Pharmacogenomic Approach Reveals a Role for the $x_c^-$ Cystine/Glutamate Antiporter in Growth and Celastrol Resistance of Glioma Cell Lines

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ABSTRACT

The $x_c^-$ cystine/glutamate antiporter has been implicated in GSH-based chemoresistance because it mediates cellular uptake of cystine/cysteine for sustenance of intracellular GSH levels. Celastrol, isolated from a Chinese medicinal herb, is a novel heat shock protein 90 (Hsp90) inhibitor with potent anticancer activity against glioma in vitro and in vivo. In search of correlations between growth-inhibitory potency of celastrol in NCI-60 cell lines and microarray expression profiles of most known transporters, we found that expression of SLC7A11, the gene encoding the light chain subunit of $x_c^-$, showed a strong negative correlation with celastrol activity. This novel gene-drug correlation was validated. In celastrol-resistant glioma cells that highly expressed SLC7A11, sensitivity to celastrol was consistently increased via treatment with $x_c^-$ inhibitors, including glutamate, (S)-4-carboxyphenylglycine, sulfasalazine, and SLC7A11 small interfering RNA. The GSH synthesis inhibitor, buthionine sulfoximine, also increased celastrol sensitivity, whereas the GSH booster, N-acetylcysteine, suppressed its cytotoxicity. Furthermore, the glioma cell lines were dependent on $x_c^-$-mediated cystine uptake for viability, because cystine omission from the culture medium resulted in cell death and treatment with sulfasalazine depleted GSH levels and inhibited their growth. Combined treatment of glioma cells with sulfasalazine and celastrol led to chemosensitization, as suggested by increased celastrol-induced cell cycle arrest, apoptosis, and down-regulation of the Hsp90 client protein, epidermal growth factor receptor. These results indicate that the $x_c^-$ transporter provides a useful target for glioma therapy. $x_c^-$ inhibitors such as sulfasalazine, a Food and Drug Administration-approved drug, may be effective both as an anticancer drug and as an agent for sensitizing gliomas to celastrol.

Chartered by widespread invasion and chemoresistance, malignant gliomas represent one of the most difficult types of cancer to understand and to treat (Furnari et al., 2007). Approximately 18,000 new cases are reported annually, and these cases account for over 12,000 deaths in the United States (Sontheimer, 2008). More recently, molecular-targeted therapy against a single oncogene or pathway has produced promising results for various solid tumors. However, clinical trials using this approach, e.g., inhibition of epidermal growth factor receptor (EGFR), have had minimal benefit (Furnari et al., 2007). One plausible explanation for the failure of the targeted therapy is that gliomas have multiple genetic alterations, and an agent hitting a single molecular target may not be sufficient to treat this disease effectively. However, glioma therapy may benefit from targeting the molecular chaperone heat shock protein 90 (Hsp90) because it simultaneously regulates activity of multiple oncogenic proteins and signaling pathways (Neckers, 2007).
Most Hsp90 client proteins, including Akt, EF-2 kinase, mutant p53, vascular endothelial growth factor receptor, and EGFR, are directly implicated in the development, growth, and invasion of gliomas (Furnari et al., 2007).

Celastrus is a quinone methide triterpene extracted from *Tripterygium wilfordii*, the Chinese herbal medicine commonly referred to as “God of Thunder Vine” (or lei gong teng) and used for treatment of rheumatoid arthritis and other inflammatory conditions (Corson and Crews, 2007). Celastrus was recently identified as a novel Hsp90 inhibitor, acting by blocking the interaction of Hsp90 with its cochaperone Cdc37 (Zhang et al., 2008). It exhibits antitumor effects in several in vitro and in vivo cancer models, including leukemia, glioma, melanoma, and prostate cancer (Abbas et al., 2007; Huang et al., 2008b). Besides Hsp90, several other molecular targets have been proposed to explain its antitumor effects, including nuclear factor κB (NF-κB), proteasome, and topoisomerase II (Sethi et al., 2007). As celastrus moves into clinical studies, it will be important to better understand its mechanism of biochemical action and the development of chemoresistance to this drug and to investigate whether its efficacy may be synergistically increased by existing anticancer agents.

Membrane transporters perform important cellular functions; they provide nutrients, remove unwanted materials, and establish electrochemical gradients across membranes (Huang and Sadée, 2006). They also play key roles in pharmacology, affecting the entry of drugs into cells and their extrusion. In addition to direct effects on drug transport, they may also indirectly modulate drug effects, for example, and affect chemosensitivity of cancer cells by mediating uptake of nutrients, such as amino acids and glucose (Huang et al., 2004). The \( x^*_c \) cystine/glutamate antiporter is an important plasma membrane transporter involved in uptake of cysteine in exchange for intracellular glutamate. It is best known for its mediation of cellular uptake of cysteine/cysteine required for synthesis of GSH, a tripeptide thiol consisting of glutamate, cysteine, and glycine, essential for cellular protection from oxidative stress (Lo et al., 2008b). The \( x^*_c \) transporter is a heterodimeric protein complex encoded by two genes, SLC7A11 and SLC3A2. The light chain protein subunit of \( x^*_c \), SLC7A11, mediates the transport activity, whereas SLC3A2, the heavy chain subunit, leads to cell surface expression of the transporter (Verrey et al., 2004). The expression of SLC7A11 is more directly correlated with \( x^*_c \) activity (Sato et al., 1999). The majority of primary gliomas and cell lines express high amounts of SLC7A11 (Savaskan et al., 2008). We previously found that the Hsp90 inhibitor, geldanamycin (GA), is susceptible to drug resistance mediated by the \( x^*_c \) transporter (Huang et al., 2005; Liu et al., 2007). Cancer cell lines expressing high levels of SLC7A11, including some lung cancer and glioma cell lines, are resistant to GA and many other anticancer agents (Huang et al., 2005; Dai et al., 2007). Inhibition of \( x^*_c \) activity can considerably enhance sensitivity to GA (Huang et al., 2005).

