Correlations between the activities of 19 standard anticancer agents, antioxidative enzyme activities and the expression of ATP-binding cassette transporters: comparison with the National Cancer Institute data

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The aim of this work was to determine the functional activities of four different antioxidative enzymes (glutathione reductase, glutathione-*S*-transferase, glutathione peroxidase, thioredoxin reductase) and the protein expression of three ATP-binding cassette transporters (P-glycoprotein, multidrug resistance protein 1, multidrug resistance protein 2) in a panel of 14 human cancer cell lines. Enzyme activities and transporter expression were then correlated with the in-vitro cytotoxic activities (GI_{50} values) of 19 standard antitumor drugs. Analogous data from the National Cancer Institute were used for comparison. The GI_{50} values of the platinum complexes, alkylating agents, antimetabolites, topoisomerase inhibitors and antimitotic drugs were determined by crystal violet or 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide assay. Standard enzymatic assays employed to measure the glutathione peroxidase, glutathione-*S*-transferase, glutathione reductase and thioredoxin reductase activities. The protein expression of the ATP-binding cassette transporter proteins was investigated by the Western-blot method. The δ method was used to normalize the data before bivariant correlation analysis. Only a few correlations between enzyme and cytotoxic activities of the antitumor agents were found. The GI_{50} values for melphalan and camptothecin correlated positively with the activity of glutathione-*S*-transferase, whereas GI_{50} values for methotrexate correlated positively with the cellular activities of both glutathione reductase and thioredoxin reductase. A significant correlation between glutathione reductase and thioredoxin reductase activities was found in our panel of cell lines. Neither P-glycoprotein nor multidrug resistance protein 2 expression could be detected by Western blot analysis in any cell lines investigated, but multidrug resistance protein 1 was consistently observed in all but four lines. Multidrug resistance protein 1 expression correlates positively with the GI_{50} values of several drugs, e.g. vinblastine and etoposide, and negatively with the GI_{50} values of 5-fluorouracil. The results confirm the complexity of resistance to antitumor agents and show that the GSH–thioredoxin system alone is not a good indication of intrinsic resistance for many of these anticancer drugs. *Anti-Cancer Drugs* 18:389–404 © 2007 Lippincott Williams & Wilkins.

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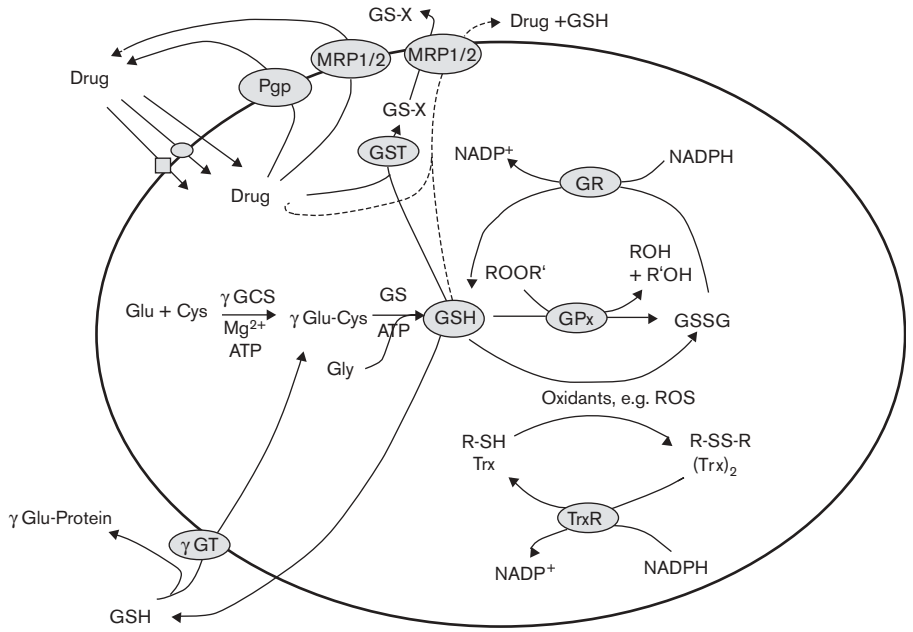
Introduction

It is now generally accepted that resistance is the major obstacle in cancer therapy [1,2]. Resistance is either intrinsic or acquired under therapy, but leads to a failure of therapy in both cases with fatal consequences for the patient. It is therefore important to understand how resistance develops towards a special drug and in return whether a prognosis can be made from the expression of a physiological parameter towards the probable response to a chosen therapy.

The mechanisms underlying resistance are complex and manifold, e.g. decreased drug accumulation owing to impaired cellular uptake or increased export of drugs, activation of antiapoptotic pathways like bcl-2 or loss of normal apoptotic control (e.g. loss of p53 function), enhanced repair systems, higher biotransformation rates of cytotoxic drugs, increased reactive oxygen species detoxification through the glutathione (GSH)–thioredoxin (Trx) redox system, a mutation of target structures (e.g. beta-tubulin) to name a few. We investigated two of these systems: the ATP-binding cassette (ABC) transporters and the GSH–Trx redox system. The interconnection between their individual parts is shown in Fig. 1.

ABC transporters belong to a family of 48 transmembrane transporters [3], divided into seven subfamilies. Twelve transporters are associated with drug resistance, the most

Fig. 1



Thiol and ATP-binding cassette transporter systems. See text for abbreviations

prominent are Pgp (P-glycoprotein; MDR1; ABCB1), MRP1 (multidrug resistance protein 1; ABCC1) and MRP2 (multidrug resistance protein 2; cMOAT; ABCC2). The first one consists of two transmembrane domains with six α helices and a nucleotide-binding domain each [4]; the other two have an additional transmembrane domain consisting of only five α helices resulting in an extracellular orientation of the N-terminus [5]. All three play an important physiological role as pharmacological barriers [6], but they differ in their transport patterns. Pgp is mainly localized in the intestine, liver and kidney, and transports many drugs that are important for chemotherapy such as doxorubicin, etoposide, taxol, vinblastine and methotrexate. In contrast, MRP1 is located in many organs but not in the liver and transports mainly organic anions or GSH conjugates. It transports some of the Pgp substrates like doxorubicin, but also other substances such as few of the alkylating drugs. MRP2 is similar to Pgp in its localization, but resembles MRP1 in its substrate spectrum. A major difference is that MRP2 is able to transport cisplatin, which MRP1 cannot, and that MRP1 and MRP2 also show a cotransport with GSH [4–7]. They reduce the intracellular concentration of drugs by enhancing their transport out of the cells, thus protecting the cellular target structures and finally preventing the cells from undergoing apoptosis.

The GSH–Trx redox system plays an important role for the redox balance and biotransformation of drugs in cells. It allows cells to defend themselves against oxidative stress that might lead to general toxicity and cancer caused by oxidative species that are produced by the cancer cells or by the cytotoxic drugs given to the patient, thus preventing cells from undergoing apoptosis and making a contribution to resistance. The different partners in this system are regulated closely and complement one another (Fig. 1).

GSH is the most abundant low-molecular-weight thiol in the cells with concentrations between 1 and 12 mmol/l in cancer cells [8], nearly 1000-fold higher than those in the plasma or urine [9]. It can be either coupled with electrophilic substrates as part of the phase II biotransformation or serve as a reducing agent in the detoxification of peroxides and oxidants, resulting in oxidized glutathione (GSSG). These reactions occur either spontaneously or enzymatically. GSSG is reduced by glutathione reductase (GR), an enzyme specific for the reduction of GSSG at the expense of nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH) [10] and thus responsible for the high GSH:GSSG ratio in the cells. The GR activity is regulated in dependence of the GSSG and NADPH concentrations in the cells [11].

Trx reductase (TrxR) has a similar function and structure [12,13] as GR, and catalyzes the reduction of Trx, a class of small thiol-containing proteins that also act as antioxidants, similar to GSH. Two different isoenzymes are present in mammals, a cytosolic and a mitochondrial form.

The glutathione peroxidase (GPx) is a selenocysteine protein like TrxR, and catalyzes the decomposition of hydroperoxides to water and alcohol under the expense of GSH [14], thus protecting the cells from the formation and propagation of free radicals that might lead to severe membrane damage and the oxidation of proteins and DNA [15,16]. The GPx exists in several isoforms [14,17,18], mainly a cytosolic, mitochondrial and gastrointestinal form.

