Cystine-glutamate transporter SLC7A11 mediates resistance to geldanamycin but not to 17-AAG

Ruqing Liu, Paul E. Blower, Anh-Nhan Pham, Jialong Fang, Zunyan Dai, Carolyn Wise, Bridgette Green, Candee H. Teitel, Baitang Ning, Wenhua Ling, Beverly D. Lyn-Cook, Fred F. Kadlubar, Wolfgang Sadée and Ying Huang

Division of Pharmacogenomics and Molecular Epidemiology (R.L., C.W., B.G., C.H.T., B.N., B.D.L, Y.H.), and Division of Biochemical Toxicology (J.F.), National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas; School of Public Health, Sun Yat-sen University, Guangzhou, China (R.L., W.L.); Program of Pharmacogenomics, Department of Pharmacology, Comprehensive Cancer Center, College of Medicine and Public Health, The Ohio State University, Columbus, Ohio (P.E.B., Z.D., W.S.); Department of Epidemiology, University of Arkansas for Medical Sciences, Little Rock, Arkansas (F.F.K.); Department of Pharmaceutical Sciences, College of Pharmacy, Western University of Health Sciences, Pomona, California (A.P., Y.H.)

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Running Title: SLC7A11 and geldanamycin analogues

Correspondence and requests for reprints: Ying Huang, Department of Pharmaceutical Sciences, College of Pharmacy, Western University of Health Sciences, Pomona, CA 91766. Phone: (909) 469-5220, fax: (909) 469-5600, e-mail: yhuang@westernu.edu

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Abbreviations: NCI, the National Cancer Institute; DTP, Developmental Therapeutics Program; MOAs, mechanisms of action; PCA, principal component analysis; siRNA, small interfering RNA; SRB, sulforhodamine B; GA, geldanamycin; GSH, glutathione; 4-S-CPG, (S)-4-carboxyphenylglycine; Hsp90, heat shock protein 90; ROS, reactive oxygen species; NAC, N-acetylcysteine; AsA, ascorbic acid.

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Abstract

The cystine-glutamate transporter SLC7A11 has been implicated in chemoresistance, by supplying cystine to the cell for glutathione maintenance. In the NCI-60 cell panel, SLC7A11 expression shows negative correlation with growth inhibitory potency of geldanamycin, but not with its analogue 17-AAG, which differs in the C-17 substituent by replacing the methoxy moiety of geldanamycin with an amino group. Structure and potency analysis classified 18 geldanamycin analogues into two subgroups, "17-O/H" (C-17 methoxy or unsubstituted) and "17-N" (C-17 amino), showing distinct SLC7A11-correlation. We used three 17-O/H analogues and four 17-N analogues to test the role of the 17-substituents in susceptibility to SLC7A11-mediated resistance. In A549 cells, which are resistant to geldanamycin and strongly express SLC7A11, inhibition of SLC7A11 by (S)-4-carboxyphenylglycine or siRNA increased sensitivity to 17-O/H, but had no effect on 17-N analogues. Ectopic expression of SLC7A11 in HepG2 cells, which are sensitive to geldanamycin and express low SLC7A11, confers resistance to geldanamycin, but not to 17-AAG. Antioxidant N-acetylcysteine, a precursor for glutathione synthesis, completely suppressed cytotoxic effects of 17-O/H, but had no effect on 17-N analogues, while the prooxidant ascorbic acid had opposite effects. Compared to 17-AAG, geldanamycin led to significantly more intracellular reactive oxygen species (ROS) production, which was quenched by addition of N-acetylcysteine. We conclude that SLC7A11 confers resistance selectively to 17-O/H (*e.g.*, geldanamycin) but not to 17-N (*e.g.*, 17-AAG) analogues partly due to differential dependence on ROS for cytotoxicity. Distinct mechanisms could significantly affect antitumor response and organ toxicity of these compounds *in vivo*.

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Introduction

Chemoresistance is a major cause of treatment failure in cancer patients. Multiple mechanisms may contribute to resistance to chemotherapy, including alterations of drug transport and metabolism, drug target, DNA repair, cell proliferation and death (Huang and Sadee, 2003). Systematic approach in screening anticancer drugs is needed for identifying drug candidates among a series of congeners to avoid chemoresistance. Previously we have developed a method to study the potential pharmacological interactions between transporter proteins and anticancer drugs (Huang et al., 2004). We applied a custom designed microarray to analyze gene expression of a majority of human membrane transporters and ion channels (namely, the transportome) in the NCI-60 cell panel, which is used by the Developmental Therapeutics Program (DTP) of the National Cancer Institute (NCI) for anticancer drug screening. Correlating gene expression levels with the growth inhibitory potencies of anticancer drugs in the NCI-60 cells, we have identified known drug–transporter interactions and suggested novel ones (Dai et al., 2006; Huang et al., 2005a; Huang et al., 2004). For a given gene–drug pair, high expression of a given gene in drug-sensitive cell lines yields positive correlation coefficients whereas high expression in resistant cells gives negative correlation coefficients. Expanding this methodology, we also developed data mining techniques for identifying structural features of compounds showing activity patterns highly correlated with specific mRNA expression patterns (Blower et al., 2002). The approach was used to discover novel associations between compound classes and drug transporters (Dai et al., 2007; Huang et al., 2005b).

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SLC7A11 (or xCT), together with SLC3A2 (or 4F2hc), encodes the heterodimeric amino acid transport system x_c^-, which mediates entry of cystine into the cell coupling to efflux of glutamate (Sato et al., 2000). SLC7A11, the light chain of system x_c^-, is thought to mediate the transport activity, while SLC3A2, the heavy chain, leads to the surface expression of the system x_c^- (Verrey et al., 2004). Once inside the cell, cystine is rapidly reduced to cysteine, the rate limiting amino acid for glutathione (GSH) biosynthesis (Gatti and Zunino, 2005). In tumor cells the amino acid transport system x_c^- plays a crucial role in regulating intracellular GSH levels (Okuno et al., 2003). GSH has been broadly implicated in resistance to chemotherapy (Gatti and Zunino, 2005). Driven by a reactive sulfhydryl group, GSH conjugation is a major detoxification pathway (Yang et al., 2006). Many xenobiotics are susceptible to GSH conjugation and detoxification. In addition, GSH protects against cellular damage caused by free radicals and other reactive oxygen species (ROS) such as superoxides and H_2O_2 (Wu et al., 2004). In a previous study (Huang et al., 2005a), SLC7A11 expression in the NCI-60 negatively correlated with activity of multiple anticancer drugs. The number of significant SLC7A11-drug correlations was much greater than those of other genes tested, suggesting that SLC7A11 plays a critical role in chemoresistance. The expression levels of SLC7A11 showed strongest correlation with the activity of geldanamycin (GA, NSC 122750), whereas no correlation was observed with the GA analogue 17-(allylamino)-17-demethoxygeldanamycin (17-AAG, NSC 330507) (Huang et al., 2005a). This indicates that structural changes can abolish SLC7A11-mediated chemoresistance as the only difference between 17-AAG and GA is at the C-17 position.

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GA is a benzoquinone ansamycin antibiotic produced by yeast (Smith et al., 2005; Whitesell et al., 1994). Its target is heat shock protein 90 (Hsp90), an essential protein that maintains stability of "client proteins" implicated in tumor growth and survival, including protein kinases, transcription factors and mutated oncogenic proteins (Workman, 2004). Treatment of tumor cells with GA results in proteasome-mediated degradation of Hsp90 client proteins. In addition, cytotoxicity of GA on tumors has also been attributed to reactive oxygen species (ROS) generation (Dikalov et al., 2002; Lai et al., 2003). Despite potent cytotoxicity on tumors, lethal hepatotoxicity in animals limits the promise of GA as a drug candidate. Synthetic GA analogues have been developed by replacing the C-17 side chains of GA, a potential mediator of liver toxicity (Tian et al., 2004). Two C-17-substituted analogues, 17-AAG and 17-DMAG (17-(2-dimethylaminoethyl)amino-17-demethoxygeldanamycin, NSC 707545) which have reduced liver toxicity but retain cytotoxic potency against tumor cells, have shown encouraging results in clinical trials (Tian et al., 2004).

