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Optimization of the Antitumor Efficacy of a Synthetic Mitochondrial Toxin by Increasing the Residence Time in the Cytosol

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Plasma membrane drug efflux pumps of the multidrug resistance associated protein (MRP) family blunt the effectiveness of anticancer drugs and are often associated with drug resistance. GSAO, a tripeptide trivalent arsenical that targets a key mitochondrial transporter in angiogenic endothelial cells, is an example of a compound whose efficacy is limited by tumor cell expression of MRP isoforms 1 and 2. A cysteine mimetic analogue of GSAO was made, PENAO, which accumulates in cells 85 times faster than GSAO due to increased rate of entry and decreased rate of export via MRP1/2. The faster rate of accumulation of PENAO corresponds to a 44-fold increase in antiproliferative activity in vitro and ~20-fold better antitumor efficacy in vivo. This information could be used to improve the efficacy of other small molecule cancer therapeutics.

Introduction

Mitochondria are an attractive cancer drug target because their proper functioning is fundamental to cell survival.[1] 4-(*N*-(*S*-Glutathionylacetyl)amino)phenylarsonous acid (GSAO[a]) represents a novel class of organoarsenical compound that selectively targets a key mitochondrial transporter in tumor endothelial cells.[2−4] It is currently being tested in a phase I/IIa clinical trial in cancer patients.

GSAO is processed at the cell surface and in the cytosol before the trivalent arsenical moiety reacts with mitochondria. The γ-glutamyl residue of GSAO is cleaved at the cell surface by γ-glutamyl transpeptidase and the product of this reaction, 4-(*N*-(*S*-cysteinylglycylacetyl)amino)phenylarsonous acid (GCAO), is transported across the plasma membrane by an organic ion transporter where it may be further processed by dipeptidases to 4-(*N*-(*S*-cysteinylacetyl)amino)phenylarsonous acid (CAO).[4] Cytosolic concentrations of GCAO and CAO are balanced by export from the cell by the multidrug resistance associated protein isoforms 1 and 2 (MRP1/2)[3] and rate of reaction with the mitochondrial inner membrane transporter, adenine nucleotide translocase (ANT) (Figure 1A).

ANT is the most abundant protein of the inner mitochondrial membrane.[5] It is a 30 kDa protein that spans the membrane six times and has two primary functions. The transporter plays an important role in oxidative phosphorylation by exchanging newly formed ATP in the matrix with spent ADP located in the inner membrane space across the inner membrane. It is also a component of the mitochondrial permeability transition pore.[6]

The transition pore allows passage of molecules smaller than 1500 Da across the inner mitochondrial membrane and is induced by matrix Ca^{2+} overload, particularly when accompanied by oxidative stress, elevated phosphate concentration, and depletion of adenine nucleotides. Transition pore opening is thought to be triggered by Ca^{2+}-dependent binding of cyclophilin-D to ANT.[6] The trivalent arsenical of GCAO or CAO reacts with two cysteine thiols located on the peptide loops of ANT that protrude into the mitochondrial matrix, forming a stable cyclic dithioarsinite complex in which both sulfur atoms are bound to the arsenic atom.[2,7] Calcium content within the mitochondrial matrix increases several-fold in proliferating cells,[8] which sensitizes proliferating cells to CAO-induced pore formation.[2] Formation of the pore coupled with the higher respiration rate in proliferating cells and associated elevated levels of O_2^- are likely responsible for the proliferation arrest observed in endothelial cells.[2]

The cell-surface cleavage of GSAO by γ-glutamyl transpeptidase is the rate-limiting step in its antimitochondrial action. Our aim was to improve the efficacy of GSAO by making analogues that have enhanced rate of accumulation in the cytosol. A cysteine mimetic analogue of CAO, 4-(*N*-(*S*-penicillaminylacetyl)amino) phenylarsonous acid (PENAO), was made that bypasses the extracellular and cytosolic processing of GSAO. PENAO accumulates in cells much more rapidly than GSAO, which translates to more potent effects

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[a] Abbreviations: ANT, adenine nucleotide translocase; BRAA, 4-(2-bromoacetylamino)benzenearsonic acid; BRAO, 4-(2-bromoacetylamino)benzenearsonous acid; CAO, 4-(*N*-(*S*-cysteinylacetyl)amino)-phenylarsonous acid; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; GCAO, 4-(*N*-(*S*-cysteinylglycylacetyl)amino)phenylarsonous acid; GSAO, 4-(*N*-(*S*-glutathionylacetyl)amino)phenylarsonous acid; MRP, multidrug resistance associated protein; OATP, organic anion transporting polypeptide; PENAA, 4-(*N*-(*S*-penicillaminylacetyl)-amino)phenylarsonic acid; PENAO, 4-(*N*-(*S*-penicillaminylacetyl)-amino)phenylarsonous acid.

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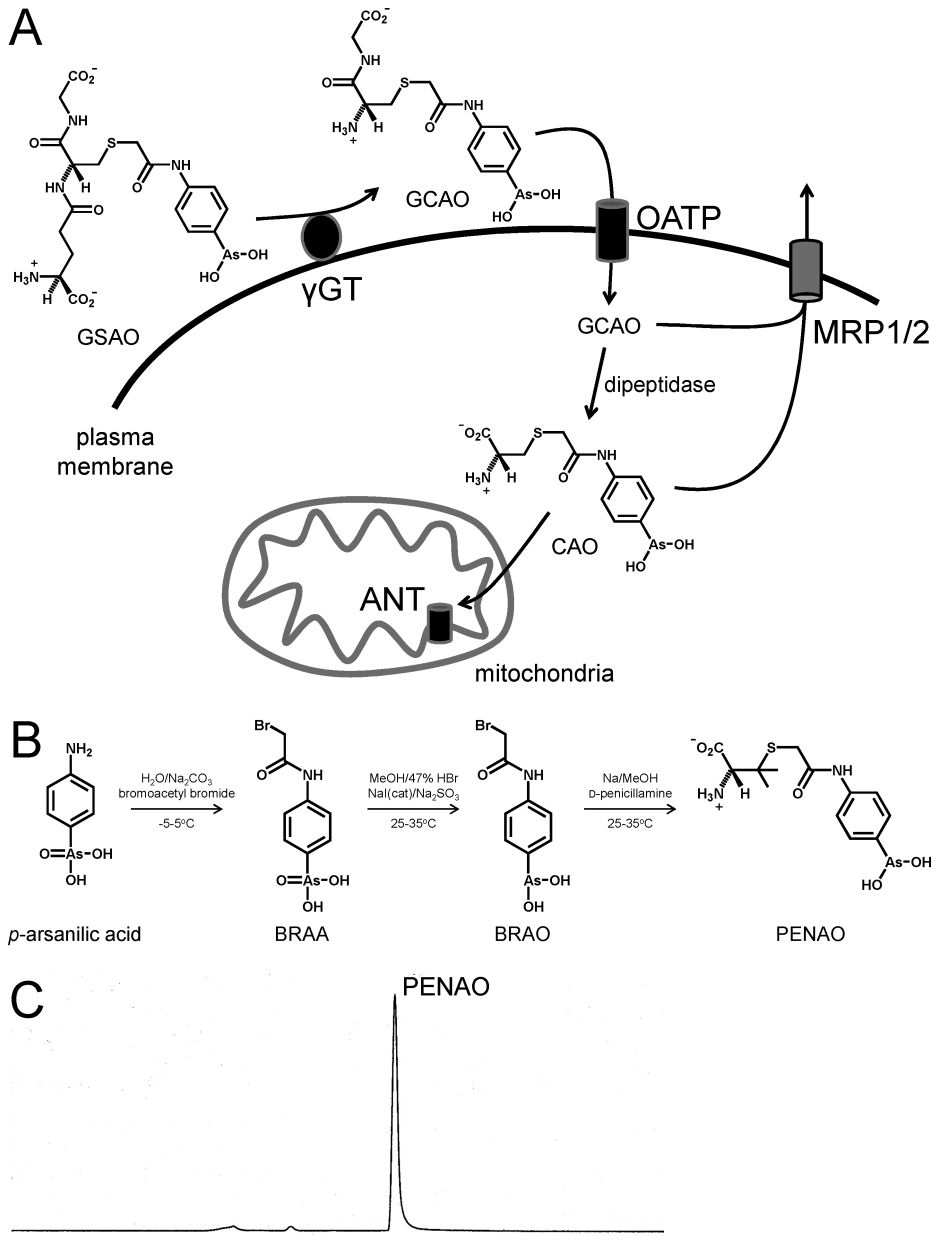

