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Modulation of GSH levels in ABCC1 expressing tumor cells triggers apoptosis through oxidative stress

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ABSTRACT

The over-expression of ABCC1 transmembrane protein has been shown to cause multidrug resistance in tumor cell lines. ABCC1 is a member of the ABC transmembrane proteins that function as efflux pumps with diverse substrate specificity. Several endogenous cell metabolites, including the leukotriene C4 (LTC_4) and glutathione (GSH) are substrates for ABCC1 protein. ABCC1 expression in certain tumor cells was demonstrated to confer hypersensitivity to glutathione modulating agents. In this report we have investigated the mechanism of collateral sensitivity seen in tumor cells over-expressing ABCC1 protein. The results of this study show that ABCC1 expression in tumor cells correlates with their hypersensitivity to various glutathione modulating agents, as demonstrated in H69AR-drug selected and HeLa/ABCC1-transfectant cells. This effect was triggered either through inhibition of GSH synthesis with BSO or by increasing ABCC1-mediated GSH transport with verapamil or apigenin. In addition, our results show that the hypersensitivity of ABCC1-expressing cells to BSO, verapamil or apigenin was preceded by an increase in reactive oxygen species (or ROS). A decrease in GSH level is also observed prior the increase in ROS. In addition, we show that hypersensitivity to the BSO, verapamil or apigenin leads to tumor cell death by apoptosis. Together, the results of this study demonstrate that ABCC1 potentiates oxidative stress in tumor cells through reductions in cellular GSH levels.

1. Introduction

Drug resistance is a major cause of treatment failure in clinical oncology [1]. Multidrug resistance (MDR) involves cross-resistance to a range of chemically unrelated agents with different cellular targets. The over-expression of ATP binding cassette (ABC) membrane proteins as causative proteins of MDR have been studied intensely over the past two decades [1]. These ABC proteins are, the P-glycoprotein 1 (P-gp1 or ABCB1), the breast cancer resistance protein (BCRP or ABCG2), and the multidrug resistance protein 1 (MRP1 or ABCC1) [2]. Although the molecular mechanism by which the latter ABC proteins mediate the transport of structurally diverse drugs remains elusive, ABC proteins have been shown to bind and transport various ligands, including anti-cancer drugs, in an ATP-dependent manner.

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Abbreviations: MRP1 or ABCC1, multidrug resistance associated protein; ROS, reactive oxygen species; GSH, glutathione; ABC, ATP binding cassette; MDR, multidrug resistance; BSO, L-buthionine (S,R)-sulfoximine; API, apigenin; VRP, verapamil; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

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ABCC1 was first isolated from a doxorubicin-resistant small lung cancer line H69AR [3–6]. The protein consists of 1531 amino acids, and when fully glycosylated, has a molecular mass of 190 kDa. ABCC1 is ubiquitously expressed in most normal tissues, with higher levels in lung, kidney, testis and blood mononuclear cells [6,7]. ABCC1 causes resistance to a broad spectrum of compounds including natural product drugs such as epipodophyllotoxins, vinca alkaloids, and certain anthracyclines [8–10]. Furthermore, it transports a variety of substrates that are conjugated to glucoronide, sulfate, or glutathione [11–14]. Leukotriene C_4 (LTC_4), a conjugate of glutathione is the highest affinity substrate of ABCC1 [11,12]. Moreover, ABCC1 transports both reduced (GSH) and oxidized (GSSG) glutathione. GSH transport was observed in ABCC1-expressing H69AR cells, consistent with an earlier report by Cole et al. [15] which have shown that ABCC1-expressing H69AR cells have reduced cellular GSH levels compared to parental H69 cells. Later studies showed that GSH affinity for ABCC1 increases (from K_m 10–20 mM to 10–100 μM) in the presence of certain drugs such as daunorubicin and vincristine [16,17]. This increase in GSH transport in the presence of these drugs appears to be mediated by co-transport mechanism [18]. The importance of GSH in ABCC1 activity is illustrated by the reduction in transport of many substrates when GSH production is inhibited with L-buthionine (*S*,*R*)-sulfoximine (BSO) [18–20]. BSO is the most specific and least toxic inhibitor of γ-glutamylcysteine synthetase (γ-GCS), thereby preventing *de novo* synthesis of GSH [21]. Several other compounds not transported by ABCC1 have been shown to stimulate ABCC1-mediated GSH export. Two such molecules that stimulate GSH transport are the calcium channel blocker verapamil (VRP) and the flavonoid apigenin (API) [22,23].

All aerobic organisms are subject to physiological oxidant stress as a consequence of aerobic metabolism. The production of ATP through oxidative phosphorylation leads to the formation of superoxide radicals ($O_2^{-\bullet}$). This radical can then form other reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and the hydroxyl radicals ($OH^\bullet$) [24]. These reactive compounds cause lipid peroxidation and can disrupt metabolic processes. GSH is the predominant defense against these toxic products of oxygen, particularly in mitochondria [25]. GSH is a major non-protein intracellular thiol that participates in redox reactions maintaining a reducing environment in the cell. Consequently, low GSH levels are sometimes associated with mitochondrial dysfunction and induction of apoptosis [26,27].

The present study explores some of the cellular consequences of GSH modulation in ABCC1 expressing cells. Our findings show that ABCC1-expressing tumor cell lines (H69AR or $Hela^{ABCC1}$ transfectant) are hypersensitive to a reduction in intracellular GSH. ABCC1-mediated drop in GSH levels appears to trigger apoptosis and is preceded by a burst of reactive oxygen species. This effect was triggered either by reducing GSH synthesis with BSO or by increasing ABCC1-mediated GSH transport with verapamil or apigenin. These observations demonstrate the mechanism by which ABCC1 expressing cells are hypersensitive to reductions in cellular GSH levels that can lead to avenues of targeting ABCC1 positive tumor cells.