In the present study, we studied 18 GA analogues to understand the 17-substituent effects of these compounds on their differential susceptibility to SLC7A11-mediated resistance as well as mechanisms of antitumor activity. According to chemical structures and potency across the NCI-60, the 18 compounds can be classified into two distinct groups, "17-O/H" and "17-N", which yield varying correlations with SLC7A11 expression levels. We further showed that SLC7A11 mediates chemoresistance to 17-O/H analogues (*e.g.*, GA), while 17-N analogues (*e.g.*, 17-AAG) bypass this resistance mechanism. This study

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not only improves the understanding of mechanisms for chemoresistance, but also demonstrates the power of our approach to revealing novel drug-transporter relationship and defining structural features associated with SLC7A11-mediated chemoresistance.

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Materials and Methods

Compound potency databases for NCI-60

The September 2003 release of the National Cancer Institute (NCI) antitumor drug screening database was obtained from the NCI's DTP website (Human Tumor Cell Line Screen: http://dtp.nci.nih.gov/docs/cancer/cancer_data.html), containing non-confidential screening results and chemical structural data from the DTP. For each compound and cell line, growth inhibition after 48 h of drug treatment had been assessed from changes in total cellular protein using a sulforhodamine B (SRB) assay (Weinstein et al., 1997). The data provide GI_{50} values for each compound-cell line pair (GI_{50}, the concentration causing 50% growth inhibition). We clustered the 18 compounds by agglomerative nesting using the complete linkage method (Blower et al., 2004; Everitt, 1993). The compound distance matrix - a measure of similarities or differences in chemical structures - was calculated using the Tanimoto coefficient based on the Leadscope™ feature set (Leadscope, Columbus, OH) (Blower et al., 2004).

Gene expression databases of NCI-60

A customized oligonucleotide microarray containing probes targeting 461 transporter and 151 channel genes, as well as 100 probes for unrelated genes was used to measure transporter gene expression in NCI-60. Array hybridization, data analysis and database were described in previous study (Huang et al., 2004). A second gene expression database, the Novartis microarray dataset, was also employed for comparison. This data set contains the average of triplicate expression measurements for 59 NCI cell lines based

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on 12,626 oligonucleotide probes from Affymetrix U95Av2 arrays, available at NCI's Developmental Therapeutics Program (DTP) website (http://dtp.nci.nih.gov/mtargets/download.html).

Correlation of gene expression profiles with compound potency patterns

Pearson correlation coefficients were calculated to correlate gene expression profiles with patterns of compound potency across the NCI-60 as described previously (Huang et al., 2005b). Correlation analysis was performed for expression profiles measured with microarrays against the potency of GA analogues. Unadjusted *p* values were obtained using Efron's bootstrap resampling method (Efron and Tibshirani, 1993), with 10,000 bootstrap samples for each gene-drug comparison.

Principal component analysis (PCA)

PCA was conducted by ArrayTrack version 3.2.0 (NCTR/FDA, AR) (Tong et al., 2003) (www.fda.gov/nctr/science/centers/toxicoinformatics/ArrayTrack/) to explore the relationships of 18 GA analogues based on the potency data (GI50) on NCI-60. PCA maps multidimensional data into a low dimensional graph to visually inspect the drug-drug relationships.

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Chemicals

GA, 17-AAG, 17-DMAG, 17-AEP-GA and 17-DMAP-GA were purchased from InvivoGen (San Diego, California). (S)-4-carboxyphenylglycine (4-S-CPG) were purchased from Tocris (Ballwin, MO). NSC 658514 and 661581 were obtained from NCI DTP. Others were purchased from Sigma Chemical Co. (St. Louis, Missouri).

Cell culture

HepG2 and 293T cells were purchased from the American Type Culture Collection (Manassas, VA) and were cultured in William's Medium E and DMEM, respectively, supplemented with 10% fetal calf serum (FBS), 100 U/ml penicillin G and 100 μg/ml streptomycin. Other cell lines were obtained from Division of Cancer Treatment and Diagnosis at NCI and cultured in RPMI 1640 medium containing 5 mM L-glutamine, supplemented with 10% FBS, 100 U/ml penicillin G and 100 μg/ml streptomycin. Cells were grown in tissue culture flasks at 37°C in a 5% CO_2 atmosphere.

Cytotoxicity assay

Growth inhibitory potency was tested using a proliferation assay with sulforhodamine B (SRB), a protein-binding reagent (Sigma), as described before (Huang et al., 2004). 3000-4000 cells/well were seeded in 96-well plates and incubated for 24 h. Before exposure to test compounds, cells were treated individually with 4-S-CPG, AsA, NAC or medium (as control) for 10 min. Test compounds were added in a dilution series in three replicated wells for 4 days. 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, California). Each experiment was performed independently at least twice. Student's *t* test was used to determine the degree of significance.

siRNA-mediated downregulation of SLC7A11

siRNA downregulation of SLC7A11 expression was described before (Huang et al., 2005a). In brief, siRNA duplexes for SLC7A11 were synthesized using the Silencer siRNA construction kit (Ambion, Austin, TX). The target sequences were 5'-AAATGCCCAGATATGCATCGT-3' which target nt 1237-1257 of the SLC7A11 mRNA sequence NM_014331. Chemically synthesized mock siRNA (fluorescein-labeled, non-silencing) was purchased from QIAGEN. Transfection was performed with HiPerFect Transfection Reagent (QIAGEN) using 5 nM siRNA. To measure cytotoxic drug potency, 24 h after transfection cells grown in 60 mm plates were subcultured into 96-well plates. After 24 h of incubation, the cells were further incubated with the test compounds for 4 days before cytotoxicity assay.

Cloning and expression of SLC7A11 in HepG2 cells

cDNAs encoding full-length human SLC7A11 were obtained from Open Biosystems (Huntsville, AL) and subcloned into the pBabe-Puro expression vector (Addgene, Cambridge, MA) and packaged into retrovirus by transient transfection of 293T cells as described (Venkateswaran et al., 2000). HepG2 cells were infected at 50% confluence with the recombinant retrovirus. Stable cell lines were selected with puromycin (2 μg/ml).

Real-time quantitative reverse transcription-PCR

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The effects of siRNA down-regulation and ectopic expression were detected by real-time RT-PCR and described previously (Huang et al., 2005a). The primers for SLC7A11 were 5'-TGCTGGGCTGATTTATCTTCG-3' (forward) and 5'-GAAAGGGCAACCATGAAGAGG-3' (reverse). The primers for ß-actin were 5'-CCTGGCACCCAGCACAAT-3' and 5'-GCCGATCCACACGGAGTACT-3'. Relative gene expression was measured with the ABI 7000 Sequence Detection system (Applied Biosystems). All amplification controls and samples were performed in triplicates and repeated at least twice.

Western Blot

Cells were lysed in lysis buffer [20 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 10% glycerol, 1% NP40, and 0.42% NaF] containing 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na_3VO_4 2 µg/mL aprotinin, and 5 µg/mL leupetin. Proteins were separated by gel electrophoresis on 7.5% polyacrylamide gels transferred to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), and detected by immunoblotting using an enhanced chemiluminescence system (Alpha Innotech, San Leandro, CA). SLC7A11 antibody (1:400 dilution) was purchased from Novus (Littleton, CO). The data was analyzed by scanning densitometry and quantified using Chemiimager 4400 software (Alpha Innotech). SLC7A11 expression levels were quantified in comparison with the ß-actin bands.