Figure 1. Mechanism of action of GSAO. (A) GSAO is processed by γ-GT at the cell surface, and the resulting GCAO is transported across the plasma membrane by an OATP. GCAO is likely further processed to CAO in the cytosol before it reacts with ANT of the innermitochondrial membrane. The cytosolic levels of GCAO and CAO are controlled by export from the cell by MRP isoforms 1 and 2. (B) Summary of the synthesis of PENAO. (C) C18 reverse phase HPLC analysis of PENAO. Purity of PENAO by peak area is 97%.

on endothelial and tumor cells in culture and markedly better antitumor efficacy in mice.

Results

Synthesis and Characterization of PENAO. The synthesis of PENAO is summarized in Figure 1B. Briefly, 4-(2-bromoacetylamino)benzenearsonic acid (BRAA) was synthesized from *p*-arsanilic and bromoacetyl bromide and reduced to 4-(2-bromoacetylamino)benzenearsonous acid (BRAO) using sodium sulphite with sodium iodide as catalyst.[9] PENAO was produced by coupling BRAO and D-penicillamine and purified by C18 silica gel chromatography. Purity by HPLC was 97% (Figure 1C).

PENAO Accumulates More Rapidly in Endothelial Cells than GSAO or Its Metabolites. PENAO accumulated in BAE cells at a 10-fold faster rate than CAO, a 9-fold faster rate than GCAO, and an 85-fold faster rate than GSAO (Figure 2A). Cellular concentration of the arsenicals was measured from the arsenic content by inductively coupled plasma spectrometry.

The organic anion transporting polypeptide (OATP) family of transporters mediate uptake of glutathione-*S*-conjugates into cells,[10] including GCAO.[4] 4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid (DIDS),[10] an inhibitor of OATP's, reduced PENAO uptake (Figure 2B), which implies that this transporter is involved in PENAO translocation across the plasma membrane.

The plasma membrane MRP isoforms 1 and 2 mediate export of GCAO and CAO from endothelial cells.[3,4] To determine if PENAO is also a substrate for MRP's, mammalian cells overexpressing MRP1, 2, 3, 4, 5, or 6, or MDR1 or BCRP were tested for resistance to PENAO. MRP1,

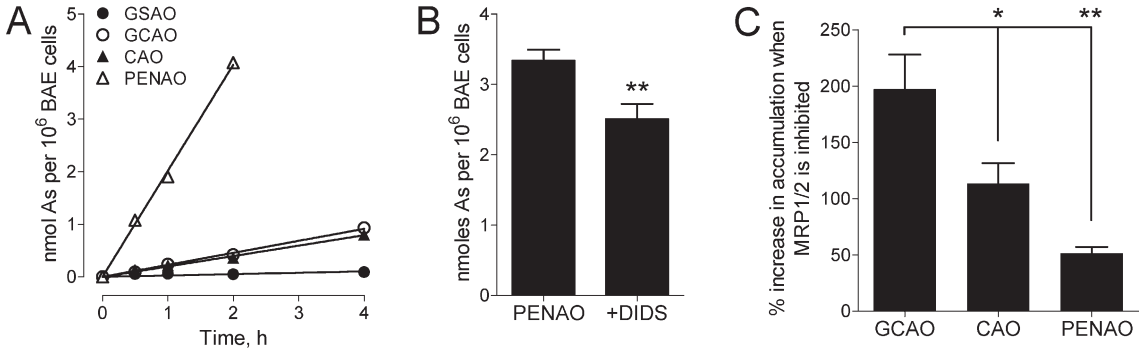

Figure 2. PENAO accumulates more rapidly in endothelial cells than GSAO or its metabolites. (A) BAE cells were incubated with 50 μM GSAO, GCAO, CAO, or PENAO for up to 4 h, and cytosolic arsenic was measured by inductively coupled plasma spectrometry. The rates of accumulation of GSAO, GCAO, CAO, and PENAO are 0.4 ± 0.1, 3.8 ± 0.1, 3.3 ± 0.1, and 34 ± 0.6 pmol As atoms per 10^6 cells per min. Data points are the mean $\pm$ SD of three determinations, and the solid line is the linear least-squares fit of the data. The results are representative of two experiments. (B) BAE cells were pretreated or not with the OATP inhibitor DIDS (500 μM) for 30 min and then incubated for 2 h with 20 μM PENAO. Cytosolic arsenic was measured by inductively coupled plasma spectrometry. (C) BAE cells were incubated for 2 h with 50 μM GCAO, CAO, or PENAO in the absence or presence of 5 μM of the MRP1/2 inhibitor Reversan and cytosolic arsenic was measured by inductively coupled plasma spectrometry. The data is expressed as the % increase in accumulation in the presence of Reversan. The values are the mean $\pm$ SD of triplicate determinations and are representative of two experiments. ** is $p < 0.01$ and * is $p < 0.05$.

