

# Cystine-Glutamate Transporter *SLC7A11* in Cancer Chemosensitivity and Chemoresistance

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## Abstract

*SLC7A11* (*xCT*), together with *SLC3A2* (*4F2hc*), encodes the heterodimeric amino acid transport system  $x_c^-$ , which mediates cystine-glutamate exchange and thereby regulates intracellular glutathione levels. We used microarrays to analyze gene expression of transporters in 60 human cancer cell lines used by the National Cancer Institute for drug screening (NCI-60). The expression of *SLC7A11* showed significant correlation with that of *SLC3A2* ( $r = 0.66$ ), which in turn correlated with *SLC7A5* ( $r = 0.68$ ), another known partner for *SLC3A2*, and with *TIA-2* ( $r = 0.60$ ; all  $P < 0.0001$ ). Linking expression of *SLC7A11* with potency of 1,400 candidate anticancer drugs identified 39 showing positive correlations, e.g., amino acid analogue, L-alanosine, and 296 with negative correlations, e.g., geldanamycin. However, no significant correlation was observed with the geldanamycin analogue 17-allylamine, 17-demethoxygeldanamycin (17-AAG). Inhibition of transport system  $x_c^-$  with glutamate or (S)-4-carboxyphenylglycine in lung A549 and HOP-62, and ovarian SK-OV-3 cells, reduced the potency of L-alanosine and lowered intracellular glutathione levels. This further resulted in increased potency of geldanamycin, with no effect on 17-AAG. Down-regulation of *SLC7A11* by small interfering RNA affected drug potencies similarly to transport inhibitors. The inhibitor of  $\gamma$ -glutamylcysteine synthetase, buthionine sulfoximine, also decreased intracellular glutathione levels and enhanced potency of geldanamycin, but did not affect L-alanosine. These results indicate that *SLC7A11* mediates cellular uptake of L-alanosine but confers resistance to geldanamycin by supplying cystine for glutathione maintenance. *SLC7A11* expression could serve as a predictor of cellular response to L-alanosine and glutathione-mediated resistance to geldanamycin, yielding a potential target for increasing chemosensitivity to multiple drugs. (Cancer Res 2005; 65(16): 7446-54)

## Introduction

The development of resistance to anticancer drugs represents a main problem of cancer chemotherapy. Contributing mechanisms include changes in drug uptake and efflux, DNA repair, apoptosis,

and cellular detoxification pathways, such as those mediated by reduced glutathione (GSH; ref. 1). Understanding these mechanisms at the molecular level is critical to prediction of drug response and in design of effective chemotherapy.

*SLC7A11* (or *xCT*) encodes the transporter subunit of the heterodimeric amino acid transport system  $x_c^-$  (2, 3). System  $x_c^-$ , consisting of *SLC7A11* and *SLC3A2* (or 4F2hc, 4F2 heavy chain, CD98), is selective for cystine and glutamate, mediating cystine entry into the cell in exchange for glutamate. Once inside the cell, cystine is rapidly reduced to cysteine, the limiting amino acid for GSH synthesis (4, 5). The synthesis of GSH from glutamate, cysteine, and glycine is catalyzed sequentially by two cytosolic enzymes,  $\gamma$ -glutamylcysteine synthetase ( $\gamma$ -GCS) and GSH synthetase (6). GSH synthesis is regulated primarily by the activity of the rate-limiting  $\gamma$ -GCS, cysteine availability, and GSH feedback inhibition (6). Increase in  $\gamma$ -GCS activity and stimulation in cysteine (cystine) uptake generally increase intracellular GSH concentrations (7). Although system  $b^{0,+}$  represents an alternative mammalian transport system of cystine (8), uptake in human cancer cell lines is largely mediated by system  $x_c^-$  (9). Therefore, system  $x_c^-$  plays an important role in regulating the intracellular GSH level, thought to mediate resistance against multiple drugs. Specifically, the  $x_c^-$  amino acid transport system has been shown to maintain intracellular GSH and consequently results in cisplatin resistance in ovarian cancer cells (9).

Our laboratory has previously used 70-mer oligonucleotide arrays to analyze gene expression of membrane transporters and channels in the 60 human cancer cell lines used by the National Cancer Institute for drug screening (NCI-60). Correlating gene expression with the potencies of 119 standard anticancer drugs identified known drug-transporter interactions and suggested novel ones (10). Relatively high expression of a given gene in drug-sensitive cell lines yields a positive correlation, whereas high expression in resistant cells gives negative correlations. We found that *SLC7A11* expression positively correlates with L-alanosine [L-2-amino-3-(N-hydroxy-N-nitrosamino)propionic acid], an amino acid analogue drug, suggesting the hypothesis that *SLC7A11* mediates cellular uptake of L-alanosine (10). On the other hand, *SLC7A11* expression showed significant negative correlations with multiple drugs of distinct structural features, suggesting that *SLC7A11* also plays a pervasive role in chemoresistance.

In the present study, we show that in the NCI-60 cell panel, *SLC7A11* expression showed significant correlation with that of *SLC3A2*, encoding the other subunit of system  $x_c^-$ . We also validate positive and negative correlations between *SLC7A11* expression and cytotoxic potencies of L-alanosine and three geldanamycin analogues, using inhibition of cystine transport with glutamate, (S)-4-carboxyphenylglycine (4-S-CPG), or small interfering RNA (siRNA) specifically targeting *SLC7A11* mRNA. L-Alanosine represents an apparent substrate of *SLC7A11*, being sensitized in cytotoxic potency by expression of the transporter. In contrast, the

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geldanamycin analogues were variably sensitized by inhibition of SLC7A11, which leads to glutathione depletion. Results with buthionine sulfoximine (BSO), a specific inhibitor of  $\gamma$ -GCS, further supported the hypothesis that SLC7A11 (or transport system  $x_c^-$ ) functions in chemoresistance via maintaining intracellular glutathione. In addition, we have studied multiple cell lines with various *SLC7A11* expression levels to determine whether the observed results are cell line-specific phenomena or are broadly applicable. This affords the opportunity to test whether a cytotoxic agent is subject to SLC7A11 (or system  $x_c^-$ )-mediated drug resistance, and to select congeners that could potentially avoid GSH-mediated resistance for drug development.

## Materials and Methods

**Gene expression using oligonucleotide microarrays.** A spotted 70-mer microarray was used to measure transporter and channel gene expression (10). Total RNA was extracted from cell cultures maintained at the NCI under conditions and with passage numbers as used in a previous cDNA microarray study (11). Expression of each gene was assessed by the ratio of expression level in the sample against a pooled control sample from 12 diverse cell lines of the NCI-60 (11). Each of the 60 cell lines was studied by one array, with four replicate probes spotted per array and compared with a separate replication of the experiment. The expression data for *SLC7A11* and *SLC3A2* showed good concordance with one repeated array experiment. Array hybridization and data analysis were previously described (10).

