these cells, it became important to see if cells that overexpress an MRP would have lower intracellular accumulation levels of SF. In addition, the role of Pgp in the export of intracellular SF/GS-SF was also assessed. Two pairs of cell lines were selected for the experiments: 1) human leukemia HL60/S cells, and the adriamycin-resistant derivative HL60/AR cells which overexpress MRP-1 [24,25]; and 2) human myeloma 8226/S cells and its adriamycin-resistant derivative 8226/Dox40 cells which overexpress Pgp-1 [26,27]. Pgp-1 was not detected in either HL60/S cells or HL60/AR cells in our Western blots, whereas a similar level of MRP-1 was seen in both 8226/S cells and 8226/Dox cells (Figure 6). When these cells were incubated with 50 or 100 μ M SF for 1 h at 37 $^{\circ}$ C, SF accumulation levels in HL60/AR cells and 8226/Dox40 cells were 16–23 % and 84 %

Figure 7 SF accumulation in cells with or without the expression of MRP-1 or Pgp-1

Each of the four cell lines (HL60/S, HL60/AR, 8226/S and 8226/Dox40) was incubated with 50 or 100 μ M SF for 1 h at 37 $^{\circ}$ C. In order to reduce cellular GSH levels, HL60/S cells and 8226/Dox40 cells were also incubated with 20 and 5 μ M BSO respectively for 24 h prior to, and during, the SF incubation. At the end of the SF incubation, cells were harvested for intracellular SF/GS-SF accumulation by the cyclocondensation assay. Each value is the mean of four determinations (standard errors were less than 6.3% of the means). The values in HL60/AR cells or 8226/Dox40 cells were significantly different from those in their parent cell lines ($P < 0.05$; Student's t test).

Table 1 GSH and GST levels in HL60 cells and 8226 cells with or without BSO treatment

Values are means $\pm$ S.D.; $n = 3$.

Cell line	BSO treatment	GSH levels (nmol/mg of protein)	GST activity (nmol/min per mg of protein)
HL60/S	0	42.24 $\pm$ 1.96	122.73 $\pm$ 11.75
	20 μ M, 24 h	15.58 $\pm$ 1.52	122.73 $\pm$ 11.75
HL60/AR	0	15.19 $\pm$ 1.55	170.73 $\pm$ 14.94
	5 μ M, 24 h	17.39 $\pm$ 1.31	Not detected
8226/S	0	30.31 $\pm$ 1.59	Not detected
	19.68 $\pm$ 1.53	Not detected	Not detected

of those in their parent cell lines respectively (Figure 7). However, because both intracellular GSH and GST were known to drive cellular accumulation of isothiocyanates, including SF, it was important that these cellular elements be adjusted to similar levels in the cell lines to be compared. As shown in Table 1, both GSH and GST differ among the cell lines. The cellular GSH level

in HL60/AR cells was 36 % of that in HL60/S cells but its GST level was 39 % higher than in the latter cells. The GSH level in 8226/Dox40 cells was 30 % higher than in 8226/S cells whereas the GST activity in both cell lines were non-detectable under our assay conditions. We have only focused on adjusting cellular GSH levels since the difference in GST activity was either small or not present. BSO treatment of HL60/S cells (20 μ M, 24 h) and 8226/Dox cells (5 μ M, 24 h) reduced their GSH content to a very similar level to that in their paired cell lines without changing GST activity. BSO depletes cellular GSH by inhibiting γ -glutamylcysteine synthetase, the rate-limiting enzyme in GSH biosynthesis. When BSO-treated HL60/S cells and 8226/Dox40 cells were then incubated with SF, there was less accumulation of SF in these cells than in BSO-untreated cells. The intracellular SF accumulation levels in BSO-treated HL60/S cells were still 2.3 times that in HL60/AR cells when the cells were incubated with 50 or 100 μ M SF, even though the GST activity was higher in the latter cells (Figure 7A). It is likely that there will be even less SF accumulation in HL60/AR cells if their GST activity is reduced to that in HL60/S cells. Likewise, when BSO-treated 8226/Dox40 cells were incubated with 50 or 100 μ M SF for 1 h, the intracellular SF accumulation was less than that in the untreated cells and was 56 % of that in 8226/S cells (Figure 7B). Because HL60/AR cells that overexpress MRP-1 and 8226/Dox40 cells that overexpress Pgp-1 were derived from HL60/S cells and 8226/S cells respectively, the above results strongly suggest that both MRP-1 and Pgp-1 may be involved in the export of intracellular SF/GS-SF. The results involving MRP-1 are consistent with the inhibitor studies in LNCaP cells as described above.

GSH conjugate pumps (also expressed as GS-X pumps) are members of a large family of ATP-binding cassette (ABC) transporters known as multidrug-resistance P-glycoprotein and multidrug-resistance associated protein (MRP). Two members of the multidrug-resistance proteins (Pgp-1 and Pgp-2) [28] and seven members of the MRP proteins (MRP 1–7) [21] have been reported in humans so far. Pgp-1 is the only multidrug-resistance gene product that has been shown to contribute to drug resistance in cancer cells, and MRP-1 is the most widely distributed MRP member. Pgp-1 and MRPs appear to transport a wide variety of structurally diverse and relatively lipophilic drugs. While MRP subfamily proteins have been shown to transport drugs either unmodified or conjugated to anionic ligands such as GSH, glucuronate, or sulphate, the P-glycoproteins are thought to transport drugs in an unmodified form [29,30]. But, it has recently been suggested that P-glycoproteins may also transport drugs that are conjugated with anionic ligands [31]. The exact chemical form of SF which is transported by Pgp-1, free SF or both free SF and GS-SF, is unknown at the present time. Moreover, although our studies have implicated both MRP-1 and Pgp-1 in the cellular export of SF/GS-SF, it remains to be determined whether some of their related members are also capable of such action. In addition, many analogues of SF, some of which are also potent anticarcinogens, are known to accumulate in cells [8]. It will be interesting to see if they are also removed from the cells by these membrane transporters.

In summary, SF was rapidly accumulated in LNCaP cells and other cells by undergoing conjugation with cellular GSH. But, intracellularly accumulated SF/GS-SF was also rapidly exported. Our results suggest that the continuous uptake of SF is necessary to maintain its intracellular accumulation in order to offset the rapid export. The accumulated SF/GS-SF was exported principally as GS-SF. Evidence presented in this paper suggests the involvement of a membrane transporter(s) in the cellular elimination of SF/GS-SF, including MRP-1 and Pgp-1.

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