The glutathione-S-transferase (GST) plays an important role in the biotransformation of substances, which are either conjugated with GSH and then excreted from the cell (e.g. via MRP1/2) or further converted resulting in mercapturic acid derivatives [19–21]. GST also shows peroxidase activity towards lipid peroxides [19]. Several different isoenzymes exist, each having a specific substrate and expression pattern. Together they constitute about 5% of soluble cell protein.

Both the ABC transporters and the GSH–Trx redox system are reported to be involved in the development of resistance towards cytotoxic drugs. The former is responsible for decreased drug concentrations in the cells, whereas the latter for an improved detoxification of the drugs themselves or their effects. Taken together, both systems prevent damage to the cells/target structures and thus protect cancer cells from apoptosis.

One method to investigate dependencies between biochemical parameters and drug activity is with correlation analysis [22], which measures the association for two or more variables. In a previous publication [23], we looked for correlations between the cytotoxic activities of standard antitumor agents in a panel of 14 diverse human cancer cell lines. In addition, we specifically looked for correlations between potency of standard antitumor agents and the intracellular levels of GSH in those cell lines.

Here, we aim to assess whether there are correlations in the same 14 cell panel (1) among the enzyme activities of GR, GST, GPx and TrxR, (2) among the enzyme activities and the potencies of standard antitumor agents, (3) among the level of expression of Pgp, MRP1 and MRP2 proteins and the potencies of the antitumor agents, and (4) whether the same correlations or lack of correlations can be found in a larger database from the National Cancer Institute (NCI).

Materials and methods

Cell types and drugs

The cell lines employed in this study are shown in Table 1, and were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany). The panel consists of 12 adherent growing cell lines of different origin and two suspension cell lines. Cells were grown in RPMI 1640 medium containing 10% fetal calf serum and supplemented with penicillin G and streptomycin (all from Sigma, Taufkirchen, Germany). The culture medium for MCF-7 was enriched with MEM non-essential amino acid solution (Sigma, M7145) and sodium pyruvate. The adherent cells were passaged weekly (nearly reaching confluence) and the suspension cells were regularly diluted in fresh medium. All cells were incubated at 37°C, humidified atmosphere and 5% CO_2 and used for a maximum of 20 passages.

Determination of cellular enzyme activities

Shortly before reaching confluence, the adherent cells were treated with a 1× trypsin–ethylenediaminetetraacetic acid (Sigma; T3924) solution to obtain a single-cell suspension. The HL-60 and U-937 cell lines could be used directly. Three identical volumes of cell suspension were centrifuged at 800*g* for 5 min at room temperature and two of the resulting pellets were resuspended in isotonic NaCl solution; the cell diameter and number were determined with a Z2 Coulter Counter (Coulter-

Table 1 Human cancer cell lines used in this study

Cell line	Origin	Patient (sex, age)	Doubling time (h)	Diameter (μm)	Treatment
5637	Primary urinary bladder carcinoma	M; 68	30	16.05	None
RT-4	Transitional urinary bladder carcinoma	M; 63	44	17.88	Gold
RT-112	Transitional urinary bladder carcinoma	F; unknown	44	15.99	None
DAN-G	Pancreatic carcinoma	Unknown, unknown	42	18.74	Unknown
YAP-C	Pancreatic carcinoma	M; 43	41	15.12	None
Kyse-70	Esophagus squamous carcinoma	M; 77	35	15.52	None
Kyse-510	Esophagus squamous carcinoma	F; 67	28	18.64	Cisplatin, radiation
Kyse-520	Esophagus squamous carcinoma	F; 58	38	17.44	None
MCF-7	Breast adenocarcinoma	F; 69	63	18.53	Hormone therapy, radiation
A-427	Lung carcinoma	M; 52	38	19.84	Unknown
LCLC-103H	Large cell lung carcinoma	M; 61	28	24.78	Chemotherapy, radiation
Siso	Cervical adenocarcinoma	F; 67	48	16.14	Chemotherapy (resistant)
HL-60	Leukemia (AML)	F; 35	22	11.67	Doxorubicin, vincristin, prednison, ara C
U-937	B-cell-lymphoma	M; 37	22	11.77	Unknown

AML, acute myeloid leukemia; F, female; M, male.

Beckman, Fullerton, California, USA). The remaining pellet was lysed in 0.1% Triton in the according assay buffer; the volume so chosen gave $3–5 \times 10^{-6}$ cells/ml and the lysate was stored on ice. We used standards of known enzymatic activity to calibrate each assay.

For the assay of GR [24–26], 10 standard concentrations of GR (Sigma; G 8404) in the range of 0.00125–0.1 IU/ml were prepared in Triton lysis buffer. In a microtiter plate, 100 µl/well of Triton lysis buffer (blank), standard or sample was added to eight (blank, sample) or four (standards) wells. To all wells, 30 µl of NADPH mixture (0.17 mmol/l NADPH, regenerated by the recycling system reported in [27]) was added. To four of the eight wells containing the sample, 30 µl buffer (143 mmol/l $NaPO_4$; 6.3 mmol/l EDTA; pH 7.4) was added. To all other wells of the plate, 30 µl of GSSG solution (0.5 mmol/l in buffer) was added. The assay was started by addition of 50 µl/well 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) solution (20 mmol/l in buffer) and the optical density of the thionitrobenzoic acid (TNB) formed was measured at $\lambda = 405$ nm every 60 s for 10 min. The values were corrected for the blank, representing the nonenzymatic product formation. As there are other systems in the cell lysate that can result in TNB formation (e.g. TrxR cleaves DTNB directly in the presence of NADPH), the differences between the product formation rates in the samples with and without GSSG were used to determine the GSSG-dependent reaction and thus the GR activity. To the best of our knowledge, this additional control in the traditional assay is reported here for the first time. The activity is calculated by linear regression from a calibration curve constituted from the standards and expressed in IU/ml cell volume.

The assay of TrxR has been described by Holmgren [28] and is also based on the cleavage of DTNB to TNB in the presence of NADPH. The standards contain of 0.02–0.1 IU/ml of TrxR (Sigma, T9698) in lysis buffer. In this case the TrxR activity can be inhibited by auranofin [29], and the difference between the sample with and without auranofin (25 µmol/l in DMSO, resulting concentration is 40 nmol/l) is used to calculate the TNB production by TrxR and therefore the TrxR activity. To 100 µl/well of blank, standard or sample (with and without auranofin) 75 µl of NADPH mix (0.33 mmol/l NADPH, generated from $NADP^+$) and 75 µl of DTNB [10 mmol/l in buffer (100 mmol/l KPO_4; 10 mmol/l EDTA; pH 7.4)] are added, and the optical density is recorded at $\lambda = 405$ nm every 60 s for 10 min and analyzed as described above.

The activities of GST were determined by a method described by Habig [30], which is based on the addition of GSH as nucleophilic substrate to CDNB (1-chloro-2,4-dinitrobenzene). This reaction is catalyzed by GST and results in a product with an absorption maximum at $\lambda = 340$ nm. The standards contain 0.05–0.5 IU/ml of GST (Sigma, G4385). Briefly, to 1.9 ml buffer (100 mmol/l KPO_4; 1 mmol/l EDTA; pH 6.5), 100 µl of GSH (55 mmol/l in buffer), 100 µl of CDNB (22 mmol/l in 96% ethanol) and 100 µl of blank, standard or lysate are added, mixed and the increase in optical density is recorded at 25°C every 30 s for 10 min with a Spekol photometer (Carl Zeiss Technologie, Oberkochen, Germany). The GST activities are plotted from a calibration curve constituted of the standards and is expressed in IU/ml cell volume.

GPx activity was measured according to a method described by Paglia [31]. It is based on the GPx catalyzed decomposition of H_2O_2 to water under the consumption of GSH and production of GSSG. The latter is reduced by GR under NADPH consumption, which is measured at $\lambda = 340$ nm. To ensure a sufficient H_2O_2 concentration, catalase (contained in the cells) is inhibited with sodium azide. The standards for the calibration curve contain 0.005–0.1 IU/ml of GPx (Sigma; G 6137). Briefly, to 1.5 ml buffer (55 mmol/l KPO_4; 1.1 mmol/l EDTA; pH 7.0) 100 µl of each GSH (22 mmol/l in buffer), NADPH (3.7 mmol/l in buffer), NaN_3 (22 mmol/l in buffer) and blank/standard/sample are added and incubated for 5 min at 37°C. Then 200 µl of H_2O_2 (5.5 mmol/l in water) are added and the decrease in optical density is monitored every 30 s for 5 min at 37°C in a Spekol photometer. The GPx activities are plotted from a calibration curve constituted of the standards and is expressed in IU/ml cell volume.

