Mechanism of Selectivity of an Angiogenesis Inhibitor From Screening a Genome-Wide Set of Saccharomyces cerevisiae Deletion Strains


Background: The synthetic tripeptide arsenical 4-(N-(S-glutathionylacetyl)amino) phenylarsenoxide (GSAO) is an angiogenesis inhibitor that targets the mitochondria of actively dividing but not quiescent endothelial cells, arresting their proliferation and causing apoptosis. Normal endothelial cells are much more sensitive to GSAO than tumor cells. To elucidate the mechanism of tumor cell resistance, we identified yeast genes that are necessary for resistance to GSAO.

Methods: We screened a genome-wide set of 4546 Saccharomyces cerevisiae deletion strains to identify GSAO-sensitive strains. We then examined GSAO accumulation in and proliferation activity of endothelial cells (BAECs) and tumor cells treated with GSAO and modulators of pathways and proteins identified in the yeast screen. We also examined GSAO effects on proliferation of mammalian cells transfected with transporter protein constructs.

Results: Eighty-eight deletion strains were sensitive to GSAO. The most sensitive strains had deletions of genes whose products are involved in vacuolar function (corresponding to drug transport in mammalian cells) and glutathione synthesis. BAECs were more sensitive to GSAO than tumor cells, and cell sensitivity to GSAO was approximately proportional to cellular glutathione levels. Treatment of BAECs and tumor cells with MK-571, an inhibitor of multidrug resistance–associated protein (MRP), or with buthionine sulfoximine, an inhibitor of glutathione synthesis, increased their sensitivity to GSAO. Mammalian cells transfected with MRP1 or MRP2 were resistant to GSAO, whereas cells transfected with MRP3, MRP4, MRP5, P-glycoprotein, or breast cancer resistance protein were not.

Conclusions: Differences in MRP activity and cellular glutathione levels contribute to the selectivity of GSAO for endothelial versus tumor cells. MRP1 and/or MRP2 may transport GSAO from resistant cells, with glutathione acting as a cotransporter. Genetic screening in yeast is a powerful tool for understanding drug action in mammalian cells. [J Natl Cancer Inst 2005;97:1539–47]

Tumor expansion and metastasis depend on tumor blood vessel formation, or angiogenesis. We recently described a synthetic tripeptide trivalent arsenical (GSAO; 4-(N-(S-glutathionylacetyl)amino) phenylarsenoxide) that causes increases in superoxide levels, proliferation arrest, mitochondrial depolarization, and apoptosis of proliferating, but not quiescent, endothelial cells in vitro and that inhibits angiogenesis in vivo (1). GSAO inactivates the mitochondrial adenine nucleotide translocase (ANT) by cross-linking the matrix-facing thiols of Cys-160 and Cys-257 (1,2). ANT exchanges matrix ATP for cytosolic ADP across the inner mitochondrial membrane and is the key component of the mitochondrial permeability transition pore (3,4).

The strong selectivity of GSAO for proliferating relative to nonproliferating endothelial cells is a consequence of the higher mitochondrial calcium ion levels in proliferating cells. ANT is a calcium receptor whose conformation and activity changes on binding of calcium ions; calcium-bound ANT has a much stronger affinity for GSAO than the calcium-free form. This mechanism is supported by the crystal structure of the calcium-free form of bovine ANT (5). The cysteine pair (Cys-160 and Cys-257) that GSAO cross-links in the calcium-replete form are 18.2 Å apart in the calcium-free structure, which is at least twice the distance required for them to interact with trivalent arsenicals.

GSAO is also a selective inhibitor of proliferating endothelial cells compared with tumor cells (1): The IC50 (i.e., concentration required for 50% inhibition) for proliferation arrest of bovine or human primary endothelial cells was 10–15 μM in a 3-day proliferation assay, whereas 3- to more than 32-fold higher concentrations of GSAO were necessary to induce proliferation arrest in all tumor cell lines tested. These cell culture observations were borne out in vivo (1); we found that systemic administration of GSAO to immunodeficient mice bearing human pancreatic BxPC-3 carcinoma tumors reduced tumor vascularity but had no effect on the proliferative index of the tumor cells.

