Correlations between the activities of 19 anti-tumor agents and the intracellular glutathione concentrations in a panel of 14 human cancer cell lines: comparisons with the National Cancer Institute data

Karin Bracht[a], Boubakari[a], Renate Grünert[a] and Patrick J. Bednarski[a]

The aim of this work was 2-fold: (i) to identify correlations between the activities of pairs of 19 anti-tumor agents in a mini-panel of 14 human cancer cell lines of diverse origins with the goal of validating the panel, and (ii) to look for correlations between the activities of 19 standard anti-tumor agents and the intracellular concentrations of glutathione (GSH). Validation with analogous data from the National Cancer Institute (NCI) Developmental Therapeutics Program was made. The cell growth inhibition potencies of the anti-tumor agents [cisplatin, carboplatin, oxaliplatin, DACH-Pt, melphalan, chlorambucil, thiotepa, busulfan, doxorubicin, etoposide, camptothecin, vinblastine, podophyllotoxin, colchicine, taxol, hydroxyurea, methotrexate, 5-azacytidine and 5-fluorouracil (5-FU)] were estimated in 14 cancer cell lines by their GI_{50} values. An enzymatic assay based on the method of Tietze was employed to measure intracellular total GSH concentrations. The Δ method was used to compare pairs of anti-tumor agents; similarities and differences in activity profiles (mean graphs) were evaluated by regression analysis. Most, but not all, of the correlations could be explained based on similarities in the mechanisms of action and many correlations/non-correlations were also observed in the NCI data. Some correlations were unexpected however, and not seen in the NCI data. For example, strong positive correlations ($P < 0.01$) were found between the GI_{50} values of melphalan/chlorambucil and the anti-mitotic agents. Similarly unexpected, a strong positive correlation was observed between methotrexate and cisplatin ($P < 0.01$). Interestingly, moderate to strong negative correlations ($P < 0.01$–0.05) were found between the GI_{50} values of 5-FU and the anti-mitotic agents/melphalan/chlorambucil. Significant positive correlations between intracellular GSH concentrations and GI_{50} values were found only for thiotepa ($P < 0.05$) and doxorubicin ($P < 0.01$). Data from a NCI panel of 34 cancer cell lines showed no correlations between GSH levels and the GI_{50} values of the same 19 compounds. In conclusion, a panel of 14 human cancer cell lines of diverse origin was used to identify similarities and differences in the activities of standard anti-tumor agents. The level of significance was stronger with the 34 cell lines of the NCI, however. Our results indicate that GSH intracellular concentrations correlate with resistance only with doxorubicin and thiotepa in these cell lines. *Anti-Cancer Drugs* 17:41–51

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[a]Department of Pharmaceutical and Medicinal Chemistry, Institute of Pharmacy, University of Greifswald, Greifswald, Germany.

Correspondence to P.J. Bednarski, Department of Pharmaceutical and Medicinal Chemistry, Institute of Pharmacy, University of Greifswald, 17487 Greifswald, Germany.
Tel: +49 3834 86 48 83; fax: +49 3834 86 48 02;
e-mail: bednarsk@pharmazie.uni-greifswald.de

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Introduction

The National Cancer Institute (NCI) Developmental Therapeutics Program uses a very large panel of cell lines (e.g. up to 60) to search for in-vitro correlations between the activity profiles of pairs of chemical substances or between the activity profile of a chemical substance and the cellular levels of a molecular target [1,2]. Analysis is automated by a web-based program known as COMPARE [3], which is free for use by the public (dtp.nci.nih.gov/docs/cancer_search.html). COMPARE uses the Δ method to generate an activity Δ plot ('mean graph') of a seed compound and then sequentially carries out simple linear regression analysis on the Δ plots of all other compounds in the data bank. Similarities in activity profiles of the seed compound and all compounds/biological targets are ranked according to either positive or negative Pearson correlation coefficients (r). Typically, a COMPARE analysis takes less than 1 min over the internet. The power of COMPARE is the ability to predict mechanisms of anti-tumor action for new substances only just screened in the panel of 60 cell lines [4,5] or to identify already tested substances that might be interacting with a newly identified biological target [6,7]. The program has gained great popularity and is widely utilized by the international cancer research community.

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In an effort to implement a smaller, in-house, in-vitro screening program for anti-tumor substances, we have used the NCI program as a conceptual model. Our panel consists of just 14 human cancer cell lines – 12 adherent and two suspension cell lines have been chosen based on three considerations: (i) they all grow in the same medium, (ii) doubling times are roughly 24–48 h and (iii) a minimum of overlap exists between the cell lines used by the NCI to generate complementary results. Like the NCI, we use GI_{50} values (50% cell growth inhibition) as endpoints for the potencies of 19 standard anti-tumor agents of diverse mechanisms of action. In contrast to the NCI, however, which uses sulforhodamine B (SRB) to stain cells, we adopted a method that stains adherent cells with Crystal Violet. In the case of the two suspension cell lines, the well-known MTT method was used. A further difference between the two methods is the number of cell lines used: the NCI typically uses 60 cell lines, while we used here only 14. We analyzed our results with a method analogous to the COMPARE program and validated our results with data obtained from the NCI databank.

Furthermore, we have quantified the intracellular total glutathione (GSH) levels in all 14 cell lines and carried out linear regression analysis with the ΔGI_{50} values obtained in 14 cell lines for the 19 test substances. Our results indicate that even a small panel of cancer cell lines is sufficient to identify similarities between drugs consistent with known mechanisms of action and resistance.

Materials and methods

Cell types and drugs

All cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) (Braunschweig, Germany). Table 1 is a listing of the 12 adherent cell lines [cervical (SISO), breast (MCF-7), bladder (RT-4, RT-112, 5637), pancreas (DAN-G, YAPC), lung (A-427, LCLC-103H) and esophageal (KYSE-70, KYSE-510, KYSE-520)] and two suspension cell lines (HL-60 and U-937) used. Cells were grown in medium containing 90% RPMI 1640 medium (Sigma, Taufkirchen, Germany) and 10% FCS (Sigma), and supplemented with penicillin G/streptomycin. Adherent cell lines were passaged shortly before confluence, whereas suspension cell lines were diluted regularly, depending on cell number, in fresh medium.