**Correlation analysis.** Growth inhibition data ( $GI_{50}$  values for 60 human tumor cell lines) were those obtained by the Developmental Therapeutics Program (<http://dtp.nci.nih.gov>). Values were expressed as potencies using the negative log of the molar concentration calculated in the NCI screen. We focused on 1,400 drugs that has been used in Scherf et al. (ref. 11; data are available online <http://discover.nci.nih.gov/nature2000/natureintomain.jspdatabases>). Pearson correlation coefficients were calculated for assessment of gene-to-gene and gene-to-drug relationships. Confidence intervals and unadjusted *P* values were obtained using Efron's bootstrap resampling method (12), with 10,000 bootstrap samples for each gene-to-drug comparison. This study involves multiple comparisons between gene expressions (same array experiments and different experiments) and between gene expression and drug activity. For different types of comparisons, 5% false discovery rate was accepted to determine the cutoff point of statistical significance (13). For correlation between gene expression and drug activity, the dual criteria of  $P < 0.05$  and  $|r| > 0.3$  were used to identify significant correlations as previously described (10).

**Compounds.** Melphalan, cisplatin, and BSO were purchased from Sigma Chemical Co. (St. Louis, Missouri). Geldanamycin (NSC 122750) and 17-allylamino, 17-demethoxygeldanamycin (17-AAG; NSC 330507) were purchased from InvivoGen (San Diego, CA). 4-S-CPG was purchased from Tocris (Ellisville, MO). Other compounds were obtained from the Developmental Therapeutics Program at NCI.

**Cell culture.** The cell lines were cultured in RPMI 1640 containing 5 mmol/L L-glutamine, supplemented with 10% fetal bovine serum, 100 units/mL sodium penicillin G, and 100  $\mu$ g/mL streptomycin. Cells were grown in tissue culture flasks at 37 °C in a 5% CO<sub>2</sub> atmosphere.

**Sulforhodamine B cytotoxicity assay.** Drug potency was tested using a proliferation assay with SRB, a protein-binding reagent (Sigma). In each experiment, 3,000 to 4,000 cells per well were seeded in 96-well plates and incubated for 24 hours. Then, the culture medium was changed to fresh medium containing 5 mmol/L glutamate or BSO (10  $\mu$ mol/L for A549 cells, 100  $\mu$ mol/L for SK-OV-3 cells) 24 hours before and during exposure to test drugs. In addition, the cells were treated with 50  $\mu$ mol/L 4-S-CPG, together with anticancer drugs, for 4 days. Anticancer drugs were added in a dilution series in three replicate wells per concentration. After 3 days, incubation was terminated by replacing the medium with 100  $\mu$ L of 10% trichloroacetic acid (Sigma) in 1 $\times$  PBS, followed by incubation at 4 °C for at least 1 hour. Subsequently, the plates were washed with water and air dried. The plates were stained with 50  $\mu$ L 0.4% SRB (Sigma) in 1% acetic acid for 30 minutes

at room temperature. Unbound dye was washed off with 1% acetic acid. After air drying and resolubilization of the protein-bound dye in 10 mmol/L Tris-HCl (pH 8.0), absorbance was read in a microplate reader at 570 nm and background absorbance at 690 nm was subtracted. To determine  $IC_{50}$  values, the absorbance of control cells without drug was set at 1. Dose-response curves were plotted using Prism software (San Diego, CA). Each experiment was done independently at least twice. Student's *t* test was used to determine the degree of significance.

**Small interfering RNA-mediated down-regulation of gene expression.** siRNA duplexes for SLC7A11 were synthesized using the Silencer siRNA construction kit (Ambion, Austin, TX). The two target sequences were as follows:

5'-AATCTTCATCTCTCCTAAGGG-3' (SLC7A11\_183).

5'-AAATGCCAGATATGCATCGT-3' (SLC7A11\_1237).

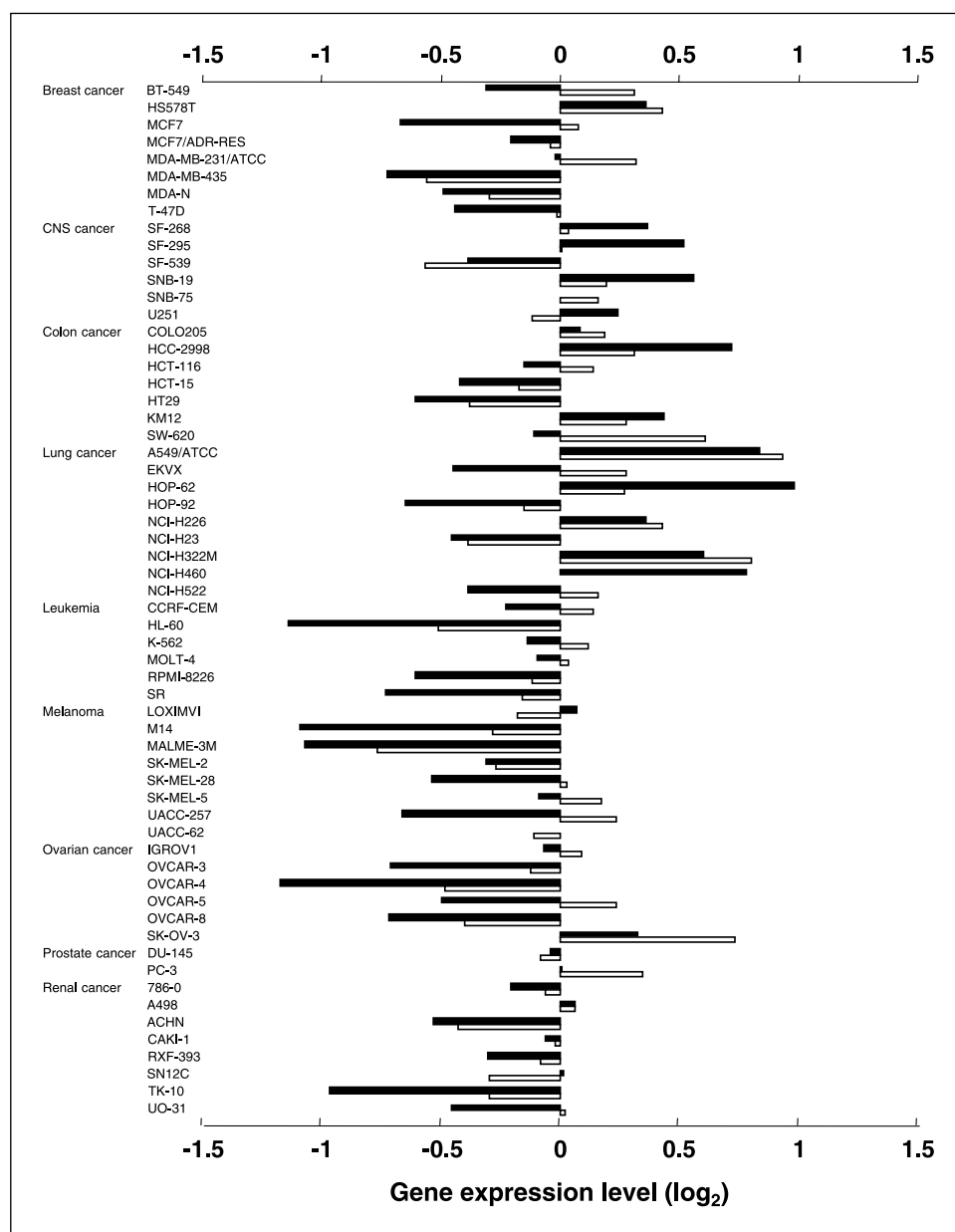
These sequences target nt 183 to 203 and 1,237 to 1,257 of the SLC7A11 mRNA sequence NM\_014331, respectively. Negative control was chemically synthesized mock siRNA (fluorescein-labeled, nonsilencing) purchased from Qiagen, Inc. (Valencia, CA). Transfection was done with TransMessenger Transfection Reagent (Qiagen). To down-regulate SLC7A11, cancer cells were transfected with 0.3  $\mu$ mol/L siRNA. For RNA extraction for reverse transcription-PCR (RT-PCR), cells were harvested 48 hours after transfection. To measure cytotoxic drug potency, cells grown in six-well plates were subcultured into 96-well plates 24 hours after transfection and incubated for 4 days before SRB assay.