2. Materials and methods

2.1. Materials

Protein-A Sepharose CL-4B and carrier-free Na^{125}Iodine (100 mCi/mL) were purchased from Amersham Biosciences (Baie d'Urfe, Quebec). The ABCC1 polyclonal Ab was produced as describe below; polyclonal anti-actin [20–33] was purchased from Sigma (St. Louis, MO). NHS-ASA and ImmunoPure immobilized protein G were purchased from PIERCE (Rockford, IL). RPMI 1640 and α-MEM media were purchase from Gibco-BRL. Verapamil, apigenin, GSH, propidium iodide and MTT were purchase from Sigma–Aldrich. BSO was purchase form MP Biomedicals (Aurora, OH). H_2DCFDA and FITC-conjugated Annexin V were purchase from molecular probes. Z-VAD-FMK was purchase from BD Pharmingen. C18 SepPak was purchase from Waters (Milford, MA). All other chemicals were of the highest possible quality.

2.2. Cell culture and transfection

The small cell lung cancer cell lines (H69, H69AR, and H69PR; from Dr. S. Cole at the Cancer Research Laboratories, Queen's University, Canada) were grown in RPMI 1640 media supplemented with 5% fetal bovine serum (or FBS). For the $H69AR^{Bcl\text{-}2}$ transfected cell line, G418 was added regularly at a concentration of 0.l mg/mL. Human cervical carcinoma cells (HeLa) and the ABCC1-transfected variants ($HeLa^{ABCC1}$; from Dr. P. Gros at Department of Biochemistry, McGill University, Canada) were cultured in α-MEM media supplemented with 10% FBS; while $HeLa^{ABCC1}$ was supplemented with 600 μg/mL G418 (Gibco-BRL) in addition to α-MEM media. $H69/AR^{Bcl\text{-}2}$ transfectant cells were generated by transfecting full-length human Bcl-2 cDNA. Briefly, H69/AR cells were plated (0.5×10^6) into 60 mm plates and transfected with a mammalian expression vector (Rc/RSV; Invitrogen, San Diego, CA) alone or containing full-length human Bcl-2 cDNA according to manufacturer's instructions using LipofectAMINE (Gibco-BRL). Twenty-four hours later, plates were treated with 0.1 mg/mL G-418 and the expression of Bcl-2 protein was monitored by Western blotting.

2.3. ABCC1 anti-sera and immuo-detection

A polypeptide encoding 15 amino acids from the C-terminal of ABCC1 (N-QRGLFYSMAKDAGLV-C) was synthesized using F-moc chemistry as previously described [28]. New Zealand Rabbits were immunized subcutaneously (sc) with a 1:1 ratio (v/v) of immunogen (thyroglobulin–peptide conjugate) and Freunds incomplete adjuvant (Sigma–Aldrich Inc.). Subsequent booster injections were administered at 2 weeks intervals and blood samples were drawn from each rabbit as previously described [29]. Antibody titer was checked by ELISA and Western blot analyses. For immuno-detection of ABCC1 and actin, 10 μg of total cell lysate was resolved on 10% acrylamide gels using the Laemmli gel system [30]. Proteins separated by SDS-PAGE were then transferred to nitrocellulose membrane [31] and probed with anti-ABCC1 (at 1:5000, v/v dilution in PBS), or anti-actin antibody (at 1:100, v/v dilution in PBS). Nitrocellulose membranes were washed with PBS and

incubated with a 1:3000 (v/v) dilution of either goat anti-rat or goat anti-rabbit antibodies conjugated to horseradish peroxidase. SuperSignal West Pico chemoluminescent substrate (Pierce, Rickport, IL) was used to detect and develop the signal in Western blotting. For immuno-detection of ABCC1 protein in cell lysate, ELISA was performed on total cell lysate with 1 μg/well in 96-well plates. Wells were blocked for 2 h with 3% BSA in PBS at room temperature, followed by the addition of the first antibody (ABCC1 anti-peptide anti-serum at 1:500, v/v dilutions) for 16 h at 4 °C. Plates were washed with PBS and incubated with a HRP conjugated second antibody (goat anti-rabbit at 1:3000, v/v dilution) in blocking buffer minus sodium azide. The signal was developed with the addition of TMB substrate solution (Sigma–Aldrich), and then quantified by measuring the absorbance at 450 nm.

2.4. Cell survival assays

The effect of drugs on tumor cell growth was determined using an MTT colorimetric assay [32]. Cells were seeded in 96-well plates (at 2.5×10^3 cells/well for HeLa or $HeLa^{ABCC1}$ and 1×10^4 cells/well for H69, H69AR, $H69AR^{Bcl\text{-}2}$, and H69PR cells) prior to the addition of various drugs. Cells were allowed to grow for 44 or 72 h in the absence or presence of increasing concentrations of BSO, VRP or API. The MTT dye was added to a final concentration of 50 μg/mL and cells were then incubated for another 4 h at 37 °C. The media containing residual MTT dye was carefully aspirated from each of the wells and a 200 μL aliquot of DMSO was added to each well to solubilize the reduced formazan dye. The effect of drugs on the growth of cells was determined from differences in absorbance between drug-treated cells versus untreated or solvent control. For cell rescue experiments, H69AR and $HeLa^{ABCC1}$ cells were incubated with increasing molar concentrations of API, VRP or BSO in media supplemented with various concentration of GSH, *N*-acetylcysteine (NAC) or Z-VAD-FMK, a generic Caspase inhibitor (BD Pharmingen).

2.5. Measurements of apoptosis by flow cytometry

Cell death from apoptosis was determine by quantifying the increase in Annexin V binding to surface exposed phosphotidylserine using Fluorescein isothiocyanate (or FITC) conjugated Annexin V. Briefly, cells were seeded in six-well plates (5×10^4 cells/well) and allowed to grow for 24 h in plates prior to the addition of API, BSO, or VRP. Cells were exposed to each drug for 1, 3, 9 or 24 h prior to cells harvesting and washing with buffer A (10 mM HEPES, 140 mM NaCl and 2.5 mM $CaCl_2$, pH 7.4). Washed cells were then re-suspended in 100 μL of Annexin-binding buffer containing 5 μL FITC-conjugated Annexin V and allowed to incubate for 15 min. To gate-out dead cells due to necrosis, propidium iodide dye (PI, 1 μg/mL) was added to the cells prior to the FACS analysis, using an argon-ion laser source with detection filters set at 617 or 575 nm for PI or FITC-conjugated Annexin V detection, respectively.