In the present study, we applied a pharmacogenomic approach in a search for correlations between microarray transporter gene expression profiles and anticancer activity of celastrus, tested with the NCI-60, a panel of 60 diverse human cancer cell lines from the Developmental Therapeutics Program (DTP) of the National Cancer Institute (NCI) for anticancer compound screening. The study included leukemia and melanoma as well as cancers of ovarian, breast, prostate, lung, renal, colon, and central nervous system origin. A strong negative correlation between expression of SLC7A11 and celastrus potency was observed, indicating that cells expressing relatively high levels of SLC7A11 are less sensitive to celastrus. In following up this lead, we established that the \( x^*_c \) transporter confers resistance to celastrus and that its inhibition significantly enhances the anticancer activity of celastrus in glioma cell lines.

**Materials and Methods**

**Compound Potency Databases for NCI-60.** The September 2008 release of the 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 nonconfidential screening results and chemical structural data from the DTP. For each compound and cell line, growth inhibition after 48 h of drug treatment was measured 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).

**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 a previous study (Huang et al., 2004). A secondary gene expression database, the Novartis microarray dataset, was also used for comparison. This data set contains the average of triplicate expression measurements for 59 NCI cell lines based on 12,626 oligonucleotide probes from Affymetrix U95Av2 arrays, available at NCI’s DTP website (http://dtp.nci.nih.gov/mtargets/download.html).

**Correlation of Gene Expression Profiles with Compound Potency Patterns.** We calculated Pearson correlation coefficients [using the cor() function in R software; http://www.R-project.org] between potency values of the set of 28 geldanamycin analogs plus celastrus analogs and expression levels of the custom transporter array (755 genes) both measured over the NCI-60. We also calculated a parallel matrix of \( p \) values. For this estimate, we first generated a null distribution by permuting the columns of the compound matrix (i.e., the cell lines), calculating the correlation matrix, and repeating 500 times. This result gave a null distribution of approximately 10 million values, which was used to estimate correlation values at several points in the extreme tail regions, such as \( p = 0.0001, 0.00025, \) etc. We then counted how many compound-gene correlation values (i.e., from the actual correlation matrix) were more extreme than the estimated values, and this result gave estimates of false discovery rates at cutoff values in the two tail regions.

**Chemicals.** Celastrus was purchased from Cayman Chemical (Ann Arbor, MI). GA was purchased from InvivoGen (San Diego, CA). (S)-4-Carboxyphenylglycine (4-S-CPG) was obtained from Tocris (Ballwin, MO). Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). A sulfasalazine (SASP) stock solution was prepared in 0.1 N NaOH containing phosphate-buffered saline, with adjustment of the pH to 7.4, according to a published method (Doxsee et al., 2007).

**Cell Cultures.** All cell lines were obtained from the Division of Cancer Treatment and Diagnosis at the NCI and maintained in RPMI 1640 medium containing 5 mM L-glutamine, supplemented with 10% fetal bovine serum, 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 Assays.** Growth-inhibitory activity was tested by using a proliferation assay with SRB, a protein-binding reagent (Sigma-
Alrich, as described before (Huang et al., 2004) or MTS assay (Promega, Madison, WI). Cells (3000–4000 cells/well) were seeded in 96-well plates and incubated for 24 h in RPMI 1640 medium. Before exposure to test compounds, cultures were incubated individually with SASP, 4-S-CPG, buthionine sulfoximine (BSO), N-acetylcysteine (NAC), or medium (as control) for 10 min. Test compounds were then added in a dilution series in three replicate wells for an additional 3-day incubation. To determine IC₅₀ values, the absorbance of control cultures without drug was set at 1. Dose-response curves were plotted by using Prism (GraphPad Software Inc., 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 SLC7A11.** Small interfering RNA (siRNA)-induced down-regulation of SLC7A11 expression was described previously (Huang et al., 2005). In brief, siRNA duplexes for SLC7A11 were synthesized by using the Silencer siRNA construction kit (Ambion, Austin, TX). The target sequences were 5′-AAATGCCCCAGATATGCATCGT-3′ (forward) and 5′-GAAAGGGCAACCATGAAAGG-3′ (reverse). The primers for β-actin were 5′-AGCCAGACGCTCAAGAC-3′ (forward) and 5′-GCCCAATACGACAAATCC-3′ (reverse).