**Real-time quantitative reverse transcription-PCR.** Total RNA was prepared by using the RNeasy Mini kit (Qiagen), following the protocol of the manufacturer. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis (visual presence of sharp 28S and 18S bands). The RNA was quantitated by spectrophotometry. One microgram of total RNA was incubated with DNase I and reverse transcribed with oligo(dT) with Superscript II RT-PCR (Life Technologies, Carlsbad, CA). One microliter of reverse transcription product was amplified by primer pairs specific for *SLC7A11*. *ACTB* ( $\beta$ -actin) was used as a normalizing control. Primers were designed with Primer Express software (Applied Biosystems, Foster City, CA). The primers for *SLC7A11* were 5'-TGCTGGGCTGATTATCTTCG-3' (forward) and 5'-GAAAGGGCAACCATGAAGAGG-3' (reverse). The primers for  $\beta$ -actin were 5'-CCTGGCACCCAGCACAAAT-3' and 5'-GCCGATCCACACGAGTACT-3'. Relative gene expression was measured with the GeneAmp 7000 Sequence Detection system (Applied Biosystems). *SLC7A11* expression level was assessed by the ratio of the expression level in an individual sample against mean expression in all the four samples.

**Determination of intracellular glutathione.** Cells ( $3 \times 10^5$ ) were plated into six-well plates. After 24 hours, the cells were treated with 5 mmol/L glutamate or 100  $\mu$ mol/L 4-S-CPG. After 24 hours, the cells were trypsinized and the total number of cells was counted. The intracellular total glutathione level was measured using ApoGSH Glutathione Colorimetric Detection kit (Biovision, Mountain View, CA) following the instruction of the manufacturer. The absorbance was measured at 405 nm. The absorbance of each sample was normalized by cell number.

## Results

**Expression of SLC7A11, SLC3A2, and related genes in cancer cells.** We studied mRNA expression of ~200 solute carrier genes, including *SLC7A11* and *SLC3A2*, in NCI-60 human cancer cell lines using oligonucleotide microarrays. Hierarchical cluster analysis of gene expression showed that *SLC7A11* and *SLC3A2* display a similar profile across the NCI-60 (data not shown). Figure 1 depicts expression levels of *SLC7A11* and *SLC3A2* in each of the 60 cell lines relative to the expression levels of a pooled control (12 cell lines of the NCI-60). The Pearson correlation coefficient (*r*) between expression of the two genes was 0.66 (95% confidence interval, 0.51-0.78;  $P < 0.0001$ ). This was the highest correlation between



**Figure 1.** Relative expression levels of *SLC7A11* and *SLC3A2* in NCI-60 cancer cell lines determined by DNA arrays. Filled columns, *SLC7A11*. Open columns, *SLC3A2*. Expression of each gene was assessed by the log ratio of expression level in the sample against a pooled control sample from 12 diverse cell lines of the NCI-60.

*SLC7A11* and any of the other measured transporters (10), consistent with previous findings that the two proteins form a functional heterodimeric complex (2) and, moreover, validating our array analysis.

Some cells express high levels of both *SLC7A11* and *SLC3A2* (such as lung cancer A549 cells), some express both genes at low levels (such as melanoma MALME-3M), and some show differential expression of the two genes (such as breast cancer BT-549, lung cancer EKVX, and ovarian cancer OVCAR-5). Whereas the level of *SLC3A2* expression required to complement the transport activity of *SLC7A11* is unknown, *SLC7A11* and *SLC3A2* show relatively high expression levels in lung, colon, and central nervous system (CNS) cancer cells (Fig. 1). Among nine lung cancer cell lines, five showed high expression of *SLC7A11*, including A549, HOP-62, NCI-H226, NCI-H322M, and NCI-H460. Of these, HOP-62 showed the highest *SLC7A11* expression, whereas A549 showed high expression of both *SLC7A11* and *SLC3A2*. Cell lines from

other tissue origins expressed less of both genes. In breast cancer cells, only HS578T expressed both genes, but at a lower level. In ovarian cancer cell lines, only SK-OV-3 expressed both genes, whereas in leukemia, melanoma, and renal cancer cell lines, *SLC7A11* expression is relatively low. Thus, *SLC7A11* and *SLC3A2* genes are expressed in a subset of human cancer cell lines, suggesting that transport system  $x_c^-$  activity may be higher in these cells.

To search for more potential partners of these genes, we did a gene-to-gene Pearson correlation analysis among the 750 genes measured by our transporter array in the NCI-60 (for gene expression data, see ref. 10). Controlling the false discovery rate at a level of 5%, the cutoff point for any significant correlation is  $|r| \geq 0.49$  and  $P < 0.0001$ . Using this criterion, 0.7% of the gene pairs showed significant correlation ( $\sim 4,000$  pairs). Table 1 lists genes showing high correlation with *SLC3A2*. *SLC3A2* is also coexpressed with *SLC7A5*, with a correlation coefficient 0.68 (95% confidence

**Table 1.** Pearson correlation coefficients between gene expression levels of *SLC3A2* and the three genes showing the highest correlation with *SLC3A2*

	<i>SLC3A2</i>	<i>SLC7A11</i>	<i>SLC7A5</i>	<i>T1A-2</i>
<i>SLC3A2</i>	1			
<i>SLC7A11</i>	0.66*	1		
<i>SLC7A5</i>	0.68*	0.36	1	
<i>T1A-2</i>	0.60*	0.34	0.47	1

\* $P < 0.0001$ .

interval, 0.51-0.81;  $P < 0.0001$ ). This is consistent with previous finding that *SLC7A5* (or *E16*, *LATI*) encodes the other light chain of SLC3A2 and forms part of amino acid transport system L (14). Coexpression of SLC3A2 is similarly required for surface expression of SLC7A5 and activity of system L. *T1A-2*, encoding a lung type I cell membrane-associated glycoprotein, is another gene whose expression highly correlated with *SLC3A2* (Table 1), suggesting potential functional interaction. There was a moderate correlation between *SLC7A11*, *SLC7A5*, and *T1A-2*, which may represent an indirect relationship because of their common relations with *SLC3A2*.

SLC3A2 serves as a common component of several amino acid transporter system, including system L, system  $\gamma^+L$ , and system  $x_c^-$  (15). However, we did not observe significant correlations between *SLC3A2* and other light chains, e.g., *SLC7A6*, *7A7*, and *7A8* (components of system  $\gamma^+L$ ), probably because expression levels of these genes do not vary sufficiently or system  $\gamma^+L$  is not prominently expressed in the 60 cell lines.