To elucidate the mechanism of the selectivity of GSAO for endothelial versus tumor cells, we screened a genome-wide set of Saccharomyces cerevisiae deletion strains to identify yeast genes that are necessary for resistance to GSAO. We used a bank of yeast homozygous diploid deletions for all nonessential genes from the Saccharomyces Gene Deletion Project (6). The availability of the deletion strains has enabled the identification of mechanisms by which yeast can protect themselves against oxidants such as inorganic hydroperoxide (H2O2), organic peroxide (cumene hydroperoxide), lipid linoleic-13-hydroperoxide, and a superoxide generating agent (menadione) (7,8), and it seemed likely that this approach would identify yeast genes necessary for GSAO resistance as well. The sheer number of

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deletion strains in the bank that we used (n = 4546) makes it likely that functions or pathways that confer resistance to GSAO will be identified. After we identified the yeast genes that were most important for GSAO resistance, we probed the function of corresponding genes and pathways in mammalian cells with pharmacologic interventions and transfected cells. We also used inhibitor studies to investigate the basis of the differential sensitivity of endothelial and tumor cells.

**MATERIALS AND METHODS**

**Chemicals**

GSAO (Mr = 548) was produced as described previously (1,9) to a purity greater than 94% (as determined by high-performance liquid chromatography). The solid was dissolved in phosphate-buffered saline (PBS; 8.1 mM Na2HPO4, 1.5 mM KH2PO4, 138 mM Na Cl, 2.7 mM KCl, pH 7.0), and the concentration of active trivalent arsenical was measured by titrating with dimercaptopyrrolopan and calculating the remaining free thiols with 5,5'-dithiobis(2-nitrobenzoic acid) (1). The titrated GSAO was sterile-filtered and stored at 4 °C in the dark until use. Stock solutions of GSAO were stable for at least 1 month and were diluted in sterile PBS just before use. Stock solutions of GSAO, buthionine sulfoximine (BSO; Sigma-Aldrich, St. Louis, MO), reduced glutathione (Sigma-Aldrich), reduced glutathione ethyl ester (Sigma-Aldrich), and sodium orthovanadate (Sigma-Aldrich) were made in PBS. The pump inhibitors MK-571 (Cayman Chemical, Ann Arbor, MI) and pGp-4008 (Sigma-Aldrich) were dissolved in dimethyl sulfoxide.

**Yeast Strains and Growth Conditions**

BY4743 yeast with homozygous diploid deletions for all non-essential genes from the *Saccharomyces* Gene Deletion Project were obtained from EUROSCARF (Frankfurt, Germany) (6). Cells were grown in liquid YEPD (2% wt/vol glucose, 2% wt/vol bacteriological peptone, and 1% yeast extract). Plates (96 well) containing 0.5 and 2 mM GSAO were prepared and stored overnight at 4 °C. Cells were seeded in the plates using a 96-pin replicator and incubated at 30 °C for 24 hours. Growth was measured by light scattering at 600 nm using a microplate reader (Thermomax Plus; Molecular Devices, Palo Alto, CA). Deletion strains that showed no or impaired growth relative to wild type at either concentration were scored as sensitive. GSAO at 2 mM had no effect on growth of wild-type yeast. These strains were then tested on plates containing a range of GSAO concentrations (0.001–1 mM).

**Cell and Culture Conditions**

Bovine aortic endothelial cells (BAECs) were from Cell Applications (San Diego, CA). HT1080 fibrosarcoma cells, Panc-1 and BxPC-3 pancreatic cancer cells, and Caco-2 cells were from the American Type Culture Collection (Manassas, VA). BAEC, HT1080, Panc-1, and Caco-2 cells were cultured in Dulbecco's modified Eagle medium, and BxPC-3 cells were cultured in RPMI 1640 medium. All cells were supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 5 U/mL penicillin–streptomycin (Gibco, Gaithersburg, MD). Trypsin–EDTA solution, which was used to detach cells from culture plates, was also from Gibco.

Madin-Darby canine kidney II (MDCKII) polarized epithelial cell lines and transfectedants that overexpress multidrug resistance–associated proteins (MRPs) 1, 2, or 3 (10,11) were obtained from P. Borst, The Netherlands Cancer Institute, Amsterdam, as were human embryo kidney (HEK293) cells and transfectedants overexpressing MRP4 (12) or MRP5 (13). The mouse embryo fibroblast line MEF3.8, which lacks functional P-glycoprotein (P-gp) and Mrp1 and expresses very low levels of the breast cancer resistance protein Bcrp1 (14), and the MEF/Bcrp1 clone BA2 (15), which overexpresses mouse Bcrp1, were kindly supplied by A. Schinkel, The Netherlands Cancer Institute, Amsterdam. The MEF/MDR1 clone H4, which overexpresses human P-gp, was produced in a manner directly analogous to the MEF/Bcrp1 clone (15)—i.e., by transducing MEF3.8 cells with the retroviral construct LZRS-MDR1-IRES-GFP vector and selecting clones by flow cytometry for GFP expression and fluorescent substrate (rhodamine 123). Cells were grown and maintained as adherent monolayers in Dulbecco’s modified Eagle medium containing 10% supplemented calf serum (Cosmic; Hyclone, Tauranga, New Zealand), 100 μg/mL penicillin, and 60 μg/mL streptomycin.