Drugs were obtained from the following suppliers and used as received: cisplatin was from Heraeus (Hanau, Germany), carboplatin was from Chempur (Karlsruhe, Germany), oxaliplatin was from Sanofi-Synthelabo (Malvern, Pennsylvania, USA), doxorubicin was from Pharmacia-Upjohn (Stockholm, Sweden), melphalan, chlorambucil, busulfan, thiotepa, etoposide, camptothecin, vinblastine, podophyllotoxin, colchicine, taxol, hydroxyurea, 5-azacytidine (5-AC), methotrexate and 5-fluorouracil (5-FU) were all purchased from Sigma (Tauf-Kirchen, Germany). D,L-DACH-Pt was synthesized as previously described [8]. With the exception of carboplatin, 1000-fold stock solutions of drugs were prepared in DMF (UltraSolv; Merck, Darmstadt, Germany). For carboplatin, 1000-fold stock solutions were prepared in a 1:1 mixture of DMF and water (Millipore-Q; Millipore, Eschborn, Germany). Stocks solutions were stored at $-30^{\circ}C$ and discarded when they became older than 6 months. Immediately prior to testing, stock solutions were removed from the freezer and serially diluted in DMF to concentrations 500-fold the desired concentrations, giving the series of five dilutions. Two-fold dilutions were normally done, but for some of the more potent compounds, i.e. vinblastine and taxol, 10-fold dilutions were made.

Screening methods

Testing was done with all cell lines growing in 96-well microtiter plates. For the adherent cell lines, cells were

Table 1 **Human cell lines used in this study (all cell lines were cultured in the same medium: 90% RPMI 1640 + 10% FCS)**

Cell line	Patient (sex, age)	Origin	Clinical treatment	Doubling time (h)	[GSH] ± SD (mmol/l)	Cell volume (pl)	Reference
SISO	F, 67	cervix adenocarcinoma	chemotherapy resistant	48	2.5 ± 0.3	2.2	[47]
RT-4	M, 63	urinary bladder carcinoma	gold grains	44	12.3 ± 5.7	3.0	[48]
RT-112	F, unknown	urinary bladder carcinoma	none	44	1.3 ± 0.2	2.1	[48]
5637	M, 68	urinary bladder carcinoma	none	30	2.5 ± 0.7	2.2	[49]
A-427	M, 52	lung carcinoma	unknown	38	1.3 ± 0.3	4.1	[50]
LCLC-103H	M, 61	large cell lung carcinoma	chemotherapy	28	4.7 ± 0.4	8.0	[51]
Kyse-70	M, 77	esophageal cell carcinoma	none	35	7.7 ± 1.5	2.0	[52]
Kyse-510	F, 67	esophageal squamous cell carcinoma	cisplatin, radiotherapy	28	6.4 ± 1.4	3.4	[52]
Kyse-520	F, 58	esophageal squamous cell carcinoma	none	38	4.5 ± 0.4	2.8	[52]
MCF-7	F, 69	breast adenocarcinoma	hormone therapy, radiotherapy	63	2.5 ± 0.2	3.3	[53]
DAN-G	unknown, unknown	pancreas carcinoma	unknown	33	9.4 ± 1.1	3.4	DSMZ[a]
YAP-C	M, 43	pancreas carcinoma	none	48	4.2 ± 0.1	1.8	[54]
HL-60	F, 36	acute myeloid leukemia	Doxorubicin, vincristine, prednisone, AraC	22	4.8 ± 1.3	0.83	[55]
U-937	M, 37	histocytic lymphoma	unknown	30	5.8 ± 1.4	0.85	[56]

[a]Originally from the German Cancer Research Center (DKFZ).

plated out 24 h prior to testing at a density of 1000 cells/well in 100 µl medium, except for the LCLC-103H cell lines, which was plated out at 500 cells/well in 100 µl medium. For the suspension cell lines HL-60 and U-937, cells were seeded out at 5000 cells/well in 50 µl medium. At the time the substances were added to the cells, one untreated plate for each cell line was removed and this plate served later as the 'C,0' control (see below). The dilution series of substances were diluted 500-fold into culture medium to give concentrations 2-fold the test concentration. In the case of the adherent cell lines, 100 µl of medium containing test substance as added to each well; for the suspension cell lines, 50 µl of test medium was added. The final DMF concentration in all wells was 0.1%. Two drugs at five concentrations per drug and eight wells per concentration were tested on each plate. Each plate contained two rows of control wells (i.e. 16 wells) for adherent cells, and a row for each blank and control in the MTT assay (see below). Adherent cells were exposed continuously to all drugs for a period of 96 h, whereas suspension cells were exposed to all drugs for a period of 48 h.

The method for measuring growth inhibition of adherent cell lines by Crystal Violet staining has been described in detail elsewhere [8]. Briefly, after a 96-h incubation with substance, the medium was discarded and replaced for 20 min with a 1% glutaraldehyde buffer solution to fix the cells. The fixing solution was discarded and the cells stored under PBS at 4°C until staining. Staining was done for 30 min with a 0.02% solution of Crystal Violet dissolved in water. After discarding the excess dye and washing the cells for 30 min in water, the cell-bound dye was re-dissolved in 70% ethanol/water and the optical density measured at $\lambda = 570$ nm with an Anthos 2010 plate reader (Anthos, Salzburg, Austria).

Growth inhibition of cells in suspensions (i.e. HL-60 and U-937) was measured with a MTT method as previously described [9]. Briefly, 20 µl of a freshly prepared solution of MTT in PBS (2.5 mg/ml) was added to each well 48 h after the test substance was added and the plates were returned to the incubator under protection from light. Following a 4-h incubation, 100 µl/well of 0.04 N HCl in isopropanol was added to dissolve the MTT-formazan product and the optical density was measured at $\lambda = 570$ nm with the Anthos plate reader.

It should be noted that with the Crystal Violet assay all 12 adherent cell lines were grown in 200 µl of medium, which as sufficient to keep the untreated cells in exponential growth over the 96 h drug exposure time. A shorter drug exposure time of 48 h with the HL-60 and U-937 cell lines however, was necessary to assure exponential cell growth over the entire period of drug exposure (i.e. 48 h); because the maximum volume of cell medium that could be added to each well of the MTT method was limited to only 100 µl during the drug exposure period, there was not enough medium to keep the cells growing in their rapid growth phase for 96 h.