ROS Determination

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Dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes, Eugene, OR), a nonpolar compound, can be converted into its nonfluorescent polar derivative H_2DCF by cellular esterases after incorporation. H_2DCF is membrane-impermeable and rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein in the presence of intracellular ROS (Lai et al., 2003). Cells were treated with 1.25 μM GA or 17-AAG for 16 h followed by incubation with 5 μM H_2DCFDA in medium for 20 min at 37 °C in the dark. The cells were trypsinized, washed and resuspended in PBS. Cellular fluorescence was measured using flow cytometry performed on a fluorescence-activated cell sorter (FACScan, Becton-Dickinson, San Jose, CA) (excitation at 488 nm and emission at wavelengths 530±15 nm) and analyzed by using CellQuest software. To study the effects of NAC on ROS production induced by GA and 17-AAG, cells were treated in combination with NAC (1 mM) and GA analogues. To determine intracellular superoxide production, we used dihydroethidium (Molecular Probes), a cell-permeant dye that is oxidized by superoxide to yield fluorescent ethidium bromide which intercalates with nuclear DNA (Dikalov et al., 2002). Cells were rinsed with PBS and incubated with 10 μM dihydroethidium in PBS for 30 min at 37 °C in the dark before FACS analysis.

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Results

Clustering of eighteen GA analogues based on structure and activity

Searching through ~43,000 compounds in the September 2003 release of the NCI antitumor drug screening database, we identified 18 GA analogues according to a common chemical substructure, the benzoquinone ansamycin moiety (Table 1). We performed cluster analysis for the 18 GA analogues based on similarities or differences in chemical structures (see Materials and Methods). They were grouped into two clusters which were characterized by C-17 substituents (Table 1). The first cluster has 10 compounds containing a methoxy group or unsubstituted at C-17, named as the "17-O/H" cluster (*e.g.*, GA, NSC 658514 and 661581); while the second cluster has 8 compounds containing various amino groups at C-17, the "17-N" cluster (*e.g.*, 17-AAG and 17-DMAG).

Patterns of growth inhibitory activity of compounds against the NCI-60 panel have been shown to be associated with mechanisms of action (MOAs), modes of resistance, and molecular structures (Keskin et al., 2000). We therefore used principal component analysis (PCA) to classify the 18 GA analogues based on their GI_{50} values on the NCI-60. This also resulted in a separation of the GA analogues by their C-17 substituents, with 17-O/H clearly separated from 17-N analogues (Figure 1). While all GA analogues similarly inhibit Hsp90, the different activity patterns for the subgroups 17-O/H and 17-N may result from the differential interactions of compounds with one or more molecular

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targets. This suggests that 17-O/H and 17-N analogues may have different MOAs and mode of resistance.

Five compounds were available for experimental investigation, including three 17-O/H analogues, *i.e.*, the parent compound GA (NSC 122750), NSC 658514 and 661581, and the two 17-N analogues 17-AAG (NSC 330507) and 17-DMAG (NSC 707545). 17-AAG carries an alkylamino group in place of the methoxy moiety at C-17 (R1) of GA (Table 1). Compound 17-DMAG also differs from GA in the side chain at C-17, carrying a diamine with a two-carbon spacer between the nitrogen atoms. NSC 658514 and 661581 differ from GA in the side chain of R6, but retain the methoxy moiety at C-17. Also included in our experimental investigations are two novel derivatives of 17-AAG, 17-(2-(pyrrolidin-1-yl)ethyl)amino-17-demethoxygeldanamycin (17-AEP-GA) and 17-(dimethylaminopropylamino)-17-demethoxygeldanamycin (17-DMAP-GA) (not shown in Table 1); growth inhibitory activity data for these two compounds are not available in the NCI-60 database. 17-AEP-GA and 17-DMAP-GA, similar to 17-AAG and 17-DMAG, have been synthesized by replacing the C-17 methoxy moiety with alkylamino groups, therefore belonging to the 17-N group (Tian et al., 2004).

Correlation of SLC7A11 gene expression with growth inhibitory potency of GA analogues

The mRNA expression was previously measured in the NCI-60 cell panel with a customized microarray containing oligonucleotide probes targeting the majority of transporter genes presently known to be relevant to drug transport (Huang et al., 2004).

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To identify genes potentially involved in sensitivity and resistance to GA analogues, we firstly performed correlation analysis between the microarray gene expression profiles and growth inhibitory activity of six GA analogues across the NCI-60. SLC7A11 and SLC3A2 were two of the five transporter genes showing significant GA correlations identified from the systematic screening (Huang et al., 2007). We next performed correlation analysis between gene expression of SLC7A11 and growth inhibitory potency of 18 GAs across the NCI-60. This yielded Pearson correlation coefficients (*r*) for each gene-drug pair. The statistical significance of the correlation for each gene-drug pair was assessed by computing unadjusted bootstrap *p* values (Efron and Tibshirani, 1993).

All 18 analogues showed negative SLC7A11 correlations (Figure 2A). Significant negative correlations indicative of chemoresistance occur between SLC7A11 gene expression and 8 out of 10 17-O/H compounds. However, only 1/8 17-N analogues showed significant negative correlations with SLC7A11. The drug correlation profiles were similar for SLC3A2 which showed significant negative correlations with 6/10 17-O/H analogues, but not with 17-N analogues (data not shown). Figure 2B shows a representative relationship between SLC7A11 level and growth inhibitory potency for GA and 17-AAG. Several cell lines, A549, HOP-62, H322M and SK-OV-3, which have relatively high expression of SLC7A11 and are resistant to GA, are highlighted. In contrast, these cell lines are not resistant to 17-AAG. The mean SLC7A11 correlation was -0.40 for the 17-O/H cluster, and -0.10 for the 17-N cluster. A Student *t*-test supported the alternative hypothesis, $\text{mean}_{17\text{-O/H}} < \text{mean}_{17\text{-N}}$ with *p* value of 0.0012. If there is a connection between drug resistance and SLC7A11 expression level, we would

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expect to detect a difference in compound potency between cell lines over- and under-expressing this gene. To corroborate GA-SLC7A11 correlations, we calculated the difference in cytotoxic potency between two lung cancer cell lines HOP-62 and HOP-92. HOP-62 expresses relatively high levels of SLC7A11, while HOP-92 does not (Huang et al., 2005a). For both compound clusters, we calculated the mean difference in potency between the two cell lines. On average, the 17-O/H analogues are approximately 6.6 times less potent against HOP-62 than HOP-92, while the 17-N analogues are approximately 7.2 times more potent against HOP-62 than HOP-92. A student *t*-test supported the alternative hypothesis ($p < 0.0001$). Therefore, the structural and potency classification (shown in Figure 1 and Table 1) may relate to SLC7A11 correlations (shown in Figure 2) and associate with the pharmacological features of these compounds due to the importance of the C-17 position. As small differences in chemical structure may determine whether a compound is subject to SLC7A11 and GSH mediated resistance (Dai et al., 2007), we subsequently studied representative compounds in the two clusters regarding to the interactions with SLC7A11.

Evidence that SLC7A11 confers resistance to 17-O/H but not to 17-N analogues

To validate various correlations between SLC7A11 and GA analogues, we used lung cancer A549 cells, which express relatively high levels of SLC7A11 and are relatively more resistant to GA (Figure 2B), and compared the drug sensitivity with and without inhibition of SLC7A11 activity. The cells were first exposed to the specific inhibitor of system x_c^-, (S)-4-carboxyphenylglycine (4-S-CPG) (Patel et al., 2004) to inhibit cystine influx and lower the intracellular GSH levels (Huang et al., 2005a). Shown in Figure 3A

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and Table 2, the presence of 50 uM 4-S-CPG sensitized the cells to GA, NSC 658514 and NSC 661581, representatives for the 17-O/H analogues. For 17-N analogues, such as 17-AAG, 17-DMAG, 17-AEP-GA, and 17-DMAP-GA, 4-S-CPG had no impact on their potencies. Cells were also exposed to 5 mM Glutamate to inhibit SLC7A11 activity (Huang et al., 2005a), yielding the same results as 4-S-CPG (data not shown). We next compared drug potencies of SLC7A11 siRNA-treated cells with those of non-silencing control siRNA-treated cells. SLC7A11 siRNA resulted in > 80% reduction in SLC7A11 mRNA levels revealed by real-time RT-PCR analysis (Supplementary Figure 1). For siRNA downregulation we have obtained similar results as SLC7A11 inhibitors (Figure 3B and Table 2).

