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Characterization of New Potential Anticancer Drugs Designed To Overcome Glutathione Transferase Mediated Resistance

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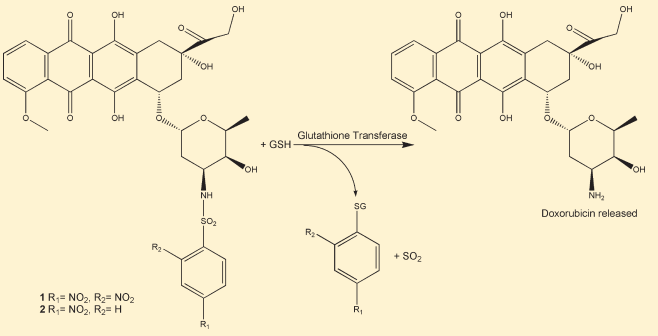
ABSTRACT: Resistance against anticancer drugs remains a serious obstacle in cancer treatment. Here we used novel strategies to target microsomal glutathione transferase 1 (MGST1) and glutathione transferase pi (GSTP) that are often overexpressed in tumors and confer resistance against a number of cytostatic drugs, including cisplatin and doxorubicin (DOX). By synthetically combining cisplatin with a GST inhibitor, ethacrynic acid, to form ethacraplatin, it was previously shown that cytosolic GST inhibition was improved and that cells became more sensitive to cisplatin. Here we show that ethacraplatin is easily taken up by the cells and can reverse cisplatin resistance in MGST1 overexpressing MCF7 cells. A second and novel strategy to overcome GST mediated resistance involves using GST releasable cytostatic drugs. Here we synthesized two derivatives of DOX, 2,4-dinitrobenzenesulfonyl doxorubicin (DNS-DOX) and 4-mononitrobenzenesulfonyl doxorubicin (MNS-DOX) and showed that they are substrates for MGST1 and GSTP (releasing DOX). MGST1 overexpressing cells are resistant to DOX. The resistance is partially reversed by DNS-DOX. Interestingly, the less reactive MNS-DOX was more cytotoxic to cells overexpressing MGST1 than control cells. It would appear that, by controlling the reactivity of the prodrug, and thereby the DOX release rate, selective toxicity to MGST1 overexpressing cells can be achieved. In the case of V79 cells, DOX resistance proportional to GSTP expression levels was noted. In this case, not only was drug resistance eliminated by DNS-DOX but a striking GSTP-dependent increase in toxicity was observed in the clonogenic assay. In summary, MGST1 and GSTP resistance to cytostatic drugs can be overcome and cytotoxicity can be enhanced in GST overexpressing cells.



KEYWORDS: glutathione transferase, drug resistance, cytostatic drug

INTRODUCTION

Cancer is the second leading cause of premature death in privileged countries. Failure of chemotherapy is a significant problem often caused when malignant cells develop resistance against anticancer drugs. Drug resistance is correlated to many different biochemical changes, including decreased influx and increased efflux of the cytotoxic drugs as well as altered expression of genes that control the cell cycle and apoptosis. Cytostatic drugs can also be detoxified by enzymes (e.g., glutathione transferases, GSTs that can be induced during chemotherapy). Some cancer cells have intrinsic resistance (before drug treatment) while others develop resistance during chemotherapy. This acquired resistance frequently involves cross-resistance to other drugs.

Another obstacle in cancer therapy is the severe side effects that are induced by antineoplastic agents. Rapidly dividing cells are especially affected by anticancer drugs, in particular, cells in the bone marrow, hair follicles and gastrointestinal mucosa. There are also specific side effects for different kinds of cytostatic drugs; doxorubicin (DOX), for example, is cardiotoxic,[1] and cisplatin has several known side effects, including nephrotoxicity,[2] ototoxicity[3] and neurotoxicity.[4] These side effects could be reduced by the use of prodrugs that have a higher degree of tumor specificity. Prodrugs are derivatives of cytotoxic drugs that are activated *in vivo* to release the parent drug to exert a desired pharmacological effect.[5] Since GSTs are often overexpressed in

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cancer cells, these enzymes are potential (pro-)drug targets; for a recent review see ref 6.

Glutathione transferases are enzymes that are involved in the cellular conjugation or reduction of hydrophobic, electrophilic substances of both xenobiotic and endobiotic origin. Several cytostatic drugs are detoxified by GSTs.[7] In this study we have used cell lines that overexpress the membrane bound microsomal glutathione transferase 1 (MGST1) and glutathione transferase pi (GSTP) respectively. Both MGST1 and GSTP can be overexpressed in cancer cells compared to nonmalignant cells and have also been linked to anticancer drug resistance.[7−9] For excellent reviews consult refs 7 and 10−12.

Microsomal GST1 is a membrane bound enzyme primarily located in the outer membrane of mitochondria and in the endoplasmic reticulum (ER) membrane. We have previously shown that cells that overexpress MGST1 are more resistant to several common anticancer drugs.[9] MGST1 is distinct from cytosolic GST forms and displays unique features regarding enzymatic properties and its activation by sulfhydryl reagents, such as *N*-ethylmaleimide.[13] It has been shown that several commonly used anticancer drugs are substrates for MGST1 and can also activate MGST1.[14,15] MGST1 is upregulated in many different malignant tissues compared to normal tissues (e.g., testicular, brain, lung, Ewing sarcoma and colorectal tumors).[8,16−20] Microsomal GST1 has also been suggested to be an early marker for tumorigenesis.[19] By performing a comprehensive gene expression analysis it was recently shown that a poor prognosis for Ewing sarcoma patients, due to DOX resistance, is correlated to higher MGST1 expression in tumors.[8]

GSTP is mainly located in the cytosol, but there are indications that GSTP also can be translocated to the nucleus, especially in cancer cells.[21] GSTP has been suggested to have several roles in drug resistance. The protein is involved in direct detoxification, but also plays an indirect role in blocking apoptosis via c-Jun N-terminal kinase 1 (JNK1) inhibition by direct protein−protein interaction.[22] In turn, JNK1 has been shown to be involved in cellular stress response, apoptosis and proliferation. Experiments with the GSTP inhibitor Telintra (TLK199) indicated that TLK199 is involved in dissociating GSTP from JNK.[23] JNK then initiates the signal transduction pathway by phosphorylating c-Jun. TLK 199 is presently being used to stimulate the production of blood cells in the bone marrow in phase II studies on patients with myelodysplastic syndrome (MDS).[24] Another example of a prodrug is Telcyta (TLK-286), which releases toxic phosphorodiamidate to spontaneously form alkylating aziridinium species upon catalytic conversion by GSTP.[25,26]

In this article we have investigated three potential anticancer drugs designed to overcome GST mediated resistance by distinct mechanisms. The first drug, called ethacraplatin, is cisplatin conjugated to ethacrynic acid (EA).[27,28] Ethacrynic acid is a potent GST inhibitor[29] and a diuretic used in the clinic.[30] Ethacraplatin is more hydrophobic than cisplatin and is therefore suggested to have a higher bioavailability. Ethacraplatin is thought to enter cells by passive diffusion where it is cleaved to release cisplatin and GST inhibiting EA. Ang et al. have previously shown that several cancer cell lines are more sensitive to ethacraplatin compared to cisplatin. In these same studies, adducts formed between ethacraplatin and GSTA1-1 and GSTP were observed.[27,28] Here we have further investigated the mechanism of ethacraplatin, focusing on the ability of ethacraplatin to overcome MGST1 mediated cisplatin resistance.[9]

We also tested the cytotoxicity of two different derivatives of DOX, 2,4-dinitrobenzenesulfonyl DOX (DNS-DOX) and 4-mononitrobenzenesulfonyl DOX (MNS-DOX; Figure 1). It has previously been shown that GST enzymes display sulfonamidase activity against other activated sulfonamides.[31,32] The design envisaged that the prodrugs enter the cells via passive diffusion (facilitated by a large increase in lipophilicity) and are then converted into the active parent drug (e.g., DOX) by overexpressed GSTs. Since the mono- and dinitrobenzenesulfonamide moieties are blocking an amino group on DOX suggested to be important for toxicity, the prodrugs are predicted to be less toxic before cleavage,[33,34] achieving not only target specificity but potentially lower side effects as well.