MRP2 or MRP3 was overexpressed in the canine kidney epithelial MDCKII cell line,[11,12] MRP4 or MRP5 in the human embryonic kidney HEK293 cell line,[13] while MRP6, MDR1, or BCRP was overexpressed in the murine embryo fibroblast MEF3.8 line.[3] The IC_{50} (concentration of compound that inhibits proliferation by 50%) for PENAO was determined for each transfected cell line and the value divided by the IC_{50} for proliferation arrest of nontransfected parental cells to give the resistance factor (Table 1). The resistance factor for MRP1 was 3.7, while for MRP2 it was 4.6. The resistance factors for MRP3, MRP4, MRP5, MRP6, MDR1, and BCRP were in the range 0.4−1.3. These results indicate that PENAO, like GCAO and CAO, is exported from cells by MRP isoforms 1 and 2.

To confirm the role of MRP1/2 in regulating cytosolic levels of PENAO, endothelial cells were incubated for 2 h with 50 μM GCAO, CAO, or PENAO in the absence or presence of 5 μM of the MRP1/2 inhibitor, 5,7-diphenylpyrazolo[1,5-*a*]pyrimidine-3-carboxylic acid (3-morpholin-4-ylpropyl)amide (Reversan),[14] and cellular arsenic was measured by inductively coupled plasma spectrometry. MRP1/2 inhibition increased the cellular levels of GCAO by $197 \pm 31\%$, CAO levels by $114 \pm 18\%$, and PENAO levels by only $52 \pm 5\%$ (Figure 2C). This result indicates that export of PENAO from the cell by MRP1/2 is less efficient than for GCAO or CAO.

The Rate of Accumulation of PENAO in Endothelial Cells Correlates with Antiproliferative Activity. The faster rate of accumulation of PENAO in endothelial cells corresponded to a 44-fold increased antiproliferative activity compared to GSAO, an 8-fold increased antiproliferative activity compared to GCAO, and a 6-fold increased antiproliferative activity compared to CAO (Figure 3A, 24 h assay). The marked time-dependence of the antiproliferative effect of GSAO is due to the requirement for processing of GSAO at the cell surface before it can react with mitochondrial ANT. There was a small decrease in the GCAO, CAO, and PENAO IC_{50} with time of incubation but not nearly to the same extent as for GSAO.

Blocking entry of PENAO into endothelial cells with the OATP inhibitor DIDS (Figure 3B) or blocking export from the cytosol via MRP1/2 using the inhibitors Reversan or (*E*)-3-[[[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl]-[[3-dimethylamino)-3-oxopropyl]thio]methyl]thio]-propanoic acid (MK571)[15] (Figure 3C) correlated with decreased or increased antiproliferative action, respectively. The inhibitors alone at the concentrations used had no effect on BAE cell proliferation (data not shown). There is a strong correlation between the antiproliferative activity of GSAO, GCAO, CAO, and PENAO and the rate of cellular accumulation of the arsenicals (Figure 3D). These results indicate that the antiproliferative efficacy of the arsenicals is proportional to their cytosolic levels.

Table 1. Resistance of Mammalian Cells Overexpressing Different Drug Transporters to PENAO[a]

cell type/drug transporter	resistance factor
MDCKII/MRP1	3.7
MDCKII/MRP2	4.6
MDCKII/MRP3	0.8
HEK293/MRP4	0.5
HEK293/MRP5	0.4
MEF3.8/MRP6	1.3
MEF3.8/MDR1	1.2
MEF3.8/BCRP	1.1

[a] Resistance factor is the ratio of the PENAO IC_{50} value for proliferation arrest of transfected cells and the IC_{50} value for non-transfected parental cells.

Blocking de novo synthesis of glutathione in BAE cells with buthionine sulfoximine (BSO), an inhibitor of γ-glutamyl cysteine synthase, enhanced GSAO's antiproliferative activity by almost 100-fold.[3] Similarly, treating BAE cells with BSO enhanced the PENAO IC_{50} for proliferation arrest by 28-fold (Figure 3E). IC_{50} values for proliferation arrest decreased from 440 ± 130 to 16 ± 10 nM in the presence of 100 μM BSO. BSO alone at this concentration had no effect on cell growth (data not shown).

Inhibiting MRP1/2 activity and blocking de novo synthesis of glutathione in endothelial cells resulted in additive enhancement of PENAO's antiproliferative activity. The IC_{50} value for proliferation arrest decreased from 1100 to 130 nM in the presence of 3 μM Reversan, to 70 nM in the presence of 20 μM BSO, and to 20 nM in the presence of both 3 μM Reversan and 20 μM BSO (Figure 3F). Reversan