Hepatocarcinoma cell line HepG2 showed a higher sensitivity to GA (IC_{50} was 4.2 $\pm$ 0.027 nM) in comparison to A549 (IC_{50} was 24 $\pm$ 0.022 nM). The expression of SLC7A11 in A549 was 4 and 1.7 times of that in HepG2 cells at mRNA and protein levels, respectively (Figure 4A and 4C). To confirm the role of SLC7A11 in cellular resistance to cytoxicity, HepG2 cells were infected with the retrovirus containing pBabe expression vector to create a cell line stably expressing either vector alone or SLC7A11 cDNA. Real-time quantitative RT-PCR showed that the HepG2/SLC7A11 cells expressed 12-fold SLC7A11 mRNA of HepG2 cells infected with the vector (Figure 4B). Western blot analysis showed a 6.5-fold increase of SLC7A11 protein in the HepG2/SLC7A11 cells (Figure 4C). The cytotoxicity of GA and 17-AAG in HepG2/SLC7A11 cells was compared to that of vector-infected HepG2 cells. The sensitivity of HepG2/SLC7A11 cells to GA was significantly reduced

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[IC_{50}(HepG2/SLC7A11)/IC_{50}(HepG2/vector) = 3.2, $P < 0.05$] (Figure 5A). However, for 17AAG, the cytotoxicity to HepG2/SLC7A11 cells and the parent cells did not show significant difference [IC_{50}(HepG2/SLC7A11)/IC_{50}(HepG2/vector) = 1.6, $P = 0.36$] (Figure 5B). These data supported that SLC7A11 plays a different role in the resistance to GA and 17AAG.

The role of SLC7A11 in chemoresistance appears to be mediated by GSH. In a previous study, we have treated the A549 and SK-OV-3 cells with buthionine sulfoximine (BSO), the specific inhibitor of the rate-limiting enzyme in the synthesis of GSH, γ-glutamylcysteine synthetase (γ-GCS), to compare the effects of suppressing SLC7A11 transport activity with those of inhibiting GSH synthesis via a different path. BSO treatment strongly sensitized the A549 cells to GA, but the effects on 17-AAG was much smaller (Huang et al., 2005a). Here we further performed a study evaluating the effects of N-acetylcysteine (NAC), a precursor of cysteine and GSH synthesis, in modulating the chemosensitivity to GA analogues. As shown in Figure 3C and Table 2, the presence of NAC (1 mM) completely blocked the cytotoxic effects of GA, NSC 658514, and 661581, but with no or much smaller protective effect on 17-AAG and other 17-N compounds. To know whether the effects of NAC are broadly observed, we performed the similar study on HepG2 cells as well and obtained similar results as in A549 cells (data not shown).

The functions of GSH and NAC involve a variety of mechanisms, among which is scavenging reactive oxygen species (ROS). We therefore tested the effects of ascorbic acid (AsA, or vitamin C), a prooxidant rather than antioxidant (D'Agostini et al., 2005),

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on the sensitivity to GA analogues. AsA has been shown to increase intracellular ROS levels and induce apoptosis of tumors, which was preventable by pretreatment with NAC (Inai et al., 2005). In contrast to the effects of NAC, administration of AsA (1 mM) considerably and significantly potentiated the cytotoxicity of GA, NSC 658514, and 661581, but for 17-N compounds it had no sensitizing effect, or mildly reduced the sensitivity for some of them (Figure 3D and Table 2). Thus, the antioxidant NAC and the prooxidant AsA, displayed opposite modulation of chemosensitivity to GA analogues. The effects of NAC and AsA appear to correlate with classification of GA analogues based on chemical structure and potency. To determine whether the observed results are cell line-specific phenomena or are broadly applicable, we similarly tested the effects of 4-S-CPG, glutamate, NAC and AsA on lung cancer HOP-62 and H322M cells and ovarian cell line SK-OV-3, which express relatively high levels of SLC7A11 (Figure 2B). The results were consistent in all cell lines tested (data not shown).

Effects of GA and 17-AAG on ROS production in A549 and HepG2 cells

SLC7A11/GSH-mediated chemoresistance and protection of cytotoxicity by the antioxidant NAC suggest a role for ROS in induction of cytotoxicity by the 17-O/H analogues. This hypothesis led us to identify and quantitate intracellular ROS levels after administration of GA or 17-AAG. The cell-permeable fluorescent dye, dichlorodihydrofluorescein diacetate (H_2DCFDA) was used to measure intracellular ROS levels. The dye can be oxidized by intracellular ROS to generate a fluorescent product measurable to give an estimation of intracellular ROS status. ROS production was low in untreated cells at basal level. However, it becomes significantly elevated at 16 h after GA

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treatment of A549 and HepG2 cells ($P < 0.05$) (Figure 6). Induction of ROS by GA was significantly higher than that by 17-AAG in both cell lines ($P < 0.05$). 17-AAG did not increase ROS level in A549 cells, but in HepG2 cells ROS was elevated. ROS induction by GA was completely suppressed by NAC, which therefore confirmed the blocking effect of NAC on cytotoxicity of GA. The effects of GA and 17-AAG on superoxide production were also studied with an established method involving oxidation of dihydroethidium (DHE). Treatment of A549 and HepG2 cells with GA significantly increased the amount of superoxide detected by DHE in comparison to 17-AAG treatment and the untreated controls (data not shown). Thus, the tumor cytotoxic effects of the 17-O/H analogues, but not the 17-N analogues, might be ROS-dependent. This may account for differential relationship of 17-O/H and 17-N analogues with SLC7A11 and indicate that 17-N analogues can bypass the resistance mechanism.

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Discussion

This study emerged from a pharmacogenomic investigation, in which we analyzed the correlations between mRNA expression profiles of human transporter genes and patterns of growth inhibitory potency of the anticancer drug GA in the NCI-60 panel (Huang et al., 2007; Huang et al., 2004). The power of this pharmacogenomic approach has been demonstrated by identification of several transporter genes conveying varying degrees of chemoresistance to different anticancer drugs with the aid of *in vitro* manipulation of transporter activities using transporter-specific inhibitors or siRNA (Dai et al., 2007; Huang et al., 2005a; Huang et al., 2005b). The present study followed up on our previous work (Huang et al., 2007) and focused on the amino acid transport system x_c^-, a heterodimer encoded by two genes in the solute carrier (SLC) superfamily, SLC7A11 and SLC3A2, for the light (or catalytic chain) and heavy chain of system x_c^-, respectively. System x_c^- plays an important role in maintaining cellular GSH levels and mediating chemoresistance to cytotoxic agents (Huang et al., 2005a).