**Western Blotting.** Western blot analysis was conducted to detect the amount of Hsp90 client protein, EGFR. Cells were lysed in lysis buffer [20 mM Tris (pH 8.0), 150 mM NaCl, 1% glycerol, 1% Nonidet P40, and 0.42% NaF] containing 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, 2 μg/ml aprotinin, and 5 μg/ml leupeptin. Twenty-five micrograms of protein for each sample was separated by gel electrophoresis on 10% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The signals were detected by immunoblotting with antibody against EGFR (1:300) (Cell Signaling Technology Inc., Danvers, MA) or β-catenin (1:500) (C4; Millipore Corporation, Billerica, MA) and using a chemiluminescence system (Western Blot Luminol Reagents; Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

**GSH Assay.** Total GSH levels were measured using the fluorometric glutathione assay kit (Sigma-Aldrich) according to the manufacturer’s recommendations. In brief, 1.0 × 10⁶ cells were seeded in 10-cm dishes in maintenance medium overnight. After 24 h, cells were incubated in Fischer’s medium overnight. The fluorescence was measured by using 390 nm for excitation and 478 nm for emission.

**Cell-Cycle Analysis.** Cell-cycle distribution after drug treatment was determined via flow cytometric analysis based on the amount of cellular DNA content using propidium iodide (PI) staining. Cells were plated at a density of 1 × 10⁶ cells per plate in 6-cm² dishes. Drugs were added to the medium after 24 h of incubation, and the cells were further incubated for another 24 h. The cell-cycle distribution of cells stained with PI was analyzed by fluorescence-activated cell-sorting analysis (FACS) by exciting PI at 488 nm and measuring the emission at 600 nm using a FACS flow cytometer.

**Apoptosis Analysis.** Flow cytometry was used to assess loss of membrane asymmetry (externalization of phosphatidylserine) and membrane integrity by fluorescein isothiocyanate-labeled annexin-V (Invitrogen, Carlsbad, CA) and PI, respectively, as described previously. In brief, 5 × 10⁵ cells were seeded in 60-mm dishes for 24 h. The cells were then incubated with drugs for 72 h. Fluorescence was detected in fluorescence channels FL1 (488-nm excitation and 530-nm emission for fluorescein isothiocyanate-labeled annexin V) and FL3 (488-nm excitation and 600-nm emission for PI). For a single analysis, gated fluorescence properties of 10,000 cells were collected. Data acquisition and analysis were performed using the FlowJo program (TreeStar Inc., Ashland, OR). Early apoptotic cells are annexin V-positive and PI-negative, whereas late apoptotic cells are both annexin V- and PI-positive.

**Results**

**Gene Expression of SLC7A11 Negatively Correlated with Anticancer Potency of Celastrol Established via NCI-60 Cell Line Screening.** Celastrol (NSC70931) is a quinone methide triterpene extracted from *T. wilfordii* Hook.

**TABLE 1**
Structure of Hsp90 inhibitors GA and celastrol and genes, the expression levels of which correlated with potency of celastrol and GA in NCI-60 cell lines

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alias</th>
<th>NSC70931</th>
<th>NSC122750</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCB1</td>
<td>MDR1</td>
<td>0.20</td>
<td>−0.19</td>
</tr>
<tr>
<td>ABCG2</td>
<td>MRP1</td>
<td>−0.45**</td>
<td>−0.53**</td>
</tr>
<tr>
<td>SLC7A11</td>
<td>xCT</td>
<td>−0.56**</td>
<td>−0.52**</td>
</tr>
<tr>
<td>SLC3A2</td>
<td>4F2hc</td>
<td>−0.43**</td>
<td>−0.40**</td>
</tr>
<tr>
<td>SLC29A1</td>
<td>ENT1</td>
<td>0.27</td>
<td>0.36*</td>
</tr>
<tr>
<td>NTnporter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADAM9</td>
<td>MDC9</td>
<td>−0.25</td>
<td>−0.50**</td>
</tr>
<tr>
<td>EGFRE</td>
<td>ERBB1</td>
<td>−0.36</td>
<td>−0.34*</td>
</tr>
<tr>
<td>ATP1B1</td>
<td></td>
<td>−0.41*</td>
<td>−0.49**</td>
</tr>
</tbody>
</table>