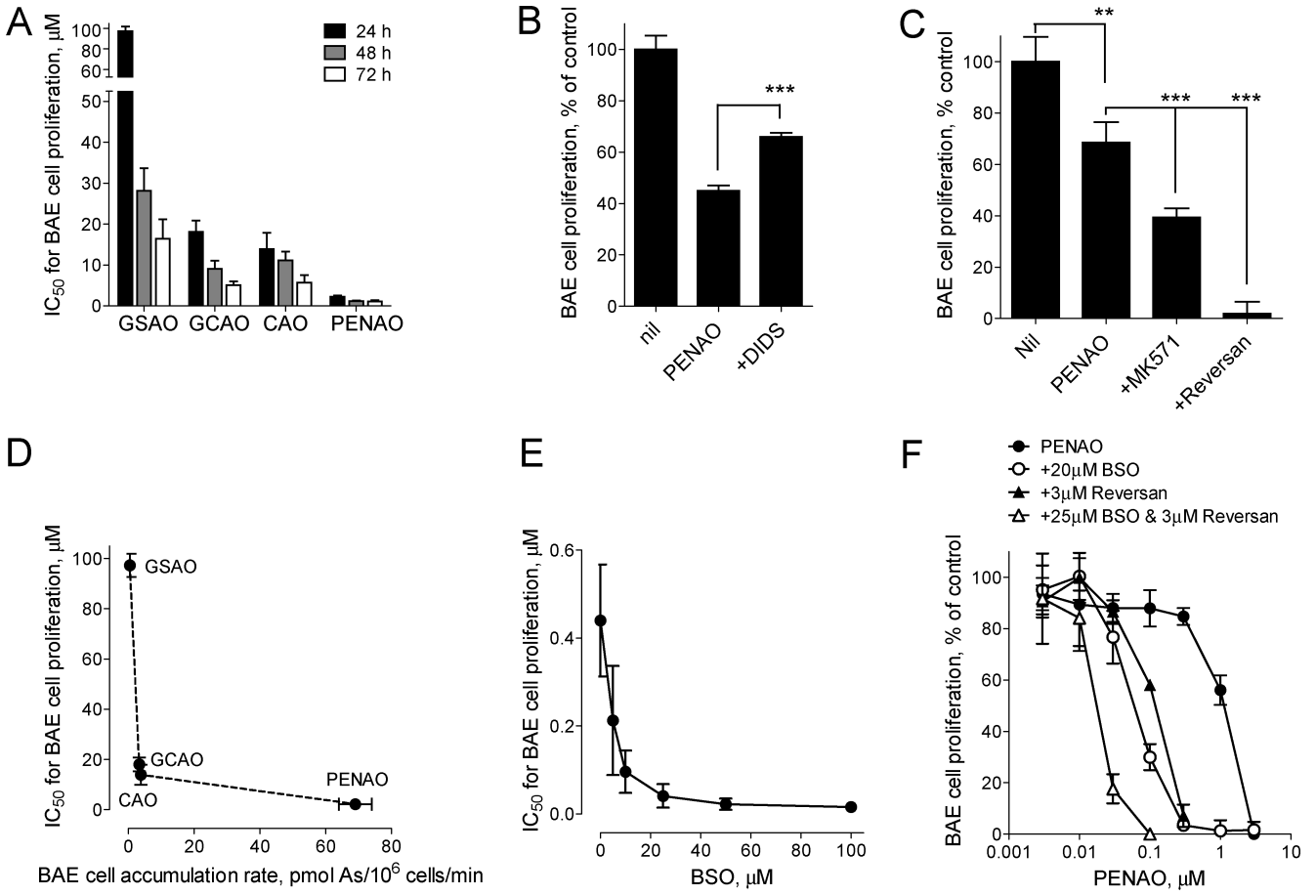

Figure 3. Rate of accumulation of PENAO in endothelial cells correlates with antiproliferative activity. (A) Comparison of the GSAO, GCAO, CAO, and PENAO IC_{50} (concentration of compound that inhibits proliferation by 50%) values for proliferation arrest of endothelial cells. BAE cells were incubated with 0.8−250 μM of the compounds for 24, 48, or 72 h and cell viability determined using MTT. Values are mean ± SD of triplicate determinations. The results are representative of three experiments. (B) BAE cells were untreated or pretreated with 500 μM DIDS for 30 min and then incubated with 3 μM PENAO for 24 h. Cell viability was determined using MTT and results expressed as percentage of control. Values are mean ± SD of triplicate determinations. Results are representative of two experiments. (C) BAE cells were incubated for 30 min with 15 μM MK571 or 2 μM Reversan prior to incubation with 0.3 μM PENAO for 72 h. Cell viability was determined using MTT and results expressed as percentage of control. The values are the mean ± SD of triplicate determinations. (D) Correlation between the IC_{50} for proliferation arrest of endothelial cells and rate of cellular accumulation of GSAO, GCAO, CAO, and PENAO. (E) BAE cells were treated with 0.001−3 μM PENAO and the indicated concentrations of BSO for 72 h and the IC_{50} for proliferation arrest was calculated. The data points and error bars are the mean ± SD from two experiments performed in triplicate. (F) BAE cells were untreated or pretreated with 20 μM BSO and/or 3 μM Reversan for 30 min and then incubated with 0−3 μM PENAO for 24 h. Cell viability was determined using MTT and results expressed as percentage of control. Values are mean ± SD of triplicate determinations. *** is $p < 0.001$ and ** is $p < 0.01$.

and/or BSO alone at these concentrations had no effect on cell growth (data not shown).

The trivalent arsenical moiety of PENAO was responsible for its antiproliferative activity as the corresponding pentavalent arsenical (4-(*N*-(*S*-penicillaminylacetyl)amino)phenylarsonic acid, PENAA) had no effect on BAE cell proliferation (see Supporting Information).

Prolonged incubation of endothelial cells with GSAO results in apoptosis of the cells.[2] Incubation of BAE cells with PENAO also resulted in loss of viability, with an IC_{50} of ~3.5 μM in a 48 h assay (see Supporting Information). This IC_{50} is 21-fold lower than for GSAO.[2]

PENAO is a More Effective Inhibitor of Proliferating Cells than GSAO. PENAO is a 5- to 17-fold better inhibitor than GSAO of proliferation of both primary and transformed cells in 72 h assays (Table 2). The antiproliferative activity for bovine, human, and murine endothelial cells, canine epithelial cells, and seven different tumor cell lines is within a factor of 19 for PENAO and a factor of 27 for GSAO. The most resistant tumor cell line tested was human pancreatic carcinoma BxPC-3 cells. The IC_{50} for proliferation arrest of BxPC-3 cells by PENAO is 19-fold higher than for endothelial cells and 27-fold higher for GSAO.

The Antimitochondrial Action of PENAO is Comparable to that of the GSAO Metabolite, CAO. Like GSAO, GCAO, and CAO,[4] PENAO triggered swelling of isolated rat liver mitochondria in a time- and concentration-dependent manner (Figure 4A). Comparison of the time for 25% maximal swelling as a function CAO or PENAO concentration indicates that the two compounds have comparable effects on mitochondrial integrity (Figure 4B).

PENAO is ~20-Fold More Efficacious than GSAO at Inhibiting Growth of Human Pancreatic Carcinoma Tumors in Mice. BalbC nude mice bearing subcutaneous human BxPC-3 pancreatic carcinoma tumors in the proximal midline were implanted with micro-osmotic alzet pumps subcutaneously in the flank. The pumps delivered vehicle control or 0.25 or 0.5 mg/kg/day PENAO. The tumor doubling times were 9.4 ± 1.4, 15.6 ± 2.1, and 13.8 ± 2.1 days for

Table 2. Comparison of PENAO vs GSAO IC_{50} Values for Proliferation Arrest of Endothelial and Tumor Cells. [a]

cell type	cell line	PENAO IC_{50}, μM	GSAO IC_{50}, μM
bovine aortic endothelial	BAE	1.1 ± 0.4	10 ± 1.3
human microvascular endothelial	HMEC-1	2.0 ± 0.3	23 ± 1.0
murine cerebral cortex endothelial	bEnd.3	2.5 ± 0.2	42 ± 2.1
canine kidney epithelial	MDCKII	2.4 ± 0.2	13 ± 2.7
human chronic myelogenous leukemia	K562	1.8 ± 0.4	14 ± 0.8
human fibrosarcoma	HT1080	4.1 ± 0.1	25 ± 4.8
mouse lung carcinoma	LLC	5.3 ± 0.1	41 ± 3.5
human colorectal carcinoma	HCT1116	5.9 ± 0.5	43 ± 5.3
human pancreatic carcinoma	PANC-1	6.4 ± 0.1	43 ± 6.2
human mammary carcinoma	MCF-7	9.4 ± 0.5	51 ± 1.5
human pancreatic carcinoma	BxPC-3	21 ± 0.7	270 ± 54

[a] IC_{50} values were determined from a 72 h proliferation assay.