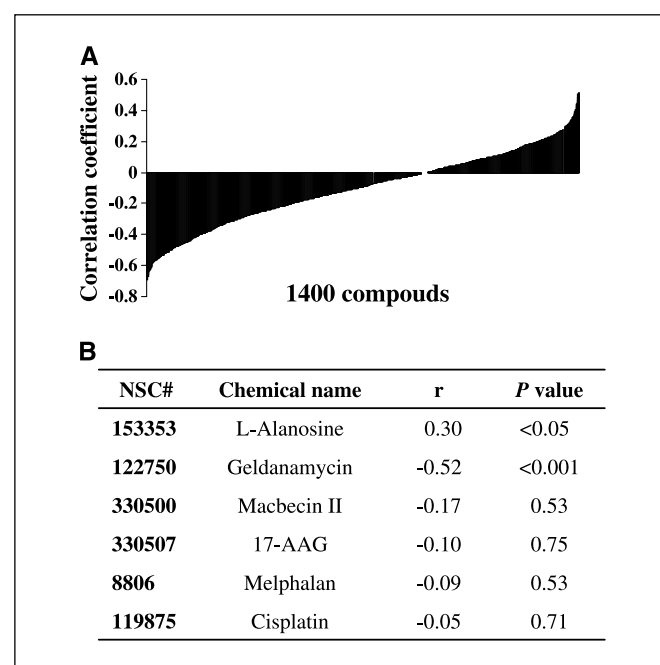
**Correlations between genes involved in transport and synthesis of glutathione and its precursor cystine, and intracellular reduced glutathione levels.** Intracellular GSH levels and the transcript levels of  $\gamma$ -GCS have been previously measured in the NCI-60 panel (16). The expression level of both *SLC7A11* and *SLC3A2* correlated with  $\gamma$ -GCS ( $r = 0.29$  and  $0.32$ , respectively;  $P < 0.05$  for both). This association may not be significant because in controlling false discovery rate at 5%, the cutoff point for significance was  $P = 0.01$ . We did not observe significant correlation between either *SLC7A11* or *SLC3A2* gene expression and GSH levels in NCI-60 reported by Tew et al. (16).  $\gamma$ -GCS expression did not correlate with levels of intracellular GSH either (16). The lack of correlation could have resulted from multiple factors determining GSH levels, including activities of SLC7A11, SLC3A2, and  $\gamma$ -GCS, as well as other as-yet poorly characterized regulatory mechanisms.

**Correlation analysis between *SLC7A11* gene expression and drug activity of 1,400 compounds.** To identify drugs that show significant correlation with the expression of *SLC7A11*, we correlated *SLC7A11* gene expression across the 60 cancer cell lines with growth inhibitory potencies of 1,400 compounds. This yielded Pearson correlation coefficients ( $r$ ) for each *SLC7A11*-drug pair. If we use  $|r| \geq 0.3$  as a heuristic criterion for potentially significant correlations, with unadjusted bootstrap  $P$  values  $< 0.05$  considered statistically significant, as previously described (10), 39 drugs showed positive correlation with *SLC7A11* (for a complete list, see <http://pharmacogenomics.osu.edu>; Fig. 2A). One of the positively correlated drug, L-alanosine ( $r = 0.30$ ,  $P$  value  $< 0.05$ ), was selected

for further study (Fig. 2B), because as an amino acid analogue it is a likely substrate of SLC7A11.

Two hundred ninety-six compounds showed negative correlation with *SLC7A11* (Fig. 2A). We hypothesized that the negative correlations could be due to the fact that SLC7A11 (or transport system  $x_c^-$ ) functions to maintain intracellular GSH levels as a chemoresistance mediator. We selected geldanamycin (NSC 122750) and its analogues for further study because they show widely diverging correlation coefficients, their mechanism of action has been well studied, and they are currently in clinical trials. Geldanamycin showed a significant negative correlation with *SLC7A11* expression ( $r = -0.52$ ,  $P < 0.001$ ; Fig. 2B), whereas the geldanamycin analogues, such as macbecin II (NSC 330500) and 17-AAG (NSC 330507), did not (Fig. 2B), indicating that the relationship between *SLC7A11* and cytotoxic drug potency is sensitive to small changes in the chemical structure of the drug. Figure 2B also highlights *SLC7A11* correlations for cisplatin and melphalan, resistance to which has been previously connected with GSH levels (9, 17). These drugs did not show significant correlations with *SLC7A11*, possibly because of the presence of other predominant mechanisms of chemoresistance in the NCI-60 that weaken the correlation for any single factor in our analysis. Therefore, we need to establish the relative importance of SLC7A11 experimentally in target cell lines.

**Effect of inhibitors of system  $x_c^-$ , glutamate, and (S)-4-carboxyphenylglycine on chemosensitivity.** To validate the positive and negative correlations between *SLC7A11* expression and potencies of L-alanosine, geldanamycin, and other anticancer drugs, we selected lung cancer cell lines A549 and HOP-62, and ovarian cancer cell line SK-OV-3, which express relatively high level of *SLC7A11*, and exposed these cells to glutamate (5 mmol/L) or



**Figure 2.** Relationship between *SLC7A11* gene expression and drug activity in the NCI-60. A, plot of sorted correlation coefficients between *SLC7A11* gene expression and cytotoxic potencies of 1,400 drugs (the complete list of drugs, correlation coefficients, and  $P$  values are available at <http://pharmacogenomics.osu.edu>). B, correlation coefficients ( $r$ ) and corresponding  $P$  values between *SLC7A11* expression and drugs tested experimentally in this study.

the more specific and potent inhibitor of system  $x_c^-$ , 4-S-CPG (18, 19), to inhibit cystine influx. In the culture medium used for these cells (RPMI 1640), the concentration of glutamate is only 0.14 mmol/L. At a concentration >2.5 mmol/L, glutamate was shown to inhibit cystine uptake competitively and decrease intracellular GSH levels (9). Shown in Table 2 and Fig. 3A, the presence of 5 mmol/L glutamate reduced the cytotoxic effects of L-alanosine to between 14% and 39% of the control in three cell lines tested, supporting the hypothesis that L-alanosine is an SLC7A11 substrate. In contrast, 5 mmol/L glutamate sensitized all three cells to geldanamycin, which showed a significant negative correlation with *SLC7A11*, with a 2.3- to 3.3-fold shift in potency, indicating that SLC7A11 functions as a resistance factor for geldanamycin (Fig. 3A). For macbecin II, lacking any significant correlation with *SLC7A11*, glutamate exerted a smaller effect (the shift in potency was 1.4-2.4-fold), indicating that the role of SLC7A11 as resistance factor may be minor but cannot be excluded for this drug. Moreover, treatment of all three cells with glutamate had no impact on potency of another geldanamycin analogue, 17-AAG, which did not correlate significantly with *SLC7A11* expression (Fig. 3A; Table 2). Glutamate generally had no effect on the potencies of melphalan and cisplatin. Whereas this result is consistent with a lack of correlation between *SLC7A11* expression and potency of these two drugs, it is also possible that glutamate may not have sufficiently depleted GSH levels, because

an inhibitor of GSH synthesis, BSO, did show sensitizing effects (see below). Therefore, we measured intracellular GSH levels for A549 and HOP-62 cells after incubation with 5 mmol/L glutamate for 24 hours, and found that glutamate treatment reduced GSH levels to  $79 \pm 3\%$  and  $75 \pm 1\%$ , respectively. The effects of inhibition of SLC7A11 and GSH synthesis in Table 2 are consistent in three cell lines tested, except for some variance in the magnitude of the change.