**GSAO Flux in BAECs Treated With Drug Transport Inhibitors and Glutathione Modulators**

BAECs were seeded at a density of 7 × 10⁴ cells per well in 12-well plates and allowed to adhere overnight. Cells were then treated with buthionine sulfoximine (BSO) (200 μM) or reduced glutathione (2 mM) for 24 hours at 37 °C or with MK-571 (25 μM), pGp-4008 (5 μM) or sodium o-vanadate (500 μM) for 10 min at 37 °C. GSAO was then added to a final concentration of 100 μM, and the cells were incubated for 4 hours at 37 °C. The cells were then washed three times with ice-cold PBS, lysed in 0.3 mL of 70% nitric acid and 0.7 mL of PBS, and kept at 4 °C until analysis. Lysates were diluted 10-fold in PBS and analyzed for arsenic atoms (which are released from GSAO in the presence of 70% nitric acid) using an Elan 6100 inductively coupled plasma spectrometer (Perkin Elmer Sciex Instruments, Shelton, CT). Results are from triplicate determinations from two or three separate experiments (see figure legends for details).

**Cell Proliferation Assays**

BAEC, BxPC-3, HT1080, and Panc-1 cells were seeded at densities of 1.5 × 10³, 1 × 10⁴, 3 × 10³, and 6 × 10³ cells per well, respectively, in 96-well plates and allowed to adhere overnight. The medium was then changed, and the cells were cultured for an additional 72 hours. Viable attached cells were determined by incubating cells with the tetrazolium salt MTT (Sigma, St. Louis, MO), which is metabolized by viable cells to form insoluble purple formazan crystals. Dimethyl sulfoxide was added to lyse the cells, the contents of the wells were homogenized by shaking, and the absorbance at 550 nm was measured. Cell growth in the untreated control sample was normalized as 100%, and growth for all treatments was expressed as percentage of control. IC₅₀ values (i.e., the drug concentration that inhibits cell proliferation by 50%) for GSAO were determined from these plots. Results are from duplicate determinations from four to seven separate experiments (see figure legends for details).
Measurement of Cellular Glutathione

BAEC, HT1080, Panc-1, and BxPC-3 cells were seeded in 75-cm² flasks and allowed to adhere overnight. The medium was replaced with fresh medium containing 10% fetal calf serum, and the cells were cultured for 72 hours. Cells were detached, counted, and centrifuged at 3000 g for 5 min at 4 °C. Dried pellets of 2 x 10⁶ cells were kept at −80 °C until use. Reduced glutathione concentration was measured using a Bioxitych GSH-400 Assay Kit (Oxis Health Products, Portland, OR) according to the manufacturer’s instructions.

Statistical Analyses

Data are presented as means with 95% confidence intervals (CIs). All variables were examined for normality and homogeneity of variance. Normality of the distributions was assessed using the Shapiro-Wilk test. One-way analysis of variance and post-hoc tests, which were parametric or nonparametric as appropriate, were used to compare mean values. All analyses were performed using the statistical package SPSS version 13 (SPSS, Chicago, IL). All tests of statistical significance were two-sided, and P values < .05 were considered statistically significant.

RESULTS

Identification of Sensitive Yeast Strains

The growth of the S. cerevisiae deletion strains was compared in the absence and presence (of 0.5 or 2 mM) GSAO for 24 hours at 30 °C. Of the 4546 deletion strains, 88 (1.9%) were found to be sensitive to GSAO (Supplementary figure available at http://jncicancerspectrum.oxfordjournals.org/jnci/content/vol97/issue20). The IC₅₀ for GSAO was determined for each of the 88 strains using a range of GSAO concentrations from 0.001 to 1 mM. For reference, the IC₅₀ for GSAO ranged from greater than 4 mM (wild type, BY4743) to 0.027 mM (ycf1 deletion strain). The 27 most sensitive strains, in order of sensitivity, are shown in Fig. 1. For clarity of presentation, the different categories of yeast gene(s) and the corresponding mammalian homologue(s) necessary for resistance to GSAO have been discussed together.