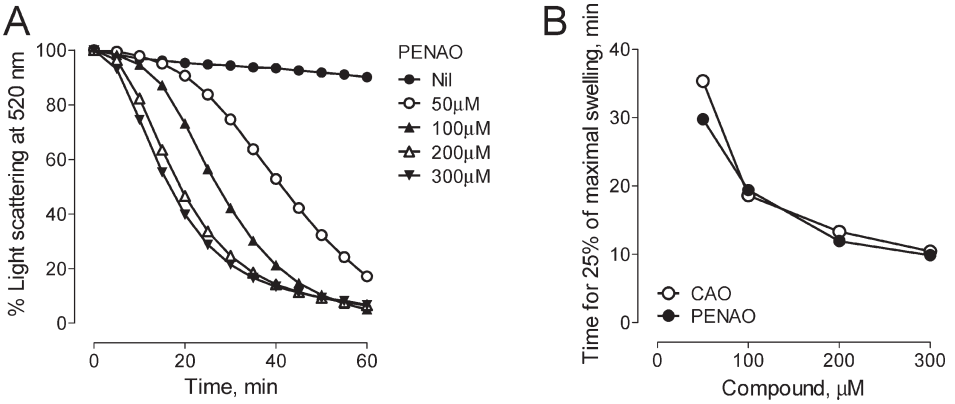

Figure 4. Antimitochondrial action of PENAO is comparable to that of the GSAO metabolite, CAO. (A) Mitochondrial swelling induced by 0−300 μM PENAO as measured by decrease in light scattering at 520 nm over 60 min. The traces are representative of two experiments performed in duplicate on two different mitochondrial preparations. (B) Time for 25% maximum swelling as a function of CAO or PENAO concentration. The results are representative of two experiments.

groups treated with vehicle or 0.25 or 0.5 mg/kg/day PENAO, respectively (Figure 5A).

There were no obvious signs or symptoms of toxicity of the compounds at the doses tested. There was a small accumulation of connective tissue at the delivery site of the osmotic pump in 7 of the 10 mice at the higher dose of PENAO. This is likely the result of a local inflammatory response triggered by the high concentrations of PENAO at the delivery site.

Immunohistochemical analysis of the vehicle and 0.25 mg/kg/day PENAO tumors following 18 days of treatment indicated a significant reduction in both blood vessel density in the treated tumors ($p < 0.01$, Figure 5B) and the proliferative index of the tumor cells ($p < 0.05$, Figure 5C). These findings indicate that PENAO has both antiangiogenic and antitumor cell activity in vivo.

Dose-dependence and comparison of the antitumor efficacy of GSAO versus PENAO is shown in Figure 5D. PENAO is ~20-fold more efficacious that GSAO at inhibiting growth of human pancreatic carcinoma tumors in immunodeficient mice. Comparable antitumor activity was observed with administration of 5 mg/kg/day GSAO and 0.25 mg/kg/day PENAO.

Discussion and Conclusions

The rate-limiting step in GSAO's antimitochondrial action is cleavage at the cell surface by γ-glutamyl transpeptidase. The resulting GCAO then enters the cell via OATP and is converted by dipeptidases in the cytoplasm to CAO. CAO is either exported from the cytosol by MRP1/2 or enters the mitochondrial matrix where it reacts with ANT, triggering proliferation arrest. We reasoned that CAO would be a more effective mitochondrial toxin than GSAO because it would enter the cell more rapidly and would bypass the dipeptidase processing in the cytoplasm.

In our hands, CAO was difficult to produce synthetically, mostly because of incompatibility with the solvents used for GSAO production. We also observed that CAO was somewhat unstable in aqueous solutions at 4 or 37 °C when compared to GSAO. We instead chose to make an analogue of CAO, PENAO, in which the cysteine residue has been replaced with the cysteine mimetic, D-penicillamine. Penicillamine contains two methyl groups in place of the hydrogens at the β-carbon atom of cysteine. PENAO was produced to a purity of 97% by HPLC. The compound was stable in phosphate-buffered saline for at least 2 weeks at 4 or 37 °C.

PENAO accumulated in endothelial cells at an 85-fold faster rate than GSAO and a ~10-fold faster rate than GCAO or CAO. Like for GCAO and CAO,[4] PENAO uptake was mediated, at least in part, by the OATP and export was via MRP1 and MRP2. The faster rate of accumulation of PENAO compared to CAO is mostly due to decreased rate of export by MRP1/2. It is not known why the two additional methyl groups in PENAO would influence its handling by MRP1/2. Presumably, the cysteine pendant of CAO binds with higher affinity to MRP1/2 than the cysteine mimetic of PENAO. PENAO and CAO had comparable effects on the integrity of isolated mitochondria, which implies that rate of entry into the mitochondrial matrix and reactivity with ANT is similar for the two compounds.