These results were further confirmed by exposing A549 and HOP-62 cells to 4-S-CPG to selectively inhibit system  $x_c^-$ -mediated cystine-glutamate exchange. The presence of 50  $\mu\text{mol/L}$  of 4-S-CPG reduced the sensitivity to L-alanosine, sensitized the two cells to geldanamycin and macbecin II, but had no effect on potency of 17-AAG, and showed a minor impact on potencies of melphalan and cisplatin, which is highly consistent with the results that were obtained using glutamate (Fig. 3B; Table 2). Changes in GSH levels in the two cells incubated with 100  $\mu\text{mol/L}$  4-S-CPG for 24 hours were measured. For the GSH measurement, the cells were cultured using cystine-restricted RPMI 1640 (0.05 mmol/L) as suggested by Okuno et al. (9), because cystine concentration in regular RPMI 1640 is high (0.2 mmol/L). In A549 and HOP-62 cells, treatment with 4-S-CPG reduced the GSH levels to  $28 \pm 0.2\%$  and  $41 \pm 0.3\%$ , respectively.

We treated HS578T cells, which express low levels of *SLC7A11*, with glutamate and 4-S-CPG. For L-alanosine, we did not observe

**Table 2.** Effects of inhibition of SLC7A11 by glutamate, 4-S-CPG, or siRNA, and inhibition of GSH synthesis by BSO, on drug cytotoxicity in lung cancer cell lines A549 and HOP62 and ovarian cancer cell line SK-OV-3

Cell	Treatment	IC <sub>50</sub> ( $\mu\text{mol/L}$ )					
		L-Alanosine	GA	Macbecin II	17-AAG	Melphalan	Cisplatin
A549	(-) Glu	0.86 $\pm$ 0.07	0.045 $\pm$ 0.005	7.1 $\pm$ 0.9	0.34 $\pm$ 0.01	13 $\pm$ 1.2	8.3 $\pm$ 0.3
	(+) Glu	2.2 $\pm$ 0.2 (0.39)*	0.013 $\pm$ 0.001 (3.3)*	2.9 $\pm$ 0.2 (2.4)*	0.28 $\pm$ 0.02 (1.2)	11 $\pm$ 1.2 (1.3)	7.0 $\pm$ 0.6 (1.2)
	(-) 4-S-CPG	0.28 $\pm$ 0.03	0.026 $\pm$ 0.001	0.23 $\pm$ 0.01	0.086 $\pm$ 0.01	6.1 $\pm$ 0.6	6.9 $\pm$ 0.15
	(+) 4-S-CPG	1.3 $\pm$ 0.2 (0.23)*	0.004 $\pm$ 0.000 (7.1)*	0.06 $\pm$ 0.002 (3.9)*	0.062 $\pm$ 0.002 (1.4)	5.3 $\pm$ 1.1 (1.2)	4.1 $\pm$ 0.59 (1.7) <sup>†</sup>
	(-) siRNA	0.16 $\pm$ 0.01	0.025 $\pm$ 0.001	0.42 $\pm$ 0.01	0.03 $\pm$ 0.00	11 $\pm$ 0.4	5.5 $\pm$ 0.2
	(+) siRNA	0.74 $\pm$ 0.53 (0.3) <sup>†</sup>	0.013 $\pm$ 0.000 (1.8) <sup>†</sup>	0.32 $\pm$ 0.02 (1.3) <sup>†</sup>	0.04 $\pm$ 0.00 (0.9)	9.0 $\pm$ 0.9 (1.2) <sup>†</sup>	3.7 $\pm$ 0.2 (1.5) <sup>†</sup>
	(-) BSO	0.61 $\pm$ 0.02	0.024 $\pm$ 0.00	3.0 $\pm$ 0.2	0.31 $\pm$ 0.03	19 $\pm$ 1.1	11 $\pm$ 0.1
	(+) BSO	0.65 $\pm$ 0.04 (0.94)	0.001 $\pm$ 0.000 (21)*	0.43 $\pm$ 0.05 (7.2)*	0.14 $\pm$ 0.00 (2.3)*	3.6 $\pm$ 1.6 (6.2)*	11 $\pm$ 1.5 (1.1)
HOP-62	(-) Glu	7.2 $\pm$ 1.0	0.041 $\pm$ 0.004	1.5 $\pm$ 0.2	0.11 $\pm$ 0.01	9.7 $\pm$ 0.5	4.1 $\pm$ 0.2
	(+) Glu	53 $\pm$ 11 (0.14)*	0.014 $\pm$ 0.002 (2.9)*	1.2 $\pm$ 0.4 (1.4)	0.14 $\pm$ 0.02 (0.8)	7.3 $\pm$ 0.7 (1.4)	2.4 $\pm$ 0.2 (1.7) <sup>†</sup>
	(-) 4-S-CPG	3.2 $\pm$ 0.1	0.058 $\pm$ 0.003	0.18 $\pm$ 0.006	0.099 $\pm$ 0.002	3.3 $\pm$ 0.1	3.6 $\pm$ 0.2
	(+) 4-S-CPG	13 $\pm$ 1 (0.25)*	0.008 $\pm$ 0.000 (6.9)*	0.12 $\pm$ 0.005 (1.5) <sup>†</sup>	0.098 $\pm$ 0.008 (1.0)	3.0 $\pm$ 0.1 (1.1)	1.9 $\pm$ 0.1 (1.9) <sup>†</sup>
	(-) siRNA	3.1 $\pm$ 0.4	0.10 $\pm$ 0.01	0.55 $\pm$ 0.03	0.06 $\pm$ 0.01	8.8 $\pm$ 0.2	3.2 $\pm$ 0.2
	(+) siRNA	6.3 $\pm$ 0.9 (0.5) <sup>†</sup>	0.02 $\pm$ 0.00 (4.7)*	0.26 $\pm$ 0.01 (2.1)*	0.05 $\pm$ 0.00 (1.2)	5.4 $\pm$ 0.1 (1.6) <sup>†</sup>	1.8 $\pm$ 0.2 (1.8) <sup>†</sup>
SK-OV-3	(-) Glu	53 $\pm$ 22	0.040 $\pm$ 0.003	1.9 $\pm$ 0.1	0.29 $\pm$ 0.02	15 $\pm$ 0.7	12 $\pm$ 0.3
	(+) Glu	>150 (<0.35)*	0.019 $\pm$ 0.001 (2.3)*	1.1 $\pm$ 0.1 (1.7) <sup>†</sup>	0.29 $\pm$ 0.02 (1.0)	18 $\pm$ 1.1 (0.8)	12 $\pm$ 0.4 (1.0)
	(-) BSO	69 $\pm$ 10	0.04 $\pm$ 0.00	1.4 $\pm$ 0.1	0.32 $\pm$ 0.04	9.9 $\pm$ 1.2	3.8 $\pm$ 0.7
	(+) BSO	77 $\pm$ 16 (1.1)	0.02 $\pm$ 0.00 (2.4)*	0.93 $\pm$ 0.12 (1.5) <sup>†</sup>	0.13 $\pm$ 0.01 (2.5)*	2.7 $\pm$ 0.3 (3.7)*	2.5 $\pm$ 0.6 (1.6) <sup>†</sup>