2.6. Quantification of intracellular ROS

Cellular ROS were measured using the fluorogenic reagent 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). Briefly, cells seeded in six-well plates (5×10^4 cells/well) were incubated with API, BSO, or VRP for 1, 3, 9 or 24 h. ROS levels were determined by loading drug-treated cells with 10 μM H_2DCFDA 30 min prior to cell harvesting. Cells were harvested, washed twice with PBS and then analyzed by FACS with fluorescence detection at 530 nm (BD FACS Aria™). Estimates of ROS following drug treatment were determined by measuring the change in mean fluorescence intensity using only live cells. Cells were gated using forward and side light scatter (FSC versus SSC) as well as propidium iodide exclusion.

2.7. Measurement of GSH levels

To determine the effect of drugs on GSH levels, a glutathione (GSH) detection kit from Chemicon international® was used. The experiment was performed according to manufacturer instructions with some modifications. Briefly, cells were incubated with each drug for 1 or 3 h, then washed and lysed. The cell lysates were centrifuged and supernatants were carefully removed and incubated with monochlorobimane (mcb) for 10 min. GSH levels were determined by measuring the fluorescence at 380/460 nm (ex/em).

2.8. Plasma membrane preparation and photoaffinity labeling

HeLa and $HeLa^{ABCC1}$ cells were cultured as described earlier. Cells were detached with trypsin-EDTA and washed with phosphate-buffered saline, pH 7.4 (PBS). The cell pellet was then resuspended in hypotonic buffer (1 mM $MgCl_2$, 10 mM KCl, and 10 mM Tris–HCl, pH 7.4) containing protease inhibitors (2 μg/mL leupeptin, 2 μg/mL aprotinin, and 1 μg/mL pepstatin A, 1 mM PMSF). Cells were lysed in a Dounce homogenizer and centrifuged at $10{,}000 \times g$ for 5 min followed by a second centrifugation at $100{,}000 \times g$ for 1 h. The final plasma membrane pellet was resuspended in 5 mM Tris–HCl, pH 7.4 containing 250 mM sucrose. Protein concentrations were quantified by Lowry et al. [33] and membranes were stored at −70 °C if not immediately used. Photoaffinity labeling of ABCC1 in plasma membranes with ^{125}I labeled photoreactive analogue of GSH, IAAGSH, was performed as previously described [34]. Briefly, 1 μL of IAAGSH was added to 25 μg aliquots of plasma membrane in a final volume of 20 μL of 5 mM Tris–HCl, pH 7.4, with 250 mM sucrose. Samples were incubated in the dark at room temperature for 30 min, followed by 10 min on ice and then UV irradiated at 254 nm for 10 min [35]. Photoaffinity labeled proteins were immunoprecipitated with anti-ABCC1 anti-serum or pre-immune serum and photoaffinity labeled proteins were immunoprecipitated with Protein-A conjugated Sepharose beads as previously described [36]. Proteins were eluted from Protein A beads in SDS-containing buffer and resolved by SDS-PAGE using Fairbanks gel system [37]. Gels with radiolabeled proteins were fixed, dried and exposed to Kodak BIOMAX MS film at −80 °C.

3. Results

The initial characterization of the drug resistant H69AR cell line demonstrated that these cells displayed collateral

sensitivity to BSO relative to their parental H69 cells. In light of more recent evidence for the role of ABCC1 in GSH transport, these findings implied that the over-expression of ABCC1 may reduce endogenous GSH levels rendering the cells hypersensitive or collaterally sensitive to BSO. The results in Fig. 1A shows that BSO has no effect on the H69 parental cells up to a concentration of 50 μM. Conversely, the H69AR cell line is extremely sensitive to BSO with IC_{50} of 0.6 μM. In order to confirm the role of ABCC1 in collateral sensitivity to BSO, the H69PR cell line was employed. H69PR are partial revertant from H69AR cells, selected without drugs for more than 36 months with very low ABCC1 expression [38]. When exposed to BSO, the H69PR cells were ten times less sensitive than H69AR, with an IC_{50} of 6.0 μM (Fig. 1A).

Although BSO is a highly specific inhibitor of γ-glutamylcysteine synthetase, it was of interest to determine if other modulators of cellular GSH levels also affect the survival of ABCC1-expressing tumor cells. Two such drugs, verapamil and apigenin, are known to decrease intracellular GSH through ABCC1-mediated efflux. Consistent with this premise, previous studies have demonstrated that ABCC1 transfected cells are more sensitive to verapamil, possibly due to increased GSH efflux [39]. To determine the effect of ABCC1-mediated GSH efflux on the growth of tumor cells, it was of interest to investigate the effects of both verapamil and apigenin on the above three cell lines. The results in Fig. 1B and C shows the effects of increasing concentrations of verapamil or apigenin on the growth of ABCC1-expressing drug resistant tumor cells (H69/AR) or ABCC1-negative parental (H69) and revertant cells (H69/PR). The IC_{50} values obtained with verapamil for H69, H69AR and H69PR cells were 100, 2.5 and 105 μM, respectively. Similarly, these cell lines displayed collateral sensitivity to apigenin with IC_{50} values of 23, 2.2 and 18 μM for H69, H69AR and H69PR, respectively. The