*P < 0.05; **P < 0.001.
F (structure shown in Table 1). Geldanamycin (NSC122750) is a benzoquinone ansamycin antibiotic produced by yeast Streptomyces hygroscopicus. Although they do not have structural similarities, celastrol and GA induced similar changes in gene expression in human cancer cell lines (Hirerøynus et al., 2006; Lamb et al., 2006), suggesting that celastrol is also an Hsp90 inhibitor. The in vitro growth-inhibitory property of celastrol was compared with that of GA in the NCI-60 cell line panel. The mean GI50 for celastrol (36 nM, ranging from 10 to 500 nM) is lower than that of GA (127 nM, ranging from 15 to 2900 nM) in the 60 human cancer cell lines. To identify genes potentially involved in sensitivity and resistance to celastrol, we performed a correlation analysis between the microarray gene expression profiles for the majority of transporter genes and growth-inhibitory activities of celastrol and GA across the NCI-60 panel. This result yielded Pearson correlation coefficients (r) for each gene-drug pair. Genes that exhibited significant correlations (r > 0.35 and p < 0.05) for at least one of the two drugs were selected (Table 1). Five transporter genes were extracted: the membrane efflux transporter genes ABCB1 (MDR1 or P-glycoprotein) and ABCC1 (MRP1) and the cystine/glutamate antiporter subunit genes SLC7A11 and SLC3A2 displayed negative correlations with celastrol and/or GA, suggesting a role for these genes in chemoresistance; the nucleoside transporter gene SLC29A1 (ENT1, encoding an equilibrative nucleoside transporter) showed positive correlations. In addition, three nontransporter genes showed negative correlations: ATP1B1 (encoding the β1 subunit of Na,K-ATPase), EGRF, and ADAM9 (encoding one member of the family of transmembrane disintegrin-containing metalloproteinases). To validate the results of the correlation analyses, we queried a genome-wide gene expression database of the NCI-60 cell line panel based on the Novartis microarray dataset obtained by the Affymetrix U95Av2 array, and we calculated the correlation coefficients between the potency of the two compounds and expression of the eight genes. The results from two microarray platforms were highly consistent (data not shown).

Overall, celastrol and GA show similar gene correlation profiles (Table 1). However, unlike GA, which was confirmed to be an MDR1 substrate (Huang et al., 2007), celastrol does not show a negative correlation with the ABCB1 gene. ABCB1(-/-) K562 and ABCB1(+/+) K562/DOX cell lines demonstrated the same sensitivity to celastrol (data not shown), suggesting that celastrol is unaffected by P-glycoprotein-mediated efflux. Among all of the genes included in the microarray, SLC7A11 showed the strongest negative correlation with celastrol (Table 1). The Pearson correlation coefficient (r) was −0.56 (P < 0.0001) for the SLC7A11-celastrol pair. The strong negative SLC7A11-celastrol correlation indicates that cell lines expressing relatively high levels of the SLC7A11 gene are less sensitive to celastrol and that SLC7A11 may be involved in chemoresistance to celastrol. In the NCI database, we identified two other compounds with a common quinone methide substructure of celastrol, hydroxypristimerin (NSC 270057) and hydroxytingenone (NSC 684506). They also showed a negative correlation with SLC7A11, r = −0.39 (P > 0.05) and r = −0.54 (P < 0.001), respectively. This result suggests that SLC7A11-mediated resistance may be related to the quinone methide moiety. If there is a connection between the celastrol resistance and SLC7A11 expression level, we would expect to detect a difference in the potency of the compound in cell lines over- and underexpressing the SLC7A11 gene. To investigate celastrol-SLC7A11 correlations, we determined the cytotoxic potency of celastrol in two lung cancer cell lines, i.e., HOP-62, which expresses relatively high levels of SLC7A11, and HOP-92, which does not (Huang et al., 2005). It was found that the HOP-62 line is one of the most celastrol-resistant cell lines (GI50 = 234 nM), whereas the HOP-92 line is the most sensitive one (GI50 = 10 nM). Figure 1A shows the correla-

![Image](image-url)

**Fig. 1.** Validation of the negative correlation between SLC7A11 expression and growth-inhibitory potency of celastrol in NCI-60 cell lines. A, scatter plot showing a correlation between SLC7A11 expression and growth-inhibitory potency of celastrol in NCI-60 cell lines. Celastrol had a significant negative correlation with SLC7A11 expression (r = −0.56, P < 0.001). A log10GI50 value of 1 for sensitivity indicates a 10-fold difference in the sensitivity to the drug. The SLC7A11 level is plotted as the abundance (log2) of the SLC7A11 mRNA transcript, relative to its abundance in the reference pool of 12 cell lines. The circles indicate three glioma cell lines, which have different levels of SLC7A11 expression and were selected for further study. Expression analysis of SLC7A11 in glioma cell lines was confirmed by RT-PCR and gel-electrophoresis. B, growth inhibition curves for SNB-19 cells in response to celastrol with or without treatment with the system x̄ inhibitor, 4-S-CPG (50 μM) or glutamate (5 mM). C, growth inhibition curves are shown for SF-295, SF-539, and SNB-19 cultures with or without treatment with SASP (150 μM). D, concentration-dependent inhibition of SLC7A11 by SASP in SNB-19 and SF-539 cells. SNB-19 cells were incubated with 50, 150, 300, and 600 μM SASP; SF-539 cells were incubated with 150, 300, and 600 μM SASP. Cells were incubated with various drugs for 3 days, and cell growth was measured using the MTS assay. Results are expressed as percentage viability of control cells (no celastrol treatment; means ± S.D. of three replicates).
tion between SLC7A11 expression and growth-inhibitory potency of celastrol. Among the 60 cell lines, glioma cell lines were relatively more resistant to celastrol, and we focused on such cell lines for further studies on SLC7A11 and celastrol.