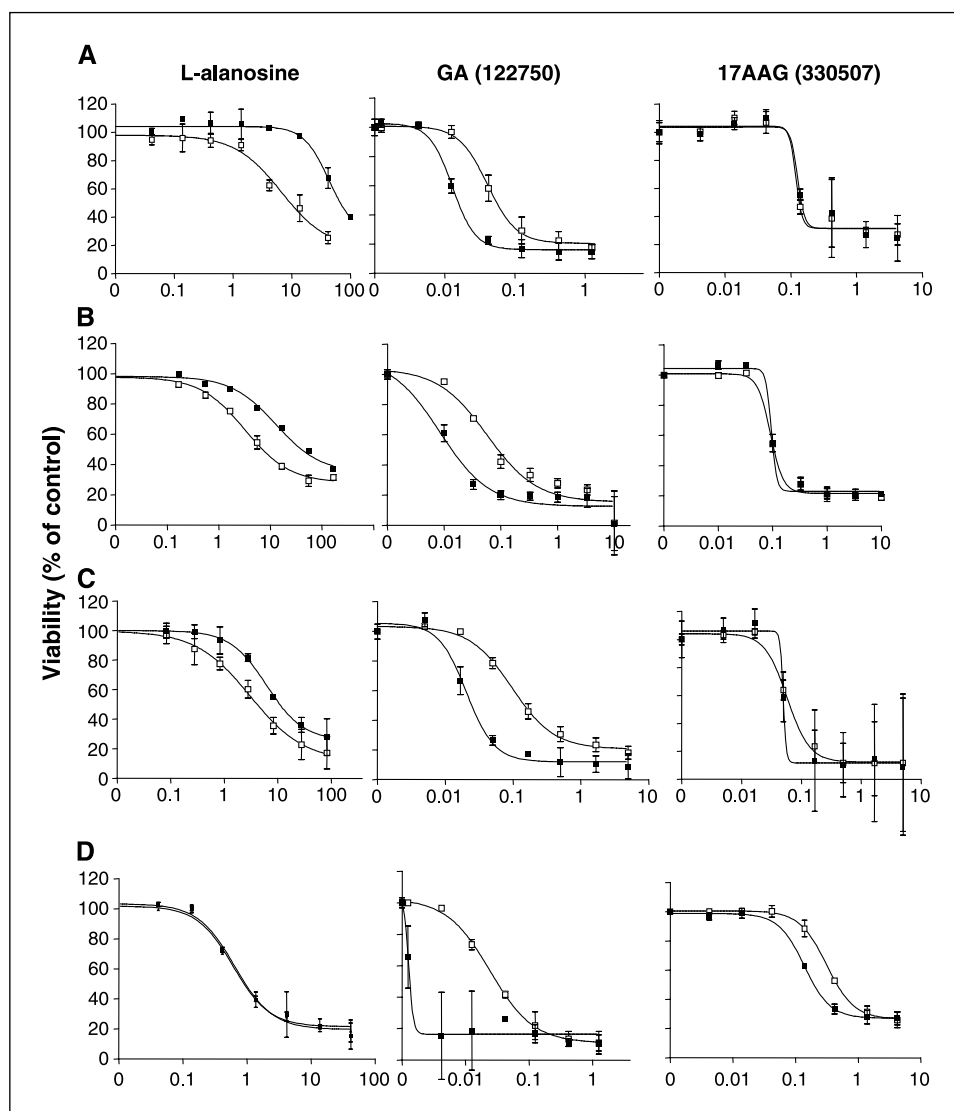
NOTE: Glutamate (5 mmol/L) or 4-S-CPG (50  $\mu\text{mol/L}$ ) was used to suppress transport activity mediated by SLC7A11 (or system  $x_c^-$ ). *SLC7A11*-specific siRNA suppressed *SLC7A11* mRNA levels by 65% and 80% in A549 and HOP-62, respectively. BSO (100  $\mu\text{mol/L}$  for SK-OV-3, 10  $\mu\text{mol/L}$  for A549) was used to suppress synthesis of GSH. IC<sub>50</sub> is the concentration that produced 50% inhibition of cell growth compared with controls. Results represent mean  $\pm$  SD of at least three experiments. Numbers in the parentheses represent fold reversal, which is the IC<sub>50</sub> for the cytotoxic drug in control cells divided by the IC<sub>50</sub> for drug in siRNA or in inhibitor-treated cells. The IC<sub>50</sub> values for glutamate in all the three cells was >5.9 mmol/L. The IC<sub>50</sub> values of 4-S-CPG for both A549 and HOP62 were >100  $\mu\text{mol/L}$ . The IC<sub>50</sub> value of BSO was  $35.6 \pm 2.5$   $\mu\text{mol/L}$  for A549 cells, and > 140  $\mu\text{mol/L}$  for SK-OV-3 cell. Abbreviations: Glu, glutamate; GA, geldanamycin.

\*Both criteria  $P < 0.05$  and fold-reversal  $\geq 2$  (or  $\leq 0.5$ ) are satisfied.

<sup>†</sup> $P < 0.05$  or fold-reversal  $\geq 2$  (or  $\leq 0.5$ ).



**Figure 3.** Validation of NCI-60 correlations between *SLC7A11* expression and activity of L-alanosine, geldanamycin, and 17-AAG. **A**, effect of inhibiting *SLC7A11* with glutamate on cytotoxic drug potency in HOP-62 cells. Cells were first treated with 5 mmol/L glutamate (■) or medium (□), and after 24 hours exposed to L-alanosine, geldanamycin, and 17-AAG for 3 days. **B**, effect of inhibiting *SLC7A11* with 4-S-CPG on drug potency in HOP-62 cells. Cells were treated with 50  $\mu$ mol/L 4-S-CPG (■) or medium (□) together with three cytotoxic drugs for 4 days. **C**, effect of siRNA transfection targeting *SLC7A11* on drug potency in cell line HOP-62. Cells were transfected with siRNA targeted against *SLC7A11* (■) or with mock-siRNA (□). After 24 hours, cells were exposed to three cytotoxic drugs for 4 days and cell growth was measured. **D**, effect of BSO on chemosensitivity of A549 cells. Cells were first treated with 10  $\mu$ mol/L BSO (■) or medium (□). After 24 hours, cells were exposed to L-alanosine, geldanamycin, and 17-AAG for 3 days, and cell growth was measured. Cell growth was measured with the SRB assay. Results are expressed as percent of control cells with no drug treatment. Points, means from three replicates; bars, SD.

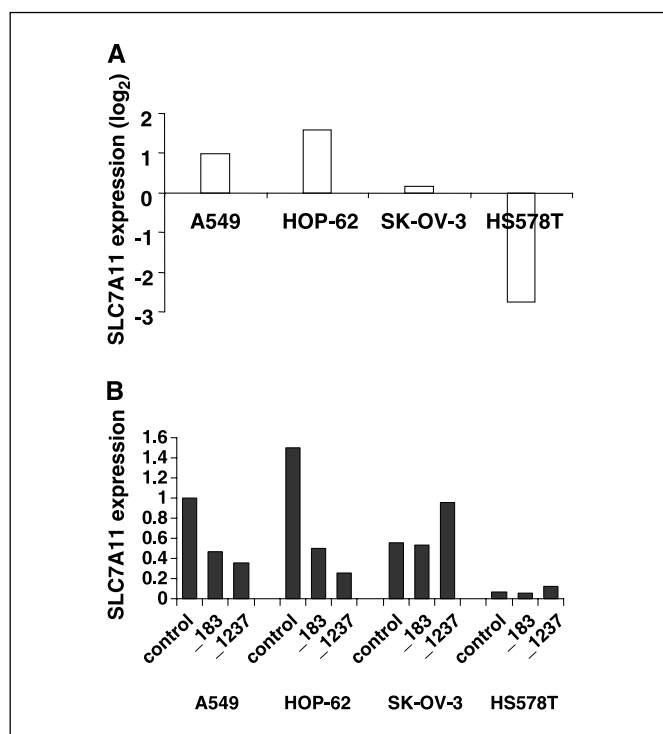


significant changes in potency, whereas for geldanamycin treatment with both inhibitors sensitized HS578T cells. Glutamate treatment enhanced geldanamycin potency 1.9-fold ( $\pm 0.1$ ,  $P < 0.05$ ), substantially less than in A549 and HOP-62 cells. Treatment of HOP-92 cells, another lung cancer cell line expressing low levels of *SLC7A11*, with 4-S-CPG did not cause significant change in potency of L-alanosine and geldanamycin.