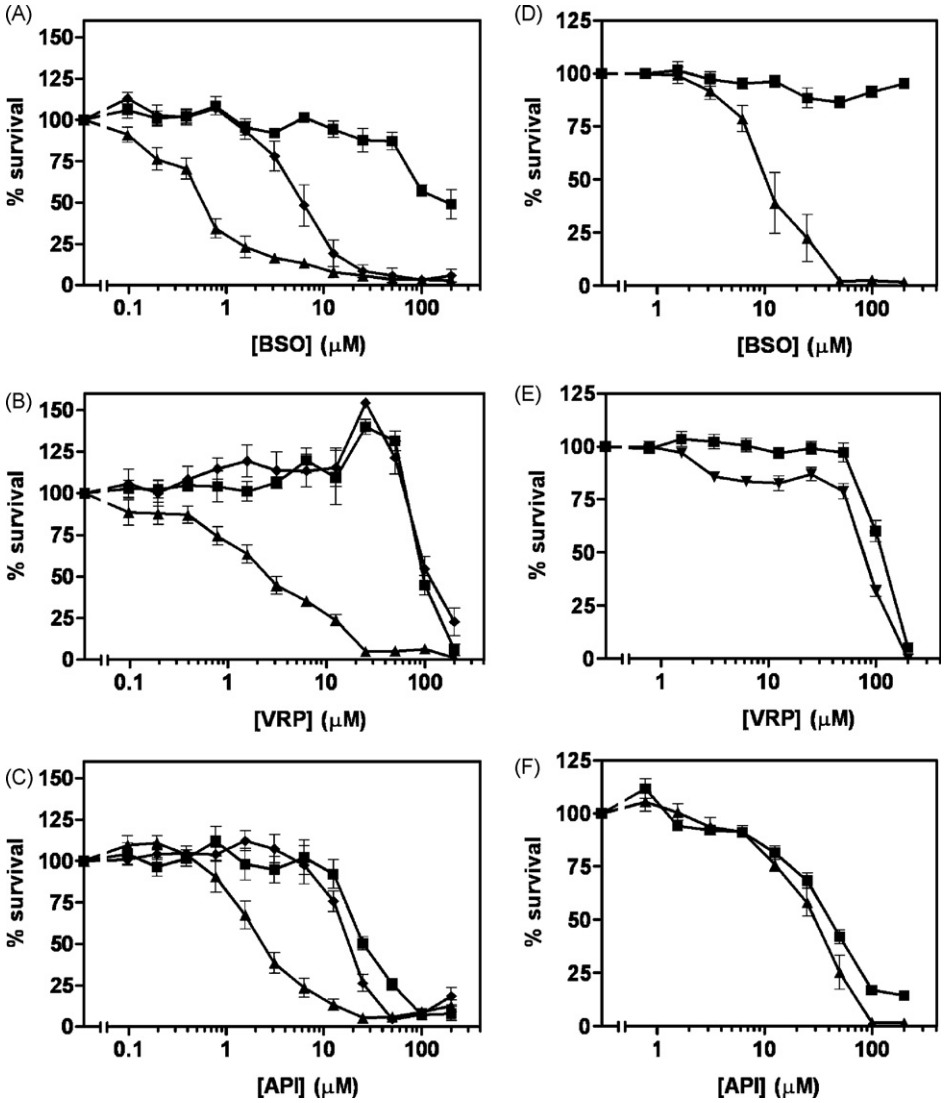

Fig. 1 – Effects of BSO, VRP or API on the survival of tumor cells with or without ABCC1 expression. Cell growth with or without drugs was assessed by MTT assay. Parental or drug sensitive cells (H69 or HeLa cells; squares), ABCC1-expressing tumor cells (H69AR or $HeLa^{ABCC1}$ cells; triangles), and ABCC1-null H69PR cells (diamonds) were cultured in the presence of increasing concentrations of BSO (panels A and D), VRP (panels B and E) or API (panels C and F) for 48 h. Error bar represent standard deviation from three different experiments done in triplicate.

differences in collateral sensitivity of H69PR and H69 in the presence of BSO is noteworthy and argues for other cellular changes in these selected cells, in addition to increases in ABCC1 expression. However, given that other cellular changes than ABCC1 alone may be responsible for the observed effects of BSO, verapamil or apigenin, it was important to evaluate the effects of the latter compounds on ABCC1-transfected HeLa cells. Cell growth assays were performed on HeLa cells transfected with human ABCC1 ($HeLa^{ABCC1}$) to determine their sensitivity to BSO, verapamil and apigenin (Fig. 1D–F). The results in Fig. 1 shows $HeLa^{ABCC1}$ cells more sensitive to all three compounds, as compared to non-transfected HeLa cells. Surprisingly, however, BSO was dramatically more toxic to ABCC1-transfectants than verapamil or apigenin. The IC_{50} for BSO, verapamil, and apigenin in $HeLa^{ABCC1}$ cells were 9, 100 and 30 μM, respectively. By contrast, the IC_{50} of HeLa cells to the same drugs were 200, 125 and 50 μM, respectively. These results demonstrate that collateral sensitivity in these tumor cell lines can be mediated solely by ABCC1 expression. A two-way ANOVA test was used to determine the significance of the differences between HeLa and $HeLa^{ABCC1}$ cells exposed to verapamil and apigenin. These analyses found that overall the survival of each cell line was significantly different from one another. The effects of GSH modulating compounds shown in Fig. 1A–F are summarized in Table 1, where by the IC_{50} values for each GSH modulating compound in the different parental and ABCC1 expressing tumor cells are shown. Together these results indicate that ABCC1 plays an important role in collateral sensitivity to GSH modulating drugs. However, the effect of BSO on ABCC1-positive cells ($HeLa^{ABCC1}$ and H69/AR) appears similar, while that of verapamil or apigenin was significantly different between the two ABCC1-positive cell lines (see Fig. 1). Such differences are likely due to differences in ABCC1 expression between the two cell lines. The results in Fig. 2 shows ABCC1 expression in H69, H69AR, H69PR, HeLa and $HeLa^{ABCC1}$ as determined by Western blot (Fig. 2A) and ELISA (Fig. 2B). Fig. 2 shows the relative levels of ABCC1 expression and demonstrates that ABCC1 expression is highest in H69AR cells, followed by lower levels in $HeLa^{ABCC1}$ cells. ABCC1 was not detectable in H69, H69PR, and HeLa cells (Fig. 2A). Collectively, the latter results demonstrate a correlation between the degree of collateral sensitivity and the level of ABCC1 expression, with H69AR cells expressing the highest levels of ABCC1 and the greatest sensitivity to verapamil, and apigenin.