To construct the dose–response curves the corrected T/C values were calculated:

$$(T/C)_{corr}(\%) = (OD_T - OD_{C,0})/(OD_C - OD_{C,0}) \times 100 \quad (1)$$

where OD_T is the mean optical density of the treated cells after staining, OD_C is the mean optical density of the controls and $OD_{C,0}$ is the mean optical density at the time the drug was added. The GI_{50} values were estimated by a linear least-squares regression of the T/C_{corr} values versus the logarithm of the substance concentration and extrapolating to T/C_{corr} values of 50%; only concentrations that yielded T/C_{corr} values between 10 and 90% were used in the calculations.

Determination of intracellular GSH

A recently improved version of the GSH reductase method of Tietze was used to determine the intracellular total GSH concentrations in all 14 cell lines, as described in detail elsewhere [10]. GSH concentrations in adherent cells were determined just before the cells reached confluence in 24-well plates. For the two suspension cell lines, the cells were first isolated by centrifugation before they were lysed and assayed for GSH. For the adherent cell lines, nuclei were automatically counted with a Coulter Counter *Z2* (Beckman-Coulter, Fullerton, California, USA) to estimate the number of cells [10]. For the suspension cell lines, the cells were counted directly. Cell sizing was done with the *Z2* fitted with a 100-µm capillary ($K_d = 58.06$). The calculated volumes are reported in Table 1

NCI data

The GI_{50} values for the 19 standard anti-tumor agents were downloaded from the website of the NCI (dtp.nci.nih.gov/docs/cancer/searches/standard_mechanism.html). Only the 34 cell lines that were common to all substances were used in the regression analysis (Table 2). The GSH levels (nmol/mg protein) were determined with the Tietze method by Dr Anne Monks at the Frederick Cancer Research and Development Center and kindly provided by Dr Susan Holbeck of the NCI (dtp.nci.nih.gov/mtweb.targetinfo?moltid=MT1501&moltnbr=44; ExperimentId:44).

Correlation analysis

Analogous to the method used by the NCI [3], correlations were made with data normalized according to the equations (Δ method):

$$\Delta GI_{50} = \log(\text{average } GI_{50} \text{ over all cell lines}) - \log(\text{individual } GI_{50} \text{ for each cell line}) \quad (2)$$

Table 2 The 34 cell lines of the NCI used for the regression analysis

Origin	Cell line
Leukemia	CCRF-CEM
	HL-60(TB)
	K-562
	MOLT-4
Large cell lung cancer	A549/ATCC
	HOP-62
	HOP-92
	NCI-H226
	NCI-H23
	NCI-H460
	NCI-H522
Colon carcinoma	HCC-2998
	HCT-116
	HCT-15
	HT29
	KM12
	SW-620
CNS	SF-295
	SF-539
	U251
Melanoma	LOX IMVI
	SK-MEL-2
	SK-MEL-28
	SK-MEL-5
	UACC-62
Ovarian cancer	IGROV1
	OVCAR-3
	OVCAR-4
	OVCAR-5
	OVCAR-8
Renal carcinoma	A498
	CAKI-1
	RXF 393
	SN12C

$$\Delta[\mathrm{GSH}] = \log(\text{average [GSH] over all cell lines}) - \log(\text{individual [GSH] for each cell line}) \quad (3)$$

Linear regression analysis of ΔGI_{50A} versus ΔGI_{50B}, where A and B represent two different substances, or ΔGI_{50A} versus Δ[GSH] were performed with Microsoft Excel. The Pearson correlation coefficient (r) was used to establish the level of significance in a two-tailed test with $(n-2)$ degrees of freedom [11]. $P < 0.05$ was considered significant.

Results

Correlations between activities of anti-tumor agents

Table 3 presents the average GI_{50} values for the 19 substances in 14 cell lines. By using the Δ method, GI_{50} values obtained with the individual cell lines are normalized to the average GI_{50} over all cell lines (Eqs 2 and 3). In the first analysis, the ΔGI_{50} values for the pairs of anti-tumor agents were compared by regression analysis. Figure 1 shows the results of representative analyses for cisplatin and carboplatin, for vinblastine and chlorambucil, and for 5-FU and colchicine. Each data point represents a cell line. The correlation coefficients (r) obtained by this analysis are shown in Table 4.

As expected, cisplatin and carboplatin show a very strong correlation due to the similarity of their mechanism of

Table 3 Average GI_{50} values (μmol/l ± SD; results are from three to six independent determinations)