**Effect of *SLC7A11* small interfering RNA on chemosensitivity.** To specifically down-regulate *SLC7A11* expression, we treated cancer cells with two sets of siRNA duplexes (*SLC7A11*\_183 and *SLC7A11*\_1237) designed to target nucleotides 183 to 203 and 1,237 to 1,257, respectively, of the *SLC7A11* mRNA sequence NM\_014331. We selected four cancer cell lines expressing various levels of *SLC7A11* gene, A549, HOP-62, SK-OV-3, and HS578T cells. Figure 4A shows the relative expression levels of *SLC7A11* in the four cell lines using real-time RT-PCR. The result agrees with those obtained using DNA microarrays (Fig. 1), showing the order of relative expression level of HOP-62 > A549 > SK-OV-3 > HS578T. Real-time RT-PCR showed that 48 hours after transfection, *SLC7A11*\_1237 was the more effective siRNA in down-regulating *SLC7A11* mRNA expression in A549 and HOP-62 cells, resulting in >65% and >80%

reduction in *SLC7A11* mRNA levels, respectively (Fig. 4B). However, none of the two siRNA sets affected *SLC7A11* expression in SK-OV-3 and HST578T cells, possibly because of the relatively low expression of *SLC7A11* in these cells (Fig. 4B). Therefore, we used *SLC7A*\_1237 siRNA in the A549 and HOP-62 cells for further experiments.

To assess how siRNA-directed suppression of *SLC7A11* affects drug sensitivity, we compared drug potencies of siRNA-treated cells with that of mock-treated control cells. The sensitivity to L-alanosine was reduced to between 30% and 50% of the control in siRNA-treated cells (Table 2). In contrast, sensitivity to geldanamycin was increased 1.8- to 4.7-fold in siRNA-treated cells. We also observed 1.3- to 2.1-fold increased sensitivity to macbecin II. However, for 17-AAG, siRNA had no impact on potency. Figure 3C illustrates the effects of siRNA on drug potencies. For cisplatin and melphalan, we observed a small increase of sensitivity after treatment with siRNA, but changes were <2-fold (Table 2). The results obtained using siRNA paralleled those using glutamate and 4-S-CPG. We treated the HS578T cells by siRNA. For both L-alanosine and geldanamycin, we did not observe significant change in potency.



**Figure 4.** RT-PCR analysis of *SLC7A11* in selected cells. **A**, relative *SLC7A11* expression in four cancer cell lines determined by real-time RT-PCR. Expression level is represented by the log ratio of expression level in the test sample over mean expression in all the four samples. **B**, effect of siRNA targeting *SLC7A11* in four cancer cell lines. The cells were transfected with siRNA duplexes *SLC7A11*\_183 and *SLC7A11*\_1237. After 48 hours, cell lysates were prepared from siRNA- or mock-treated control cells. *SLC7A11* expression level in the control of A549 cells was set as 1. Expression levels were normalized to  $\beta$ -actin levels.

#### Effect of buthionine sulfoximine on drug chemosensitivity.

To compare the effects of suppressing *SLC7A11* transport activity with those of inhibiting GSH synthesis via a different path, we treated the cells with BSO, a potent and specific inhibitor of  $\gamma$ -GCS, the rate-limiting enzyme in the synthesis of GSH (20). We selected A549 and SK-OV-3 cells for the experiments using BSO concentration of 10 and 100  $\mu$ mol/L, respectively, which did not affect cell viability but enhanced the sensitivity of the two cells to melphalan, a drug known to be sensitized by BSO treatment (21).

BSO treatment had no effect on potency of L-alanosine (Table 2), indicating that the sensitivity to L-alanosine is unrelated to GSH synthesis. This is consistent with the hypothesis that *SLC7A11* is a chemosensitivity factor by serving as a carrier for cellular uptake of the drug. In contrast, BSO treatment sensitized A549 and SK-OV-3 cells to all three geldanamycin analogues, although for 17-AAG the shift in potency was small (Table 2). Figure 3D compares the effects of BSO on concentration-response curves of these compounds. For 17-AAG, BSO treatment increased sensitivity, which did not occur with glutamate, 4-S-CPG, and siRNA (Fig. 3A-C). Both BSO and siRNA sensitized melphalan and cisplatin to a small extent (Table 2). In A549 cells, the GSH level was reduced to  $43 \pm 2\%$  of the control in the presence of 10  $\mu$ mol/L of BSO. Therefore, GSH-mediated detoxification seems to play various roles with all the drugs tested in this study, except for L-alanosine. The geldanamycin analogues macbecin II and 17-AAG and cisplatin and melphalan are likely less influenced by GSH-mediated chemoresistance than geldanamycin in the cells tested.

## Discussion

We used oligonucleotide DNA microarrays to analyze gene expression of the light chain of transporter system  $x_c^-$  encoded by *SLC7A11* and related transporter proteins in the 60 human cancer cell lines used by the NCI for drug screening (NCI-60). In NCI-60, *SLC7A11* shows a similar gene expression pattern to that of *SLC3A2*, which encodes the heavy chain of transporter system  $x_c^-$ . Expression of *SLC3A2* also correlates with *SLC7A5*, which encodes the light chain of another amino acid transporter, system L. Indeed, *SLC3A2* is the large subunit of several heterodimeric transporter systems,  $x_c^-$ , L, and  $y^+L$  (14). Coexpression of *SLC3A2* is required for surface expression of its counterpart light chains. Therefore, gene-to-gene correlations in the NCI-60 panel reveals known functional interactions between transporters. We also found a significant correlation between *SLC3A2* with *TIA-2*. One possibility is that *TIA-2* represents a novel light chain of *SLC3A2*. Alternatively, the *TIA-2* transporter is incidentally coexpressed with *SLC3A2*, which is highly expressed in rapidly proliferating cells (15).