Depletion of cellular GSH pools, through ABCC1-mediated GSH efflux (e.g. verapamil or apigenin) or inhibition of GSH *de novo* synthesis is thought to increase oxidative stress leading to cell death. To confirm this possibility, it was of interest to directly measure the levels of ROS and GSH in these cells and determine if they correlate with progression of cells to apoptosis. The results in Fig. 3A and D shows the effects of BSO, verapamil, and apigenin on H69AR and $HeLa^{ABCC1}$ cells as determined by Annexin V staining of tumor cells following a time course drug treatment. The results in Fig. 3A shows that apoptosis appeared quickly in H69AR treated with apigenin (1 h) and later with BSO and verapamil in $HeLa^{ABCC1}$ (9 h). As GSH function is to maintain a reductive state in mammalian cells, cellular levels of ROS were measured under the same conditions used to evaluate Annexin V staining or apoptosis. The amount of ROS was determined with the fluorescent reagent, H_2DCFDA, as mean fluorescence of intact cells. The results in Fig. 3B and E shows a time-dependent increase in cellular ROS in H69AR and $HeLa^{ABCC1}$ after exposure to BSO, verapamil and apigenin, respectively. It is note worthy that the increase in ROS always appears before an increase in Annexin V staining, suggesting that accumulation of ROS may proceed the cellular changes leading to the induction of apoptosis. To confirm the effect of drugs on cellular GSH pools and the accumulation of ROS with GSH reduction, intracellular GSH levels were determined using the GSH reactive fluorescent dye, monochlorobimane. The results in Fig. 3C and F shows that all three drugs caused a significant decrease in cellular GSH levels. Again, this decrease in cellular GSH levels appeared before or simultaneously with the accumulation of ROS. Therefore, the reduction of GSH may be directly responsible for the accumulation of ROS and the eventual induction of apoptosis.

Given the correlation between the effects of drugs (BSO, verapamil and apigenin) on GSH depletion, rise in ROS and induction of apoptosis, it was important to determine if an increase in GSH pool in these cells could reverse BSO, verapamil and apigenin-induced collateral sensitivity. The results in Fig. 3 are summarized in Table 2, showing the time

Table 1 – Effects of GSH modulating drugs on tumor cells growth

Tumor cells	IC_{50} in μM		
	BSO	VRP	Api
H69	178	108	25
H69AR	0.6	2.4	2.3
H69PR	6.1	98.4	17.7
HeLa	–	114	40.1
$HeLa^{MRP1}$	10.1	74.4	29.0

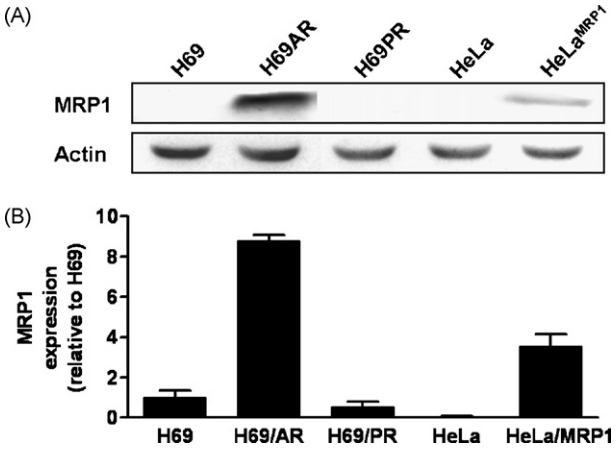

Fig. 2 – ABCC1 protein expression in tumor cell lines. Total cell extracts from H69, H69AR, H69PR, HeLa, and $HeLa^{ABCC1}$ were resolved on SDS-PAGE and the expression of ABCC1 and actin were determined by Western blotting (panel A) or ELISA (panel B) using ABCC1 or actin-specific antibodies (see Section 2).

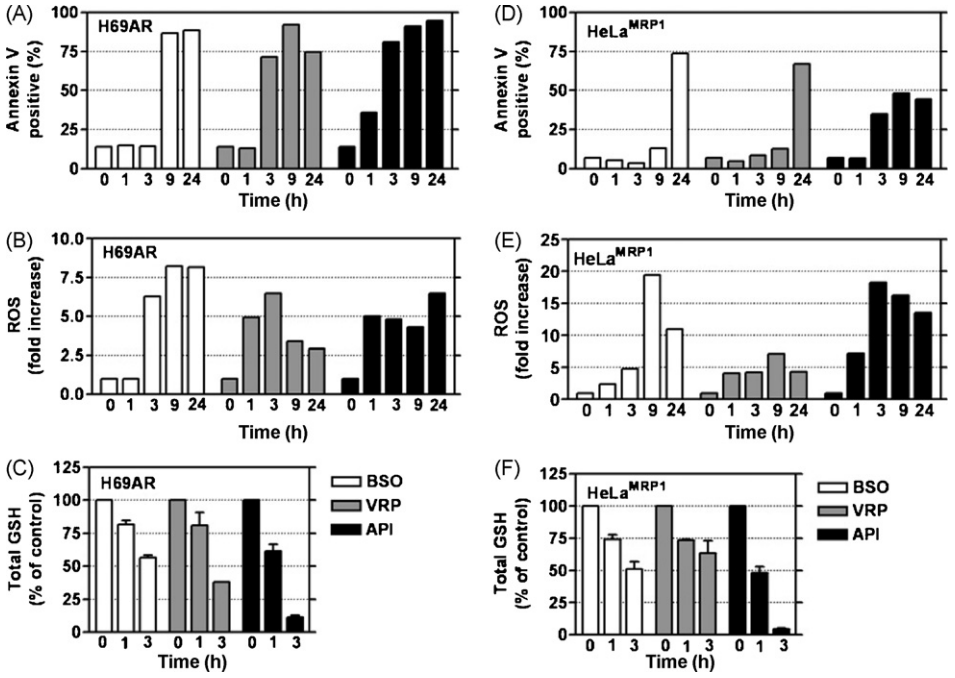

Fig. 3 – Estimates of relative levels of apoptosis, ROS and GSH in H69AR or HeLa^{ABCC1} cells without or with drug treatment. H69AR or HeLa^{ABCC1} cells were treated with BSO (50 μM), VRP (50 μM) or Api (25 μM) for 1–24 h. Annexin V staining of cells was determined by flow cytometry (panels A and D). Panels B and E show relative levels of ROS in cells as determined from mean fluorescence of live cells loaded with H_2DCFDA dye. The results from a single representative flow cytometry experiment are presented in panels A, B, D and E. Panels C and F show cellular GSH levels in drug treated relative to non-treated cells. Error bars represent standard deviation from results obtained in triplicate. The results above show a representative experiment repeated three times.

(h) whereby exposure of tumor cells to BSO, VRP, or Api, results in a significant drop in cellular GSH levels, a five-fold increase in ROS and apoptosis in >40% of tumor cells.