	HL-60	U-937	LCLC	5637	A-427	RT-112	SISO	RT-4	DAN-G	YAPC	Kyse-70	Kyse-510	Kyse-520	MCF-7
Cisplatin	0.407 ± 0.090	0.81 ± 0.19	0.90 ± 0.19	0.35 ± 0.10	1.96 ± 0.54	1.22 ± 0.13	0.24 ± 0.06	1.61 ± 0.16	0.73 ± 0.34	4.09 ± 0.69	0.63 ± 0.14	0.44 ± 0.07	3.61 ± 1.86	1.38 ± 0.29
Carboplatin	6.80 ± 0.80	22.1 ± 5.1	14.59 ± 5.67	4.34 ± 1.70	25.28 ± 4.05	15.81 ± 5.51	3.08 ± 1.16	29.03 ± 16.8	12.79 ± 6.81	57.4 ± 12.4	23.3 ± 12.1	5.03 ± 2.19	34.5 ± 23.0	29.4 ± 9.8
Oxaliplatin	0.254 ± 0.118	0.42 ± 0.19	0.51 ± 0.32	1.13 ± 0.33	0.76 ± 0.086	1.97 ± 0.85	2.86 ± 0.67	0.16 ± 0.01	22.58 ± 6.60	15.1 ± 8.3	0.46 ± 0.22	14.75 ± 0.95	3.10 ± 1.71	0.32 ± 0.04
DACH-Pt	0.227 ± 0.163	0.18 ± 0.07	0.57 ± 0.23	0.82 ± 0.19	0.93 ± 0.27	0.55 ± 0.27	0.10 ± 0.05	0.17 ± 0.11	0.95 ± 0.74	0.53 ± 0.40	0.32 ± 0.10	0.68 ± 0.45	1.84 ± 0.61	0.23 ± 0.13
Melphalan	1.04 ± 0.48	0.88 ± 0.33	4.00 ± 0.42	1.32 ± 0.14	5.13 ± 1.93	4.69 ± 2.59	1.00 ± 0.32	14.25 ± 9.54	2.65 ± 1.02	5.95 ± 1.24	16.16 ± 1.82	8.18 ± 3.49	10.49 ± 3.38	3.71 ± 1.19
Chlorambucil	3.11 ± 0.42	5.10 ± 2.09	14.5 ± 7.1	6.55 ± 3.46	9.50 ± 3.47	29.5 ± 13.0	9.02 ± 2.93	35.8 ± 15.2	278 ± 5.0	29.5 ± 4.5	38.4 ± 1.7	37.41 ± 8.15	48.3 ± 16.5	18.4 ± 5.5
Busulfan	61.9 ± 28.1	47.99 ± 23.3	240.9 ± 122.6	79.41 ± 14.4	78.7 ± 23.6	142.5 ± 7.5	65.4 ± 4.0	1085 ± 460	60.8 ± 24.6	103.0 ± 36.0	72.1 ± 10.8	138.9 ± 17.4	275.4 ± 125.8	103.1 ± 17.7
Thiotepa	1.840 ± 0.447	4.13 ± 2.09	6.97 ± 0.98	2.0 ± 0.38	1.58 ± 0.66	3.40 ± 0.89	1.40 ± 0.29	18.27 ± 9.92	1.66 ± 0.11	2.66 ± 0.27	5.40 ± 2.04	4.31 ± 0.38	6.44 ± 0.68	3.23 ± 0.52
Doxorubicin	0.050 ± 0.023	0.020 ± 0.02	0.013 ± 0.0016	0.0097 ± 0.0013	0.0015 ± 0.0003	0.0049 ± 0.0031	0.0039 ± 0.0020	0.03 ± 0.015	0.011 ± 0.0042	0.03 ± 0.01	0.046 ± 0.022	0.02 ± 0.0072	0.035 ± 0.024	0.017 ± 0.005
Etoposide	0.314 ± 0.072	0.097 ± 0.025	0.42 ± 0.13	0.54 ± 0.30	0.127 ± 0.101	0.23 ± 0.08	0.155 ± 0.023	35.76 ± 15.18	0.358 ± 0.082	1.36 ± 0.01	0.94 ± 0.23	0.70 ± 0.05	0.413 ± 0.071	0.50 ± 0.19
Camptothecin	0.0019 ± 8.4E-04	0.0048 ± 0.0025	0.0077 ± 0.0025	0.0052 ± 0.0014	0.0066 ± 0.0007	0.0072 ± 0.0028	0.0054 ± 0.0025	0.0145 ± 0.0023	0.021 ± 0.013	0.0098 ± 0.0022	0.0048 ± 0.0024	0.03 ± 0.01	0.026 ± 0.014	0.0035 ± 0.0003
Vinblastine	0.00022 ± 6.2E-05	6.8E-05 ± 3.9E-05	0.0011 ± 0.0013	0.0004 ± 0.00015	0.00018 ± 0.00013	0.0008 ± 0.0004	0.0004 ± 0.0001	0.0008 ± 0.0003	0.0005 ± 4.2E-05	0.0003 ± 0.00008	0.0011 ± 0.0002	0.0006 ± 0.0002	0.0015 ± 0.0008	0.0003 ± 0.0001
Podophyllotoxin	0.0033 ± 0.001	0.0020 ± 0.0011	0.0041 ± 0.0015	0.0064 ± 0.0012	0.0071 ± 0.0045	0.0142 ± 0.0082	0.0085 ± 0.0012	0.0075 ± 0.0016	0.0055 ± 0.0005	0.012 ± 0.0023	0.03 ± 0.001	0.01 ± 0001	0.014 ± 0.011	0.0043 ± 0.0027
Colchicine	0.0035 ± 0.0021	0.0011 ± 0.0006	0.0067 ± 0.0031	0.01 ± 0.0032	0.008 ± 0.002	0.34 ± 0.06	0.019 ± 0.017	0.0146 ± 0.0043	0.054 ± 0.018	0.0158 ± 0.0129	0.32 ± 0.05	0.073 ± 0.044	0.0015 ± 0.0008	0.31 ± 0.06
Taxol	0.00097 ± 0.00031	0.0007 ± 7.5E-05	0.0007 ± 7E-05	0.0012 ± 0.0002	0.0017 ± 0.0008	0.0027 ± 0.0006	0.0022 ± 0.0015	0.0010 ± 0.0004	0.0011 ± 0.0004	0.0017 ± 0.0004	0.0032 ± 7.7E-05	0.0014 ± 0.0003	0.0042 ± 0.001	0.0011 ± 0.0006
Hydroxyurea	65.6 ± 13.9	75.9 ± 36.5	131 ± 21	164 ± 10	226 ± 33	122 ± 32	140 ± 51	366 ± 122	111 ± 14	276 ± 5	142 ± 14	170 ± 15	148 ± 33	165 ± 54
5-AC	1.92 ± 0.67	1.94 ± 0.73	2.24 ± 0.40	1.73 ± 1.20	0.63 ± 0.05	3.25 ± 4.18	2.86 ± 1.16	2.73 ± 1.20	1.21 ± 0.57	3.77 ± 0.80	1.59 ± 1.93	3.03 ± 0.93	1.89 ± 1.039	6.78 ± 5.21
Methotrexate	0.017 ± 0.017	0.059 ± 0.053	0.025 ± 0.012	0.0155 ± 0.009	5.52 ± 3.55	0.44 ± 0.04	0.049 ± 0.021	0.04 ± 0.02	0.077 ± 0.005	0.65 ± 0.05	0.05 ± 0.02	0.058 ± 0.002	70.6 ± 5.3	0.05 ± 0.02
5-FU	17.0 ± 12.9	12.18 ± 4.67	2.66 ± 1.055	2.54 ± 2.19	1.46 ± 0.42	1.67 ± 1.12	2.02 ± 0.98	2.70 ± 0.81	1.04 ± 0.133	2.94 ± 0.28	0.55 ± 0.09	1.28 ± 0.14	3.99 ± 3.35	2.15 ± 0.40