Drug Transporters

The yeast strain lacking YCF1 was the most sensitive to GSAO, with an IC₅₀ of 0.027 mM (Fig. 1). Given that GSAO resembles a glutathione S-conjugate of phenylarsenoxide and that Ycf1 mediates transport of glutathione S-conjugates into the yeast vacuole, it is possible that Ycf1 recognizes GSAO as a detoxified phenylarsenoxide. Other highly sensitive deletion strains include those lacking several other genes implicated in vacuole biogenesis (especially PEP3, but also KCS1 and VID28; Fig. 1), underscoring the crucial role of the vacuole for resistance to GSAO. The strain lacking YAP1, a transcriptional activator of YCF1 (20,21), was also sensitive to GSAO (supplementary figure).

YCF1 encodes an ATP-binding cassette (ABC) protein (22). Mammalian ABC proteins include P-glycoprotein, P-gp (ABCB1), the breast cancer resistance protein BCRP (ABCG2), and the multidrug resistance–associated proteins (MRP) 1 to MRP7. In mammalian cells, MRPI has been characterized as a transporter of multivalent organic anions, preferentially glutathione S-conjugates (23,24) but also sulfate conjugates, glucuronides, oxidized glutathione, and complexes of reduced glutathione with As(III) (24,25). Ycf1, therefore, is considered the structural and functional homologue of human MRPI (26). P-gp, the product of the MDR1 gene (27,28), and BCRP transport amphiphilic compounds with apparently no other defining features (29), whereas the MRP class members are predominantly transporters of organic amines (37).

To determine if GSAO is transported from mammalian cells by an MRP class transporter, BAECs were treated with GSAO for 4 hours in the presence or absence of the MRP inhibitor...
MK-571 (23). Treatment with MK-571 resulted in 2.2-fold increase in accumulation of arsenic in BAECs (Fig. 2, B). MRP activity requires energy in the form of ATP, and a similar increase in arsenic accumulation was observed in cells treated with sodium o-vanadate, an ATPase type P inhibitor (Fig. 2, B). By contrast, treatment with pGp-4008, a specific inhibitor of P-gp (30), did not result in arsenic accumulation. These results are consistent with the idea that GSAO is a substrate for MRP.

The observation that inhibition of MRP activity resulted in accumulation of GSAO (as measured by an increase in arsenic) in BAECs implies that blocking MRP activity should also decrease the IC₅₀ of GSAO for inhibition of BAEC proliferation. Indeed, treatment of BAECs with MK-571 caused a dose-dependent decrease in the GSAO IC₅₀ (Fig. 2, C). The IC₅₀ decreased by 10-fold, from 10.2 μM in the absence of MK-571 to 1.1 μM at the highest concentration of MK-571 (mean difference = 9.2 μM; 95% CI on the difference = 7.8 to 10.6 μM). Treatment with the P-gp inhibitor pGp-4008 did not change the GSAO IC₅₀ (Fig. 2, D). In the absence of GSAO, neither MK-571 nor pGp-4008 alone affected BAEC proliferation at the highest concentration used (data not shown). The results presented in Fig. 2 demonstrate that GSAO is pumped from endothelial cells by MRP.

To identify the MRP subclass proteins that are responsible for exporting GSAO from mammalian cells, we treated transfected cells that overexpress MRP1, MRP2, MRP3, MRP4, MRP5, P-gp, or BCRP with increasing concentrations of GSAO and compared their proliferation with that of GSAO-treated parental cells (Figs. 3, A–C). Transfected cells overexpressing MRP1 or MRP2 were more resistant to GSAO than untransfected cells, whereas overexpression of MRP3, MRP4, MRP5, P-gp, or BCRP did not confer resistance to GSAO. However, expression of MRP1 and MRP2 in endothelial and tumor cells as detected by immunoblotting was not consistent with their patterns of GSAO resistance. The IC₅₀s for GSAO inhibition of BAEC, HT1080, Panc-1, and BxPC-3 cells were 10, 25, 43, and 270 μM, respectively (see below). MRP1 protein was expressed at highest levels in Panc-1 tumor cells, at intermediate levels in BAEC endothelial cells, and at lowest levels in HT1080 and BxPC-3 tumor cells, and only BxPC-3 cells contained detectable levels of MRP2. In this context, it is important to note that the expression level of MRPs may not coincide with their functional activity. For instance, Miller et al. (31) have shown that MRP1 expression in Panc-1 does not necessarily correlate with activity, perhaps due to the presence of nonfunctional MRP1 or MRP1 located within the membranes of intracellular organelles.