Both MGST1 and GSTP have been shown to be overexpressed in tumor cells compared to nonmalignant tissues, suggested to be early markers of tumorigenesis and connected to resistance against several anticancer drugs.[11,16−19,35] Thus, these enzymes are interesting targets for developing improved anticancer strategies. Here we show that GST inhibitor and GST prodrugs in combination with established cytostatic drugs could be promising strategies overcoming drug resistance at the cellular level. Further, we show that a modification of the drug affecting reactivity and GST turnover can have a large impact on toxicity.

MATERIALS AND METHODS

Chemicals. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline and penicillin/streptomycin were purchased from GIBCO; cisplatin, reduced glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), DOX and EA, all reagents for SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and Western blot analysis were purchased from Sigma Aldrich. Ethacraplatin was synthesized as described.[27,28] DNS-DOX and MNS-DOX were synthesized essentially as described for ref 36.

Synthesis of Prodrug. Prodrug was synthesized as depicted in Scheme 1.

DNS-DOX (1). *N*,*N*-Diisopropylethylamine (60 μL, 340 μmol) was added to a solution of doxorubicin (15 mg, 26 μmol) in dimethylformamide (520 μL) at 0 °C. 2,4-Dinitrobenzensulfonyl chloride (23 mg, 86 μmol) was added to the reaction mixture. The mixture was stirred 35 min at the same temperature and then warmed to room temperature. After stirring for 30 min, the reaction mixture was diluted with ethyl acetate and washed with saturated $NaHCO_3$ aq. The organic layer was dried over Na_2SO_4 and evaporated in vacuo. The residue was purified by flash column chromatography (chloroform: acetone = 3:1) to give desired compound **1** (9.9 mg, 13 μmol, 49%).

^{1}H NMR (400 MHz, DMSO): δ 8.73 (1H, s, DNs), 8.49−8.47 (1H, d, DNs, *J* = 8.7), 8.27−8.24 (1H, d, DNs, *J* = 8.7), 7.89 (2H, S, dox), 7.64 (1H, s, dox), 5.17 (1H, s, dox), 4.89 (1H, s, dox), 4.54 (2H, s, dox), 4.21−4.02 (2H, m, dox), 3.99 (3H, s, dox), 3.62−3.59 (1H, d, dox, *J* = 12.1), 3.34 (1H, s, dox), 2.94 (2H, s, dox, *J* = 6.3), 2.13−1.89 (2H, m, dox), 1.40−1.38 (1H, d, *J* = 8.7), 1.10−1.08 (3H, d, dox, *J* = 6.2). ^{13}C NMR (125 MHz, DMSO): δ 161.39, 156.56, 154.90, 149.85, 147.69, 139.55, 137.56, 136.74, 135.90, 135.14, 134.61, 133.79, 131.72, 127.61, 120.38, 120.19, 119.46, 111.18, 100.27, 75.41, 70.46, 69.27, 67.19, 64.14, 57.10, 50.97, 37.24, 32.61, 30.81, 17.45.

HR-ESI-MS (ESI) *m*/*z*: calcd for $C_{33}H_{30}N_3O_{17}S^-$ ($[M-H]^-$) 772.12656, found 772.12833.

MNS-DOX (2). Synthesis of compound **2** was carried out in a similar manner as described for compound **1** from doxorubicin (15 mg, 0.026 mmol), *N*,*N*-diisopropylethylamine (60 μL, 0.34 mmol), and

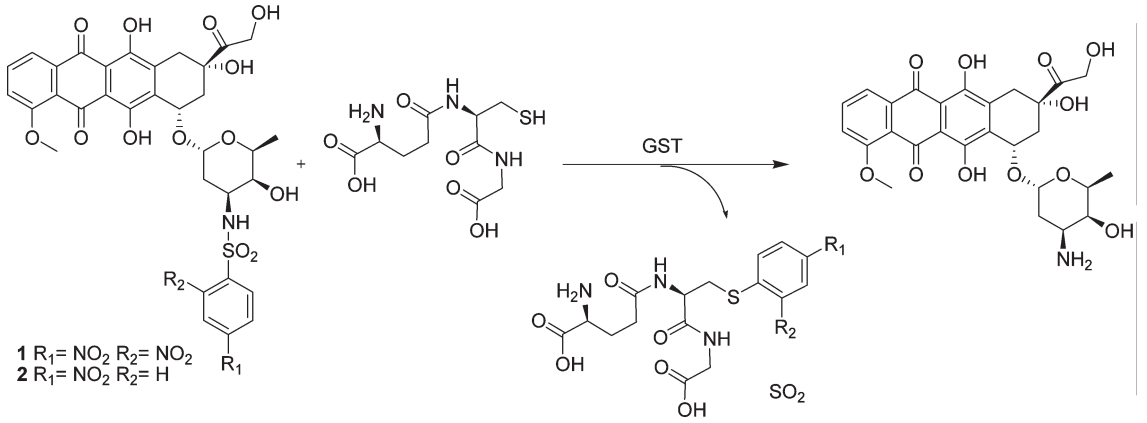


Figure 1. Chemical structures and proposed conversion of DNS-DOX and MNS-DOX by GSTs. Conversion of DNS/MNS-DOX to free DOX, sulfur dioxide and a di/mononitrobenzene moiety conjugated to GSH. DNS-DOX and MNS-DOX consist of DOX conjugated to a dinitrobenzene or mononitrobenzene sulfonamide moiety. These two lipophilic prodrugs are hypothesized to enter the cell via passive diffusion. Within the cell GSTs are able to activate the prodrugs to DOX via their sulfonamidase activity.

4-nitrobenzensulfonyl chloride (16 mg, 73 μmol). Desired compound **2** was purified by reverse-phase HPLC (0−85% acetonitrile/50 mM triethylammonium acetate gradient) to give 4.7 mg (6.4 μmol, 25%).