Table 4 Correlation coefficients from a panel of 14 cell lines

	GSH	Platinum complexes				Alkylating agents				Topoisomerase inhibitors			Anti-mitotics				Anti-metabolites			
		Cisplatin	Carboplatin	Oxaliplatin	DACH-Pt	Chlorambucil	Melphalan	Thiotepa	Busulfan	Camptothecin	Etoposide	Doxorubicin	Colchicine	Vinblastine	Taxol	Podophyllotoxin	5-FU	5-AC	Methotrexate	Hydroxyurea
GSH	1.0	−0.042	0.126	0.045	−0.123	0.331	0.307	**0.542**	0.313	0.435	0.135	**0.750**	−0.048	0.264	−0.254	−0.024	−0.004	0.019	−0.300	0.059
Cisplatin	1.0	**0.911**	0.073	0.412	0.493	**0.560**	0.357	0.437	0.342	0.127	0.119	0.267	0.152	0.265	0.225	0.011	0.060	**0.730**	0.486	
Carboplatin		1.0	−0.128	0.193	0.443	**0.555**	0.436	0.320	0.179	0.129	0.274	0.225	0.064	0.139	0.157	−0.013	0.064	0.517	0.370	
Oxaliplatin			1.0	0.492	0.412	0.063	−0.370	−0.209	**0.626**	0.241	−0.129	0.263	0.145	0.229	0.345	−0.356	−0.002	0.288	0.065	
DACH-Pt				1.0	0.377	0.348	−0.061	0.087	**0.532**	0.001	−0.101	0.322	0.366	0.399	0.304	−0.272	−0.412	**0.607**	0.100	
Chlorambucil					1.0	**0.866**	0.519	**0.543**	**0.789**	0.296	0.220	**0.766**	**0.732**	0.519	**0.724**	−0.648	0.232	0.393	0.511	
Melphalan						1.0	**0.646**	**0.638**	**0.609**	0.265	0.266	**0.612**	**0.684**	0.493	**0.703**	**−0.566**	0.043	0.402	**0.630**	
Thiotepa							1.0	**0.831**	0.388	−0.093	0.520	0.184	0.510	0.090	0.159	−0.008	0.261	0.017	0.359	
Busulfan								1.0	0.479	−0.068	0.235	0.218	**0.618**	0.176	0.223	−0.063	0.248	0.171	**0.615**	
Camptothecin									1.0	0.149	0.099	0.402	**0.536**	0.292	0.430	−0.436	−0.074	0.428	0.385	
Etoposide										1.0	0.265	0.356	−0.058	−0.064	0.444	−0.178	0.133	0.147	0.332	
Doxorubicin											1.0	0.096	0.233	−0.100	0.038	−0.415	0.293	−0.179	−0.094	
Colchicine												1.0	**0.658**	**0.644**	**0.708**	−0.593	0.307	0.392	0.155	
Vinblastine													1.0	**0.695**	**0.670**	−0.516	0.096	0.188	0.286	
Taxol														1.0	**0.785**	−0.452	−0.066	**0.584**	0.128	
Podophyllotoxin															1.0	**−0.684**	0.016	0.400	0.435	
5-FU																1.0	0.101	−0.070	−0.415	
5-AC																	1.0	−0.268	0.129	
Methotrexate																		1.0	0.228	
Hydroxyurea																			1.0	

Pearson *r* (two-tailed test): $n=14$; $f=12$; level of significance: $P<$ **0.05:** 0.532; $P<$ **0.02:** 0.612; $P<$ **0.01 :** 0.661.

Table 5 Correlation coefficients from a panel of 34 cell lines (NCI data)

	GSH	Platinum complexes						Alkylating agents				Topoisomerase inhibitors			Anti-mitotics				Anti-metabolites			
		Cisplatin	Carboplatin	Oxaliplatin	DACH1	DACH2	DACH3	Chlorambucil	Melphalan	Thiotepa	Busulfan	Camptothecin	Etoposide	Doxorubicin	Colchicine	Vinblastine	Taxol	Podophyllotoxin	5-FU	5-AC	Methotrexate	Hydroxyurea
GSH	1.000	−0.067	0.016	−0.067	−0.197	−0.193	−0.020	0.017	−0.013	0.038	0.297	0.107	−0.019	−0.111	−0.254	−0.310	−0.265	−0.334	0.053	0.127	−0.058	0.014
Cisplatin		1.000	**0.891**	0.097	0.296	0.151	**0.533**	**0.759**	**0.781**	**0.776**	**0.483**	**0.563**	**0.596**	**0.554**	0.075	0.194	0.167	0.122	0.055	0.103	0.039	**0.576**
Carboplatin			1.000	0.052	0.193	0.029	**0.442**	**0.671**	**0.667**	**0.637**	**0.483**	**0.443**	**0.459**	**0.491**	0.216	0.329	0.271	0.275	0.004	0.155	−0.072	**0.494**
Oxaliplatin				1.000	**0.746**	**0.740**	**0.662**	**0.340**	**0.374**	0.298	0.306	0.330	**0.403**	**0.397**	0.073	0.133	0.214	0.157	0.230	0.249	0.029	0.336
DACH1					1.000	**0.917**	**0.807**	0.265	**0.358**	0.305	0.153	0.235	0.307	**0.347**	0.079	0.235	0.313	0.230	0.192	0.189	0.049	0.329
DACH2						1.000	**0.749**	0.141	0.256	0.213	0.116	0.152	0.227	0.248	−0.053	0.108	0.151	0.100	0.182	0.164	0.031	0.285
DACH3							1.000	**0.559**	**0.663**	**0.529**	**0.403**	**0.381**	**0.452**	0.325	0.005	0.096	0.097	0.062	0.150	0.192	0.006	**0.574**
Chlorambucil								1.000	**0.956**	**0.992**	**0.666**	**0.759**	**0.835**	**0.648**	0.235	0.262	0.230	0.144	0.118	0.171	0.183	**0.795**
Melphalan									1.000	**0.919**	**0.706**	**0.659**	**0.797**	**0.613**	0.182	0.222	0.216	0.131	0.124	0.268	0.120	**0.825**
Thiotepa										1.000	**0.640**	**0.770**	**0.865**	**0.735**	0.225	0.290	0.281	0.141	0.281	0.257	0.219	**0.795**
Busulfan											1.000	**0.354**	**0.577**	**0.447**	0.098	0.045	−0.054	0.126	0.234	0.307	−0.003	**0.517**
Camptothecin												1.000	**0.811**	**0.742**	0.280	**0.359**	0.259	0.172	0.137	0.092	0.192	**0.613**
Etoposide													1.000	**0.847**	**0.392**	**0.406**	0.335	0.226	0.241	0.208	0.169	**0.668**
Doxorubicin														1.000	**0.454**	**0.560**	**0.514**	**0.359**	0.216	0.239	0.156	**0.464**
Colchicine															1.000	**0.902**	**0.744**	**0.717**	0.216	0.269	0.071	0.215
Vinblastine																1.000	**0.869**	**0.664**	0.161	0.299	0.102	0.252
Taxol																	1.000	**0.472**	0.238	0.318	0.214	0.233
Podophyllotoxin																		1.000	0.218	0.324	−0.028	0.123
5-FU																			1.000	**0.349**	0.264	0.172
5-AC																				1.000	−0.092	0.205
Methotrexate																					1.000	0.220
Hydroxyurea																						1.000