Previous studies have shown that free GSH is able to enter epithelial cells by an unknown mechanism [40]. Fig. 4A and B shows that co-incubation of HeLa^{ABCC1} and H69AR cells with drugs together with increasing concentrations of GSH was able to counteract the toxicity of these drugs. In H69AR cells, 25 mM GSH was able to completely rescue cells from 12.5 μM BSO and 25 μM verapamil. The survival of cells exposed to 25 μM apigenin was increased from 0% to 35%. The survival of HeLa^{ABCC1} cells exposed to 125 μM verapamil was completely rescued by 3.13 mM GSH. At 6.25 mM, GSH only partially improved the survival of HeLa^{ABCC1} cells exposed to 25 μM BSO and 125 μM apigenin. Similar rescue effects were observed with the membrane-permeable GSH precursor, NAC (Fig. 4C and D), with near complete rescue in H69AR and a partial rescue in HeLa^{ABCC1} cells.

To investigate further the molecular mechanism(s) of BSO, verapamil and apigenin on MRP-1 expressing tumor cells, the effect of the general caspase inhibitor Z-VAD-FMK on collateral sensitivity of drug treated H69AR cells was determined. The results in Fig. 5A shows that 100 μM Z-VAD-FMK completely reversed or inhibited verapamil-induced apoptosis; whereas the survival of H69AR cells exposed to 12.5 μM BSO was increased from 15% to 45%, while survival in the presence of 25 μM apigenin was increased from 10% to 48%. The effect of Z-VAD-FMK was also investigated in the H69AR cell line using H69AR cells transfected with human *Bcl-2* full-length cDNA, H69AR^{Bcl-2}. The results in Fig. 5B shows that the increased expression of Bcl-2 in H69AR cells was protective from BSO, verapamil, and

Table 2 – Relative effects of GSH modulating drugs, a time-course, on GSH cellular levels, reactive oxygen species and apoptosis in tumor cells expressing ABCC1, relative to control

	Tumor cell lines					
	H69AR			HeLa^{MRP1}		
	BSO	VRP	Api	BSO	VRP	Api
Time leading to >20% drop of GSH levels (h)	1	1	1	1	1	1
Time leading to five-fold increase in ROS (h)	3	3	3	9	9	1
Time leading to 40% increase in apoptosis	9	3	3	24	24	9

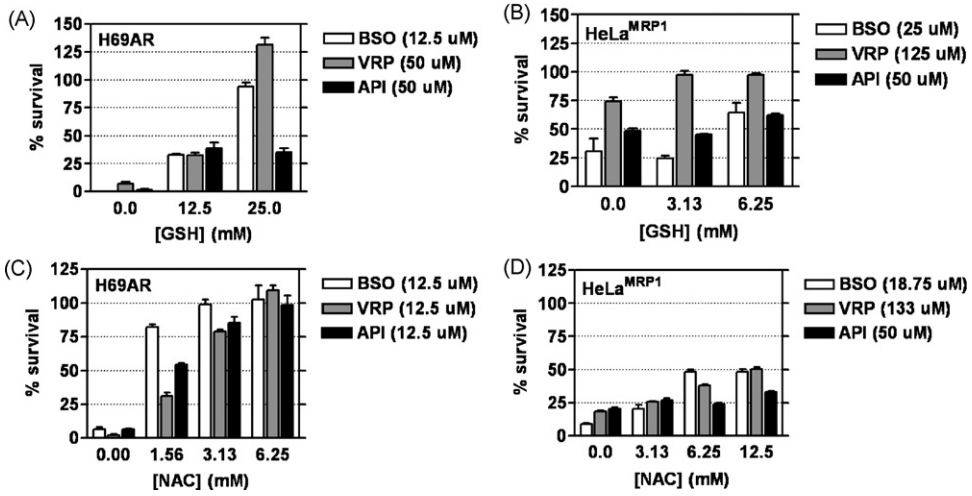

Fig. 4 – Reversal of BSO, VRP or API effects on H69AR and HeLa^{ABCC1} cells with GSH or NAC supplemented media. The effects of BSO, VRP and API alone or with exogenously supplemented GSH or NAC on the growth of tumor cells. Panel A shows the survival of H69AR cells without and with 12.5 or 25 mM GSH supplemented media. Similarly, Panel B shows the survival of HeLa^{ABCC1} cells without and with 3.13 or 6.25 mM GSH supplemented media. Panel C shows the survival of H69AR cells without and with 1.56, 3.125 or 6.25 mM NAC supplemented media. Similarly, Panel D shows the survival of HeLa^{ABCC1} cells without and with 3.13, 6.25 or 12.5 mM NAC supplemented media. Cell survival was determined with an MTT assay (see Section 2). Error bars represent standard deviation from results obtained in triplicate. The results above show a representative experiment repeated three times.

apigenin-induced apoptosis. Survival was increased by 20–40%, depending on the toxic drug. The greatest effect on survival (increase from 35% to 68%) was evident in cells exposed to BSO. These observations show that collateral sensitivity to drugs that reduce GSH levels in ABCC1 expressing cells may be in part due to an apoptotic pathway. It is important to point out that BCL2 transfection in H69AR cells (H69AR$^{Bcl\text{-}2}$) did not alter the levels of ABCC1 relative to H69AR untransfected cells (results not shown), while Bcl2 expression was increased by two-fold (Fig. 5C).

Several studies [22,41] have shown that both verapamil and apigenin stimulate ABCC1-mediated efflux of GSH. We have previously demonstrated that verapamil-mediated increase of GSH efflux results in a significant increase in ABCC1 binding to GSH, as determined using a photoreactive analogue of GSH (or IAAGSH [34]). In light of our results in this study, together with our earlier observation with IAAGSH photoaffinity labelling of ABCC1, it was of interest to examine the effect of increasing concentrations of BSO, verapamil and apigenin on ABCC1 binding or photoaffinity labelling with IAAGSH. The results in Fig. 6A shows a significant increase (2.3-fold) in IAAGSH photolabeling of ABCC1 with 100-fold molar excess of verapamil. However, surprisingly, there was no increase in the photoaffinity labelling of ABCC1 with IAAGSH in the presence of apigenin, as it has been previously shown [42] to increase ABCC1-dependent efflux of GSH. The absence of effect of BSO on ABCC1 photoaffinity labelling with IAAGSH is not surprising since BSO is not known to be a substrate of ABCC1 nor does it affect GSH efflux. Taken together, the results in Fig. 6 shows that verapamil and apigenin, although both enhance GSH efflux via ABCC1, do so by interacting with different sites on ABCC1.