^{1}H NMR (400 MHz, DMSO): δ 8.73 (1H, s, DNs), 8.49−8.47 (1H, d, DNs, J = 8.7), 8.27−8.24 (1H, d, DNs, J = 8.7), 7.89 (2H, S, dox), 7.64 (1H, s, dox), 5.17 (1H, s, dox), 4.89 (1H, s, dox), 4.54 (2H, s, dox), 4.21−4.02 (2H, m, dox), 3.99 (3H, s, dox), 3.62−3.59 (1H, d, dox, J = 12.1), 3.34 (1H, s, dox), 2.94 (2H, s, dox, J = 6.3), 2.13−1.89 (2H, m, dox), 1.40−1.38 (1H, d, J = 8.7), 1.10−1.08 (3H, d, dox, J = 6.2). ^{13}C NMR (100 MHz, DMSO): δ 186.38, 181.02, 149.79, 164.65, 161.60, 160.78, 156.12, 154.46, 142.12, 139.18, 136.21, 135.50, 134.54, 134.17, 129.27, 126.20, 119.974, 119.01, 116.60, 110.62, 106.62, 99.98, 74.86, 69.83, 69.33, 66.91, 63.59, 56.53, 53.03, 49.67, 20.59, 16.97.

HR-ESI-MS (ESI) m/z: calcd for $C_{33}H_{31}N_2O_{15}S^-$ ($[M-H]^-$) 727.14451, found 727.14734.

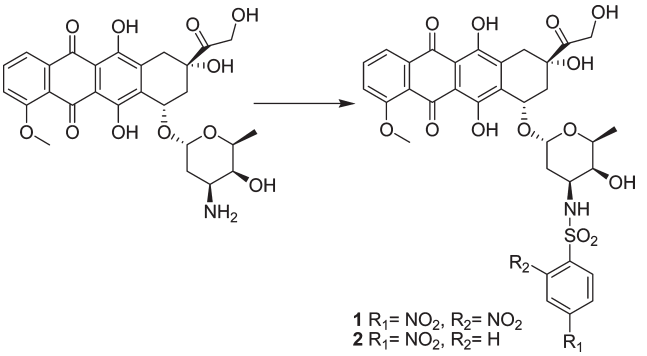
Cell Lines. MCF7wt (human breast carcinoma cells) were transfected with a vector for overexpressing rat-MGST1, or a vector control with antisense against rat-MGST1 (for characterization of the cell lines see refs 9 and 37). V79 cells (Chinese hamster lung fibroblast cells) were purchased from the American Type Culture Collection (ATCC) and transfected with vector overexpressing GSTP1-1 or empty vector.

Culture. MCF7 cells and V79 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), without sodium pyruvate, supplemented with 10% FBS, 100 units penicillin/mL, 100 μg streptomycin/mL and 1 mM sodium pyruvate at 37 °C and 5% CO_2 in a humidified environment. Transfected cells were maintained in the above media supplemented with 1 mg/mL Geneticin for MCF7 transfected cells and 100 μg/mL Hygromycin for V79 transfected cells.

Stable Transfection of Cells Overexpressing GSTP. *Construction of Expression Vectors.* The mammalian expression vector pCEP4 (Invitrogen, Inc.) was modified (pCEP4delta) to remove the sequence required for episomal replication in mammalian cells, in order to allow for selection of clonal transfectant cell lines expressing hygromycin resistance with the vector stably integrated into cellular DNA; this was prepared by *Cla*I digestion as previously described.[38,39]

PCR reactions were performed in 1.5 mM $MgCl_2$, 20 mM dNTP, 10% DMSO, buffer provided and 1 unit of PfuTurbo DNA Polymerase (Stratagene) and were carried out in 10 cycles, each involving denaturation at 94 °C, 30 s, annealing at 40 °C, 60 s, elongation at 72 °C, 2 min, plus 20 cycles with denaturation at 94 °C, 5 s, annealing at 50 °C, 60 s, elongation at 72 °C, 2 min, followed by a final 7 min elongation at 72 °C. The PCR products were isolated by low-melting agarose electrophoresis and subsequently purified from the gel using GenElute gel extraction kit (Sigma).

Scheme 1. Synthesis of Prodrug



The cDNA for human GSTP1-1 (Val^{105}, Ala^{114}), subcloned into pKKdeltaAcc vector, was used as template for PCR reactions and generously provided by Professor Bengt Mannervik. Prior to incorporation into the vector for cytosolic and nuclear localization expression, the cDNA was modified by polymerase chain reaction amplification to include *Hin*dIII and *Bam*H1 endonuclease cleavage sites. For the nuclear expression the nuclear localization signal (NLS) sequence (PKKKRKV)[40] was included in the lower primer, immediately 5′ to the TGA stop codon (Table 1).

The full-length GSTP1-1 was subcloned into the high-copy-number pGEM-T Easy Vector (Promega), sequenced using the SP6 and T7 primers (Promega), and expanded in transformed competent *Escherichia coli* cells. The full-length GSTP1-1 subcloned into pGEM was isolated by low-melting agarose electrophoresis and subsequently purified from the gel and then subcloned into the modified pCEP4 delta vector, which had been linearized with *Bam*HI and *Hin*dIII.

Table 1. Sequences of Primers Used for PCR and Sequence Analysis

	primer	sequence[a,b]
cytosolic expression	U primer-30	aattca**aagctt**gacaaa**atg**ccgccctac
	L primer-24	tctctc**ggatcc**tcactgtttccc
nuclear expression	U primer-30	aattca**aagctt**gacaaa**atg**ccgccctac
	L primer-45	tctctc**ggatcc**tcacacctttctcttcttcttgggctgtttccc
mitochondrial expression	5BamGSTp	c**gggatcc**accggtcgccacc**atg**ccgccctacacc
	GSTpBam3	c**gggatcc**tcactgtttcccgttgccattgatggggaggtt cacgtact

[a] Restriction enzymes and the ATG start codon are bolded. [b] Nuclear and mitochondrial localization signal are underlined.

The sequence of mitochondria targeting double signals (MTS) of cytochrome C oxidase subunit VIII precursor was obtained from pKillerRed-dMito vector (Evrogen). pCEP4 delta vector and pKillerRed d-Mito vector were digested with *Nhe*I and *Bam*HI, and the fragment coding MTS was ligated to the digested pCEP4 delta vector (MTS delta) that was digested again with *Bam*HI prior to dephosphorylation by alkaline phosphatase, calf intestinal (CIP) to avoid self-ligation. In parallel, site-directed mutagenesis was performed to add *Bam*HI sites to both 5′ and 3′ end of GSTP1-1 Val^{105}, Ala^{114} cDNA (see primers in Table 1). The PCR product was subcloned into pGEM vector, and the sequence of PPVAT (overlapped with *Bam*HI partially) from MTS plus GSTP1-1 was verified by automated sequencing. The *Bam*HI fragment with correct sequence was then subcloned into MTS delta digested with *Bam*HI followed by incubation with CIP. Before sequencing of the final expression plasmid the number and direction of the insert were confirmed by key restriction enzyme digestions *Stu*I and *Kpn*I.

Correct base sequence of each construct was confirmed by sequencing (ABI model 377 DNA sequencer) using pCEP Forward and EBV Reverse primers (Invitrogen) for nuclear and cytosolic expressions vectors. For mitochondrial expression vector sequencing LS-358 (5′cgtataggcaggaggctttg3′) was also used to verify the MTS sequence prior to GSTP1-1. The DNA of expression plasmid with correct sequence was prepared by EndoFree Plasmid Maxi kit (Qiagen) for optimal transfection.