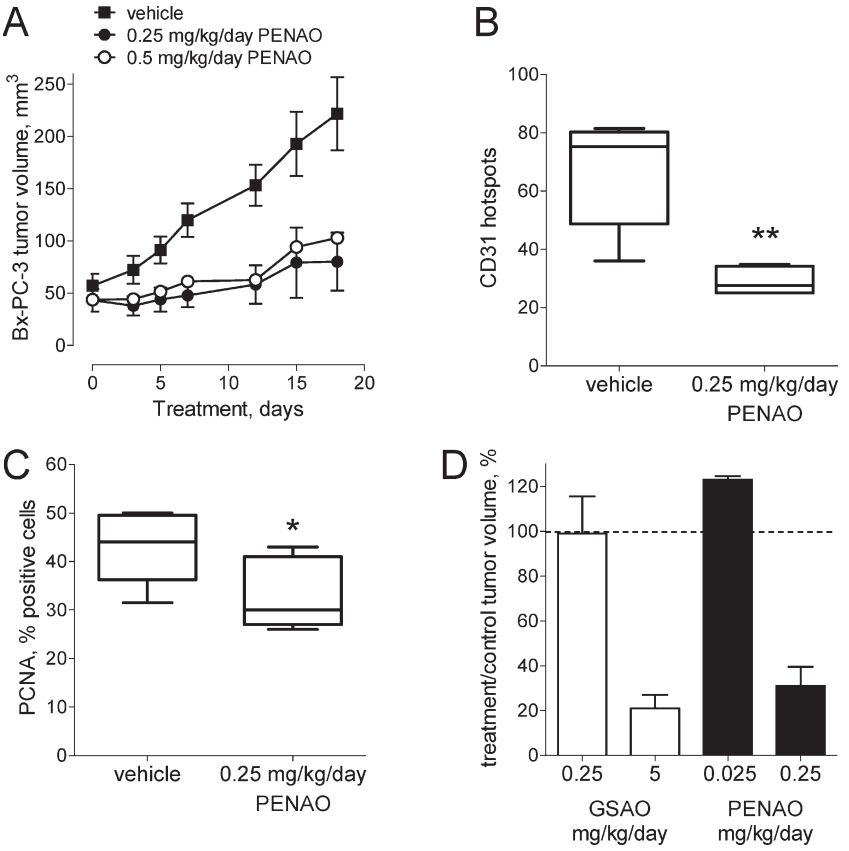

Figure 5. PENAO is ~20-fold more efficacious that GSAO at inhibiting growth of human pancreatic carcinoma tumors in mice. (A) BxPC-3 tumors were established in the proximal midline of female 7−9 week old BalbC nude mice. Mice bearing ~50 mm^3 tumors were randomized into three groups ($n = 10$) and implanted with 28 day alzet micro-osmotic pumps subcutaneously in the flank. The pumps delivered vehicle alone or 0.25 or 0.5 mg/kg/day PENAO. The data points are the mean ± SE of the tumor volumes. (B) and (C) Tumors were excised at day 18 in mice treated with 0.25 mg/kg/day PENAO (see part A), fixed, sectioned, and analyzed for vascularity (CD31 hotspots, (B)) and tumor cell proliferation (PCNA, (C)). ** is $p < 0.01$ and * is $p < 0.05$. (D) Comparison of the antitumor efficacy of PENAO and GSAO. BalbC nude mice bearing ~50 mm^3 subcutaneous BxPC-3 tumors in the proximal midline were treated for 18 days with the indicated doses of either GSAO (white bars) or PENAO (black bars). The compounds were administered subcutaneously via micro-osmotic pumps implanted in the flank. There were 5 and 7 mice, respectively, for the 0.25 and 5 mg/kg/day GSAO groups and 2 and 7 mice, respectively, for the 0.025 and 0.25 mg/kg/day PENAO groups. The data points are the mean ± SE of the treatment/control values expressed as %. The dotted line represents no change in tumor growth rate.

The 85-fold faster rate of accumulation of PENAO in endothelial cells compared to GSAO corresponded to a 44-fold increased antiproliferative activity, while the ~10-fold faster rate of accumulation compared to GCAO or CAO equated to ~7-fold increased antiproliferative activity. There is a marked time-dependence of the antiproliferative effect of GSAO, which is due to the requirement for cell surface processing of GSAO by γ-glutamyl transpeptidase before it can exert its cellular effect.[4] There was little difference in the PENAO IC_{50} value for proliferation arrest in 24 or 72 h assays, however. This finding is consistent with the direct mitochondrial action of PENAO. It is neither processed at the cell surface nor in the cytoplasm before it reacts with mitochondrial ANT.

The antiproliferative activity of PENAO is dependent on cellular glutathione levels as it is for GSAO and GCAO.[3] Depletion of endothelial cell glutathione reduced the PENAO IC_{50} for proliferation arrest from 440 to 16 nM. This strong correlation between glutathione level and antiproliferative activity likely relates to the mechanism of export of PENAO. MRP1 transports trivalent arsenic as a complex with three molecules of glutathione.[16] The arsenic atom of PENAO, therefore, is probably glutathionylated before the conjugate is exported by MRP1/2. Low cellular glutathione would translate to impaired export and, therefore, increased antiproliferative activity.

The relative antiproliferative activity of PENAO and GSAO for 11 different primary or transformed cell types in culture is within a factor of 19 for PENAO and a factor of 27 for GSAO. This finding indicates that PENAO has similar cellular selectivity as GSAO.

PENAO is a ~20-fold more effective inhibitor of tumor growth in mice than GSAO. Comparable antitumor activity was observed with administration of 0.25 mg/kg/day PENAO and 5 mg/kg/day GSAO. No systemic toxicity of either PENAO or GSAO was apparent at these doses. GSAO-treated tumors are characterized by a significant reduction in blood vessel density and no effect on the tumor cell proliferative index,[2] which is consistent with an antiangiogenesis mechanism of action. Analysis of PENAO treated tumors showed a marked reduction in vascular hot spots ($p < 0.01$) as well as significant inhibition of BxPC-3 tumor cell proliferation ($p < 0.05$). This can be explained by the difference in the effects of the two compounds on proliferation of BxPC-3 cells. The IC_{50} for proliferation arrest of BxPC-3 cells is 270 μM for GSAO[2] compared to 21 μM for PENAO.

In summary, the cytotoxic and antitumor efficacy of GSAO has been markedly improved by constructing an

analogue of a GSAO metabolite that enters cells faster and is exported slower. The increased residence time of PENAO in the cytosol correlates with its increased mitochondrial toxicity. PENAO is one of the most potent antitumor agents in mice that has been reported and is well tolerated at efficacious doses.

Experimental Section

Synthesis and Purification of PENAO. An aqueous solution of sodium carbonate (36.6 g, 345 mmol, 250 mL) was added to a suspension of *p*-arsanilic acid (50.0 g, 230 mmol) in water (250 mL) and cooled to 0 °C. Bromoacetyl bromide (92.6 g, 460 mmol) was added over a period of 10 min to the cooled solution. The reaction was maintained for 1 h below 20 °C. The precipitated product, BRAA, was collected by filtration and washed with water. The wet cake was added to a mixture of methanol (375 mL), 47% aqueous hydrobromic acid (375 mL), and sodium iodide (5.14 g, 34.5 mmol), followed by dropwise addition of a solution of sodium sulphite (86.9 g, 690 mmol) in water (325 mL) over 15 min. The heterogeneous mixture was maintained at room temperature for 2 h and filtered. The filter cake was slurried in a mixture of methanol, 47% aqueous hydrobromic acid, and water (375:150:225 mL), followed by water (375 mL) and 5% aqueous sodium bicarbonate solution (375 mL). The product was filtered and dried under vacuum at 45 °C for 12 h giving 54 g of BRAO (72.8% yield). ^{1}H NMR (CF_3COOD): δ 4.14 (s, 2H), 7.80 (d, J = 7.8 Hz, 2H), 7.95 (d, J = 7.6 Hz, 2H), 11.5 (s, 1H). ^{13}C NMR (CF_3COOD): δ 28.47, 123.69, 133.26, 134.07, 171.4.

To a freshly prepared solution of sodium (10.7 g 465 mmol) in methanol (500 mL) was added D-penicillamine (25.53 g, 171-mmol) under argon atmosphere. The reaction mixture stirred for 5 min. BRAO (50 g, 155 mmol) was added, stirred for 10 min, and pH was adjusted to 3−5 using 5% sulfuric acid in methanol and filtered. The clear filtrate was taken and dry distilled under vacuum to give crude PENAO (190 g, 125% yield). The crude PENAO was further purified by C18 silica gel chromatography using water as eluent. The pure fractions were collected and distilled completely to give 30 g of purified material. The obtained solid was dissolved in methanol (90 mL) and added to a solution of acetone and water mixture (600 mL:45 mL) and stirred for 14 h. The precipitated white product was filtered and dried at 40 °C for 4 h to give 18.1 g (30%) of PENAO. ^{1}H NMR (D_2O): δ1.40 (s, 3H), 1.61 (s, 3H), 3.61 (d, J = 6.34 Hz, 2H), 3.72 (s, 1H), 7.58 (d, J = 7.81 Hz, 2H), 7.74 (d, j = 7.81 Hz, 2H). ^{13}C NMR (D_2O): δ 25.93, 29.68, 35.91, 49.58, 64.10, 124.02, 132.77, 141.55, 146.86, 173.44 ppm.