Glutathione Synthesis Pathway Components

When the 88 genes conferring resistance to GSAO were grouped into functional categories, the most striking feature was the abundance of genes involved in sulfur metabolism and sulfate assimilation. Moreover, of the 13 S. cerevisiae strains with the highest sensitivity to GSAO, eight lacked genes whose products are involved in sulfate assimilation or in sulfur amino acid and glutathione synthesis (Figs. 1 and 4). The most sensitive deletion strains in this group (cys3, cys4, and gsh1) are all defective in the central pathway of cysteine and glutathione synthesis. No alternative pathway for de novo cysteine production is available to S. cerevisiae in the absence of these genes. All of the genes encoding enzymes involved in sulfate assimilation (from

<table>
<thead>
<tr>
<th>Deleted gene</th>
<th>Process</th>
<th>IC₅₀, mM</th>
<th>Function of the gene product</th>
</tr>
</thead>
<tbody>
<tr>
<td>YCF1</td>
<td></td>
<td>0.027</td>
<td>Homologue of the mammalian Multidrug Resistance–associated Protein (MRP1)</td>
</tr>
<tr>
<td>CYT4</td>
<td></td>
<td>0.032</td>
<td>Enzyme that converts homocysteine to cystathionine</td>
</tr>
<tr>
<td>CYT3</td>
<td></td>
<td>0.043</td>
<td>Enzyme that converts cystathionine to cysteine</td>
</tr>
<tr>
<td>PEP3</td>
<td></td>
<td>0.045</td>
<td>Vacuolar membrane protein involved in protein trafficking</td>
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<td>SOD1</td>
<td></td>
<td>0.049</td>
<td>Cu/Zn superoxide dismutase</td>
</tr>
<tr>
<td>GSH1</td>
<td></td>
<td>0.054</td>
<td>Enzyme that converts cysteine to γ-glutamylcysteine</td>
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<td>0.062</td>
<td>Unknown function</td>
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<tr>
<td>MET8</td>
<td></td>
<td>0.070</td>
<td>Transcriptional activator regulating expression of 3’-phosphoadenylylsulfate reductase and SO₃ reductase</td>
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<tr>
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<td>Enzyme that converts adenylylsulfate to 3’-phosphoadenylylsulfate</td>
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<td>0.080</td>
<td>β subunit of enzyme that converts SO₃ to H₂S</td>
</tr>
<tr>
<td>MET6</td>
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<td>Enzyme that converts 3’-phosphoadenylylsulfate to SO₃</td>
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<td>0.096</td>
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<td>Cyclin-dependent kinase involved in stress response</td>
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<td>Heat shock protein involved in mitochondorial protein import</td>
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<td>CCR4</td>
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<td>0.130</td>
<td>Transcriptional regulator for some glucose-repressed genes</td>
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<td>Enzyme that converts α-acetylimmoniumcarboxylate and H₂S to homocysteine</td>
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</table>

Fig. 1. The 27 Saccharomyces cerevisiae deletion strains most sensitive to 4-(N-(S-glutathionylacetyl)amino) phenylarsenoxide (GSAO). The 50% inhibitory concentration (IC₅₀) values for inhibition of growth and the function of the gene product are indicated. Green, yellow, pink, and red indicate genes that are involved in drug transport, cysteine and glutathione biosynthesis, oxidative stress, and general defense mechanisms, respectively.
was determined by measuring GSAO arsenic atoms using inductively coupled plasma spectrometry. The data points and error bars are means and upper 95% confidence interval from a representative of two experiments performed in triplicate. ***, P<.001 compared with control (Nil). C, D) Effect of MK-571 (C) or (pGp-4008 (D) treatment GSAO inhibition of BAEC proliferation. The IC_{50} for GSAO was determined in a 72-hour proliferation assay. The data points represent the mean values obtained from three to six experiments performed in duplicate.

SO_{4}^{2-} to γ-glutamylcysteine are included in the 27 most sensitive deletion strains (met14, met16, ecm17, met10, and met17). The MET8 gene product controls the expression of both PAPS reductase (encoded by MET16) and sulfite reductase (encoded by ECM17), and the met8 deletion strain was among the most sensitive to GSAO. The carbon backbone of homocysteine derives from L-aspartate (Fig. 4). Most of the enzymes of the homocysteine biosynthetic pathway (Hom3, Hom2, and Hom6) were required for full GSAO resistance. Met28, Cbf1, and Yap1 are transcription factors that participate in the control of sulfur metabolism by regulating the expression of γ-glutamylcysteine synthase (GSH1), which encodes the rate-limiting enzyme in glutathione synthesis (33).