Inhibition of \( \text{x}_c \) Enhanced Anticancer Efficacy of Celastrol in Glioma Cell Lines Highly Expressing SLC7A11. Based on the microarray gene expression values of SLC7A11 in the NCI-60 cell line panel, we selected three glioma cell lines to validate the correlation between SLC7A11 expression and anticancer activity of celastrol, i.e., SNB-19 and SF-295, showing high expression of SLC7A11, and SF-539, showing low expression of the gene (Fig. 1A). We first microarrayed the data for the three cell lines using RT-PCR analysis. Consistent with the microarray data, the expression levels of SLC7A11 in SNB-19 and SF-295 cells were higher than those in SF-539 cells (Fig. 1A).

We then examined the effect of glutamate, a highly specific \( \text{x}_c \) inhibitor, on the cytotoxic effect of celastrol on SNB-19 cells. Treatment of the SNB-19 cells with 5 mM glutamate was found to significantly sensitize the cells to celastrol (2.0-fold decrease of the IC\text{50}) (Fig. 1B). Another \( \text{x}_c \) inhibitor with higher potency, 4-S-S-CPG (50 \( \mu \)M), also sensitized the SNB-19 cells to celastrol, with a 2.6-fold decrease of the IC\text{50} value (Fig. 1B).

We next examined the effect of sulfasalazine [salicylazo-sulfapyridine (SASP)], a recently discovered inhibitor of the \( \text{x}_c \) transporter (Gout et al., 2001), on the cytotoxicity of celastrol in the three glioma cell lines with different levels of SLC7A11. The cells were incubated with SASP (150 \( \mu \)M) before treatment with celastrol. As shown in Fig. 1C, the presence of SASP increased the potency of celastrol in SF-295 and SNB-19 cell cultures by shifting the IC\text{50} from 2.2 ± 0.16 to 0.74 ± 0.05 \( \mu \)M (3-fold) and from 1.7 ± 0.05 to 0.66 ± 0.05 \( \mu \)M (2.6-fold), respectively (\( P < 0.05 \)). However, 150 \( \mu \)M SASP did not significantly change the celastrol activity in the SF-539 cultures. Thus, the effects of SASP inhibition are probably related to the basal expression level of SLC7A11. The result shown in Fig. 1C confirms that the SF-295 and SNB-19 cells are more resistant to celastrol than the SF-539 cells and that such resistance can be reduced by \( \text{x}_c \) inhibition.

We next examined the dose-dependent effects of SASP on the growth-inhibitory activity of celastrol in SNB-19 and SF-539 cultures. The celastrol efficacy increased in the presence of SASP in a dose-dependent manner for both SNB-19 and SF-539 cell lines (Fig. 1D). For SF-539 cells, 300 \( \mu \)M SASP resulted in a significant increase of celastrol potency, whereas 600 \( \mu \)M SASP alone led to cell death.

siRNA targeting SLC7A11 was also used to treat the SF-295 glioma and the A549 lung cancer cell lines to specifically down-regulate SLC7A11 expression. SLC7A11 mRNA expression was measured by RT-PCR 48 h after transfection (Fig. 2A). We compared the efficacy of celastrol in siRNA-treated cultures with that in control cultures treated with nonsilencing control siRNA. After down-regulation of SLC7A11, SF-295 cells showed a significant increase in sensitivity to celastrol (the IC\text{50} of celastrol decreased from 1.6 ± 0.04 to 0.9 ± 0.005 \( \mu \)M, \( P < 0.05 \)) (Fig. 2B). Similar results were found with A549 cultures. These results further confirm that SLC7A11 has a role in cellular resistance to celastrol.

The \( \text{x}_c \) Transporter Mediates Resistance to Celastrol by Supplying Cystine for GSH Synthesis. To confirm that the role of the \( \text{x}_c \) transporter in celastrol resistance was related to GSH synthesis, we suppressed GSH synthesis via a different path, using BSO, a potent inhibitor of \( \gamma \)-glutamylcysteine synthetase, the rate-limiting enzyme for GSH synthesis (Wu et al., 2004). BSO (200 \( \mu \)M) increased the potency of celastrol in SNB-19 cultures by 1.8-fold (\( P < 0.05 \)) (Fig. 3A). Furthermore, we evaluated the effects of NAC, as a precursor of GSH, in modulating the activity of celastrol. In the presence of 5 mM NAC, the IC\text{50} of celastrol increased from 1.8 ± 0.08 to 19 ± 0.06 \( \mu \)M (\( P < 0.05 \)) (Fig. 3B). These results indicate that GSH plays a role in celastrol resistance.

To confirm that chemosensitizing activity of SASP in the glioma cell cultures was based on reduction of GSH synthesis via cystine starvation, we incubated SNB-19 cells with SASP, \( \beta \)-mercaptoethanol (\( \beta \)-ME), or both for 24 h in Fischer’s medium, which contains cystine at a concentration (~84 \( \mu \)M) close to cystine concentrations in human plasma (Chawla et al., 1984). As shown in Fig. 3C, a 24-h incubation of SNB-19 cells with 0.5 mM SASP led to marked depletion of their intracellular total GSH contents from 69.6 ± 3.5 to 17.9 ± 1.4 nmol/mg protein (\( P < 0.001 \)). The SASP-induced GSH depletion was partially prevented by \( \beta \)-ME (100 \( \mu \)M) to 45.2 ± 0.9 nmol/mg protein (\( P < 0.01 \)). Because \( \beta \)-ME allows cellular uptake of cystine via a route circumventing the \( \text{x}_c \) transporter (Ishii et al., 1981), this result indicates that the GSH depletion by SASP was largely based on inhibition of cystine uptake, as previously described for another system (Gout et al., 2001).