PENAO purity was characterized by HPLC (1200 Series; Agilent Technologies, Santa Clara, CA) on a Zorbax Eclipse XDB-C18 column (4.6 mm × 150 mm, 5 μm; Agilent Technologies) using a mobile phase of acetonitrile-water (25:75 vol/vol), flow rate of 0.5 mL per min, and detection by absorbance at 256 nm. Purity of PENAO by peak area was 97%.

GSAO, GCAO, and CAO were prepared as previously described.[2,4] All arsenical compounds were dissolved in phosphate-buffered saline containing 100 mM glycine, and concentrations were determined by titrating with dimercaptopropanol and calculating the remaining free thiols with 5,5′-dithiobis-(2-nitrobenzoic acid). The solutions were stored at 4 °C in the dark until use. There was no significant loss in the active concentration of stock solutions of the arsenicals for at least a week when stored under these conditions.

Cell Culture. Bovine aortic endothelial (BAE) cells were from Cell Applications, San Diego, CA, and BxPC-3, HT1080, LLC, PANC-1, MCF-7, HCT116, bEnd.3, HMEC-1, and K562 cells were from ATCC, Bethesda, MD. MDCKII cells were from P. Borst (The Netherlands Cancer Institute, Amsterdam). BAE, HT1080, PANC-1, MCF-7, HCT116, MDCKII, bEnd.3, and LLC cells were cultured in DMEM. K562 and BxPC-3 cells were cultured in RPMI medium. HMEC-1 cells were cultured in MCDB131 medium (Gibco, Gaithersburg, MD) supplemented with 10 ng per mL EGF and 1 μg per mL hydrocortisone. The cells were supplemented with 10% fetal calf serum (FBS), 2 mM L-glutamine, and 10 units per mL penicillin and 10 μg per mL streptomycin. Cell culture plasticware was from Techno Plastic Products (Trasadingen, Switzerland). All other cell culture reagents were from Gibco.

Accumulation of the Arsenicals in BAE Cells. BAE cells were seeded in 6-well plates and allowed to attach overnight. The medium was replaced, and the cells were incubated for 30 min in the absence or presence of transport inhibitors and then with 50 μM GSAO, GCAO, CAO, or PENAO for up to 4 h. Cytosolic arsenic measured by inductively coupled plasma spectrometry.[4]

Drug Transporter Transfectants. Canine kidney epithelial MDCKII cells stably overexpressing MRP1, MRP2, or MRP3,[11,12] human embryonic kidney HEK293 cells stably overexpressing MRP4 and MRP5,[13] and murine embryo fibroblast MEF3.8 cells stably overexpressing MRP6, MDR1, or BCRP[3] were used as described. Cells were grown and maintained as adherent monolayers in DMEM containing 10% calf serum (Cosmic, Hyclone, Tauranga, New Zealand), 10 units per mL penicillin, and 10 μg per mL streptomycin. Cytotoxicity assays were performed as described previously.[17]

Cell Proliferation Assays. BAE, NB4, K562, MDCKII, HT1080, LLC, HCT116, PANC-1, MCF-7, and BxPC-3 cells were seeded at a density of 1.5×10^3, 3×10^3, 4×10^3, 5×10^2, 2×10^3, 5×10^2, 5×10^2, 6×10^3, 6×10^3, and 1×10^4 cells per well, respectively, into 96-well plates. Cells were allowed to adhere overnight at 37 °C in a 5% CO_2, 95% air atmosphere. Cells were untreated or pretreated with transporter inhibitors and then cultured for 24, 48, or 72 h in medium containing 10% fetal calf serum and PENAO. See figure legends for reagent concentrations and times of incubation. Viable 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. DMSO was added to lyse cells, the contents of the wells were homogenized, and the absorbance at 550 nm measured. Cell number in the untreated control was normalized as 100%, and viable cell number for all treatments was expressed as percentage of control.

Mitochondrial Swelling Assay. Mitochondrial permeability transition induction was assessed by light scattering using isolated rat liver mitochondria.[4]

Primary Tumor Growth Assays. Female 7−9 week old Balb C nude were used (UNSW Biological Resource Centre). Mice were held in groups of 3−5 at a 12 h day and night cycle and were given animal chow and water ad libitum. A suspension of 3×10^6 BxPC-3 cells in 0.1 mL of PBS was injected subcutaneously in the proximal midline. Tumors were allowed to establish and grow to a size of ~50 mm^3, after which they were randomized into groups of 2−10. Animals were implanted with 28 day alzet model 1004 micro-osmotic pumps (ALZA Corporation, Palo Alto, CA) subcutaneously in the flank. The pumps delivered vehicle, 0.025, 0.25, or 0.5 mg/kg/day PENAO or 0.25 or 5 mg/kg/day GSAO. Tumor volume and animal weight was measured every 2 or 3 days. Tumor volume was calculated using the relationship length × height × width × 0.523. Tumor doubling time (T_D) was calculated from the tumor growth rate curve during exponential growth using the formula $TD = 0.693/\ln(V_F/V_I)/I_D$, where V_F is final tumor volume, V_I is initial tumor volume, and I_D the interval in days.[18]

Immunohistochemistry. Blood vessel density was estimated by immunostaining sectioned tumors for CD31 (rat antimouse CD31; BD Pharmingen, Australia). Tumor cell proliferation was estimated by immunostaining for PCNA (mouse antihuman PC10; Dako, Carpinteria, CA). Excised tumors were fixed in formalin, embedded in paraffin, and 4 μm sections were cut and mounted on Superfrost slides. Sections were deparaffinized

in xylene, rehydrated in PBS using an ethanol gradient, and microwaved for 5 min to increase antigen retrieval. Sections were incubated with anti-CD31 at 1 in 50 and with anti-PCNA at 1 in 100 dilutions for 60 min at 37 °C and secondary peroxidase-conjugated antibodies for 30 min at 37 °C. Microvessels were counted in each of the five most vascularized, separately located areas or hot spots.[19] Tumor cell proliferative index was determined by counting PCNA-positive cells in 100 tumor cells in three random fields in each tumor tissue. Magnification was 400×.