The next step in our analysis involved correlation between *SLC7A11* expression and cytotoxic potency of 1,400 compounds. Among 296 negative correlated and 39 positively correlated compounds, we selected L-alanosine (positive correlation) and geldanamycin (NSC 122750; negative correlation) for further study. We hypothesized that L-alanosine is transported by *SLC7A11* into the cell, whereas cystine is transported by the same transporter, which maintains protective GSH levels as a factor in geldanamycin resistance. As no significant correlation was found for the geldanamycin analogues macbecin II (NSC 330500) and 17-AAG (NSC 330507), negative *SLC7A11*-drug correlations seem to be sensitive to small perturbations in chemical structure. The benzoquinoid ansamycin, geldanamycin, and its derivatives target heat shock protein 90 (22). 17-AAG is the first heat shock protein 90 inhibitor to enter clinical trial (22–24). 17-AAG was found to have similar cellular effects but lower hepatotoxicity than the parent compound geldanamycin. Our results here suggest that 17-AAG may bypass a chemoresistance mechanism mediated by glutathione in the cells. This could be due to the avidity of the quinone ring of geldanamycin to undergo conjugation with glutathione, compared with 17-AAG. Detailed structure-activity studies for geldanamycin analogues are needed for the identification of the mechanism of differential interaction of these compounds with *SLC7A11* and GSH.

To test the role of *SLC7A11* in chemosensitivity and resistance, we determined changes in drug activities upon treatment with glutamate and 4-S-CPG (to inhibit transport system  $x_c^-$ ) or siRNA targeting *SLC7A11*. In A549, HOP-62, and SK-OV-3 cells, these treatments decreased sensitivity to L-alanosine, but increased geldanamycin potency. Resistance of tumor cells to a variety of anticancer drugs is often associated with increased GSH levels. The function of *SLC7A11* in chemoresistance seems to be mediated by maintaining intracellular GSH levels, as treatment with glutamate, 4-S-CPG, or BSO reduced the intracellular GSH level. Using regular RPMI 1640, the change in GSH levels after 4-S-CPG treatment was not detectable. Therefore, we used cystine-restricted RPMI 1640 as suggested by Okuno et al. (9). Both glutamate or BSO treatment, however, with regular RPMI 1640, reduced cellular GSH levels to a similar extent when compared with cystine-restricted RPMI 1640. Whereas there seem to be some differences in total GSH depletion, the two inhibitors produced comparable effects on drug potency of the five compounds (Table 2). It is possible that the approach taken to measure the basal GSH level in cancer cells does not accurately reflect the rate of GSH synthesis under depletion stress, such as in

the presence of cytotoxic compounds. Our study is consistent with the previous finding of the role that SLC7A11 plays in cystine transport and maintaining intracellular GSH level, shown to mediate resistance to cisplatin (9). However, in our study, inhibitors of system  $x_c^-$  failed to sensitize the cells to cisplatin and melphalan, whereas using siRNA and BSO increased the potency of both drugs, if only to a smaller extent than geldanamycin. These results do not contradict those of Okuno et al. (9), as the potency of cisplatin or melphalan may have been less dependent than geldanamycin upon GSH in the cells studied here (25). Okuno et al. (9) used a cisplatin-resistant variant of an ovarian cell line highly expressing *SLC7A11*. The cell lines used in our study (A549, HOP-62, and SK-OV-3) may have a lower level of *SLC7A11* expression than the cisplatin-resistant variant. Furthermore, the mechanism of resistance to geldanamycin may additionally involve a glutathione conjugate export pump. MK-571, a selective inhibitor of MRP1, was able to sensitize HL-60/ADR cell, which overexpresses MRP1, to geldanamycin, but not cisplatin.<sup>3</sup> This suggests that geldanamycin may form conjugates with GSH, and the geldanamycin-GSH conjugates may be the substrates of MRP1, which is also highly expressed in the cell lines we tested. However, cisplatin and melphalan or their GSH conjugate do not seem to be substrates of MRP1.

Our analysis showed that 296 of 1,400 compounds having negative correlation with *SLC7A11*. In future studies, we plan to characterize the common structural features that determine relationship with *SLC7A11* or GSH. Expression of  $\gamma$ -GCS, the rate-limiting enzyme of GSH synthesis, was found to be inversely correlated with potency of alkylating drugs (16). In the NCI-60, the expression patterns of *SLC7A11* and *SLC3A2* correlated to a degree with that of  $\gamma$ -GCS ( $r = 0.29$  and  $0.32$ , respectively), suggesting that these genes involved in GSH synthesis may be coregulated. Moreover, *SLC3A2* expression also correlated with potency of some drug (data not shown). Because the expression of *SLC7A11* is more directly correlated with transport activity of system  $x_c^-$  than *SLC3A2* (2), which serves in multiple transport systems, this study has focused on *SLC7A11* only.

The relationship between *SLC7A11* expression and potency of L-alanosine is unrelated to GSH synthesis because BSO was unable to change sensitivity to L-alanosine. This result supports the view that SLC7A11 (system  $x_c^-$ ) is responsible for the recognition and uptake of L-alanosine, and therefore contributes to the sensitivity to this drug.  $x_c^-$  is an anionic amino acid transport system for cystine and glutamate. L-alanosine is an L- $\alpha$ -amino acid analogue derived from *Streptomyces alanosinicus* (26). Its structure is closely related to cystine by replacement of the sulfhydryl group with 3-hydroxynitrosamine (27), making it a possible substrate for system  $x_c^-$ . Inhibiting adenosylsuccinate synthetase, it interferes with the *de novo* synthesis of adenosine in both malignant and normal cells

(28). In cancer cells deficient in methyladenosine phosphorylase (required in the purine salvage pathway), L-alanosine is a highly efficient cytotoxic agent. Several amino acid transporters have already been implicated as carriers of amino acid-related drugs, playing a critical role in drug targeting and sensitivity (10). For example, *SLC7A5* (LAT1, L-type amino acid transporter 1) has been shown to mediate uptake of aromatic amino acids (thyroxine) and derivatives, including L-dopa and melphalan (29). In contrast, SLC7A11 seems to have a rather narrow substrate selectivity compared with SLC7A5. Further substrate-transport studies are needed to clarify the substrate specificity of SLC7A11.

Our study shows the utility of gene-to-drug correlations across multiple cell lines, followed by experimental validation using siRNA or transport inhibitors. The identified transporter-drug correlations are likely to reveal novel chemosensitivity and chemoresistance factors. Our findings may have clinical implications because of the relatively high expression of *SLC7A11* and high activity of transporter system  $x_c^-$  in human cancer cells and its function in regulating GSH level. Although we have selected several drugs for detailed study here, there are a large number of compounds showing strong correlation with *SLC7A11* expression. Any causative relationship for a larger set of compounds will require experimental validation, using the methodology outlined here. The predictive value of the correlations can be further improved by selecting subsets of cells with optimal distribution of *SLC7A11* expression. Hence, *SLC7A11* could serve as a biomarker for predicting the efficacy of chemotherapeutic agents and selecting optimal drug therapies. For example, L-alanosine or other amino acid-related drugs could be more effective than agents subject to GSH-mediated chemoresistance, against cancers expressing high level of *SLC7A11*. The finding that the potency of 17-AAG is only minimally affected by inhibition of GSH synthesis suggests that 17-AAG avoids GSH detoxification because of structural modifications from geldanamycin. Hence, cell-based assays can readily identify drug sensitivity to glutathione, providing one criterion for selecting a drug candidate over another. An alternative strategy is to inhibit SLC7A11 activity, or GSH synthesis with selective inhibitors, to overcome the resistance to any drugs associated with GSH detoxification. Overall, the data presented here provide an approach for mining available information on gene expression and drug potency to studying the mechanisms of chemosensitivity and chemoresistance, and providing insight for selecting optimal drug candidates.

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<sup>3</sup> Y. Huang and W. Sadée, unpublished results.

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