The finding that so many genes involved in sulfur metabolism and sulfate assimilation are necessary for GSAO resistance indicates that integrity of the cysteine and glutathione synthesis pathways (up to γ-glutamylcysteine; Fig. 4) is essential for the yeast to protect itself against this compound. It is of interest that GSH2, which encodes glutathione synthase (the enzyme that converts γ-glutamylcysteine to glutathione), does not appear to be essential for GSAO resistance. This finding is in accordance with the finding that the gsh2 deletion strain copes well with oxidative stress induced by hydrogen peroxide and superoxide anions (34); thus, it would also be expected to be tolerant of the oxidative stress induced by GSAO. Furthermore, γ-glutamylcysteine has been shown to be at least as good as glutathione in protecting yeast against an oxidant challenge (35). These findings suggest that γ-glutamylcysteine can substitute for the antioxidant properties of glutathione in eukaryotic cells. By contrast with the involvement of MET genes that are required for glutathione synthesis, the genes that are specific to the synthesis of methionine were not implicated in the resistance against GSAO.

To probe the dependence on glutathione level of the sensitivity of mammalian cells to GSAO, we treated the BAECs with exogenous glutathione (to increase glutathione levels) or with inhibitors of glutathione production (to decrease levels) before treating the cells with GSAO. Treatment with glutathione or with glutathione ethyl ester, a membrane-permeable derivative of glutathione, substantially blunted GSAO inhibition of BAEC proliferation (Fig. 5, A). Conversely, blocking de novo synthesis of glutathione with BSO, an inhibitor of γ-glutamylcysteine synthase, enhanced the sensitivity of BAECs to GSAO by almost 100-fold (Fig. 5, B). The glutathione content of BAECs following BSO treatment was 15% that of untreated cells.

These results raised the possibility that the selectivity of GSAO for endothelial versus tumor cells reflects differences in glutathione levels. Indeed, we found a striking association between the GSAO IC_{50} for inhibition of proliferation and the cellular glutathione level of the different cells (Fig. 5, C).

**Linkage Between MRPI Activity and Cellular Glutathione in Resistance to GSAO**

Our findings that the activity of MRPI and MRP2 and the glutathione synthesis machinery contribute to differences in the sensitivity of different mammalian cells to GSAO reflects the fact that these processes are not independent. For example, the yeast YAP1 gene encodes a transcription factor that controls the expression of both YCF1 and GSH1 [γ-glutamylcysteine synthase (20,36)]. In addition, efflux of drugs from mammalian cells via MRPI involves cellular glutathione, although the precise mechanism by which glutathione participates in MRPI function differs from drug to drug. The efflux of glutathione conjugates and of glucuronate conjugates by MRPI is independent of cellular glutathione content (21), whereas vincristine and anthracyclines are cotransported with glutathione (37). On the other hand, efflux of glucuronide conjugates by MRP is enhanced by glutathione, although it is not cotransported (25).

We therefore investigated whether cellular glutathione levels moderate MRP-mediated efflux of GSAO in endothelial cells. Cotreatment of BAECs with high concentrations of both BSO and MK-571 did not increase arsenic accumulation to a greater extent than treatment with either inhibitor alone (Fig. 5, D) and had no additional effect on the IC_{50} for GSAO inhibition of proliferation beyond that of treatment with either inhibitor alone (data not shown). Presumably, MRPI was not functional in cells treated with very high BSO concentrations (i.e., 200 μM) because cellular glutathione levels were insufficient for transport activity; therefore, MK-571 had no additional effect in such cells. In BAECs treated with lower concentrations of BSO, the remaining MRPI activity was inhibited by MK-571, as measured by further decreases in the IC_{50} for GSAO (data not shown).
MK-571 and BSO on the IC\textsubscript{50} of GSAO for inhibiting proliferation of endothelial and tumor cells. Treatment with MK-571 or BSO reduced the GSAO IC\textsubscript{50} in both endothelial and tumor cells (Fig. 6). In addition, the IC\textsubscript{50}s for GSAO inhibition of BAECs (IC\textsubscript{50} = 1.06 \mu M; 95% CI = 0.71 to 1.42 \mu M) and HT1080 cells (IC\textsubscript{50} = 1.22 \mu M; 95% CI = 0.58 to 2.17 \mu M) were the same when MRPI activity was blocked (Fig. 6). This result suggested that the difference in the sensitivity of BAEC and HT1080 cells for GSAO (IC\textsubscript{50} for BAEC = 10.2 \mu M, 95% CI = 8.6 to 11.7 \mu M; IC\textsubscript{50} for HT1080 = 24.8 \mu M; 95% CI = 19.6 to 29.9 \mu M) could be accounted for by a difference in the expression and/or activity of MRPI and/or MRPII. By contrast, although treatment of the Panc-1 and BxPC-3 cells with MK-571 reduced the GSAO IC\textsubscript{50} by fourfold in Panc-1 cells [from 43 \mu M (95% CI = 36 to 49 \mu M) to 11 \mu M (95% CI = 4 to 18 \mu M)] and 25-fold in BxPC-3 cells [from 270 \mu M (95% CI = 213 to 327 \mu M) to 11 \mu M (95% CI = 8 to 13 \mu M)], these IC\textsubscript{50}s were still higher than in BAECs and HT1080 cells. Thus, it appeared that some mechanism(s) other than MRPI and MRPII activity in these cell lines were contributing to their resistance to GSAO.