Determination of transporter expression

All experiments were done at least in triplicate. Cells were isolated by scraping the cells from the culture vessels, followed by centrifugation at 300*g* for 4 min. The resulting pellets were washed in PBS and then lysed by multiple freeze–thaw cycles in liquid nitrogen–water bath. The protein content of the lysate was determined by using a Roti-Quant universal kit (Roth, Karlsruhe, Germany). Positive controls (known cell lines with expression of the transporter in question, namely MDCK cells transfected with human Pgp or MRP2, respectively and HeLa cells expressing MRP1) were treated similarly. A prestained molecular weight marker (245–17 kDa; Roth) and 50 µg protein 20 µl lane were submitted to sodium dodecylsulfate–polyacrylamide gel electrophoresis, as described by Laemmli [32], using a 4% stacking gel and a 7% separation gel of 1 mm thickness. Gels were run at 80–90 V and then used for Western blotting [33] on nitrocellulose membranes (0.22 µm pore size) at 370 mA. The membranes were reversibly stained with Ponceau S (0.2% in 3% trichloroacetic acid) to control the quality of electrophoresis and blotting. After destaining, they were blocked overnight under gentle rocking in 10% non-fat dry milk in Tris-buffered saline-Tween-20 (TBST) (0.5% Tween). Incubation with primary antibody in 1% BSA in TBST [all antibodies supplied from Sigma (Taufkirchen,

Germany): anti-Pgp (P7965; 1 : 1000), anti-MRP1 (M9067; 1 : 4000), anti-MRP2 (M8316; 1 : 2000)] and a peroxidase coupled secondary antibody [anti-mouse (A0168; 1 : 6000), anti-rabbit (A 1949; 1 : 6000)] was done at room temperature under gentle rocking and separated by a washing period in TBST. The membranes were also washed prior to luminescence detection, for which Roti-Lumin (Roth) was used according to manufacturer's instruction. Detection was documented by exposure of Kodak-BioMax-XAR film (Kodak, Stuttgart, Germany) for 5–25 min to the membranes; films were developed with Kodak processing chemicals according to manufacturer's instruction.

For quantifying MRP1 expression, the signal intensity was graded by visual comparison with the positive control by two observers, who independently assigned three levels of expression: 'no signal', 'medium signal' and 'strong signal', which were assigned the grade of 0, 0.5 and 1.0, respectively. The intensity of the positive control cell line was always graded with a score of 1. Average grades were then calculated by summing the grades from all plots by both observers and then dividing this sum by the number of independent gels (3–7) run for each cell line and the number of observers (2).

National Cancer Institute data

For the substances tested by us GI_{50} values (μmol/l), GSH levels (nmol/mg protein), mRNA levels of the enzymes and MRP1, determined by using Affymetrix chips, were downloaded from the Developmental Therapeutics Program website at the NCI: http://dtp.nci.nih.gov/docs/dtp_search.html (experiment ID numbers: 6370, 44, 2511, 7504, 29194, 4553, 1154, 29470, 4082, 7933, 44869, 32714, 56357, 6139, 20445, 8156, 25575, 10846, 7816, 26399, 10480, 32152, 3223). We used only those cell lines for which data were available for all of our investigated parameters. This reduced the panel of cell lines from 60 to 30, consisting of leukemia, lung, colon, central nervous system, melanoma, ovarian and renal malignancies (see Table 2).

Table 2 NCI cell lines

Origin	Cell line
Leukemia	CCRF-CEM
	HL-60(TB)
	K-562
	MOLT-4
Large lung cell cancer	A549/ATCC
	HOP-62
	HOP-92
	NCI-H226
	NCI-H23
	NCI-H522
Colon carcinoma	HCC-2998
	HCT-116
	HCT-15
	KM12
	SW-620
CNS	SF-539
	U251
Melanoma	LOX IMVI
	SK-MEL-2
	SK-MEL-28
	SK-MEL-5
	UACC-62
Ovarian	IGROV1
	OVCAR-3
	OVCAR-4
	OVCAR-5
	OVCAR-8
Renal carcinoma	A498
	CAKI-1
	RXF-393

CNS, central nervous system.

Correlation analysis

It should be noted that a statistically significant positive or negative correlation alone is not proof of a real association between two variables, but only provides evidence of possible ones. It has, however, been shown in the past that antitumor agents with high correlations often share a common mechanism of action.

We first normalized our data by calculating δ values (see equation 1) for each parameter investigated in all available cell lines according to the method employed by the NCI COMPARE program and described in [22].

$$\delta = \log(\text{average of parameter over all cell lines}) - \log(\text{parameter in individual cell line})$$

The resulting delta values were entered into SPSS 14.0 software (SPSS, Chicago, Illinois, USA) and bivariant correlation analysis was conducted. The output contains the Pearson correlation coefficients (r), the level of significance in a two-tailed test (P) and the number of cell lines (n) used for the correlation of a given pair of parameters. $P \leq 0.05$ was considered significant; $P \leq 0.02$ was considered very significant and $P \leq 0.01$ marked highly significant correlations. Multivariant correlation analysis was also performed on the data but this did not lead to an improvement in any of the correlations.

Results

GI_{50} values

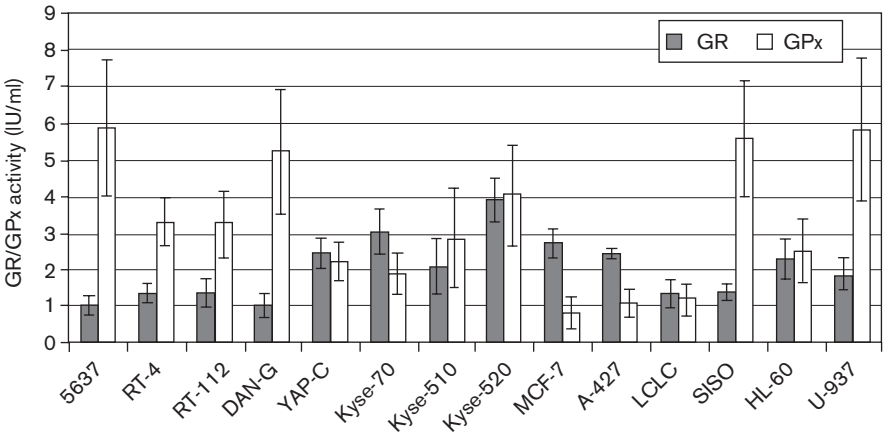
The GI_{50} values for the 19 antitumor agents have been reported by us in detail previously [23]. The suspension cells (HL-60, U-937) are generally more sensitive towards the cytotoxic drugs than the adherent growing cells originating from solid tumors. The only exception from this is 5-fluorouracil (5-FU) which has higher GI_{50} values in HL-60 and U-937. The least cytotoxic drugs are busulfan and HU (hydroxyurea), showing GI_{50} values between 50 and 360 μmol/l. Very potent drugs are the mitosis inhibitors vinblastine (VBL), podophyllotoxin, colchicine and taxol, the topoisomerase inhibitors doxorubicin and camptothecin and the antimetabolite methotrexate (MTX). They are effective in nanomolar or even sub-nanomolar concentrations. The third topoisomerase

Table 3 Intracellular GSH levels (mmol/l) and enzyme activities (IU/ml cell volume) for 14 cell lines; results are means and standard deviations from *n* independent determinations