Transfection and Selection of Colonies. Transfections were carried out in cells in suspension using Lipofectamine 2000 (Invitrogen) reagent diluted in Opti-MEM I Reduced Serum Medium (GIBCO) according to the manufacturer's instructions. Hygromycin B was added 72 h after transfection (0.7 mg/mL of growth medium), and resistant cell clones were isolated 2−3 weeks after transfection. These colonies were picked when they reached a size visible to the naked eye, by using a pipet tip to gently scrape the colony. The detached colony was then transferred into a drop of trypsin and incubated at 37 °C for 5 min before plating onto a 24-well plate. Once the colony had expanded to a confluent 10 cm tissue culture plate, the cells were harvested in order to freeze aliquots and measure GST activity. Selection of plasmid-containing V79 cells was maintained by the addition of hygromycin (100 μg/mL). Colonies transfected with cytosolic targeted expression vector were very few, and the expression was unstable (declining after a few passages). Therefore in this paper we use the nuclear and mitochondrial targeted GSTP1-1 expressing cells only.

Enzyme Assays. Enzymatic activities of homogenates, cytosolic, microsomal fractions and isolated GSTs were measured using 5 mM GSH and 0.5 mM 1-chloro-2,4-dinitrobenzene (CDNB, $\varepsilon_{340} = 9.6\ mM^{-1}\ cm^{-1}$) as second substrate in a 100 μL cuvette with a single beam Philips PU8700 UV/visible spectrophotometer (Philips Scientific & Analytical Equipment, Cambridge, U.K.) by following the change in absorbance at 340 nm. All enzymatic measurements were performed at room temperature (22 °C). The cytosolic GSTs were assayed in 0.1 M potassium phosphate buffer (pH 6.5), whereas MGST1 was assayed in 0.1 M potassium phosphate buffer (pH 6.5) containing 0.1% Triton X-100 (required for enzyme solubility). Enzyme activities were calculated after correction for the nonenzymatic reaction.

The fluorescence spectrophotometric measurements were performed on a Shimadzu RF-510LC fluorescence spectrophotometer (Analytical Instruments Division, Kyoto, Japan). Essentially the same assay protocol as that described above and in ref 41 was used with 50 μM DNS-DOX and MNS-DOX substituting for CDNB as second substrate. The excitation and emission wavelengths used were 480 and 555 nm, respectively. Initial rates were calculated from the relative increase in fluorescence intensity when DOX was formed. Standard solutions of DOX were used to quantify the signal and control for the inner filter effect. The following amounts of enzyme were used: GSTP 13/130 milliunits in the DNS- and MNS-DOX assays; MGST1 4.7/9.4 milliunits in the DNS- and MNS-DOX assays, respectively. One milliunit converts 1 nmol/min in the CDNB assay.

Enzyme Isolation. Isolation of MGST1 was performed according to Morgenstern, 2005.[42] Isolation of GSTP1-1 was achieved according to Hegazy et al., 2004.[43]

Inhibition Studies. MGST1 enzyme activity toward CDNB was measured as described above, and EA and ethacraplatin were added at different concentrations to determine the degree of inhibition. The IC_{50} value was determined by plotting data with Graphpad Prism.

Cell Uptake of Cisplatin and Ethacraplatin. MCF7 cells (sense and controls) were seeded at a density of 1×10^6 cells/well in 6-well plates and cultured overnight before they were exposed to 80 μM cisplatin or ethacraplatin for 90 min at 37 °C. After exposure cells were washed with PBS three times, trypsinized and centrifuged 1200 rpm for 3 min, the supernatant was removed and the pellet was stored at −20 °C until platinum determination.

Each sample was dissolved in 3 mL of deionized water, mixed with 1 mL of concentrated nitric acid (67% OPTIMA, Fisher Scientific) and digested under nitrogen (40*106 psi) at 250 °C for 30 min (Milestone ultraCLAVE II microwave digestion system, EMLS, Leutkirch, Germany) to obtain carbon-free solutions. The samples were brought to room temperature, transferred to polyethylene tubes (SARSTEDT, Nümbrecht, Germany) and diluted with deionized water to a final weight of 5.4 g. Platinum (m/z 195) was measured using inductively coupled plasma mass spectrometry (ICPMS; Agilent 7700x, Agilent Technologies, Japan. Platinum external standard (CPI International,

Amsterdam, Netherlands) and iridium internal standard (High-Purity Standards; Charleston, SC, USA) solutions were prepared freshly in HNO_3 (OPTIMA, Fisher Scientific, U.K.) before every run. For quality control we used reference material (NIST SRM 2670a High, Toxic Elements in Urine, National Institute of Standards and Technology, Gaithersburg, MD) with certified concentration 51.5 ± 6.6 μg/L, for which we obtained 45.6 ± 2 μg/L (n = 8). The limit of detection (LOD) was 0.001 μg/L (n = 12).

Colony Forming Efficiency. Cells were seeded at a density of 1050 cells/well for MCF7 cells and 1500 cells/well for V79 cells in a 6-well plate; after 24 h, the cells were exposed to cytotoxic agents for 3 h in different concentrations; thereafter the medium was changed to regular culture medium. After 7 days for MCF7 cells and 5 days for V79 cells, medium was removed and cells were washed with 1× PBS, fixed with 10% formaldehyde for 20 min, and stained with 0.01% crystal violet. A colony was defined as at least 16 cells, whereafter the colonies were counted with help of a light microscope.

Cell Proliferation Assay (MTT-Assay). A cell proliferation assay was used as a quantitative colorimetric assay for measurements of cellular cytotoxicity as previously described by Mosman with slight modifications.[44] MGST1 overexpressing cells, vector control cells and MCF7wt cells were run in parallel as well as V79 vector control, MIT and NLS. Briefly, cells were seeded at a density of 1×10^4 cells/well for MCF7 transfected cells and 2×10^4 cells/well for V79 transfected cells in a 96-well plate; after 24 h culturing, the medium was changed to DMEM without phenol red and serum containing cytotoxic drugs at different concentrations; four replicates were used for each concentration. After 24 h the medium was changed to medium supplemented with 0.5 mg/mL MTT, incubated at 4 h. Formazan crystals were dissolved in DMSO, and absorbance was read spectrophotometrically at 590 nm minus a reference at 650 nm.

Western Blot. Levels of MGST1 and GSTP in the cells were estimated by Western blotting. Cells were lysed in 1% SDS, 1% Triton X-100 in dH_2O. 20 μg of protein from cell lysate was separated by SDS−polyacrylamide gel electrophoresis in a 15% gel, transferred to a nitrocellulose membrane, and immunologically stained using polyclonal rabbit IgG against rat MGST1 or human GSTP as the primary antibody and horseradish peroxidase-labeled anti-rabbit IgG as the secondary antibody. Blots were developed by enhanced chemiluminescence using the ECL kit (Amersham Biosciences). Protein determination was performed by Micro BCA protein assay kit in a 96-well plate

Statistical Analysis. Raw data was analyzed with help of Student's unpaired t test.

RESULTS

Ethacraplatin Reverses MGST1 Dependent Resistance against Cisplatin. MGST1 overexpression confers robust protection from cisplatin.[9] Similarly, other GST overexpression systems have been linked to cisplatin resistance and have become the target of investigations to develop new therapies. Such investigations include the design and synthesis of cisplatin derivatives that include a releasable GST inhibitor.[27,28] The EA derivative of cisplatin was shown to be more toxic than cisplatin itself, possibly due to the inhibition of GST enzymes. In this study, both EA and ethacraplatin are shown to be inhibitors of MGST1 with IC_{50} values, in the standard CDNB inhibition assay (see above), of 50 and 10 μM, respectively.