4. Discussion

ABCC1 has been shown to mediate the transport of endogenous cell metabolites and anti-cancer drugs via an ATP-dependent efflux mechanism [43]. One such endogenous substrate of ABCC1 is the tripeptide GSH, a major cellular detoxifying compound [44,45]. In this report we demonstrate that over-expression of ABCC1 contributes to cell death by oxidative stress through drug enhanced GSH efflux. Several studies have now demonstrated that GSH plays an important role in ABCC1 functions. In one instance, GSH is effluxed by ABCC1 pump, while in another GSH plays a catalytic role enhancing the transport of non-conjugated drugs. Consistent with its role as a substrate for, and modulator of ABCC1 efflux functions, we have recently demonstrated that GSH interacts with several domains in ABCC1, including drug transport domains [34]. Moreover, there is increasing evidence that ABCC1 expression in tumor cells or its lack-of (knockout studies) modulate cellular GSH levels. The three drugs tested in this study led to oxidative-stress induced apoptosis in ABCC1-expressing cells caused by inhibition of *de novo* synthesis of GSH (e.g. BSO), or increasing ABCC1-mediated transport of GSH (e.g. verapamil or apigenin). Specifically, our results show that ABCC1-expressing H69AR cells were hypersensitive to BSO (IC_{50} = 0.6 μM) relative to ABCC1-null parental H69 cells (IC_{50} = 200 μM). The revertant cell line, H69PR, was less affected with BSO (IC_{50} = 6 μM). The reduced toxicity of H69PR cells with BSO correlates with the lower expression level of ABCC1 in H69PR compared to H69AR. These findings indicate that the level of ABCC1 expression correlates with the degree of collateral toxicity to BSO; however other cellular changes in H69AR tumor cells unrelated to ABCC1

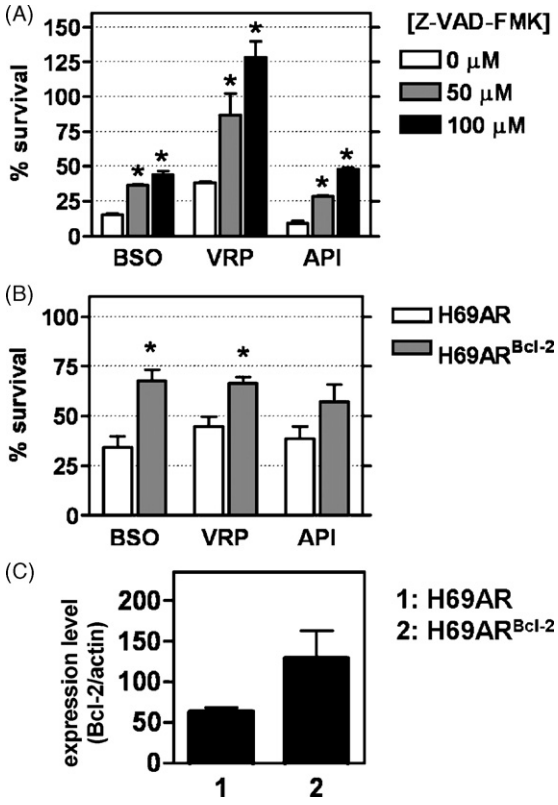

Fig. 5 – Relative inhibition of BSO-, VRP- and API-induced apoptosis in H69AR and H69AR^{Bcl2} cells. H69AR or H69AR^{Bcl-2} cells were treated with BSO (12.5 or 6.25 μM), VRP (25 or 3.13 μM) and API (25 or 3.13 μM) in the absence or presence of the general caspase inhibitor, Z-VAD-FMK (at 0, 50 and 100 μM; panels A & B, respectively). Cell growth was determined by MTT after 24 h incubation. Panel C shows the relative increase in Bcl-2 expression in H69AR transfectants, as a ratio of actin expression between H69AR and H69AR^{Bcl2} cells evaluated by densitometry of Western blot signals. Error bars represent standard deviation from results obtained in triplicate. Stared (*) bars show a significant increase ($P < 0.05$) relative to negative controls.

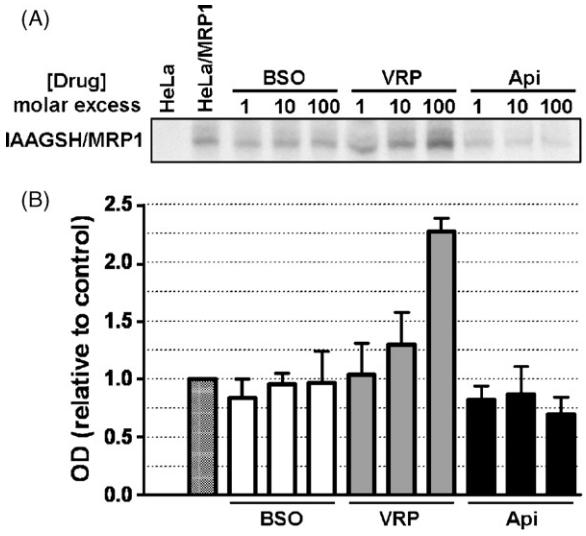

Fig. 6 – Photoaffinity labeling of ABCC1 with IAAGSH. HeLa or HeLa^{ABCC1} plasma membranes were photoaffinity labeled with 2 μM IAAGSH in the absence or presence of BSO, VRP and API. Drugs were added to plasma membranes containing IAAGSH at 1-, 10- or 100-molar excess. Panel B shows the relative change in photolabeling of ABCC1 with IAAGSH in the presence of the same drugs as evaluate by densitometry relative to solvent control. Error bars represent standard deviation.

expression in these cells could not be ruled out. Hence, it was essential to examine the effects of BSO on cells where ABCC1 alone was differentially expressed. Concordantly, results with HeLa^{ABCC1} cells display a far more pronounced sensitivity to BSO (IC_{50} = 10 μM) as compared to HeLa cells. Indeed, HeLa cells were unaffected by BSO up to a concentration of 200 μM. These findings demonstrate that BSO hypersensitivity is a consequence of ABCC1 expression in tumor cells. In this respect, it would be of interest to examine the effects of BSO on normal tissues and organs that express high levels of ABCC1, such as in lung, kidney, liver, testis and blood mononuclear cells [6,7]. Phase I clinical trials using BSO alone or in combination with melphalan to reduce GSH intracellular pools have concluded that continuous infusion of BSO is relatively non-toxic at concentrations that cause 10–30% below normal baseline values of GSH [46,47]. Interestingly, the latter studies observed considerably lesser depletion of GSH in normal peripheral lymphocytes than that in tumor sections [46,47].