	5637	RT-4	RT-112	DAN-G	YAP-C	Kyse-70	Kyse-510	Kyse-520	MCF-7	A-427	LCLC-103H	SISO	HL-60	U-937
GSH[a]														
Mean	**2.5**	**12.3**	**1.3**	**9.4**	**4.2**	**7.7**	**6.4**	**4.5**	**2.5**	**1.3**	**4.7**	**2.5**	**4.8**	**5.8**
SD	0.7	5.7	0.2	1.1	0.1	1.5	1.4	0.4	0.2	0.3	0.4	0.3	1.3	1.4
GR														
Mean	**1.0**	**1.3**	**1.3**	**1.0**	**2.5**	**3.0**	**2.1**	**3.9**	**2.8**	**2.5**	**1.3**	**1.4**	**2.3**	**1.9**
SD	0.3	0.2	0.4	0.3	0.4	0.6	0.8	0.6	0.4	0.1	0.4	0.2	0.6	0.4
n	7	8	10	6	7	6	8	4	6	5	9	7	5	5
GST														
Mean	**77.1**	**194**	**134**	**117**	**197**	**160**	**147**	**121**	**4.2**	**54.5**	**26.0**	**2.5**	**48.4**	**45.0**
SD	7.9	48.7	40.3	25.0	45.8	8.7	8.4	31.3	1.9	3.3	4.6	1.4	7.9	7.8
n	5	8	7	6	7	6	5	7	4	4	6	6	5	5
GPx														
Mean	**5.9**	**3.3**	**3.3**	**5.2**	**2.2**	**1.9**	**2.9**	**4.0**	**0.8**	**1.1**	**1.2**	**5.6**	**2.5**	**5.8**
SD	1.9	0.6	0.9	1.7	0.6	0.6	1.4	1.3	0.4	0.4	0.4	1.6	0.9	1.9
n	10	8	6	9	5	7	9	7	5	5	6	11	5	5
TrxR														
Mean	**40.4**	**28.6**	**26.6**	**24.1**	**107**	**171**	**65.4**	**190**	**21.4**	**70.4**	**72.4**	**18.6**	**35.2**	**31.1**
SD	10.5	3.0	5.9	3.9	27.9	23.6	4.4	19.8	6.6	9.1	11.1	4.4	8.1	4.5
n	5	3	4	4	6	5	5	5	4	3	4	5	5	5

GR, glutathione reductase; GPx, glutathione peroxidase; GSH, glutathione; GST, glutathione-*S*-transferase; TrxR, thioredoxin reductase.
[a]Data are from [23].

Fig. 2



Average glutathione reductase (GR) and glutathione peroxidase (GPx) activities (IU/ml cell volume).

inhibitor etoposide is about 10-fold less active then the other two drugs from this class. The alkylating agents melphalan, thiotepa and chlorambucil have GI_{50} values in a similar range, the first two being slightly more potent than the latter one (1–18 versus 3–48 µmol/l). The platinum complexes have different cytotoxicities, their potencies decrease in the following order: Pt-DACH > CDDP (cisplatin) > oxaliplatin > carboplatin. Most of the GI_{50} values were in the therapeutic range (plasma levels) of the drugs.

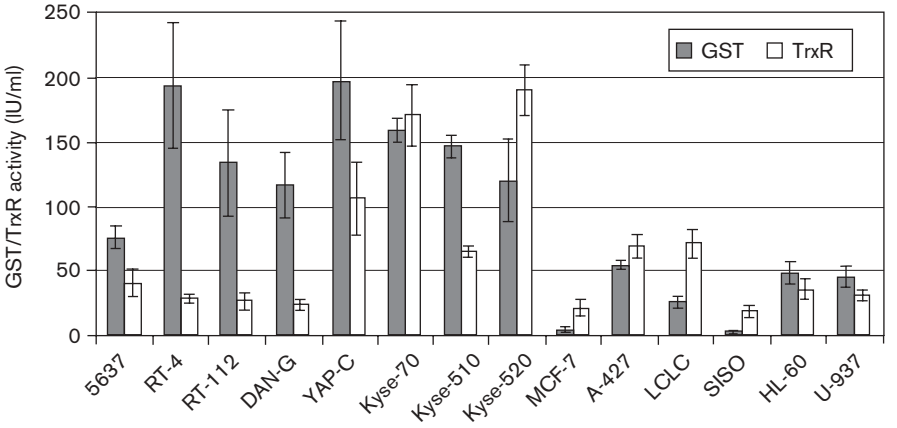
Intracellular glutathione levels and enzyme activities

The intracellular GSH levels (mmol/l) for these cell lines have been reported and discussed by us previously [23]. The GSH levels as well as the enzyme activities (IU/ml cell volume) are reported together in Table 3, and shown in Figs 2 and 3. The results are from at least three independent experiments each and the standard deviation is given in the table.

Detectable GR activity (1.0–3.9 IU/ml cell volume) was found in all cell lines, with highest activities in Kyse-520, Kyse-70 and MCF-7 and lowest in 5637 and DAN-G.

Much higher and more varying activity was found for GST in the 14 cell lines. Enzyme activities range between 2.5 IU/ml in SISO and 197 IU/ml in YAP-C. Generally, the bladder, esophageal and pancreas carcinoma cells have higher GST enzyme activities than the cells originating from breast, lung, cervix and the blood system.

Fig. 3



Average glutathione transferase (GST) and thioredoxin reductase (TrxR) activities (IU/ml cell volume).

The activities of GPx were found to be in a more narrow range, lying between 0.8 and 5.9 IU/ml, with lowest levels in the breast and lung cancer cell lines and highest levels in SISO, 5637 and DAN-G.

TrxR activity is much higher than that of GR, ranging between 18.6 IU/ml in SISO and 190 IU/ml in Kyse-520, and most cell lines lying between 25 and 70 IU/ml. All three bladder cancer cells have low TrxR activities as in HL-60 and U-937.

Expression of P-glycoprotein, MRP1 and MRP2

The expression of the MRP proteins was quantified by grading the intensity of the photographic negatives obtained by means of Western blot methods. Average gradings from multiple blots and by two independent observers are reported in Table 4 and an example radiographic film is shown in Fig. 4.

All cell lines expressed neither Pgp nor MRP2. In addition, MRP1 was not found in Kyse-510, A-427, HL-60 and U-937 cell lines. Kyse-70 generally produced strong signals, while the other nine cell lines expressed less MRP1. MRP1 expression clearly varies between the different esophageal and lung cancer cells.

Correlations between intracellular glutathione concentrations, enzyme activities and activities of antitumor agents

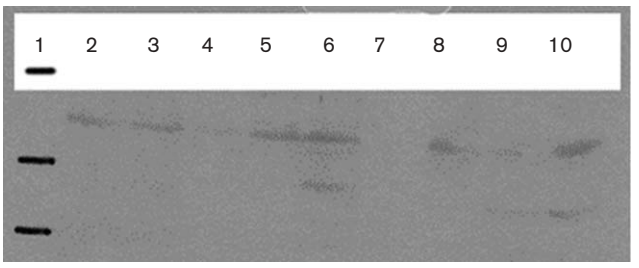
Table 5 shows the Pearson correlation coefficients (PCC, r values) for all the data collected from our panel of 14 cancer cell lines: MRP1 expression, intracellular GSH concentration, activities of GR, GPx, GST and TrxR and the sensitivity towards 19 standard anticancer drugs. The Pearson correlation coefficients were calculated as described above, and $r > 0.532$ ($P < 0.05$), $r > 0.612$

Table 4 Relative expression levels of MRP1 in 14 cell lines determined by Western blot

Cell line	MRP1[a]	*n*
5637	0.21	4
RT-4	0.33	6
RT-112	0.46	6
DAN-G	0.38	6
YAP-C	0.21	6
Kyse-70	0.70	5
Kyse-510	0.07	7
Kyse-520	0.35	5
MCF-7	0.25	4
A-427	0.04	7
LCLC-103H	0.29	6
SISO	0.35	5
HL-60	0.08	3
U-937	0	3

MRP1, multidrug resistance protein 1.
[a]Expression levels were visually graded as strong (1), medium (0.5) or no expression (0). Values are the averages results of *n*-independent Western blots, whereby in each individual experiment, two observers independently graded the expression level. The sum of all grades was then divided by the sum of all observations ($2 \times n$) for each cell line.

Fig. 4



Representative Western blot of MRP1 expression, visualized by luminescence detection. [lane 1: molecular weight marker (245, 123 and 77 kDa are marked in the blot); 2: MRP1 positive control (HeLa cells expressing MRP1); 3: SISO; 4: MCF-7; 5: Kyse-70; 6: RT-112; 7: RT-4; 8: DAN-G; 9: 5637; 10: LCLC-103H]. MRP1, multidrug resistance protein 1.