We examined the potential links between the amino acid transport system x_c^- (represented by SLC7A11) and cytotoxicity to a panel of GA analogues. In one of our previous study GA was identified as the candidate anticancer drug with negative SLC7A11 correlation (Huang et al., 2005a). Although closely related in structural properties, GA analogues differ widely in their correlations with SLC7A11 expression. The present study shows that SLC7A11 conveys resistance to GA, NSC 658514 and 661581, but not to 17-AAG, 17-DMAG, 17-AEP-GA and 17-DMAP-GA. The 18 GA analogues can be clustered into

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two distinct subgroups, *i.e.*, 17-O/H, and 17-N, according to their chemical structures and potency on the NCI-60. Most of the 17-O/H analogues showed significant correlations with SLC7A11 expression, whereas no significant SLC7A11- correlation was observed with most of the 17-N analogues. This suggested that the two subgroups have distinct MOAs and mode of resistance. This notion was supported by a variety of methods – by pharmacologic inhibition of SLC7A11, siRNA-mediated downregulation of SLC7A11, overexpression of SLC7A11, and toxicity profiles of cell lines with high *vs*. low endogenous SLC7A11 expression. The basis for this difference could be that SLC7A11 plays an important role in maintenance of cellular GSH levels and that 17-O/H drugs induce higher levels of ROS when compared to 17-N derivatives. This notion was further supported by addition of either NAC, an antioxidant, or AsA, a prooxidant, in combination with GA analogues. NAC dramatically increased the IC_{50} of GA, while having minimal effects upon 17-AAG and other 17-N analogues. AsA reduced the IC_{50} of GA, but did not reduce the values for 17-AAG and related agents. 17-AEP-GA and 17-DMAP-GA are new GA analogues with the alkylamino group in place of the methoxy moiety at C-17. They were shown to induce similar tumor cell growth inhibition as 17-AAG, but with better water solubility (Tian et al., 2004). According to their structural features, they can be grouped as the "17-N" analogues. Our data showed that they have similar SLC7A11-relationship as 17-AAG and 17-DMAG. Thus, we used 17-AAG or 17-DMAG as representatives for the 17-N group since they are currently in clinical trials. These results have important implication, as SLC7A11 may serve as a biomarker for predicting efficacy of a panel of anticancer drugs and selecting optimal drug therapies for

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individual tumors. For this purpose, the role of SLC7A11 needs to be tested against more analogues of GA or other compound classes in more cell types.

The transport system x_c^- is a heterodimeric complex comprised of SLC7A11 and SLC3A2. It is clear that SLC3A2 is required for proper cell surface localization of SLC7A11 and also for its transport function. One would expect that in HepG2 the optimal expression and proper function of SLC7A11 will require SLC3A2. We manipulated HepG2 cells only with SLC7A11 overexpression, as it was reported that in NIH3T3 cells transfected with SLC7A11 cDNA alone, the activity of cystine transport was significantly increased (Wang et al., 2003). However, this may account for the observation that although the increase of SLC7A11 in HepG2/SLC7A11 was high (12-fold), the change of potency for GA was only 3.2 fold (Figure 5). It may be important to know whether the levels of endogenous SLC3A2 are sufficient for proper localization and functioning of SLC7A11 in HepG2 cells and other cells with low level of SLC7A11.

There are two possibilities for the connection between SLC7A11/GSH and chemoresistance. First, GSH forms drug conjugates, which commonly renders the compounds less cytotoxic, facilitates transport out of the cells, or prevents binding to the target (Hsp90). Two recent reports showed that GA, 17-AAG, and 17-DMAG could react chemically (*i.e*. nonenzymatically) with GSH (Cysyk et al., 2006; Lang et al., 2006). Although the conjugation proceeds rapidly with GA, less rapidly with 17-DMAG and 17-AAG, the latter can also form GSH conjugates. In addition, we found that MRP1 encoded by the ABCC1 gene, an efflux transporter for GSH conjugated compounds, mediates

efflux and resistance to both 17-O/H and 17-N compounds (unpublished results). Therefore, GSH conjugation may not fully explain the difference. Second, GSH plays a prominent role in a cellular defense against ROS. In fact, system x_c^- can be adaptively induced in response to oxidative stress and thereby closely associated with the cellular antioxidant machinery (Sasaki et al., 2002). SLC7A11 has been shown to play a pivotal role in maintaining redox balance and protection from oxidative stress based on the results from SLC7A11-/- mice (Sato et al., 2005). In the present study, GA and 17-AAG showed difference in generation of ROS: GA produced significantly higher levels of intracellular superoxides and H_2O_2. Consistently, it has been shown that treatment of endothelial cells with GA resulted in a dramatic increase in superoxide formation by redox cycling, an effect independent Hsp90 inhibition (Dikalov et al., 2002), while to our knowledge there was no report of 17-N analogues producing ROS. Therefore, our data not only support a SLC7A11-mediated resistance mechanism that is specifically directed towards 17-O/H analogues, but also suggest a differential role of ROS in the cytotoxic activity between the two subgroups of compound. The strong correlations between SLC7A11 and 17-O/H analogues in NCI-60 panel could have resulted from the fact that cancer cells expressing high level of SLC7A11 have a high antioxidant potential, producing or maintaining more GSH, which scavenges ROS and/or forms GSH conjugates, and therefore are more resistant to cytotoxic effects of 17-O/H analogues. In contrast, cell lines with low level of SLC7A11 cannot produce sufficient GSH and might be more sensitive to ROS-producing compounds. The cytotoxic effects of 17-O/H analogues may depend on ROS production, while ROS may not be important for 17-N analogues which therefore bypass the resistance mechanism mediated by SLC7A11.

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However, to distinguish between the possibilities of GSH conjugation and ROS scavenging, further experiments should be done to examine the ability of 17-O/H analogues to target Hsp90 in the presence of GSH and perform drug transport study in the presence or absence of GSH.

Several genes and proteins have been previously associated with sensitivity to GA analogues, including expression levels or mutation status of key Hsp90 client proteins such as ERBB2, BRAF, Bcr-Abl and AKT (da Rocha Dias et al., 2005; Demidenko et al., 2005) as well as levels of Hsp90 family members such as Hsp70 (Guo et al., 2005). For 17-AAG, the metabolizing enzyme NQO1 was found important for tumor cell sensitivity (Kelland et al., 1999). Moreover, elevated expression of the ABC (ATP-binding cassette) transporter gene ABCB1 (MDR1 or P-glycoprotein) contributes to tumor resistance to some of the GA analogues (Huang et al., 2007; Huang et al., 2004). Thus, like other commonly used anticancer agents, multiple factors contribute to the cellular sensitivity and resistance to GA analogues. There is a surprising coherence between SLC7A11-correlations and the degree of hepatotoxicity of these compounds: 17-O/H analogues, such as GA, showed strong hepatotoxicity and are more hepatotoxic than 17-N analogues in preclinical studies (Uehara, 2003). Thus, system x_c^- and GSH could play a role in modulating hepatotoxicity, and therefore be factors determining the differential toxicity of the analogues observed in preclinical studies.

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Footnotes:

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Legends for figures:

Figure 1. Principal component analysis (PCA) of 18 GA analogues based on their growth inhibitory potency (GI_{50} values) across the NCI-60. The location was based on the first three principal components using the GI_{50} values for the 18 GA analogues. Compounds are labeled according to the classification obtained based on chemical structures. Each sphere represents a 17-O/H analogue. Each cube represents a 17-N analogue.

Figure 2. Correlation between SLC7A11 expression and growth inhibitory potency of 18 GA analogues in the NCI-60. *A*, Plot of sorted Pearson correlation coefficients (r) between gene expression of SLC7A11 and cytotoxic potencies of 18 GA analogues. *: *P* value < 0.05. 17-O/H compounds are in bold; *B*, Scatter plots showing the correlation (r) of SLC7A11 expression with chemosensitivity of the NCI-60 cell lines to GA and 17-AAG. A $\log_{10}GI_{50}$ value of 1 for sensitivity indicates a tenfold difference in the sensitivity to the drug. The SLC7A11 level is plotted as the abundance ($\log_2$) of the SLC7A11 mRNA transcript, relative to its abundance in the reference pool of 12 cell lines. The circles indicate four cell lines selected for the experiments which have relatively high expression of SLC7A11 and are relatively more resistant to GA.

Figure 3. Functional validation of the SLC7A11-GA correlations. *A*, Growth inhibition curves for A549 cells in response to GA, NSC 661581 and 658514, 17-AAG and 17-DMAG with or without treatment with the system x_c^- inhibitor 4-S-CPG (50 μM).