Pearson *r* (two-tailed test): $n=34$; $f=32$; level of significance: $P<$ **0.05:** 0.339; $P<$ **0.02:** 0.398; $P<$ **0.01 :**0.437.

action; both drugs form the same *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ species that platinates DNA, yielding intrastrand GpG crosslinks [12]. Cisplatin and oxaliplatin however, showed no correlation; this is due to the different diamine non-leaving groups that platinate DNA and dramatically effect the specificity of anti-tumor activity [13]. No correlations were found between vinblastine and the derivative etoposide, but this was not unexpected due to their very different mechanisms of action [14]. Strong to very strong correlations were found within the family of anti-mitotic agents (e.g. colchicine, vinblastine, taxol, vinblastine). As expected, all four alkylating agents showed strong to very strong correlations with each other, with one exception (chlorambucil and thiotepa). Cisplatin and carboplatin showed statistically significant similarities in their activities to melphalan ($P < 0.05$), but not to chlorambucil, thiotepa and busulfan, although a positive trend was noted.

The findings of strong correlations between the activities of the mitotic poisons and the two alkylating agents chlorambucil and melphalan, but not between the alkylating agents thiotepa and busulfan, were surprising. These results suggest that chlorambucil and melphalan may act to some degree as anti-mitotic agents in addition to alkylating DNA. Equally unexpected was the finding that chlorambucil ($P < 0.01$), melphalan ($P < 0.05$), oxaliplatin ($P < 0.02$) and DACH-Pt ($P < 0.05$) showed strong positive correlations with the activity of camptothecin, a topoisomerase I inhibitor.

Other unexpected findings that are difficult to explain are the significant correlations between the activity of the anti-metabolite methotrexate and cisplatin ($P < 0.01$), DACH-Pt ($P < 0.05$), as well as taxol ($P < 0.05$). Another anti-metabolite, 5-FU, showed significant negative correlations between chlorambucil ($P < -0.02$), melphalan ($P < -0.05$), and two anti-mitotic agents colchicine and vinblastine.

Comparisons with the NCI data

Table 5 shows the results of the regression analysis for the NCI data, obtained for the same anti-tumor agents over 34 cell lines, representing seven different cancer types. Only leukemia and non-small cell lung cancer cell lines were represented in both panels, and only the HL-60 cell line was common to both panels.

Not unexpectedly, the levels of significance are higher (typically $P < 0.01$) in the NCI panel of 34 cell lines compared with our panel of 14 cell lines. The NCI data gave comparable results to ours however, in many cases (*cf.* Tables 4 and 5), e.g. the correlations within the alkylating agents, or within the anti-mitotic agents, or between the platinum complexes and alkylating agents, or between camptothecin and chlorambucil/melphalan. With the exception of 5-FU in the NCI panel, no correlations were seen between 5-AC and the other agents in either panel. In both panels, significant correlations were found between the anti-mitotic agents taxol and vinblastine, which stabilize and destabilize the mitotic spinal, respectively.

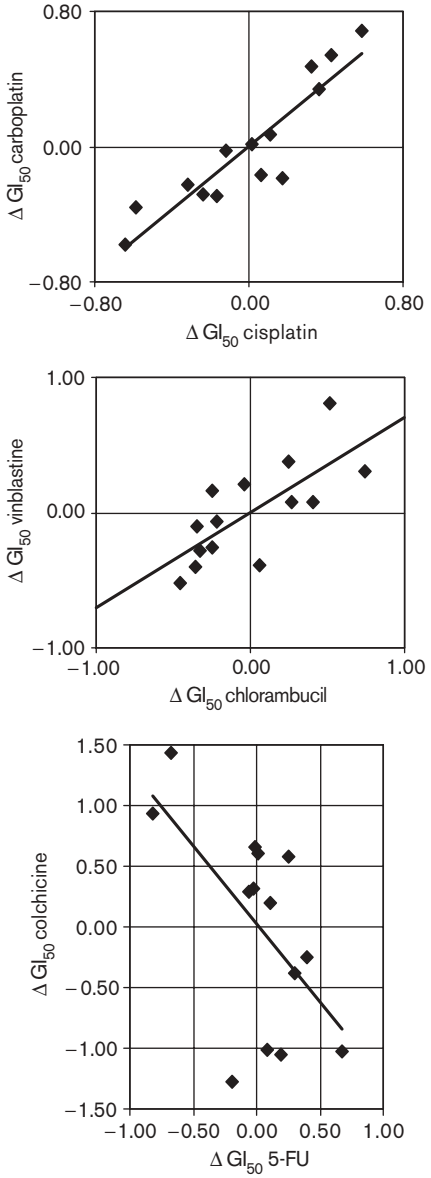
In some important cases contrasting results were obtained for the two panels. No correlations were found in the NCI data between the anti-mitotic agents and the two alkylating agents chlorambucil and melphalan, in contrast to our data. With the NCI data, doxorubicin showed significant correlations with the activities of the platinum complexes as well as the alkylating agents; none of these were seen with our data. The NCI data indicated a strong correlation ($P < 0.01$) between etoposide (topoisomerase II inhibitor) and camptothecin (topoisomerase I inhibitor), whereas our data did not. In contrast to our data, the NCI data showed not a single correlation between the anti-metabolite methotrexate and the other anti-tumor agents. The same was true for 5-FU; for this anti-metabolite we picked up some correlations (see above). For the alkylating agents chlorambucil and melphalan we saw significant correlations with the group of the anti-mitotic agents; in the case of the NCI data, no such correlations were apparent. In our data, the ribonucleotide reductase inhibitor hydroxyurea only showed correlations with melphalan ($P < 0.02$) and busulfan ($P < 0.02$), whereas in the NCI data the number of agents showing highly significant ($P < 0.01$) similarities in activity to hydroxyurea was considerably greater.