Table 5 **Pearson's correlation coefficients from the panel of 14 cell lines**

	ABC	Thiol system					Platinum complexes				Alkylating agents				Antimetabolites				Topoisomerase inhibitors			Antimitotics			
	MRP1	GSH	GR	GST	GPx	TrxR	CDDP	Carbo	Oxali	DACH-Pt	Chloram	Melph	Thio	Busul	MTX	FU	Aza	HU	Doxo	Campto	Etopo	Colch	VBL	Taxol	Podoph
MRP1	1.000	0.000	−0.085	0.051	−0.206	0.150	0.052	−0.060	0.193	0.215	0.522	0.465	0.059	0.353	0.113	**−0.572**	0.066	0.382	−0.029	0.215	**0.569**	**0.637**	**0.786**	0.477	**0.638**
GSH		1.000	−0.050	0.391	0.235	0.159	−0.045	0.122	0.043	−0.126	0.329	0.307	**0.541**	0.323	−0.308	−0.006	0.304	0.056	**0.751**	0.434	0.482	−0.054	0.266	−0.267	−0.024
GR			1.000	0.027	−0.451	**0.625**	0.468	0.498	−0.099	0.077	0.194	0.386	0.111	−0.059	**0.544**	0.099	0.206	0.032	0.324	−0.001	0.144	0.344	−0.020	0.445	0.267
GST				1.000	0.197	0.507	0.367	0.343	0.251	0.482	0.453	**0.543**	0.369	0.293	0.250	−0.137	−0.391	0.235	0.374	0.506	0.442	0.103	0.315	0.176	0.406
GPx					1.000	−0.241	−0.362	−0.430	0.334	−0.023	−0.106	−0.381	−0.112	−0.117	−0.104	0.202	−0.174	−0.254	0.104	0.218	−0.205	−0.186	−0.059	0.084	−0.016
TrxR						1.000	0.449	0.419	0.143	0.519	0.415	0.606	0.289	0.133	**0.537**	−0.191	−0.116	0.209	0.337	0.346	0.429	0.266	0.415	0.480	0.543
CDDP							1.000	**0.911**	0.073	0.377	0.493	**0.560**	0.356	0.419	**0.726**	0.011	0.001	0.491	0.103	0.343	0.230	0.265	0.152	0.253	0.225
Carbo								1.000	−0.128	0.168	0.443	**0.555**	0.435	0.307	0.503	−0.013	0.049	0.378	0.269	0.180	0.249	0.218	0.064	0.131	0.157
Oxali									1.000	0.465	0.412	0.063	−0.370	−0.227	0.299	−0.356	−0.040	0.089	−0.151	**0.626**	0.186	0.265	0.145	0.313	0.345
DACH-Pt										1.000	0.408	0.365	−0.021	0.100	**0.610**	−0.337	−0.452	0.126	−0.170	**0.567**	0.121	0.361	0.396	0.323	0.309
Chloram											1.000	**0.866**	0.519	**0.532**	0.415	**−0.648**	0.102	**0.538**	0.193	**0.789**	**0.581**	**0.766**	**0.732**	0.512	**0.724**
Melph												1.000	**0.646**	**0.630**	0.426	**−0.566**	0.003	**0.635**	0.240	**0.609**	**0.610**	**0.616**	**0.684**	0.447	**0.703**
Thio													1.000	**0.833**	0.011	−0.008	0.242	0.364	0.525	0.387	0.406	0.179	0.510	−0.064	0.158
Busul														1.000	0.175	−0.061	0.176	**0.601**	0.217	0.471	0.391	0.215	**0.611**	0.018	0.212
MTX															1.000	−0.102	−0.328	0.266	−0.198	0.438	−0.100	0.419	0.228	**0.668**	0.432
FU																1.000	0.170	−0.450	0.327	−0.436	−0.332	**−0.599**	−0.516	−0.426	**−0.684**
Aza																	1.000	0.198	0.450	−0.049	0.473	0.068	−0.036	−0.243	−0.161
HU																		1.000	−0.151	0.414	0.510	0.185	0.294	0.174	0.447
Doxo																			1.000	0.086	**0.567**	0.067	0.202	−0.096	0.003
Campto																				1.000	0.315	0.401	**0.535**	0.282	0.430
Etopo																					1.000	0.371	0.530	0.126	0.484
Colch																						1.000	**0.667**	**0.721**	**0.714**
VBL																							1.000	0.489	**0.670**
Taxol																								1.000	**0.860**
Podoph																									1.000

Bold indicates significance of the *r* values.
Two-sided test; levels of significance: ***P*<0.05** (*r*>0.532), ***P*<0.02** (*r*>0.612), ***P*<0.01** (*r*>0.661).

ABC, ATP binding cassette; Aza, 5-azacytidine; campto, camptothecin; carbo, carboplatin; CDDP, cisplatin; chloram, chlorambucil; colch, colchin; DACHII, *trans-d,l*-[$PtCl_2$(DACH)] doxo, doxorubicin; etopo, etoposide; FU, fluorouracil; GPx, glutathione peroxidase; GSH, glutathione; GST, glutathione-*S*-transferase; HU, hydroxyurea; melph, melphalan; MRP1, multidrug resistance protein 1; oxali, oxaliplatin; PCC, Pearson's correlation coefficients; podoph, podophyllotoxin; TrxR, thioredoxin reductase; thio, thiotepa; VBL, vinblastine.

($P < 0.02$) and $r > 0.661$ ($P < 0.01$) were considered significant, very significant and highly significant, respectively ($n = 14$; $f = 12$). These values differ slightly from the r values we reported in Table 4 of our previous paper for the same data [23]; this is due to the use of different statistical programs, i.e. SPSS in the present work and MS Excel in the previous.

Only one significant correlation could be found within the enzymes: the functionally similar GR and TrxR correlate ($P < 0.02$). Both enzyme activities also correlate with the sensitivity of drug action of the anti-metabolite MTX, while only TrxR activity correlates also with podophyllotoxin activity. GST activity was found to correlate significantly with the GI_{50} values to melphalan ($P < 0.05$) but not to chlorambucil or thiotepa.

Previously, we found a highly significant correlation between GSH levels and doxorubicin GI_{50} values and a significant one between GSH and thiotepa [23].

Correlations between the MRP1 expression and the other parameters investigated

The expression of MRP1 correlates negatively with 5-FU GI_{50} values and positive with etoposide GI_{50} values ($P < 0.05$). We also found significant correlations between this ABC transporter and the GI_{50} values of the anti-mitotic drugs colchicin, VBL and podophyllotoxin, but not with taxol. We were also not able to establish any correlation between MRP1 expression and the alkylating or platinating drugs (MRP1/chlorambucil just missed significance with $P = 0.055$) as well as with GSH or any of the enzymes investigated.

Comparisons with the National Cancer Institute data

In contrast to us, the NCI determined not enzyme activities or protein expression (MRP1), but mRNA levels by using Affymetrix chips. These allow for the differentiation between several isoforms, which enlarges the correlation table distinctly. In addition, several sets of results were usually available for a given isoform. The partial correlation tables for all 30 cell lines analyzed (see Table 2) are shown in Tables 6 and 7.

In the chosen NCI panel of 30 cell lines, VBL and taxol correlate with cellular GSH levels ($P < 0.05$), but thiotepa or doxorubicin do not. There is no correlation between GR and any of the cytotoxic drugs.

From the 12 GST isoforms, GST A1 shows the most significant correlations. It correlates significantly with the platinum complexes, alkylating agents, hydroxyurea, doxorubicin and camptothecin. The subtypes A2, A3, M1 and M5 do not correlate with any drug, while the remaining GST isoforms correlate randomly with different drugs. With exception of oxaliplatin/GST θ2, none of the GSTs correlate with the platinum complexes.

The GPxs correlate neither with topoisomerase inhibitors nor with antimitotic drugs. But in contrast to our results, correlations can be found between the GPx and the platinum complexes, chlorambucil, melphalan and 5-FU (the latter three correlate negatively).

There are only a few correlations between the TrxRs and various antitumor agents and they differ from the ones we found in our panel of cell lines. In the NCI panel, TrxR-1 correlates positively with chlorambucil and melphalan and negatively with 5-FU, whereas TrxR-3 correlates with HU.

Few correlations can be found between the mRNA levels of the GST and the GPxs, and with exception of TrxR 1/GST A4 none are found for GR or the TrxRs.

As in our case, GSH does not correlate with any of the mRNA levels of any of these enzymes.

Only two correlations can be found for MRP1 in the NCI data; that is, MRP1 mRNA levels correlated negatively with the mRNA levels of GST ω1 ($P < 0.05$) and positively with the GI_{50} of colchicine. The correlation between MRP1 and VBL just missed significance ($P = 0.065$), as did the correlation with GPx 2 ($P = 0.059$).