B, Effect of down-regulation of SLC7A11 by siRNA or non-silencing control siRNA on drug sensitivity of A549 cells. *C*, Chemosensitivity of A549 cells with or without treatment with the GSH precursor, an antioxidant, N-acetylcysteine (NAC) (1 mM). *D*, Chemosensitivity of A549 cells with or without treatment with the prooxidant ascorbic acid (AsA) (1 mM). Results are expressed as percentage survival of control cells with no drug treatment (means $\pm$ SD from three replicates).

Figure 4. Expression analysis of SLC7A11 detected by quantitative real-time RT-PCR and Western Blot. *A*, Real-time RT-PCR analysis to compare the basal SLC7A11 levels in A549 and HepG2 cells. Relative gene expression is shown (SLC7A11 level in A549 cells was set as 1). Expression levels were normalized to the β-actin levels. Each reaction was performed in triplicate. The SLC7A11 mRNA in HepG2 cells was 25% of that in A549 cells. *B*, Real-time RT-PCR results of SLC7A11 levels in HepG2 infected with pBabe retroviral vector containing SLC7A11 cDNA (HepG2/SLC7A11) and HepG2 infected with empty vector (HepG2/vector). HepG2/SLC7A11 expressed nearly 12 folds of SLC7A11 mRNA of the parent cells. Expression levels of β-actin were used for normalization. *C*, Expression of SLC7A11 protein was detected by Western blot analysis (a single 55 kDa band). Compared to A549 cells, HepG2 expresses less SLC7A11. The level of SLC7A11 expression in HepG2/SLC7A11 cells was much higher than the parent cells. Quantification of the band intensity is shown for each band. A mesothelioma cell line was used as the positive control.

Figure 5. Cytotoxicity of GA (*A*) and 17-AAG (*B*) in HepG2 with ectopic SLC7A11 expression and the control HepG2 cells. Cytotoxicity of GA and 17-AAG were measured by SRB assay in HepG2 cells infected with a retrovirus containing pBabe vector alone or SLC7A11 cDNA and selected with 2 μg/ml puromycin. Results are expressed as percentage survival of control cells with no drug treatment (means $\pm$ SD from three replicates).

Figure 6. Effects of GA and 17-AAG on the production of ROS in drug treated A549 cells (*A*) and HepG2 cells (*B*). Cells were respectively treated with 1.25 μM of GA and 17-AAG for 16 hr. ROS production in the treated cells was represented by intracellular fluorescent intensity determined by flow cytometry. The data are the means ± SD. Similar results were obtained in three independent experiments. *: $p<0.05$ (relative to control samples without drug treatment).

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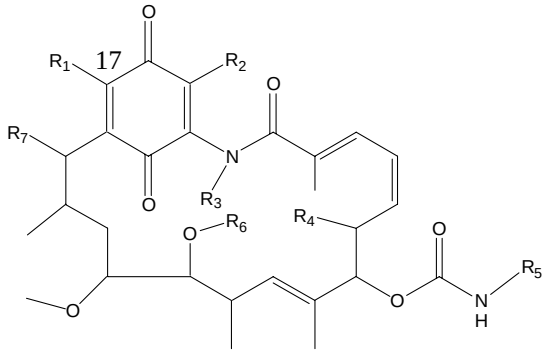
Table 1. Chemical structures of GA and its analogues. Cluster analysis based on similarities or differences in chemical structures grouped these analogues into two subgroups which were characterized by the C-17 substituents (R1): 17-O/H (in bold, 10 compounds), 17-N (8 compounds). 17-O/H analogues contain a methoxy or hydroxy group or unsubstituted at C-17, whereas the 17-N group contains various amino groups at the C-17. Five analogues highlighted in gray are those available for experimental validation.



NSC	Name	R_1	R_2	R_3	R_4	R_5	R_6	R_7
122750	GA	OMe	H	H	OMe	H	H	H
658514		OMe	H	H	OMe	H	$C(=O)(CH_2)_3NH_2$	H
661581		OMe	H	H	OMe	H	$C(=O)(CH_2)_2NH_2$	H
210753		OMe	CH=N-t-Bu	H	OMe	H	H	H
255111		OMe	$CH=N\text{-}C_5H_{10}N$	H	OMe	H	H	H
330512		OMe	H	Me	OMe	Me	H	H
661580		OMe	H	H	OMe	H	$C=(O)CH_2NH_2$	H
255104		OH	H	H	OMe	H	H	H
305978	Herbimycin	H	H	H	OMe	H	Me	OMe
330499	Macbecin I	H	H	H	Me	H	Me	OMe
330507	17-AAG	$NCH_2CH=CH_2$	H	H	OMe	H	H	H
707545	17-DMAG	$NCH_2CH_2NMe_2$	H	H	OMe	H	H	H
255109	17-AG	NH2	H	H	OMe	H	H	H
320877		NCH_2CH_2Cl	H	H	OMe	H	H	H
683661		$NCH_2CH=CH_2$	H	H	OMe	H	$C=(O)CH_2NH_2$	H
683662		$NCH_2CH=CH_2$	H	H	OMe	H	$C=(O)CH_2NMe_2$	H
683663		$NCH_2CH=CH_2$	H	H	OMe	H	$C(=O)(CH_2)_2NH_2$	H
683664		$NCH_2CH=CH_2$	H	H	OMe	H	$C(=O)(CH_2)_2NMe_2$	H

Table 2. Effects of 4-S-CPG, SLC7A11 specific siRNA, N-acetylcysteine (NAC) and ascorbic acid (AsA) on cytotoxicity of GA analogues in A549 cells. 4-S-CPG (50 μM) was used to suppress SLC7A11 transport activity. siRNA was used to downregulate SLC7A11 expression. The effects of the GSH precursor and antioxidant, N-acetylcysteine (NAC) (1 mM) and the prooxidant ascorbic acid (AsA) (1 mM) were also tested on the cytotoxicity of GA analogues. CC is the Pearson correlation coefficients between compound potency and SLC7A11 expression. IC_{50} values in μM, the concentrations that produced 50% inhibition of cell growth compared to controls, are shown for each drug in the presence or absence of 4-S-CPG, siRNA, NAC or AsA. Numbers in the rows of "Fold" are fold-reversal, which is the IC_{50} for the cytotoxic drug in control cells divided by that in inhibitor-treated cells. When IC_{50} was higher than the maximum concentration used, it is denoted as "> maximum concentration" and the fold changes was calculated using the maximum concentration. Results represent mean ± SD of at least three experiments. *: $p < 0.05$ versus controls without adding inhibitors or siRNA.

NSC #	**122750**	**658514**	**661581**	**330507**	**707545**	**-**	**-**
Name	**GA**	**-**	**-**	**17-AAG**	**17-DMAG**	**17-AEP-GA**	**17-DMAP-GA**
CC	-0.57	-0.46	-0.50	-0.10	0.06	-	-
(-) 4-S-CPG	0.026 ± 0.00	2.5 ± 0.51	0.35 ± 0.05	0.086 ± 0.01	0.15 ± 0.00	0.072 ± 0.01	0.31 ± 0.02
(+) 4-S-CPG	0.004 ± 0.00	1.6 ± 0.40	0.12 ± 0.01	0.062 ± 0.00	0.14 ± 0.02	0.082 ± 0.00	0.34 ± 0.04
Fold	**(7.1)***	**(1.6)***	**(3.0)***	**(1.4)**	**(1.1)**	**(0.89)**	**(0.94)**
(-) siRNA	0.025 ± 0.00	5.9 ± 0.33	0.92 ± 0.13	0.03 ± 0.00	0.10 ± 0.00	0.030 ± 0.00	0.11 ± 0.01
(+) siRNA	0.013 ± 0.00	2.8 ± 0.11	0.51 ± 0.03	0.04 ± 0.00	0.10 ± 0.01	0.017 ± 0.00	0.083 ± 0.03
Fold	**(1.8)***	**(2.1)***	**(1.8)***	**(0.9)**	**(1.0)**	**(1.8)**	**(1.4)**
(-) NAC	0.026 ± 0.00	2.0 ± 0.35	0.45 ± 0.05	0.033 ± 0.00	0.090 ± 0.01	0.057 ± 0.01	0.14 ± 0.01
(+) NAC	>6.25	>16.7	>16.7	0.030 ± 0.00	0.24 ± 0.01	0.15 ± 0.05	0.26 ± 0.02
Fold	**(<0.0042)***	**(<0.12)***	**(<0.027)***	**(1.1)**	**(0.37)***	**(0.42)**	**(0.54)***
(-) AsA	0.029 ± 0.00	1.9 ± 0.18	0.35 ± 0.03	0.026 ± 0.00	0.088 ± 0.01	0.047 ± 0.01	0.11 ± 0.00
(+) AsA	0.012 ± 0.00	0.063 ± 0.00	0.051 ± 0.00	0.038 ± 0.00	0.11 ± 0.01	0.057 ± 0.01	0.28 ± 0.04
Fold	**(2.5)***	**(30)***	**(6.8)***	**(0.69)***	**(0.78)**	**(0.82)**	**(0.41)***