Cystine Deficiency or SASP Treatment Inhibits Glioma Cell Proliferation. Cystine or cysteine, its reduced form, is a nonessential amino acid, because it is normally generated by the liver via the trans-sulfuration pathway.
This pathway involves metabolism of methionine (an essential amino acid) to cystathionine and its subsequent cleavage by γ-cystathionase to α-ketobutyrate and cysteine (Lo et al., 2008a). Previous studies have shown that certain cancer cells cannot generate cysteine/cystine and depend on uptake of the amino acid from the microenvironment to maintain growth and viability (Gout et al., 2001). To determine whether glioma cell lines require exogenous cystine for growth and survival, we cultured the cells in the presence and absence of methionine (as a positive control), cystine, and/or cystathionine in various combinations. Robust growth of gliomas cell lines SF-295, SF-539, and SNB-19 was only observed in cultures containing both methionine and cystine (Fig. 4A), demonstrating that the absence of either amino acid inhibited survival and proliferation of these cancer cells. Partial growth was observed for SF-295 and SNB-19 cells in medium containing methionine and cystathionine (Fig. 4A), indicating that cystathionine could not fully substitute for cystine and that these cell lines lack a fully operative trans-sulfuration pathway. These results are consistent with studies in which gliomas were found to be dependent on \( \chi_\text{cysteine} \)-mediated uptake of cystine for growth and survival and that inhibition by SASP could offer an effective treatment (Chung et al., 2005).

We next evaluated the antiproliferative activity of SASP in the same panel of glioma cell lines. SASP inhibited growth of all three cell lines, with the SF-539 line being most sensitive (Fig. 4B). The lower sensitivity of the SNB-19 and SF-295 cells to SASP correlates with a higher level of SLC7A11 expression in these cells. However, although SASP alone shows cytotoxicity in glioma cultures, the IC\(_{50}\)s were as high as \(-1 \text{ mM} \) when we used the regular RPMI 1640 medium in the study. In addition, SASP on its own only showed cytostatic activity, with minimal apoptosis and necrosis induction. These results support the notion that SASP could be more effective for glioma therapy when used in combination with other anticancer agents such as celastrol.

**SASP and Celastrol Show Synergistic Inhibitory Effects on Growth in Glioma Cell Cultures.** To evaluate the effects of combined use of SASP and celastrol, dose-response curves were generated for SASP alone, celastrol alone, and for a combination of SASP and celastrol in SNB-19 cell cultures (Fig. 5A). In SNB-19 cultures, celastrol alone had an IC\(_{50}\) of 1.8 ± 0.03 mM, whereas SASP alone had an IC\(_{50}\) of 1.03 ± 0.26 mM. When the two drugs were combined in a 1:1200 concentration ratio, the IC\(_{50}\) was reduced to 0.68 ± 0.16 mM (Fig. 5A). To evaluate the nature of the interaction between SASP and celastrol, combination analyses were performed with the combination index (CI) method (Chou and Talalay, 1984; Chou et al., 1994), using the drugs at a constant concentration ratio (celastrol: SASP = 1:1200). As can be seen from Fig. 5B, the combination produced additive or synergistic interaction at high fractional effects (CI values <1 indicate synergistic activity). We also tested other concentration ratios, and, although we consistently obtained synergism, we observed the maximal level of synergism at the 1:1200 ratio.
SASP Increases Celastrol- or GA-Mediated Down-Regulation of the Hsp90 Client Protein, EGFR, in Glioma Cell Lines. Both celastrol and GA have been shown to disrupt the Hsp90 complex in various types of tumors. To further confirm the synergistic effect of SASP with celastrol or GA, we treated SF-295 cultures with GA (1 mM), celastrol (1 or 5 μM), or SASP (1 mM) individually or with combinations of GA+SASP and celastrol+SASP. The SF-295 line was used instead of the SNB-19 line because it has higher basal levels of the Hsp90 client protein, EGFR. GA decreased the levels of EGFR protein in a concentration-dependent manner, whereas celastrol (1 or 5 μM) alone did not lead to significant EGFR down-regulation. The presence of SASP led to increased down-regulation of EGFR (Fig. 6).

SASP Increased Celastrol-Induced G2/M Arrest in Glioma Cell Lines. We performed cell cycle analyses to determine whether combined treatment of SASP and celastrol could synergistically induce arrest in the cell cycle. SNB-19 cells were incubated for 24 h with celastrol alone (1 and 5 μM), SASP alone (600 μM), or with a combination of the two drugs. Celastrol at 1 μM did not induce a significant change in the cell cycling (Fig. 7), whereas 5 μM celastrol increased the G2/M phase from 22.9% to 35.2% and increased the S phase from 6.2 to 21.1% (data not shown). SASP (600 μM) alone did not lead to a cell-cycle change. However, when cultures were treated with both celastrol (1 μM) and SASP (600 μM), the progression of cells through the cell cycle was markedly arrested in the G2/M phase to 46.6% (Fig. 7). Combined use of 5 μM celastrol and 600 μM SASP also showed increased G2/M arrest (data not shown). We similarly observed synergistic effects of SASP and GA in cell-cycle arrest of SNB-19 cells (data not shown).