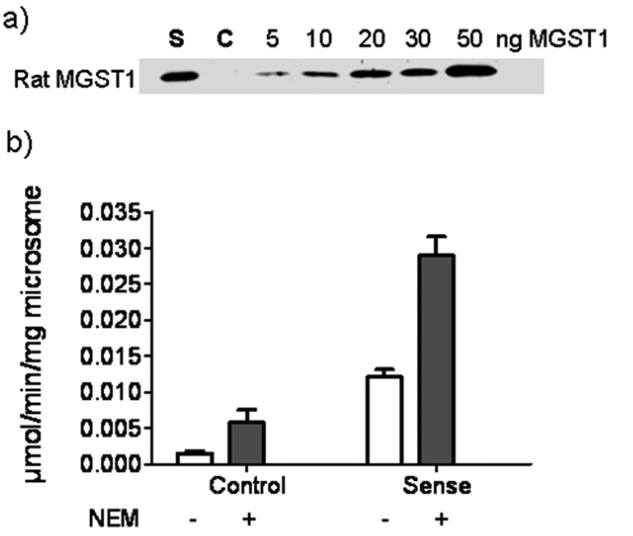


Figure 2. Characterization of the protein levels and enzyme activity in MGST1 overexpressing cells. (a) Protein levels of rat-MGST1 in isolated microsomes from MGST1 overexpressing cells (S) and vector control cells (C). Standard curve of isolated rat-MGST1 with 5, 10, 20, 30, 50 ng of MGST1. (b) Specific activity of MGST1 in microsomes isolated from MGST1 overexpressing cells (sense) and vector control cells (control) ± activation with NEM.

To further clarify the role of MGST1 in cisplatin resistance, the cytotoxicity of cisplatin, ethacraplatin, EA and the coincubation of cisplatin and ethacrynic acid was investigated using MCF7 cells transformed to stably overexpress rat-MGST1. The protein expression and activity of MGST1 were determined by Western blot and activity measurements (Figure 2).

In Figure 3 the previous observation[9] of strong MGST1 dependent protection from cisplatin in the short term MTT toxicity assay as well as with a clonogenic assay was demonstrated (Figure 3a and Figure 3b respectively). When the cisplatin derivative ethacraplatin is used, the MGST1 dependent protection is almost completely eliminated (Figure 3c and Figure 3d).

The advantage of tethering EA and cisplatin into one molecule is further demonstrated by less pronounced toxicity resulting from adding corresponding concentrations of the two parent drugs together (Figure 3e,f). Here, protection by MGST1 is still evident, but compared to cisplatin alone, the coincubation shows more marked decline in cell viability for the MGST1 overexpressing line, relative to the control, reducing the differences between the cell lines. These observations suggest that, while EA enhances cisplatin activity in the control cell line, coadministration of this complex reduces MGST1-induced cisplatin resistance. It is interesting to note that the effects of coadministration are not equivalent to tethering the complexes, suggesting there are additional factors that contribute to the cytotoxic effect, for example, uptake, enhanced MGST1 inhibition or the molecular mechanism. As yet, the molecular mechanism of this complex remains to be elucidated, however it is known that ethacraplatin is taken up effectively into the cells where it is cleaved into the cisplatin and ethacrynic acid moieties.[27,28] To investigate whether ethacraplatin is taken up efficiently also in our cell system we performed uptake experiments. MGST1 overexpressing cells took up 27 ± 2 and 930 ± 130 ng of Pt/10^6 cells (±SEM, n = 6 and 3) when exposed to cisplatin and ethacraplatin respectively. The corresponding values for control cells were 17 ± 2 and 820 ± 60 ng of Pt/10^6 cells (±SEM, n = 6 and 3).