Finally, in contrast to the NCI panel, we were surprised to find no correlation between the ΔGI_{50} values for oxaliplatin and DACH-Pt. Although we used the racemate D,L-DACH and the NCI used the pure enantiomers D- and L-DACH, this cannot explain the lack of correlation in our data. In only two of the 14 cell lines we used (A-427 and RT-4), oxaliplatin was more active than DACH, which was 3–10 times more active than oxaliplatin in the other cell lines, except in the case of DAN-G, where DACH-Pt was 100 times more active than oxaliplatin. Thus, some cell lines are very sensitive and others are very resistant to oxaliplatin. In contrast, all of our cell lines have similar sensitivity to DACH.

Correlations between intracellular GSH concentrations and activities of anti-tumor agents

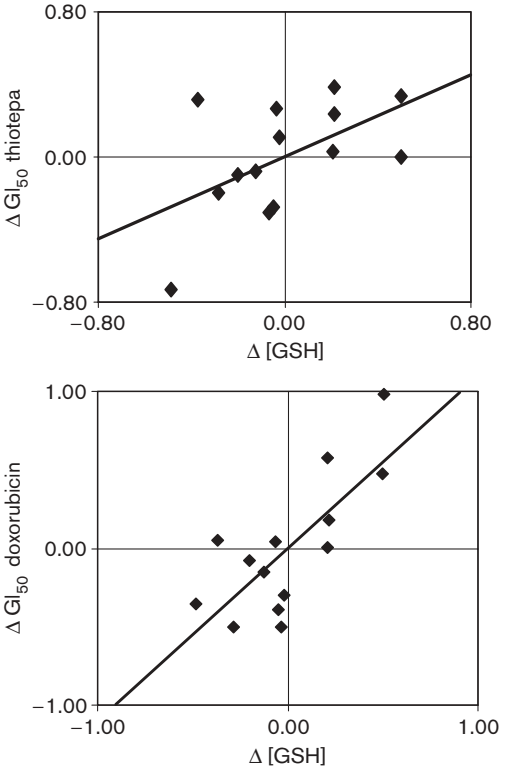
The biological parameter studied in this work was intracellular concentration of total GSH. The correlation coefficients are presented in Table 4. Although GSH has been extensively linked to resistance of cancer cells to platinating and alkylating agents, we only found a significant correlation between intracellular GSH concentrations and the GI_{50} values of thiotepa ($P < 0.05$). Interestingly, a strong positive correlation

Fig. 1



Correlations between ΔGI_{50} values for cisplatin/carboplatin, chlorambucil/vinblastine and 5-FU/colchicine in 14 cancer cell lines.

Fig. 2



Correlations between intracellular GSH concentrations and ΔGI_{50} values for doxorubicin and thiotepa in 14 cancer cell lines.

was found for doxorubicin ($P < 0.01$). Figure 2 graphically displays the data with the calculated linear regression line.

In the NCI data, not a single correlation was found between GSH levels and the activities of any of the anti-tumor agents (Table 5).

Discussion

The COMPARE program of the NCI has achieved great acceptance in the evaluation of new anti-tumor active substances. The results are very much dependent on the number and types of cell lines used in the analysis, however, as well as the concentrations used in the dilution series, leading to differing interpretations of the data for the same compound. Testing a substance in 60 or more cell lines, as is done by the NCI, is prohibitive for smaller institutions. Thus, we have attempted to validate a smaller panel of cell lines with standard anti-tumor agents to determine whether meaningful results can also be obtained with less cell lines. Our mini-panel consists of 14 cell lines; with the exception of one cell line (HL-60), they are not contained in the NCI panel of 34 cell lines we used for comparison. Furthermore, our panel highlights cancers not used in the NCI panel: bladder, esophageal and pancreatic cancer. Thus, our results are complementary to those of the NCI.

Another important difference between our test system and that of the NCI is the use of different dyes to stain the adherent cells: the NCI uses SRB to stain proteins while we use Crystal Violet to stain DNA. The length of the assays differs too: the NCI exposes all cells for 48 h (dtp.nci.nih.gov/branches/btb/ivclsp.html) to test substance while we use 96 h for adherent cell lines and 48 h for the suspension cells.

Another difference between the methods is the NCI dissolves the test substances in DMSO, whereas we use DMF as a solvent to avoid side-reactions with the Pt(II) complexes. Finally, the NCI uses five concentrations at 10-fold dilutions, whereas we used five concentrations generally at 2-fold dilutions; this gives a truer estimate of the GI_{50} value than 10-fold dilutions.

Thus, it was not surprising that our results did not completely overlap with those calculated from the data of the NCI. Nevertheless, important consistencies in the results from both panels were found, e.g. correlations of cisplatin versus carboplatin, chlorambucil versus melphalan/thiotepa/busulfan, colchicine versus vinblastine/taxol/podophyllotoxin, and cisplatin versus chlorambucil/melphalan/busulfan. Importantly, correlations were lacking in both panels for substances that have different mechanisms of action/resistance, e.g. hydroxyurea/alkylating and platinating agents versus anti-mitotic agents, pyrimidine anti-metabolites versus platinating/alkylating agents. A correlation between cisplatin/carboplatin and oxaliplatin/DACH-Pt was also lacking in both panels, as has been previously reported for the NCI data [13].

The differences in the correlations between some pairs of substances were noted however. This is likely due in part to the different types of cancers represented in the two panels. Differences in the testing methods may also play a role however. For example, our method exposes the adherent cell to test substance for 96 h, whereas the NCI uses only a 48-h exposure. The longer incubation time was chosen to give the cells more rounds of division; it is at cell division that these anti-tumor agents unfold their anti-proliferative effects. Thus, anti-tumor agents with delayed mechanisms of action might be more likely to give different results between the methods. In fact, it was with the anti-metabolites (methotrexate, 5-FU and hydroxyurea), which do not act directly but block important biochemical processes upstream of cell division, where the greatest discrepancies were seen.

Another discrepancy was the lack of a correlation between the ΔGI_{50} values of oxaliplatin and DACH-Pt. A correlation between oxaliplatin and tetraplatin has been reported in the NCI panel of 60 cell lines [13], and we observed correlations between oxaliplatin and D-DACH/L-DACH in the NCI data with 34 cell lines ($P < 0.01$). Data from our 14 cell lines however, indicates that oxaliplatin and DACH-Pt have different spectra of activities, i.e. the oxalate ligand acts not as a simple leaving group, but may influence directly the cellular pharmacology of the Pt(II) complex. In this context it is important to note that the NCI uses DMSO (dtp.nci.nih.gov/branches/btb/ivclsp.html) as a solvent, whereas we use DMF. DMSO is known to react avidly with Pt(II) complexes to yield a variety of products [15], which might have different biological activities to the parent complex. Cisplatin is inert to DMF however [16].