Earlier studies have shown that transport of GSH is enhanced by verapamil and apigenin [22,23]. Given this and the hypersensitivity to BSO, it was suspected that verapamil and apigenin could induce toxicity in ABCC1-expressing cells by reducing the intracellular concentration of GSH. Indeed, H69AR cells are 40-fold more sensitive to verapamil, and 10.5-fold more sensitive to apigenin, compared to H69 cells. Unlike BSO toxicity, the sensitivity of H69PR to these compounds was similar to H69. This highlights some of the difficulties in using MDR cell lines generated by drug selection. The drug selection process may alter the expression of other proteins. Consequently, the different levels of drug sensitivity in H69-derived cells may in-part be a product of phenotypic changes other than ABCC1 expression. To address this possibility, the effects of these compounds were tested in transfected HeLa^{ABCC1} cells. As expected, HeLa^{ABCC1} cells were more sensitive to verapamil and apigenin compared to HeLa cells. Together, these finding demonstrate clearly that collateral sensitivity to BSO, verapamil and apigenin is a direct consequence of ABCC1 expression.

As GSH plays an important role in cellular detoxification through its reducing potential [44,45], the accumulation of reactive oxygen species (ROS) may be the critical factor that initiates cell death due to BSO, verapamil and apigenin collateral sensitivity. High levels of intracellular ROS leading to intrinsic oxidative stress have been shown to cause cell death through apoptosis in different tumor model systems [48–51]. Our findings in this study show that ROS and apoptosis

are at least in part involved in cell death due to reductions in cellular GSH. Treatment of ABCC1-expressing cells with Z-FAD-FMK, a general caspase inhibitor, was able to significantly inhibit cell death. Furthermore, transfection of H69AR cells with the anti-apoptotic human *bcl-2* gene was also able to improve survival and confirm that GSH-modulating agents are causative of tumor cell death through enhanced apoptosis. Z-FAD-FMK was most effective at inhibiting toxicity caused by verapamil; indicating that verapamil-initiated apoptosis is different from that of BSO or apigenin.

The reduction of cellular GSH levels, either through inhibition of synthesis or enhanced efflux, is likely to result in increased accumulation of ROS. Consistent with this latter link, our results in this study show that treatment of tumor cells with BSO, verapamil or apigenin causes a significant dose- and time-dependent increase in ROS. One of the most notable examples was the observed 19-fold increase in ROS in HeLa^{ABCC1} cells following 24 h of treatment with BSO. Moreover, the increase in ROS production occurs prior to the appearance of apoptosis with all three compounds, in an ABCC1-dependent manner. Furthermore, reductions in GSH levels in these tumor model systems preceed apoptosis in cells exposed to BSO, verapamil or apigenin. The most striking reduction occurred in both H69AR and HeLa^{ABCC1} cells treated with apigenin after 3 h, where GSH levels were reduced by approximately 90%. Remarkably, this finding correlates with previous studies that show apigenin to be one of the most effective stimulators of GSH transport by ABCC1 [22,42]. In all cases, the reduction in GSH starts 1 h after drug addition, resulting in the subsequent increase in ROS and the eventual appearance of apoptosis. If a reduction in intracellular GSH is indeed the initiating step leading to ROS production and subsequent apoptosis, it was reasoned that increasing GSH levels should negate the effects of these compounds and reverse collateral sensitivity. In support of the latter notion, the results in Fig. 4 shows that cells treated with BSO, verapamil and apigenin can be rescued by exogenously added GSH or NAC to the cell growth media. Again, this effect was most pronounced in BSO-treated H69AR cells in that they were completely rescued with the addition of 25 mM GSH or 6.25 mM NAC. As expected, lower molar concentrations of NAC than GSH lead to a similar rescue effects, likely due to greater membrane permeability of NAC, a precursor of GSH. Indeed, it is not entirely clear how externally added GSH crosses the cell membrane. One possibility is the presence of low affinity GSH importer. Such GSH import has been described in the renal system whereby the sodium dicarboxylate transporter (SDCT-2) and the organic anion transporter OAT1/3 were demonstrated to mediate GSH import from extracellular milieu [40]. Although it is not know if these latter transporters are expressed in H69AR or HeLa cells, such transporters can mediate the GSH influx. Alternatively, high extracellular concentrations of GSH in ABCC1-expressing cells could competitively inhibit ABCC1-mediated GSH efflux or potentiate ABCC1-mediated influx of GSH. Taken together, this further demonstrates that GSH depletion is the primary or a significant mediator of collateral sensitivity in ABCC1 expressing tumor cells.

Although it is not entirely clear how verapamil and apigenin enhance GSH transport via ABCC1, it is thought that both compounds increase the ABCC1 affinity to GSH. Consistent with the latter hypothesis, we have previously shown that verapamil increases the affinity of ABCC1 for GSH binding, as evident by the substantial increase in ABCC1 photolabeling with IAAGSH [34]. However, surprisingly the results in this study show that while verapamil stimulates IAAGSH binding to ABCC1, apigenin did not increase ABCC1 binding to GSH. Although unexpected, it is possible that apigenin enhances ABCC1 efflux of GSH via a different mechanism than that seen with verapamil. Efforts are underway to compare differences in verapamil and apigenin binding on ABCC1.

In conclusion, this study points to a general oxidative stress that occurs in ABCC1-expressing cells as a consequence of ABCC1-mediated efflux of GSH. This state of oxidative stress can be further potentiated through the inhibition of the *de novo* synthesis of GSH or through enhanced GSH efflux. Together, these findings provide an enticing way of specifically targeting drug resistant tumor cells that express ABCC1.

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