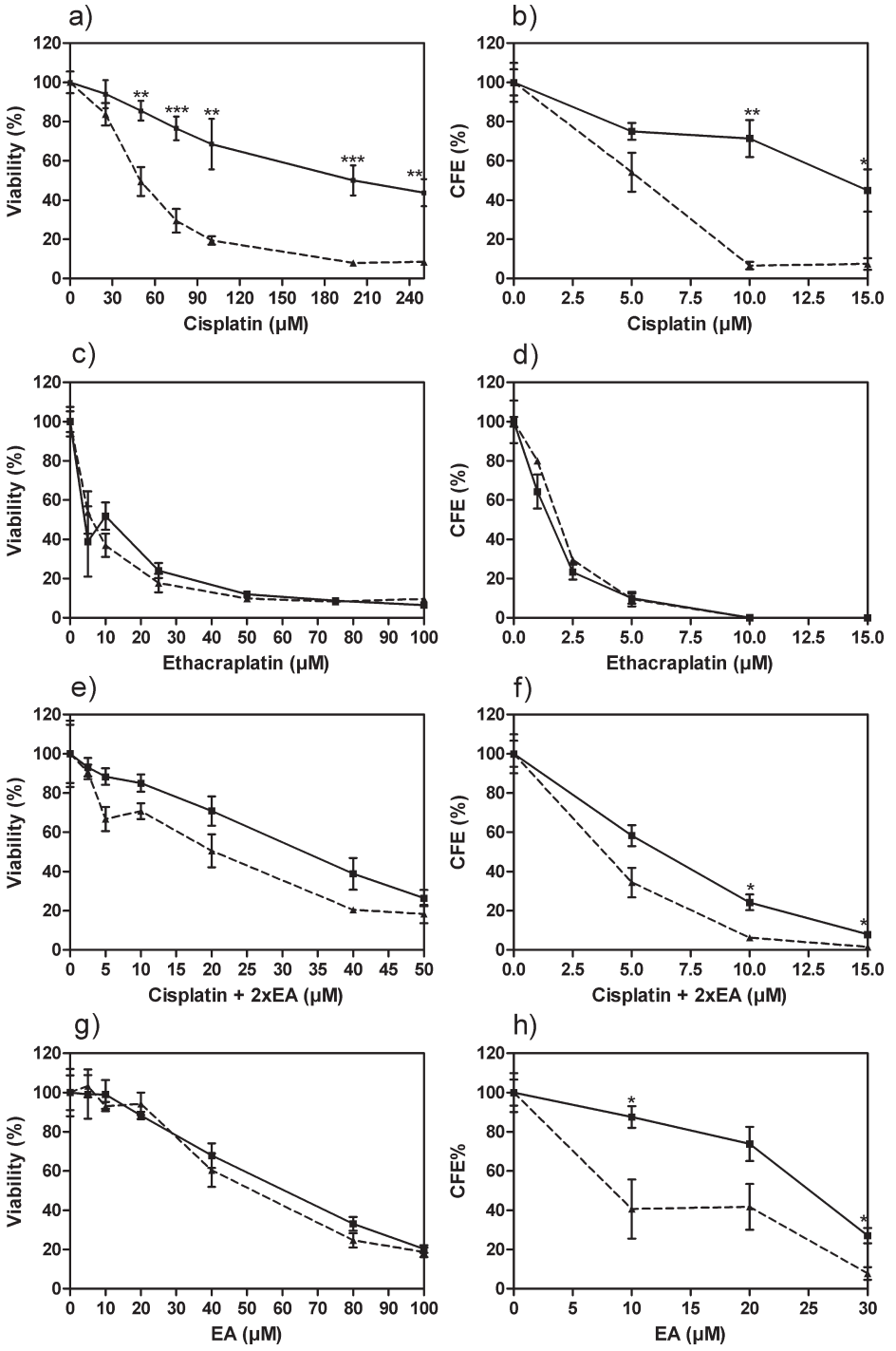


Figure 3. Ethacraplatin reverses cisplatin resistance in MGST1 overexpressing cells. Cytotoxicity was determined by viability test (MTT; a, c, e, g) and a clonogenic assay (CFE; b, d, f, h) where MGST1 overexpressing cells (filled line) and vector control (dashed line) were exposed to cisplatin (a, b), ethacraplatin (c, d), cisplatin and ethacrynic acid (EA) (e, f) (with double concentration of EA to mimic ethacraplatin concentration in b, c) and EA alone (g, h). The cells were exposed to cytotoxic drugs for 24 h for MTT ($n = 4$) and 3 h for CFE ($n = 3$). For CFE the cells were cultured for 7 days before colonies were counted. The results are expressed as mean values ± SEM. Significance levels are $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. Raw data was analyzed with Student's unpaired *t* test.

Ethacraplatin is clearly taken up much more efficiently than cisplatin.

Incubation with EA alone exerts a toxic effect, as expected. It has been shown that EA inhibits the Wnt/β-catenin signaling pathways that are overexpressed in specific cancer cell lines[45] including MCF7.[46] In the MTT assay, the toxic effect follows a similar profile to incubation of cisplatin with the sensitive cell line. However, in contrast to the cisplatin incubation, little difference in EA cytotoxicity is observed between the cisplatin-sensitive and resistant cell types. In contrast, the clonogenic assay discriminates between the two cell lines and the two complexes, with EA being markedly less toxic than cisplatin and more

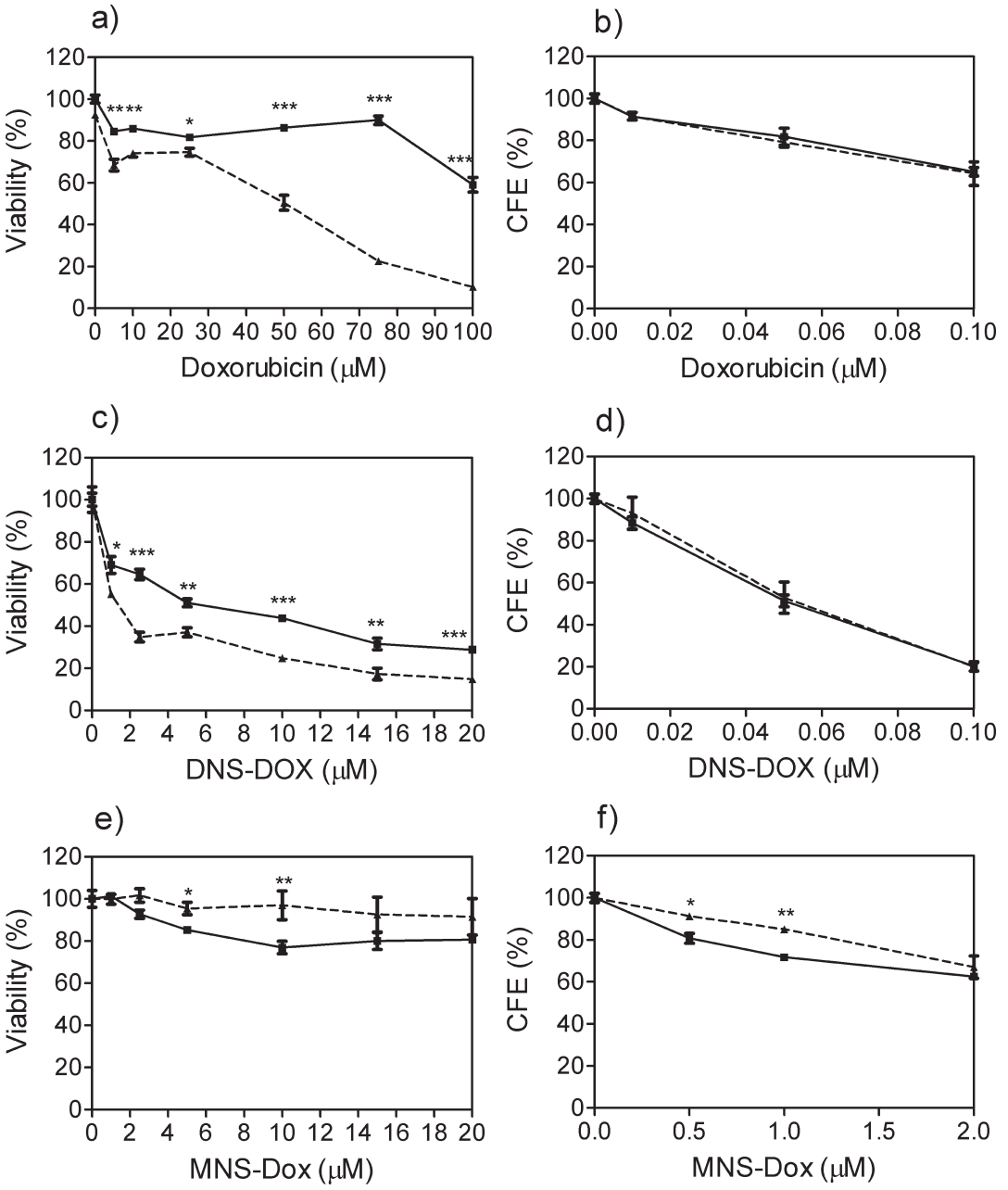


Figure 4. Prodrugs could be used to reverse MGST1 responsible resistance. Short-term cytotoxicity test (MTT; a and b; $n = 4$) and long-term clonogenic assay (CFE; c and d; $n = 3$) where MGST1 overexpressing cells (filled line) and vector control cells (dashed line) were exposed to DOX (a and b), DNS-DOX (c and d) and MNS-DOX (e and f). The cells were exposed to the cytotoxic drugs for 24 and 3 h in the MTT and CFE assays respectively. The results are expressed as mean values ± SEM. Significance levels are $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

effective against the cisplatin sensitive cell line, the latter observation suggesting a protective effect of MGST1 that could account for the enhanced activity of cisplatin on coadministration with EA.

MGST1 Displays Sulfonamidase Activity toward DNS-DOX and MNS-DOX. An alternate strategy to overcome drug resistance and actually take advantage of GST overexpression in tumors involves development of prodrugs that release toxic entities as a result of GST-dependent conversion. With this strategy in mind, two new prodrugs that are derivatives of DOX (DNS-DOX and MNS-DOX) were synthesized. First we wanted to find out if MGST1-sulfonamidase activity is able to activate the two prodrugs to DOX. The sulfonamidase activity was probed using fluorescence spectra of DOX, DNS-DOX and MNS-DOX between 480 to 700 nm with excitation at 480 nm. Peaks were observed at an emission wavelength of 550 nm for all three compounds. The fluorescence intensity was approximately 3 times lower for the prodrugs compared to DOX, which made it possible to measure the increase in fluorescence intensity as MGST1 catalyzes the conversion of the prodrugs and releases fluorescent DOX. By measuring this increase in fluorescence and comparing the fluorescence intensity to a standard with known concentrations of DOX we determined the rate of DOX formation. It could be shown that MGST1 was able to activate the

prodrugs to DOX at a rate of 150 ± 20 nmol/(min mg) and 1.33 ± 0.09 nmol/(min mg) isolated rat MGST1 for DNS-DOX and MNS-DOX respectively (n = 3).