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Figure 1

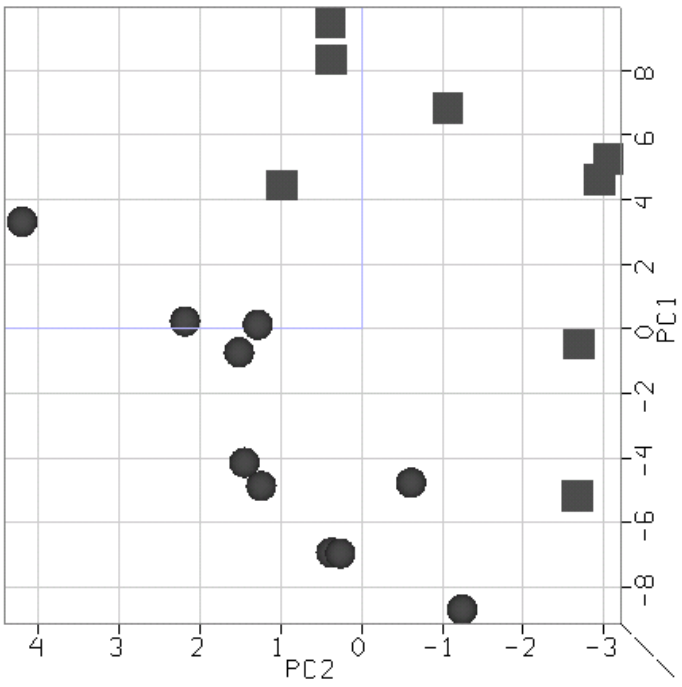

Figure 2

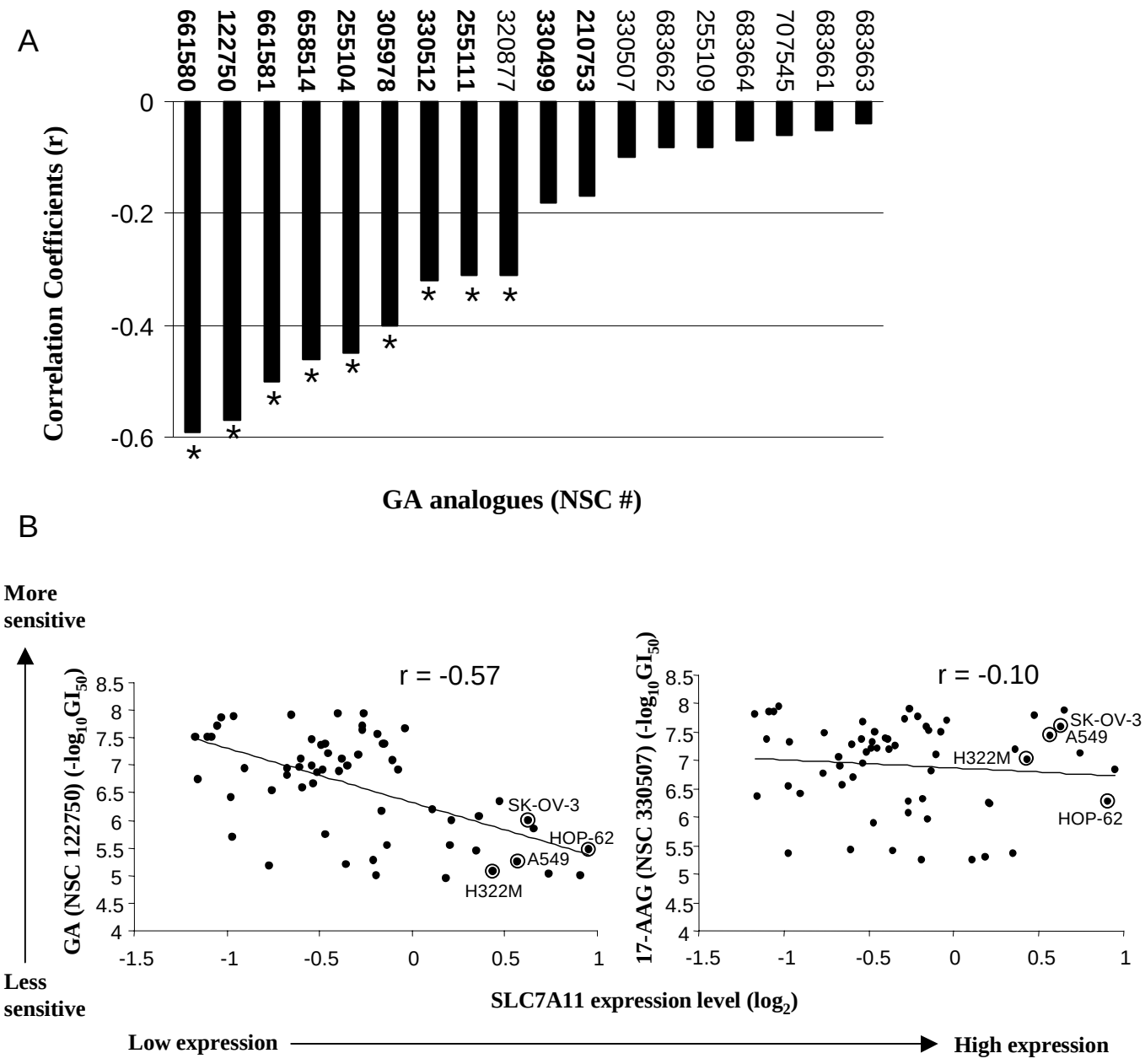

Figure 3

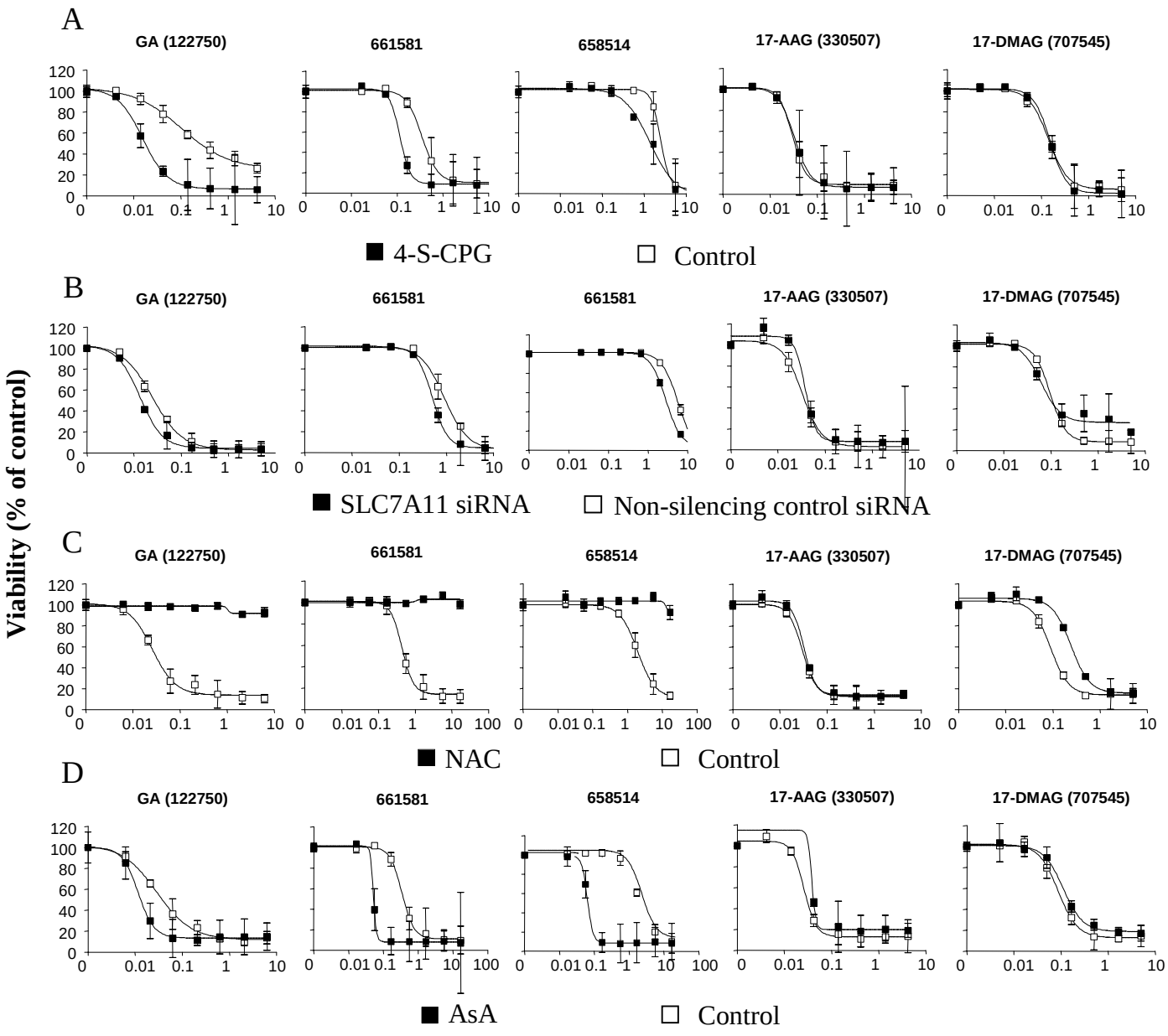