SASP Increases Celastrol-Induced Apoptosis in Glioma Cell Lines. Induction of apoptosis by celastrol (1 or 5 μM), SASP (600 μM), or by combinations of the two drugs was evaluated in SNB-19 cell cultures. The cultures were incubated for 72 h with different combinations of the drugs, and the degree of apoptosis was examined by annexin V binding and PI staining assay. As shown by representative plots (Fig. 8), celastrol at concentrations of 5 μM induced significant apoptosis in SNB-19 cells but not at a concentration of 1 μM. SASP at 600 μM, a noncytotoxic concentration, did not induce apoptosis either. However, when the cells were exposed to a combination of 1 μM celastrol and 600 μM SASP, there was a significant increase in the number of apoptotic cells. When a combination of 5 μM celastrol and SASP was used, the majority of the cells were present in late apoptotic stage (93.9%). Similar apoptosis inductions were demonstrated with the other two glioma cell lines (data not shown).

Discussion
In considering molecular targets for clinical therapy of malignant gliomas, it is still not clear which genes and/or proteins are most critical in the development of the malignancy, including its tissue invasion and resistance to chemo-
therapy. In the present study, two potential therapeutic targets for gliomas were evaluated: the molecular chaperone Hsp90 and the cystine/glutamate antiporter x<sup>c</sup>-.<br>

Hsp90 is involved in the maturation and stability of a number of proteins critical for proliferation of glioma cells. By targeting Hsp90 protein via blocking of ATP binding, the benzoquinone ansamycin antibiotic, GA, and its analogs have exhibited significant antitumor effects on glioma cell lines (Neckers, 2002). However, GA and its analogs are substrates of P-glycoprotein, which impedes their penetration across the blood-brain barrier, hence limiting their use for brain tumor therapy (Hay et al., 2004; Huang et al., 2007). Our results show that celastrol is unaffected by P-glycoprotein-mediated efflux, suggesting that it may be a better drug for such therapy. Celastrol also exerted anticancer effects on in vitro glioma models (Huang et al., 2008b). Yet, celastrol is not a specific inhibitor of Hsp90, because its actions involve multiple targets. Thus, the effects of celastrol on cell viability, apoptosis, and progression through the cell cycle shown in this study (Figs. 7 and 8) may not necessarily be due to Hsp90 inhibition. In addition, based on the results shown in Fig. 6, the effect of celastrol on EGFR regulation is not as strong as that of GA. In a previous study (Zhang et al., 2008), celastrol (0.1–100 μM), unlike GA, did not interfere with ATP binding to Hsp90 but disrupted the Hsp90-Cdc37 interaction in the pancreatic cancer cell line Panc-1. Celastrol (1–5 μM) induced Hsp90 client protein degradation (Cdk4 and Akt). However, EGFR levels were not tested in the study by Zhang et al. (2008). In the present study, we were the first to show the effects of celastrol alone or in combination with SASP on EGFR protein levels. Studies of the effects of celastrol on other client proteins of Hsp90 seem to be warranted.

The x<sup>c</sup>- transporter mediates cystine entry into the cell in exchange for intracellular glutamate. Once inside the cell, cystine is rapidly reduced to cysteine, the limiting precursor for GSH synthesis. This transporter has been shown to play an important role in glioma growth and tissue invasion, serving as an essential path for uptake of cystine for synthesis of GSH and release of neurotoxic glutamate, which promotes glioma invasion (Chung et al., 2005). Expression of the SLC7A11 gene, which encodes the light chain subunit of the x<sup>c</sup>- cystine/glutamate antiporter, showed the strongest negative correlation with anticancer activity of celastrol, as indicated by our pharmacogenomic analysis of multiple transporter genes. This SLC7A11-celastrol correlation was validated with the use of several x<sup>c</sup>- inhibitors. Such inhibitors, including SASP and in particular glutamate, a highly specific x<sup>c</sup>- inhibitor, reduced the levels of GSH in glioma cells and enhanced their sensitivity to celastrol (Fig. 1). Therefore, inhibition of x<sup>c</sup>- aimed at depletion of GSH, a compound reported to promote drug resistance via removal of free radicals and drug detoxification (Wu et al., 2004), seems to be a valid approach for sensitizing gliomas to celastrol and possibly other anticancer agents. Consistent with our results, quinone methides were known to be reactive electrophiles, which are reactive toward GSH (Kupfer et al., 2002). Our results were obtained from three selected glioma cell lines. We also used the lung cancer cell line A549 in this study and obtained consistent results (unpublished results). A wider panel of glioma cells will need to be used to provide broader representation of these findings. This finding is relevant from a translational research perspective and could lead to a new direction in glioma therapy, e.g., rendering gliomas vulnerable to Hsp90 inhibitors via pretreatment with SASP at patient-tolerated

Fig. 8. Apoptotic index analysis of SNB-19 cells exposed to SASP and celastrol. SNB-19 cells were treated with vehicle, celastrol alone (1 or 5 μM), SASP alone (600 μM) or a combination of the two drugs for 72 h. Cells were then stained with annexin V and PI and analyzed using flow cytometry. The combination of celastrol and SASP leads to enhanced apoptosis in glioma cells.
doses (~0.2 mM) (Guastavino et al., 1988), and perhaps other types of cancer with elevated \( x_c \) activities.