Statistical Analyses. Results are presented as means ± SD, except for the results shown in Figure 5 where SE are used. All variables were examined for normality and homogeneity of variance. Normality of the distributions was assessed using the Shapiro−Wilk test. Results of blood vessel density and tumor cell proliferative index were analyzed using one-way ANOVA. Posthoc tests, parametric, or nonparametric as appropriate were used to compare mean values. All analyses were performed using GraphPad Prism (GraphPad, San Diego, CA). All tests of statistical significance were two-sided, and p values < 0.05 were considered statistically significant.

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Supporting Information Available: Results are presented that demonstrate that the trivalent arsenical moiety of PENAO is responsible for its antiproliferative activity and that PENAO also reduces the viability of endothelial cells, albeit at concentrations that are higher than those that trigger proliferation arrest. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Don, A. S.; Hogg, P. J. Mitochondria as cancer drug targets. *Trends Mol. Med.* **2004**, *10*, 372–378.
(2) Don, A. S.; Kisker, O.; Dilda, P.; Donoghue, N.; Zhao, X.; Decollogne, S.; Creighton, B.; Flynn, E.; Folkman, J.; Hogg, P. J. A peptide trivalent arsenical inhibits tumor angiogenesis by perturbing mitochondrial function in angiogenic endothelial cells. *Cancer Cell* **2003**, *3*, 497–509.
(3) Dilda, P. J.; Don, A. S.; Tanabe, K. M.; Higgins, V. J.; Allen, J. D.; Dawes, I. W.; Hogg, P. J. Mechanism of selectivity of an angiogenesis inhibitor from screening a genome-wide set of *Saccharomyces cerevisiae* deletion strains. *J. Natl. Cancer Inst.* **2005**, *97*, 1539–1547.
(4) Dilda, P. J.; Ramsay, E. E.; Corti, A.; Pompella, A.; Hogg, P. J. Metabolism of the tumor angiogenesis inhibitor 4-(*N*-(*S*-glutathionylacetyl)amino)phenylarsonous acid. *J. Biol. Chem.* **2008**, *283*, 35428–35434.
(5) Halestrap, A. P.; McStay, G. P.; Clarke, S. J. The permeability transition pore complex: another view. *Biochimie* **2002**, *84*, 153–166.
(6) Halestrap, A. P. What is the mitochondrial permeability transition pore? *J. Mol. Cell. Cardiol.* **2009**, *46*, 821–831.
(7) McStay, G. P.; Clarke, S. J.; Halestrap, A. P. Role of critical thiol groups on the matrix surface of the adenine nucleotide translocase in the mechanism of the mitochondrial permeability transition pore. *Biochem. J.* **2002**, *367*, 541–548.
(8) Murgia, M.; Giorgi, C.; Pinton, P.; Rizzuto, R. Controlling metabolism and cell death: at the heart of mitochondrial calcium signalling. *J. Mol. Cell. Cardiol.* **2009**, *46*, 781–788.
(9) Adams, E.; Jeter, D.; Cordes, A. W.; Kolis, J. W. Chemistry of organometalloid complexes with potential antidotes: structure of an organoarsenic(III) dithiolate ring. *Inorg. Chem.* **1990**, *29*, 1500–1503.
(10) Kobayashi, D.; Nozawa, T.; Imai, K.; Nezu, J.; Tsuji, A.; Tamai, I. Involvement of human organic anion transporting polypeptide OATP-B (SLC21A9) in pH-dependent transport across intestinal apical membrane. *J. Pharmacol. Exp. Ther.* **2003**, *306*, 703–708.
(11) Kool, M.; van der Linden, M.; de Haas, M.; Scheffer, G. L.; de Vree, J. M.; Smith, A. J.; Jansen, G.; Peters, G. J.; Ponne, N.; Scheper, R. J.; Elferink, R. P.; Baas, F.; Borst, P. MRP3, an organic anion transporter able to transport anti-cancer drugs. *Proc. Natl. Acad. Sci. U.S.A* **1999**, *96*, 6914–6919.
(12) Evers, R.; Kool, M.; Smith, A. J.; van Deemter, L.; de Haas, M.; Borst, P. Inhibitory effect of the reversal agents V-104, GF120918 and Pluronic L61 on MDR1 Pgp-, MRP1- and MRP2-mediated transport. *Br. J. Cancer* **2000**, *83*, 366–374.
(13) Wielinga, P. R.; Reid, G.; Challa, E. E.; van der Heijden, I.; van Deemter, L.; de Haas, M.; Mol, C.; Kuil, A. J.; Groeneveld, E.; Schuetz, J. D.; Brouwer, C.; De Abreu, R. A.; Wijnholds, J.; Beijnen, J. H.; Borst, P. Thiopurine metabolism and identification of the thiopurine metabolites transported by MRP4 and MRP5 overexpressed in human embryonic kidney cells. *Mol. Pharmacol.* **2002**, *62*, 1321–1331.
(14) Burkhart, C. A.; Watt, F.; Murray, J.; Pajic, M.; Prokvolit, A.; Xue, C.; Flemming, C.; Smith, J.; Purmal, A.; Isachenko, N.; Komarov, P. G.; Gurova, K. V.; Sartorelli, A. C.; Marshall, G. M.; Norris, M. D.; Gudkov, A. V.; Haber, M. Small-molecule multidrug resistance-associated protein 1 inhibitor reversan increases the therapeutic index of chemotherapy in mouse models of neuroblastoma. *Cancer Res.* **2009**, *69*, 6573–6580.
(15) Jedlitschky, G.; Leier, I.; Buchholz, U.; Barnouin, K.; Kurz, G.; Keppler, D. Transport of glutathione, glucuronate, and sulfate conjugates by the MRP gene-encoded conjugate export pump. *Cancer Res.* **1996**, *56*, 988–994.
(16) Leslie, E. M.; Haimeur, A.; Waalkes, M. P. Arsenic transport by the human multidrug resistance protein 1 (MRP1/ABCC1). Evidence that a tri-glutathione conjugate is required. *J. Biol. Chem.* **2004**, *279*, 32700–32708.
(17) Allen, J. D.; Brinkhuis, R. F.; Wijnholds, J.; Schinkel, A. H. The mouse Bcrp1/Mxr/Abcp gene: amplification and overexpression in cell lines selected for resistance to topotecan, mitoxantrone, or doxorubicin. *Cancer Res.* **1999**, *59*, 4237–4241.
(18) Wolff, N. C.; Randle, D. E.; Egorin, M. J.; Minna, J. D.; Ilaria, R. L., Jr. Imatinib mesylate efficiently achieves therapeutic intratumor concentrations in vivo but has limited activity in a xenograft model of small cell lung cancer. *Clin. Cancer Res.* **2004**, *10*, 3528–3534.
(19) Weidner, N. Current pathologic methods for measuring intratumoral microvessel density within breast carcinoma and other solid tumors. *Breast Cancer Res. Treat.* **1995**, *36*, 169–180.