Discussion

In a previous publication we reported on the correlations between the antitumor activities of 19 anticancer agents in a panel of 14 human cancer cell lines [23]. We showed that this mini-panel of cell lines could pick up many of the same correlations in the activity of the antitumor agents that were found with a comparable but larger panel of cell lines from the NCI. Some new correlations in our panel, however, were seen that were not present in the NCI and vice versa. We also investigated the correlation between intracellular concentrations of GSH and the activities of the antitumor agents [23]; only in the cases of doxorubicin and thiotepa did we find a significant positive correlation between the GI_{50} values and the GSH levels. Here, we expand on the biochemical characterization of the 14 cell lines to include the cellular activities of four enzymes involved in the GSH system: GST, GR, GPx and TrxR. Furthermore, we have characterized all cell lines for the presence of three membrane transporter proteins, Pgp, MRP1 and MRP2, which are known to be involved in cellular resistance to antitumor agents.

Glutathione–thioredoxin reductase redox system and correlation analysis

The GSH–TrxR redox system is important for cell viability and disruptions in this system can lead to apoptosis. The total intracellular GSH concentrations of this panel of 14 cell lines lies within the reported range of

Table 6 Pearson's correlation coefficients from a panel of 30 cell lines (NCI)

	MRP1	GSH	GR	GST A1	GST A2	GST A3	GST A4	GST M1	GST M3	GST M5	GST κ1	GST ω1	GST ω2	GST θ1	GST θ2	GPx1	GPx2	GPx3	GPx4	GPX7	TrxR1	TrxR2	TrxR3
MRP1	1.000	−0.116	0.176	−0.145	−0.040	−0.260	−0.197	−0.239	0.200	−0.328	−0.102	−0.361	0.187	0.074	−0.062	−0.044	0.348	−0.133	−0.162	0.058	0.131	0.123	0.011
GSH		1.000	−0.191	−0.061	−0.060	0.127	0.203	−0.028	−0.212	0.122	0.191	0.351	−0.305	−0.116	−0.076	−0.288	−0.167	−0.263	0.238	0.158	0.118	0.136	0.044
GR			1.000	−0.051	−0.042	−0.221	−0.015	−0.152	−0.064	−0.185	0.018	0.103	0.058	−0.035	−0.099	0.006	0.042	0.074	0.123	−0.343	0.332	−0.084	0.181
GST A1				1.000	−0.024	0.299	0.178	0.237	0.204	−0.275	0.033	0.197	0.063	0.138	0.250	0.050	0.113	0.198	−0.241	−0.238	0.171	0.158	0.213
GST A2					1.000	0.104	−0.199	**0.589**	**0.419**	0.180	**0.394**	0.068	0.337	−0.271	−0.083	−0.060	−0.042	0.024	0.043	−0.048	0.108	0.074	0.112
GST A3						1.000	−0.043	**0.424**	−0.138	−0.270	−0.216	−0.020	−0.250	−0.149	0.092	−0.112	0.160	**−0.423**	−0.095	0.193	0.125	−0.211	−0.035
GST A4							1.000	−0.026	0.183	0.110	0.064	0.315	−0.154	0.133	0.185	−0.106	−0.037	0.180	0.196	−0.048	**0.413**	−0.107	0.324
GST M1								1.000	0.338	0.177	0.105	0.121	0.092	−0.287	0.251	0.039	−0.113	0.120	0.177	0.108	−0.053	−0.086	0.190
GST M3									1.000	0.015	−0.033	−0.068	0.112	0.257	0.226	−0.073	−0.269	0.090	−0.018	−0.013	−0.072	0.230	0.236
GST M5										1.000	**0.449**	0.174	0.221	**−0.379**	0.124	−0.003	−0.291	0.221	0.148	0.073	−0.117	−0.003	0.062
GST κ1											1.000	0.014	0.276	−0.203	0.109	−0.058	0.024	0.151	0.172	−0.217	0.290	0.262	0.127
GST ω1												1.000	−0.103	−0.074	−0.236	−0.126	−0.014	0.119	0.301	−0.022	0.130	−0.118	0.039
GST ω2													1.000	−0.227	−0.186	0.082	0.273	0.182	**−0.381**	−0.220	0.161	−0.212	0.117
GST θ1														1.000	−0.258	−0.028	−0.084	−0.160	−0.167	−0.104	0.048	0.311	0.067
GST θ2															1.000	0.301	−0.007	**0.388**	0.178	0.119	−0.030	0.001	0.303
GPx1																1.000	0.126	**0.455**	−0.102	0.045	−0.196	−0.120	0.130
GPx2																	1.000	−0.136	−0.188	**−0.362**	0.335	−0.142	0.117
GPx3																		1.000	0.047	0.041	−0.020	0.126	0.274
GPx4																			1.000	−0.021	0.058	−0.071	0.005
GPx7																				1.000	−0.201	−0.066	−0.299
TrxR1																					1.000	0.083	0.301
TrxR2																						1.000	0.147
TrxR3																							1.000

Bold indicates significance of the *r* values.
Two-sided test; levels of significance: ***P*<0.05**, ***P*<0.02**, ***P*<0.01**.

GPx, glutathione peroxidase; GR, glutathione reductase; GST, glutathione-*S*-transferase; GSH, glutathione; MRP1, multidrug resistance protein 1; NCI, National Cancer Institute; TrxR, thioredoxin reductase.

Table 7 **Pearson correlation coefficients from a panel of 30 cell lines (NCI)**

	CDDP	Carbo	Oxali	DACHIIa	DACHIIb	DACHIIc	Chloram	Melph	Thio	Busul	MTX	FU	Aza	HU	Doxo	Campto	Etopo	Colch	VBL	Taxol	Podoph
MRP1	−0.098	0.075	−0.048	−0.066	−0.089	−0.045	−0.080	−0.045	−0.122	0.140	−0.314	−0.269	0.293	−0.166	0.007	−0.221	−0.052	**0.448**	0.341	0.102	0.284
GSH	−0.075	0.030	−0.135	−0.318	−0.255	−0.111	−0.042	−0.049	−0.078	0.310	−0.104	−0.042	0.047	0.012	−0.261	0.056	−0.098	−0.286	**−0.367**	**−0.374**	−0.351
GR	0.060	0.114	−0.350	−0.282	−0.283	−0.265	−0.045	−0.054	−0.030	−0.109	−0.113	−0.233	−0.123	−0.080	−0.092	−0.295	−0.122	0.167	0.154	0.228	−0.020
GST_A1	**0.622**	**0.586**	**0.382**	0.308	0.200	**0.530**	**0.612**	**0.627**	**0.547**	**0.387**	0.240	−0.078	−0.172	**0.551**	**0.369**	**0.364**	0.307	−0.074	0.030	0.090	−0.038
GST_A2	−0.004	−0.089	0.185	0.117	0.014	0.123	0.089	0.096	−0.035	−0.081	−0.060	−0.240	−0.020	−0.072	0.048	0.119	0.106	0.004	−0.001	0.142	−0.059
GST_A3	0.039	0.201	−0.022	−0.010	−0.081	0.034	−0.038	−0.117	−0.193	−0.002	−0.190	−0.272	−0.296	−0.138	−0.115	−0.094	−0.270	−0.298	−0.260	−0.238	0.097
GST_A4	0.131	0.054	0.012	0.001	0.125	0.165	0.316	**0.372**	**0.370**	0.358	0.162	0.101	0.200	**0.630**	0.144	0.208	0.273	0.122	0.073	0.054	0.047
GST_M1	−0.072	−0.040	0.094	−0.021	−0.023	0.087	−0.069	−0.034	−0.160	−0.175	−0.095	−0.280	−0.108	−0.022	−0.025	−0.064	−0.136	−0.241	−0.137	−0.009	−0.179
GST_M3	0.077	0.002	0.197	0.127	0.130	0.142	0.242	0.286	0.257	0.222	0.034	0.088	0.079	0.314	**0.382**	0.246	0.338	0.346	**0.368**	0.355	0.143
GST_M5	−0.074	−0.163	0.163	0.293	0.359	0.170	−0.093	−0.024	−0.055	−0.236	0.041	0.168	0.379	0.059	−0.090	0.034	−0.020	−0.113	0.061	0.222	−0.239
GST_κ1	0.255	0.205	0.118	0.159	0.075	0.199	**0.399**	**0.376**	**0.368**	−0.003	0.107	0.015	0.110	0.288	0.181	**0.366**	**0.382**	0.205	0.226	0.350	−0.047
GST_ω1	0.352	0.249	−0.051	−0.028	−0.025	0.358	0.285	0.343	0.271	0.306	−0.133	0.081	0.144	**0.376**	−0.150	0.167	0.098	−0.330	−0.313	−0.296	−0.301
GST_ω2	0.097	−0.003	0.167	0.249	0.279	0.195	0.043	0.051	0.022	−0.310	0.187	**−0.418**	−0.111	−0.045	0.098	0.096	0.002	−0.056	0.052	0.116	−0.257
GST_θ1	0.330	0.148	−0.096	−0.096	−0.053	−0.053	0.302	0.314	**0.422**	0.321	0.066	0.186	0.106	0.244	0.244	0.238	0.271	−0.002	−0.024	−0.083	0.147
GST_θ2	0.046	0.182	**0.442**	0.281	0.316	0.252	0.214	0.202	0.223	0.139	0.002	0.199	−0.066	0.319	**0.392**	0.189	0.351	0.290	**0.384**	**0.397**	0.134
GPx1	0.015	−0.004	0.202	0.275	0.243	0.130	−0.103	−0.052	−0.070	−0.046	0.032	0.111	−0.186	−0.101	−0.114	−0.197	−0.136	0.009	0.079	0.180	−0.040
GPx2	**0.408**	**0.421**	−0.033	0.096	−0.027	0.237	0.265	0.223	0.140	0.136	−0.310	**−0.463**	−0.120	0.076	0.087	0.112	0.122	0.092	0.038	−0.154	0.181
GPx3	−0.040	−0.148	**0.557**	**0.427**	**0.522**	**0.389**	0.058	0.169	0.173	−0.073	0.244	0.235	0.081	0.297	0.092	0.052	0.190	0.068	0.119	0.208	0.004
GPx4	−0.091	−0.020	−0.141	−0.137	−0.128	−0.081	0.038	0.120	0.121	0.191	−0.298	0.101	0.217	0.160	0.099	0.105	0.162	0.124	0.111	0.100	0.103
GPx7	**−0.417**	−0.345	0.016	0.018	0.183	−0.004	**−0.403**	**−0.380**	−0.310	−0.187	−0.253	0.249	0.132	−0.249	−0.307	−0.258	−0.276	−0.238	−0.185	−0.249	−0.150
TrxR1	0.218	0.120	0.176	0.092	0.115	0.199	**0.416**	**0.389**	0.346	0.294	−0.094	**−0.431**	0.084	0.311	0.204	0.215	0.320	0.024	−0.029	−0.048	−0.008
TrxR2	0.170	0.031	0.194	0.027	−0.059	−0.058	0.274	0.257	0.319	0.279	0.194	0.178	0.187	0.152	0.251	0.303	0.353	0.134	0.151	0.064	0.201
TrxR3	0.227	0.241	0.224	0.054	0.007	−0.021	0.224	0.280	0.269	0.095	0.067	−0.154	0.215	**0.385**	0.289	0.136	0.200	0.102	0.142	0.320	0.079