One likely mechanism was increased glutathione levels. When cells were treated with the highest concentration of BSO used (200 \mu M), the IC\textsubscript{50} for GSAO inhibition of BAEC (0.13 \mu M [95% CI = 0.11 to 0.15 \mu M]), HT1080 (0.16 \mu M [95% CI = 0.05 to 0.27 \mu M]), and Panc-1 (0.19 \mu M [95% CI = 0.02 to 0.41 \mu M]) were approximately the same. However, BxPC-3 cells were still less sensitive to GSAO than the other three cell lines. This resistance may be explained by the partial resistance of BxPC-3 cells to BSO treatment. That is, the glutathione content of BAEC, HT1080, and Panc-1 cells following BSO treatment was very similar (1.0 [95% CI = 0.4 to 1.6] to 1.3 [95% CI = 0.9 to 1.8] nmol glutathione per 10\textsuperscript{6} cells), whereas the glutathione content of BxPC-3 cells was substantially, and statistically significantly, higher (2.2 [95% CI = 1.7 to 2.7] nmol glutathione per 10\textsuperscript{6} cells) (P<.01).

**Fig. 3.** Expression of multidrug associated–resistance protein (MRP)-1 and MRP2 in mammalian cells and resistance to 4-(N-(5-glutathionylacetyl)amino) phenylarsenoxide (GSAO). A–C) Mammalian cells overexpressing MRP1 to -5, P-glycoprotein (P-gp), or breast cancer resistance protein (BCRP) were exposed to the indicated concentrations of GSAO for 96 hours, and the numbers of viable cells were measured and expressed relative to the numbers of untreated cells. MRP1, MRP2, and MRP3 were each overexpressed in MDCKII canine kidney epithelial cells (A), MRP4 and MRP5 were overexpressed in HEK293 human embryo kidney cells (B), and P-gp and BCRP were overexpressed in MEF3.8 murine embryo fibroblasts (C). Untransfected cells of each line were also tested. Data points and error bars are the means with 95% confidence intervals (CIs) of two to four independent experiments, each performed in quadruplicate (n = 8 to 16) at each GSAO’s concentration tested. D) Immunoblot analysis of MRP1 and MRP2 expression in bovine aortic endothelial cells (BAEC) (lane 1), tumor cell lines (HT1080 fibrosarcoma and BxPC-3 and Panc-1 pancreatic cancer, lanes 2–4), MEF3.8 cells (lane 5), Caco-2 cells (lane 6), human kidney tissue (lane 7), and MRP1- or MRP2-transfected MDCKII cells (run together on lane 8). Blots were stained with antibodies to α- tubulin as a loading and transfer control. Position of the molecular weight markers in kilodaltons is indicated at right.

**Fig. 4.** Diagram of the cysteine and glutathione synthesis pathway and genes necessary for GSAO resistance. **Open boxes** denote gene products that were found to be important for resistance of Saccharomyces cerevisiae to GSAO. **Shaded boxes** denote gene products that, when deleted, confer the highest sensitivity to GSAO. **Ovals** denote regulatory gene products that are involved in the resistance to GSAO. Deletion strains for met3 and met2 were not available for testing. Relational MRP1 and MRP2 and Glutathione Synthesis Activity to the Selectivity of GSAO for Endothelial Versus Tumor Cells

To investigate the relative contributions of MRP1 and MRP2 activity and cellular glutathione levels to the selectivity of GSAO for endothelial versus tumor cells, we examined the effects of relationship between MRP1 and MRP2 expression in bovine aortic endothelial cells (BAEC) (lane 1), tumor cell lines (HT1080 fibrosarcoma and BxPC-3 and Panc-1 pancreatic cancer, lanes 2–4), MEF3.8 cells (lane 5), Caco-2 cells (lane 6), human kidney tissue (lane 7), and MRP1- or MRP2-transfected MDCKII cells (run together on lane 8). Blots were stained with antibodies to α-tubulin as a loading and transfer control. Position of the molecular weight markers in kilodaltons is indicated at right.