There are several commonly used \( x_c \) inhibitors. Glutamate is the main exchange substrate of the \( x_c \) transporter; as such, it is a potent, highly specific inhibitor of \( x_c \)-mediated cystine uptake. Glutamate can severely inhibit or completely arrest in vitro proliferation of malignant cells that depend on \( x_c \)-mediated uptake of cystine for growth. However, glutamate cannot be used as a therapeutic in vivo due to its neurotoxicity (Lo et al., 2008b). Another potent inhibitor is S-4-CPG (Lo et al., 2008b), which in the micromolar range has antiproliferative and cytotoxic effects in a number of glioma cell lines (Chung et al., 2005). Unlike glutamate, S-4-CPG is a nonsubstrate inhibitor for \( x_c \), which induces intracellular GSH depletion without glutamate efflux. However, S-4-CPG is also an antagonist for group I metabotropic glutamate receptors with an IC\(_{50}\) of 4 \( \times \) 10 \(^{-6}\) M (Savaskan et al., 2009). Another drug used for \( x_c \) inhibition is SASP, used for over 60 years for treatment of Crohn’s disease and rheumatoid arthritis. Although oral administration of SASP leads to degradation by intestinal bacteria to sulfapyridine and 5-aminosalicylic acid lacking \( x_c \)-inhibitory activity (Gout et al., 2001), levels of ~0.2 mM SASP in the plasma can be reached (Guastavino et al., 1988). The growth of cultures of lymphoma cells and certain breast, prostate, lung, and pancreatic cancer cells can be completely inhibited by SASP at patient tolerated levels via specific inhibition of \( x_c \) (Lo et al., 2008a,b). In animals bearing transplants of lymphomas, gliomas or prostate, lung, and pancreatic cancers, when administered intraperitoneally, SASP acts primarily as a cytostatic agent, without leading to major toxicity in the hosts (Gout et al., 2001, 2003; Chung et al., 2005; Dooxsee et al., 2007; Lo et al., 2008b). Although SASP also inhibits NF-kB and GSH S-transferase, its growth-inhibitory activity was found to be specifically based on \( x_c \)-inhibition (leading to cysteine and GSH depletion) and not on NF-kB inhibition (Chung and Sontheimer, 2009). For the synergism studies based on cell cycle, EGFR down-regulation, and apoptosis analysis, the concentration we chose (0.6–1 mM) was based on those concentrations markedly demonstrating synergic cytotoxic effects. At these concentrations, SASP may also inhibit NF-kB. Therefore, the mechanisms of SASP underlying its sensitizing effect need further investigation. Although the concentrations of SASP as low as 0.15 mM significantly increased celastrol potency (Fig. 1D), in the synergistic studies, we selected to use higher concentrations (0.5–1 mM) to prove the concept for achieving a maximal synergistic effect. Additional in vitro and in vivo studies are needed to find the minimal effective doses for tested drugs. However, this approach should not be a limitation for translational research on gliomas, because local drug delivery is a feasible method.

Multiple factors may contribute to cellular sensitivity and resistance to celastrol and other Hsp90 inhibitors. More recently, membrane transporters have been recognized as important determinants of drug disposition in the body and cellular entry and extrusion of drugs. As such, they can markedly affect efficacy of cancer therapy (Huang and Sadée, 2006). Our study was focused on the role of transporter genes in drug resistance. We used a custom-designed microarray to analyze mRNA expression of a majority of human membrane transporters in the NCI-60 cell line panel (Huang et al., 2004). By correlating transporter expression levels with the growth-inhibitory potencies of multiple anticancer drugs in the NCI-60 cell lines, we previously identified known drug-transporter interactions and suggested novel ones (Huang et al., 2008a). Expression levels of several transporter and non-transporter genes significantly correlated with growth-inhibitory potency of celastrol (Table 1). Although all identified genes will need to be investigated for their pharmacological functions in drug resistance and sensitivity, we first focused on the interactions between SLC7A11 and celastrol because of relatively high expression levels of SLC7A11 in glioma cells. Other genes will be investigated for their pharmacological functions in separate studies. Although the present study is not an unbiased genome-wide screening for chemosensitivity and chemoresistance genes, it provides an approach for associating groups of potentially novel compounds, including natural products with relevant genes or gene families. The results obtained also suggest that SLC7A11 expression could serve as a genomic biomarker for predicting cellular responses to Hsp90 inhibitors and that the \( x_c \) transporter provides a target for sensitizing target cells to such drugs.

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References


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