Prodrugs Can Be Used To Sensitize MGST1 Overexpressing Cells to DOX. Previously it has been shown that MGST1 is involved in resistance against DOX in patients suffering from Ewing sarcoma.[47] In this study, we wanted to investigate if MGST1 overexpressing cells are resistant to DOX and if this resistance could be reversed using prodrugs of DOX. Both a short-term toxicity assay (MTT) and a longer term clonogenic assay were used to evaluate the toxicity of DOX, DNS-DOX and MNS-DOX. A significant protection in MGST1 overexpressing cells compared to vector control could be seen when cells were incubated with DOX using the MTT test while no appreciable protection could be seen in the clonogenic test (Figure 4a and Figure 4b respectively). When we exposed the cells to DNS-DOX (Figure 4c and Figure 4d), the protection by MGST1 was diminished; however DNS-DOX was much more toxic to the cells compared to DOX and MNS-DOX. In contrast, when cells were exposed to MNS-DOX, the MGST1 resistance was totally reversed and vector control cells were significantly less sensitive compared to MGST1 overexpressing cells (in both the MTT test and the clonogenic assay, Figure 4e and Figure 4f respectively). Further, it was shown that MNS-DOX was less toxic to both cell lines compared to DOX and DNS-DOX.

Cells Overexpressing GSTP Are Sensitive to DNS-DOX. Since GSTP has been shown to confer DOX resistance, we tested whether, in this case, DNS-DOX could reverse resistance or even be more toxic to GSTP overexpressing cells. GSTP catalyzed the conversion of DNS-DOX to activated DOX with a rate of 84 ± 12 nmol/(min mg). We have generated cell lines that overexpress GSTP in different cellular compartments. Although stable cytosolic overexpression could not be achieved, there are reports of nuclear and mitochondrial localization of GSTP,[21,48,49] especially in cancer cells. Therefore, our cell lines serve as model systems. The NLS cells contain a nucleus targeted construct, and the MIT cells contain mitochondrial targeted construct (see Materials and Methods). The V79 cell lines generated were shown to express human GSTP and higher GST activity than controls (Figure 5a and Figure 5b respectively). Fractionation studies revealed enrichment of GSTP in the nucleus and cytosol for the NLS cells and mitochondria and cytosol for the MIT cells (data not shown). Figure 5a shows protein expression of human GSTP in the cell homogenate of the different cell lines used using a polyclonal anti-GSTP antibody. To determine the cytotoxicity of DOX and DNS-DOX in these cells both the MTT and CFE assay were used. Cells overexpressing GSTP were protected against DOX in both the MTT and CFE assay compared to control (Figure 6a and Figure 6b, respectively, show a significant albeit modest protection). However, when we exposed the cells to the prodrug DNS-DOX this protection was totally reversed. In the clonogenic test, DNS-DOX was much more toxic to cells overexpressing GSTP (Figure 6d). The rank order of protection against DOX (Figure 6b) and increased sensitivity against DNS-DOX (Figure 6d) logically follows the GSTP expression and activity levels (Figure 5).

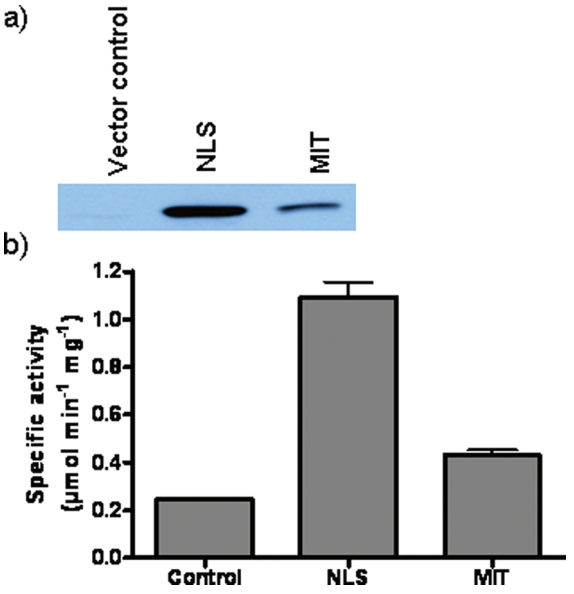


Figure 5. Characterization of GSTP overexpressing V79 cells. (a) Protein detection in NLS (GSTP overexpression with nuclear target construct), MIT (GSTP overexpression with mitochondrial target construct) and vector control cells by Western blot (20 μg protein/lane). (b) GST activity measurements of GSTP overexpressing and control cells (cell homogenates) using the CDNB assay.

■ DISCUSSION

Cisplatin and DOX are used to treat many types of cancer. Nevertheless, the use of both drugs, as well as many others, is limited by the appearance of serious side effects and the frequently observed development of tumor drug resistance. Here we used novel strategies to overcome drug resistance, using a combined prodrug either to inhibit GST or to exploit GST overexpression as a tool to activate prodrugs.

GSTs including MGST1 induce resistance against cisplatin.[9,50] In this study we used ethacraplatin in an attempt to reverse MGST1 resistance. Ethacraplatin consists of cisplatin conjugated to two EA moieties, and Ang et al. have previously shown that ethacraplatin is more toxic than cisplatin and a good GST inhibitor.[27,28] Here we show that ethacraplatin can reverse MGST1 dependent resistance. Reversal of resistance was also achieved by coincubation of the cells with cisplatin and ethacrynic acid, but the effect is not as pronounced as when the complexes are tethered as in ethacraplatin. Ethacrynic acid by itself displays toxicity to MCF7 cells regardless of the presence of MGST1 (Figure 1d), but this toxicity does not fully account for the effects of ethacraplatin. We therefore conclude that the covalent combination of EA and cisplatin does have the ability to reverse MGST1 dependent drug resistance in our model system. The mechanism most likely involved inhibition of MGST1, but the toxic effects of EA and possible enhanced uptake of ethacraplatin compared to the EA and cisplatin given together could also be important.

It should be kept in mind that ethacrynic acid is a potent diuretic agent,[51,52] and, since cisplatin is also known to be nephrotoxic, the coadministration of these two drugs is too toxic for clinical use. On the other hand, the nephrotoxicity of cisplatin is connected to a reactive platinum−glutathione conjugate, a product suggested to be formed by GST catalysis.[53] If the dose of cisplatin could be reduced due to higher potential therapeutic index (in the case of ethacraplatin) together with inhibition of GSTs by EA or other GST inhibitors, less reactive conjugate will be formed that could result in decreased nephrotoxic side effects.