**Relationship of MRP1 and MRP2 and Glutathione Synthesis Activity to the Selectivity of GSAO for Endothelial Versus Tumor Cells**

To investigate the relative contributions of MRP1 and MRP2 activity and cellular glutathione levels to the selectivity of GSAO for endothelial versus tumor cells, we examined the effects of relationship between MRP1 and MRP2 expression in bovine aortic endothelial cells (BAEC) (lane 1), tumor cell lines (HT1080 fibrosarcoma and BxPC-3 and Panc-1 pancreatic cancer, lanes 2–4), MEF3.8 cells (lane 5), Caco-2 cells (lane 6), human kidney tissue (lane 7), and MRP1- or MRP2-transfected MDCKII cells (run together on lane 8). Blots were stained with antibodies to α-tubulin as a loading and transfer control. Position of the molecular weight markers in kilodaltons is indicated at right.
DISCUSSION

Until recently, our understanding of the mechanism of drug action in mammalian cells has been limited to probing specific proteins or pathways. Gene array technology has enabled a global appreciation of the response of cells or tissues to a drug, but it does not report on how cells or tissues manage a drug prior to gene expression. The recent availability of a genome-wide set of *S. cerevisiae* deletion strains has afforded a very precise way to identify which gene products mediate or blunt the effects of a drug. In this study, we elucidated the mechanism of selectivity of an angiogenesis inhibitor for endothelial versus tumor cells by screening a set of 4546 deletion strains. Importantly, we showed that mammalian proteins that were closely related to the gene products identified as mediating resistance in yeast were of functional importance to resistance in mammalian cells. Our results indicate that the activity of the drug transporters MRP1 and/or MRP2 and of cellular glutathione levels are largely responsible for the selectivity of GSAO for endothelial cells as compared with fibrosarcoma and pancreatic carcinoma cell lines. Multidrug transporter proteins of the ABC superfamily, which include not only MRP1 through MRP7 but also P-gp and BCRP, are believed to contribute to drug resistance and chemotherapy failure by active efflux of cytotoxic agents from cancer cells (37). MRP1, MRP2, MRP3, P-gp, and BCRP have all been shown to confer resistance to a variety of natural products and anticancer drugs, collectively including the taxanes, *Vinca* alkaloids, anthracyclines, epipodophyllotoxins, and camptothecin-class topoisomerase I inhibitors, whereas MRP4 and -5 confer resistance to nucleoside analogs (29). In addition to a role in drug resistance, multidrug transporters influence the uptake, elimination, and disposition of substrate drugs by virtue of their polarized expression on epithelial and endothelial cell membranes in the intestine.
levels, which combine to enhance export of GSAO. These findings raise the possibility that GSAO could be converted from an antiendothelial to an anti–tumor cell compound by blocking MRP1 activity or glutathione synthesis in tumor cells. Alternatively, GSAO’s antiangiogenic activity could be enhanced by targeting these processes in proliferating endothelial cells. For instance, GSAO could be combined with the glutathione synthesis inhibitor BSO that has been shown to decrease glutathione levels in human tumors in a phase I clinical trial (50). The S. cerevisiae screen we have employed should have more general use in defining the mechanism of action of other human therapeutics. Such knowledge will better define a drug’s strengths and weaknesses, which will help with the optimization of its use and rational design of analogues.

Fig. 6. Roles of MRP1, MRP2, and glutathione in the selectivity of 4-(N-(S-glutathionylacetylamino) phenylarsenoxide (GSAO) for endothelial versus tumor cells. The 50% inhibitory concentration (IC50) for GSAO in BAECs and in HT1080 fibrosarcoma and Panc-1 and BxPC-3 pancreatic tumor cells was determined in a 72-hour proliferation assay in the absence (Nil) or presence of the MRP inhibitor MK-571 (20 μM) or the glutathione synthesis inhibitor BSO. BSO used was used at concentrations that caused no decrease in cell viability over the course of the assay: 200 μM for BAEC, Panc-1, and BxPC-3 cells, and 10 μM for HT1080 cells. The data points and error bars are the means with upper 95% CIs of three to seven IC50 determinations. ***, P = .001 compared with BAEC; ***, P = .003 to P = .006 compared with BAEC; *, P = .017 compared with BAEC.

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NOTES

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