Bold indicates significance of the *r* values.
Two-sided test; levels of significance: ***P*<0.05**, ***P*<0.02**, ***P*<0.01**.

Aza, 5-azacytidine; campto, camptothecin; carbo, carboplatin; CDDP, cisplatin; chloram, chlorambucil; colch, colchicine; DACHII, *trans-d,l*-[$PtCl_2$(DACH)] doxo, doxorubicin; etopo, etoposide; FU, fluorouracil; GPx, glutathione peroxidase; GSH, glutathione; GST, glutathione-*S*-transferase; HU, hydroxyurea; melph, melphalan; MRP1, multidrug resistance protein 1; NCI, National Cancer Institute; oxali, oxaliplatin; podoph, podophyllotoxin; TrxR, thioredoxin reductase; thio, thiotepa; VBL, vinblastine.

1–10 mmol/l, with only RT-4 cells showing slightly higher concentrations (12.3 mmol/l) [9,34].

GR is connected with GPx by making reduced GSH available for the GPx-mediated decomposition of peroxides. It can be seen from Fig. 2 that GR and GPx activities do not correlate with each other, which might be because of the comparatively high GSH levels so that GSH does not become a limiting factor under normal conditions. Austin *et al.* [35] found GR and GPx activities of human muscle fibers to be in a similar range to ours. GPx is very effective in decomposition of peroxides and complements with the H_2O_2-decomposing enzyme, catalase. GPx is mainly localized in the cytosol [18] and is active under lower H_2O_2 concentrations, whereas catalase is localized in the peroxisomes and shows increased activity under high levels of peroxide. It would therefore be interesting to correlate GPx activities with GI_{50} values of H_2O_2.

The functional assay we employed to determine GST activity does not allow us to distinguish between different isoforms, of which GST π is the most prevalent one [36]. GST π is also a tumor marker for a number of different cancers [9]. The differentiation between GST isoforms would only be possible with polymerase chain reaction or immuno-based methods. Therefore our results represent the total functional GST activity of the cells. The distribution pattern of the GST isoenzymes is unique for every organ [19]. In addition to their conjugation activity, GSTs also have a non-selenium-dependent organic peroxidase activity [20]. The GSTs do not interfere in the GPx assay because GST is not able to break down H_2O_2 itself. HL-60 and MCF-7 are the only two cell lines that were common to our panel and the NCI's cell panel of 60. We were able to confirm qualitatively that HL-60 cells have much higher GST activity than MCF-7 [37]. A comparatively wide variation in GST activities, in contrast to other enzyme activities (GPx, GR) and GSH, was also seen in a panel of lung cancer cell lines by Sharma *et al.* [38].

As seen with GST, the TrxR activities vary greatly between the different cell lines. It can be seen from Figs 2 and 3 that cell lines with high GR activity also tend to have higher TrxR activities and *vice versa*. This suggests that some cancer cell lines seem to have developed a generally good antioxidative defense.

Taken together our 14 cell lines show individual profiles of GSH levels and antioxidative enzyme activities, confirming the work of Castro *et al.* [39] and suggesting that the resistance pattern of the cells differs accordingly. This supports the theory that resistance towards cytotoxic drugs is multifactorial and individual for a given cell line. Sharma *et al.* [38] were also not able to detect any correlation between the activities of GPx, GR, glucose-6-phosphate dehydrogenase and the intracellular GSH concentration, adding further evidence to this theory.

It has been reported that resistance towards platinum compounds was conferred by increased GSH levels and GST activities [40–42], much of these data being obtained from cell lines made resistant *in vitro* to cisplatin. We could find no correlation between GSH levels and/or GST activities and cytotoxicity in our panel of cells to support this theory, which is in accordance with the findings Welters *et al.* [43] made in head and neck cancer cell lines. In contrast, Sharma *et al.* [38], found a positive correlation between GST activities and cisplatin GI_{50} values. They conducted their studies in a panel of small cell lung cancer cell lines and this more homogenous panel might be the reason why they found a correlation and we did not as our panel differs to a greater extent in histology. In addition the conjugation of cisplatin with GSH also occurs spontaneously and was never shown to be catalyzed by GST [38], which could explain the lack of correlation between the two.

GSH is known to be a reaction partner in biotransformation of several drugs used in chemotherapy. Some of these are cisplatin, melphalan, chlorambucil, thiotepa and busulfan. Although Komiya *et al.* [44] found that GSH levels correlate with cisplatin GI_{50} values in osteosarcoma cells, we reported previously that we could not find such a correlation in our panel [23]. This is in accordance with reports in the literature [38]. Of the alkylating agents only thiotepa correlates with GSH levels. The highly significant correlation between doxorubicin GI_{50} values and GSH levels may be explained by the oxidative damage caused by doxorubicin and the subsequently better protection from it under high GSH levels. From the drugs tested in our experiments, doxorubicin is the only one prone redox potential.

Chlorambucil, melphalan and thiotepa are all known substrates of GST [9,20,45] and GST is believed to be important for the development of resistance against antitumor drugs. Surprisingly we could only detect a correlation between melphalan and GST activity ($P = 0.045$); despite the structural similarity with chlorambucil no significant correlation was found ($P = 0.104$). Ample evidence exists for increases in GST activities in cells made resistant to alkylating agents *in vitro* [9,46]. Furthermore, a high protective effect is achieved against cytotoxic drugs after transfection with GST [47], but it should be noted that many of these studies were conducted with rodent GSTs and that data are still conflicting. It has also been discussed that an overexpression of GST cannot be responsible for resistance against alkylating agents in resistant cells as they lack cross-resistance [36].