A final discrepancy was the presence of a significant correlation between the alkylating agents chlorambucil/melphalan and the anti-mitotic agents in our data, but not in that of the NCI. This correlation is difficult to explain because these classes of anti-tumor agents are thought to have widely different mechanisms of action. One explanation could be that chlorambucil and melphalan have an as-yet unrecognized effect on microtubule assembly, as was later discovered with estramustine, another nitrogen mustard analog [17,18].

GSH is the most prevalent non-protein thiol in cells and it plays a critical role in cell physiology by detoxifying reactive oxygen species [19], and electrophiles such as alkylating agents and Pt(II) complexes [20], as well as maintaining the redox state of cells [21] and effecting apoptosis [22]. More recently, GSH has been found to be an important player in transport process carried out by the multidrug resistance-associated proteins (MRP) [23–26] – a family of at least nine ATP-binding transporters [27] that transport not only endogenous substrates such as GSH *S*-conjugated leukotriene (LTC4) [28], but also GSH conjugates of electrophilic drugs, such as cisplatin [29]. MRP transports not only GSH conjugates of drugs, but also drugs that associate with GSH during the transport, such as doxorubicin, etoposide and vincristine [30,31].

Thus, determining the intracellular concentrations of GSH in our cell lines and correlating these with the potencies of the various standard agents was a prime goal of our research. We have recently improved on the traditional GSH reductase method of Tietze for determining total GSH (GSH + GSSG) in adherent cells growing in microtiter plates by including a NADPH/G6PDH auxiliary reaction and by measuring the volume of the cellular sample as opposed to the amount of protein in the sample [10]. This method allows for the determination of the molar concentration of GSH in cells. The standard method for determining cellular GSH levels is to adjust the amount of GSH to the milligram amount of cellular protein, as is done by the NCI (see Results). Thus, these results are dependent on the protein content of the cells as well. We believe that adjusting the GSH amounts to sample volume as opposed to amounts of sample protein is a more accurate estimation of the total GSH levels in cells.

In the cases of 17 of 19 substances, regression analysis of Δ[GSH] versus ΔGI_{50} yielded no significant correlations. The two cases where significance was observed however (doxorubicin: $P < 0.01$; thiotepa: $P < 0.05$) can be explained based on known mechanisms of cellular

resistance. Doxorubicin showed a highly significant correlation between the [GSH] and GI_{50} values. Interactions between GSH and doxorubicin have been associated with resistance at several levels: (i) GSH protects cells from reactive oxygen species formed by doxorubicin [32], (ii) elevated glutathione S-transferase (GST) activity have been correlated with resistance to doxorubicin [33,34] and (iii) GSH mediates transport of doxorubicin by MRP [25]. Recently, evidence has been presented that GSH levels are correlated to the sensitivities of eight human cancer cell lines to doxorubicin [35] and overactive GSH recycling may contribute to the intrinsic resistance of doxorubicin in ovarian cancer [36].

Whether MRPs play a role in the resistance to doxorubicin will require the direct quantification of their levels in our cell lines. Others have already done this – evidence indicates that MRP mRNA levels in the NCI panel of 60 cell lines are not singularly associated with resistance to doxorubicin [7]; these authors speculate that other factors such as GSH levels may also contribute to resistance. Likewise, Doyle has reported that elevated expression of MRP is not associated with resistance to thiotepa [37]. Thiotepa acts with GSH, resulting in monoglutathionyl conjugates; the rate of this reaction is increased in the presence of GST (GST A1-1 and P1-1) [38]. One report concluded however, that GSH levels are not associated with resistance to thiotepa in urinary transitional cell carcinoma [39].

Chlorambucil [40], busulfan [41] and melphalan [42,43] are all known to react with GSH in conjugation reactions catalyzed by GST. GSH levels have also been positively correlated with the sensitivities of eight cancer cell lines to chlorambucil [35]. No significant correlations between potency and GSH levels were observed however, for these three agents; nonetheless positive trends were noted (chlorambucil: $r = 0.33$; busulfan: $r = 0.31$; melphalan: $r = 0.31$). This lack of significance may be due to lower reactivities of these alkylating agents towards GSH/GST compared with thiotepa, decreasing the contribution of that detoxification pathway below the level of significance.

As already noted in a previous publication of ours, none of the platinum complexes showed a correlation between potency and GSH levels [44]. Reaction of GSH with Pt(II) anti-tumor agents to form inactive GSH–Pt(II) conjugates has often been discussed as a mechanism for detoxification, particularly when the cells have acquired resistance to these agents [20]. As yet there is no evidence that GST can catalyze GSH conjugations with Pt(II) complexes. Correlations between GSH levels in non-pretreated cancer cells and cytotoxicity of Pt(II) complexes have also not been found [45,46]. In our case, only one cell line for sure (KYSE-510), but possibly a second (SISO), was reportedly obtained from a patient previously treated with cisplatin. Thus, our panel is composed mostly of cell lines having no prior exposure to platinum complexes. Our data would therefore suggest that intrinsic resistance to platinum complexes is not mediated by elevated intracellular levels of GSH. Earlier investigators drew similar conclusions [45,46].

With the GSH data from the NCI, no correlations between potencies of any of the anti-tumor agents and GSH levels were observed (Table 5). This may be due to the use of different cell lines, but it also may be due to the fact that the results were normalized to cellular protein levels and not to cell volume, as we have done.

Conclusions

We have used a small panel of human cancer cell lines to identify similarities and differences in the activity profiles of various anti-tumor agents. Many, but not all, of the activity profiles can be explained based on the mechanism of action of the agents. Importantly, intracellular GSH concentrations were found to correlate with the cell growth-inhibitory activities in our panel for doxorubicin and thiotepa. This result can be explained based on the known mechanisms of detoxification of these anti-tumor agents. No correlations between the activities of platinum complexes in the cell lines and the GSH levels were found however, indicating that GSH does not play a significant role in intrinsic resistance to platinum complexes. The main advantage of our small panel is that less effort and expense need to be expended to obtain meaningful results. The main advantage of the NCI panel of 34 cell lines is that the levels of significance are higher than most of the correlations found in our panel of 14 cell lines.

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