Taken together, cisplatin combined with a GST inhibitor might reverse GST dependent drug resistance and lessen the nephrotoxic

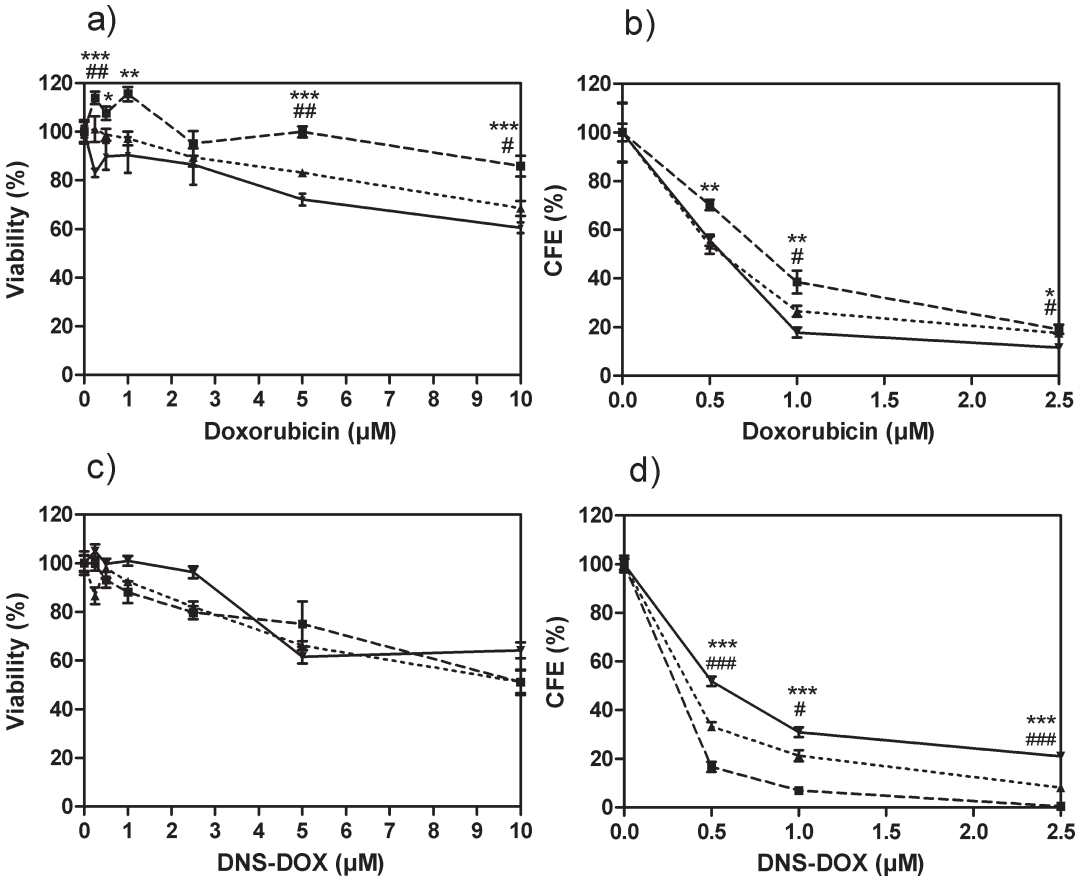


Figure 6. DNS-DOX is more toxic to GSTP overexpressing cells compared with control cells. GSTP overexpressing V79 cells with nuclear target construct (NLS; dashed line), mitochondrial target construct (MIT; dotted line) and vector control cells (filled line) were exposed to DOX (a and b) and DNS-DOX (c and d) in a short-term cytotoxicity test (a and c, $n = 4$) and long-term clonogenic assay (b and d; $n = 3$) . The results are expressed as mean values ± SEM. Significance levels for NLS vs control are $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ and for MIT vs control $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$.

effects of cisplatin−GSH conjugates. We propose that combinations with other GST inhibitors, that do not have (nephro-)toxic properties, would be interesting drug candidates, for instance, indomethacin that has been used in combination with cisplatin in vitro,[54] with an IC_{50} for isolated MGST1 of 4 μM[55] that contains a carboxylate group for synthesizing a cisplatin adduct.

As many tumors overexpress GSTs, attempts have been made to synthesize prodrugs that release cytotoxic moieties upon GST catalysis, e.g., TLK-286.[26] Here we use an alternate strategy to derivatize an existing and well-characterized cytostatic drug DOX such that it becomes less toxic (in the case of MNS-DOX also partly due to limited solubility) and a GST substrate (DNS- and MNS-DOX).

The MCF7 cell systems display low overall GST activity (both basal and transfected where the latter activity is 10-fold lower than in liver and comparable to many extrahepatic tissues[56]) whereas the V79 cell system has a much higher basal GST level (>10-fold). In MGST1 overexpressing cells DNS-DOX treatment did not result in enhanced toxicity but rather an attenuation of protection. One can speculate that nonenzymatic release of DOX can contribute when GST activity is low, but this suggestion needs to be investigated further. From our results it appears that DNS-DOX is more effective when high GST levels are present. Importantly, inasmuch as the clonogenic assay is more relevant to tumor treatment, the most pronounced GST-dependent cytotoxicity enhancement occurs in this assay (MNS-DOX for MGST1 and DNS-DOX for GSTP).

The different character of DOX, DNS-DOX and MNS-DOX was logically reflected in cellular uptake and distribution as studied in living cells using fluorescence microscopy for two hours (data not shown). DOX was rapidly transported to the nucleus. In contrast, the DNS-DOX fluorescence appeared first in the cytosol, whereafter it slowly accumulated in the nucleus. In the case of MNS-DOX, the signal remained solely in the cytosol. As both DOX and its derivatives are fluorescent (with DOX three times stronger), we interpret cytosolic staining as representing prodrug and nuclear accumulation resulting from released DOX.

Our finding of increased toxicity of the DOX derivatives dependent on GST overexpression in cells is an important first step toward realization of drugs based on this principle. Previously, sulfonamide GST substrate derivatives[31,57] have been synthesized, but these examples did not include cytostatics. Our work represents the first description of a GST releasable sulfonamide coupled cytostatic drug. The ease of synthesis opens the possibility that this strategy could be employed for amino group containing compounds in general (e.g., mitomycin). Furthermore, it could be extrapolated that from the limited range of complexes assayed in this report by suitable derivatization of the benzene ring with electron withdrawing groups the GST catalyzed release rate of the cytostatic drug could be controlled. This

derivatization is an important feature that allows fine-tuning for optimal selectivity (e.g., ref 58). In addition, the lipophilic character of the prodrug (and thereby the GSH conjugate formed) can also be altered in a systematic fashion (e.g., ref 59) allowing optimization of GST and drug efflux pump inhibitory properties.

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ABBREVIATIONS USED

BSA, bovine serum albumin; CDNB, 1-chloro-2,4-dinitrobenzene; CFE, colony forming efficiency; DNS-DOX, 2,4-dinitrobenzenesulfonyl doxorubicin; DOX, doxorubicin; EA, ethacrynic acid; FBS, fetal bovine serum; G418, Geneticin; GSH, glutathione; GST, glutathione transferase; MGST1, microsomal glutathione transferase1; GSTP, glutathione transferase pi; NEM, *N*-ethylmaleimide; MNS-DOX, 4-mononitrobenzenesulfonyl doxorubicin; MTS, mitochondria targeting signal; MTT, 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide

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