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Figure 4

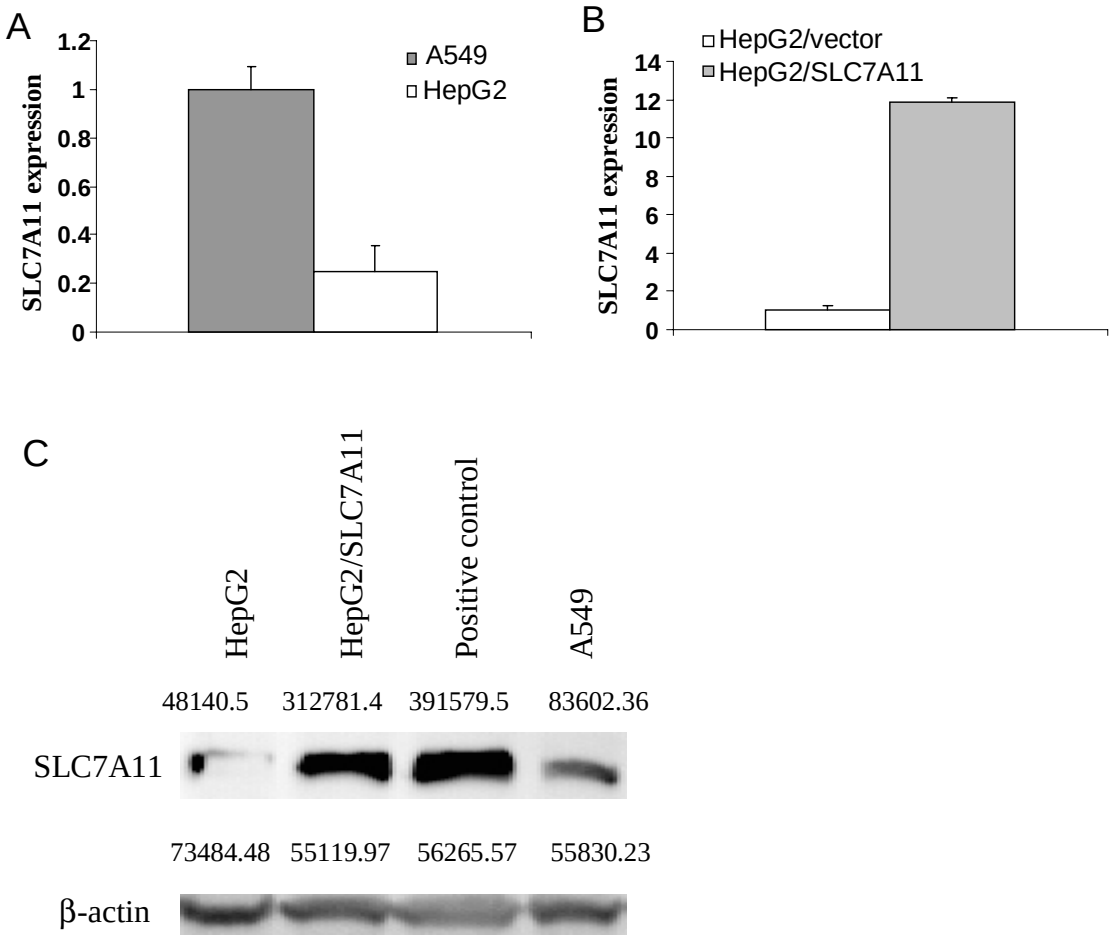

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Figure 5

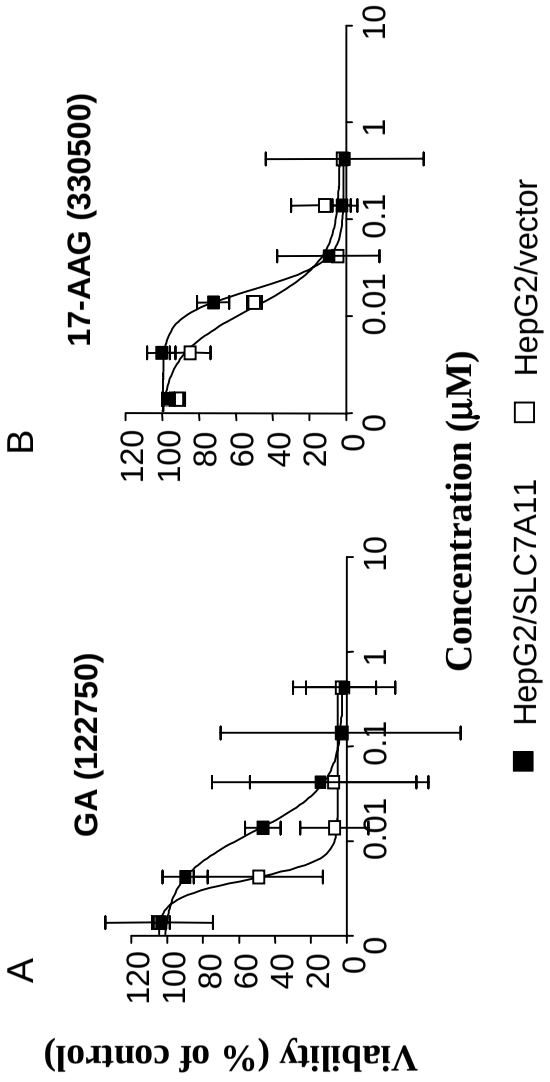

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Figure 6