Some evidence exists that increased GPx activities protect cells from oxidative damage [35,48]. Except for

doxorubicin, all drugs tested can only cause oxidative stress indirectly. This may be the reason why we found no correlations between GPx activity and cytotoxic activity of any of the drugs. It was shown by Akman *et al.* [49] that cells made resistant to doxorubicin *in vitro* exhibited a 25-fold increase in the GPx levels compared with the parental cells. In our panel, only the HL-60 cell line was created from cells obtained from a patient treated with doxorubicin [23], but they do not show a particularly high GPx activity. Cisplatin has been found to inhibit GPx and thus might be responsible for a radiosensitizing effect of cisplatin [50], but other researchers were also not able to establish a correlation between the two [38].

The correlation between TrxR and GR can be explained based on their similarity in structure and function. [51,52]. These results suggest that cells upregulate multiple parts of the GSH–TrxR redox system when becoming malignant. The reason we found only one correlation with TrxR and none with GPx and any anticancer drug might be because the cell lines are grown in a selenium-deficient environment, resulting in lower levels of these selenocysteine-containing enzymes than the cells would have *in vivo* [53]. The fact that selenium levels in human serum are 100–500-fold higher than in culture medium containing 10% fetal calf serum [12] and that selenocysteine residue is essential for TrxR function [54] support this theory.

We could not confirm the reported connection between high TrxR activity and high GI_{50} values of cisplatin, which is reported to be an inhibitor of mammalian TrxR [53,55]. As the mechanism of MTX action is not directly based on the formation of ROS, the correlation between MTX and TrxR is difficult to explain.

Transporter expression and correlation analysis

Although the MRP1 protein was consistently expressed in 10 of the 14 cell lines (Table 4), we were not able to detect either Pgp or MRP2 in any of the cell lines. Pgp and MRP2 are both physiologically expressed in colon, liver and kidney tissues [7,56], and we did not use any cell line originating from one of these organs. Cancer cells usually develop multidrug resistance under chemotherapy, probably by selection of cells carrying an ABC transporter. From our 14 cell lines only one (SISO) is known to be resistant to chemotherapy and only two other (HL-60: doxorubicin; Kyse-510: cisplatin) have been treated with Pgp/MRP2 substrates previously. This makes it unlikely that the cells were selected to express one of these transporters. In addition Pgp expression is a common finding in acute myeloid leukemia, but not all AML cells are positive for this transporter protein [57].

The situation is different for MRP1, which is expressed ubiquitous [56] in healthy tissues. It is also reported to be expressed in a wide range of clinical tumors and cell lines [58], showing high expression in a subset of patients with lung (80–90% of lung cancers are MRP1-positive), breast and ovarian cancer or acute myeloid leukemia and chronic leukocytic leukemia [59]. One of our lung cancer cell lines (LCLC-103H) and the breast cancer cell line MCF-7 show MRP1 protein expression, and we also found this transporter in a range of other cell lines (see Table 4), half of which had not been pretreated with chemotherapy previously. This can be explained by the ubiquitous physiological expression of the transporter. On the basis of measurements of mRNA levels, Alvarez *et al.* [60] were able to detect MRP1 in all cell lines from a panel of 60 cell lines of different origin with levels varying 14-fold; showing highest expression in lung and central nervous system cells, and lowest in colon and melanoma cells. Their finding of MRP1 expression in HL-60 cells could not be confirmed by our results but this might be because of the different methods employed (polymerase chain reaction versus Western blot).

Several reports suggest a correlation between MRP expression and GST activity, so that GSH conjugates are built first and then transported from the cell via MRP, also called the GS-X pump [20,60]. Unfortunately, many reports do not discriminate between MRP1 and MRP2 or even other MRP isoforms. No correlation between MRP1 and any of the enzymes or GSH could be found in our panel of cell lines. The latter one could have been expected, as cotransport with GSH as well as transport of GSH adducts by MRP1 is known to occur for several substrates [5,7,56]. But GSH levels (1–10 mmol/l) exceed the drug concentrations (nmol/l to μmol/l) manifold and may thus not become a limiting factor, which would account for not finding any correlation between MRP1 expression and intracellular GSH concentration.

Verkris *et al.* [42] found a negative correlation between oxaliplatin and MRP1 in their in-silico research. We found no correlation to support this for oxaliplatin in our studies but our results are consistent with their finding that there is no correlation between MRP1 and cisplatin or carboplatin. This is further supported by the findings of Xu *et al.* [62], who excluded an involvement of MRP1 in cisplatin resistance [61]. Besides, it is known that cisplatin is not a substrate of MRP1 but MRP2 [58].

Of the cytotoxic drugs tested in this study, several are known to be MRP1 substrates. These are chlorambucil/chlorambucil–GSH conjugate, melphalan–GSH conjugate, thiotepa–GSH conjugate, doxorubicin + GSH, etoposide + GSH, MTX, colchicine, taxol and VBL [5,7,56,58,62], whereas 5-FU and cisplatin are known not to be transported by MRP1. The positive correlations we found were between MRP1 and the substrates etoposide, colchicine and VBL. Interestingly, we observed

a significant negative correlation between MRP1 and 5-FU. On the other hand, Nishioka *et al.* [63,64] were able to show that chemosensitivity against 5-FU is independent of MRP1 expression in colorectal adenocarcinoma.

Comparisons with National Cancer Institute data

When comparing our data with that of the NCI one has to keep in mind the different methods by which these data were obtained. First, the panels themselves differ, only leukemia, breast and lung cancer being represented in both panels. This was a consideration in setting up our panel of cell lines to obtain complementary results but it might be a source for differences in the results. In addition, the NCI set is more than twice as large as our panel of 14 cell lines and thus smaller Pearson correlation coefficient values are needed to reach the same level of significance. Second, the screening for cytotoxic activity differs in the incubation period (96 versus 48 h in the adherent cell lines), the assay employed (MTT/crystal violet versus the NCI sulforhodamine method) and the dilutions tested (two-fold in *N*,*N*-dimethylformamide against 10-fold in dimethylsulfoxide) [23]. Third, GSH content has been determined by the method of Tietze, but is expressed in relation to cell volume or protein content (NCI). Fourth, the NCI used gene chip technology to measure mRNA levels, whereas we measured functional enzyme activity and transporter protein expression in the cells. It was shown by other authors, however, that MRP mRNA expression correlates with protein expression and functional activity of the transporter, determined as calcein efflux [60]. Last, as several sets of data were available for the enzymes and MRP1 on the Developmental Therapeutics Program website, one set was chosen for each enzyme randomly. Taken together, these facts might account for the differences observed in the correlations.

The correlation we found between TrxR and GR is very plausible as explained above. It is therefore interesting. that the NCI panel does not show a correlation between GR and any of the TrxR isoforms investigated. This would lead to a different conclusion, that the thioredoxin Trx and GSH systems are regulated independently.

As described in the literature, the NCI panel shows several correlations between the GSTs and the platinating and alkylating agents, suggesting that GST A1 is the most important in developing resistance towards these drugs.

In both panels no correlation can be found between the selenocysteine enzymes TrxR and GPx. This could have been suspected as they both belong to the antioxidative defense and depend on NADPH/glucose-6-phosphate pathway.

In contrast to our results, the NCI data contain only one correlation with MRP1, i.e. with the known MRP1 substrate colchicine ($P < 0.02$). It is surprising that no more correlations with other known MRP1 substrates could be found in the NCI panel of cell lines. Alvarez *et al.* [60] also found only a few strong correlations between MRP expression and drug sensitivity. Even with known substrates like doxorubicin they found only low Pearson correlation coefficients. They discussed that drug sensitivity cannot be predicted from MRP levels alone.

Conclusion

We were able to confirm some known associations between resistance to cytotoxic drugs and enzymes of the GSH–TrxR redox system and MRP1. We, however, did not find significant correlations for some of the well-known associations (e.g. a correlation between GST and chlorambucil or thiotepa). In addition, some new correlations were found (e.g. between GR/TrxR and MTX). Future work is necessary to confirm these associations.

From the differences between our findings and those from the literature, one can conclude that very different results are obtained when working with intrinsically resistant cell lines compared with cell lines with acquired resistance to antitumor agents. The latter often show increased expression of Pgp and MRP2, which are often related to resistance. In the former case, expression patterns seem to be according to the physiological role of the transporter in question.

Although both the GSH–TrxR redox and ABC transporter systems are often discussed together regarding resistance, we could not confirm that these systems alone determine the level of intrinsic resistance to antitumor agents. Correlation analysis with cancer cell lines is very sensitive to differences in the cell panel used, however, and it might be worthwhile to study more focused panels of cell lines originating from the same tumor types.

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