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Relationship between cytotoxic drug response patterns and activity of drug efflux transporters mediating multidrug resistance

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Abstract

Drug activity patterns in 10 human tumor cell lines representing defined mechanisms of drug resistance, including cell lines with high expression of P-glycoprotein and multidrug resistance associated protein (MRP), have previously been used for prediction of mechanism of drug action. In the present study, this cell line panel was analyzed for cellular accumulation of the fluorescent probe calcein/AM [4′5′-bis(*N*,*N*-bis (carboxymethyl) aminomethyl) fluorescein acetoxymethyl ester] and compared with drug response patterns of 20 standard chemotherapeutic drugs. According to degree of correlation with the ability to exclude calcein/AM, topoisomerase II inhibitors and tubulin actives were at the top of the list although the correlations were of lower magnitude than those obtained from the drug response patterns of mechanistically similar drugs. There was a significant relationship between the rank-order of drugs based on correlation with calcein/AM accumulation and Pgp/MRP mediated drug resistance suggesting that compounds being substrates for these pumps were identified. In simulated drug response profiles, the impact of Pgp and MRP expressing cell lines on the mechanistic prediction was found to be marginal. The results indicate that the differential molecular function/expression in the cell line panel may identify drugs interacting with specific biochemical pathways. Furthermore, the presence of cell lines overexpressing drug efflux mechanisms in the panel do not significantly influence the mechanistic predictions. © 1998 Elsevier Science B.V.

Keywords: Calcein/AM; Tumor cell line; Cytotoxic drug; Multidrug resistance

1. Introduction

In 1985 the NCI (National Cancer Institute) introduced a new drug discovery strategy reflecting more of a disease-oriented philosophy. The new screening system consisted of a panel of cell lines (currently >60) representing the major forms of human cancers (Alley et al., 1988). A semi-automated non-clonogenic in vitro assay was selected for the analysis of differential growth inhibition and cytotoxicity (Monks et al., 1991). The differential drug activity information provided by the panel has been shown to reveal specific patterns of in vitro response to agents with similar mechanisms of action when analyzed by correlation analysis (Paull et al., 1989; Boyd and Paull, 1995).

We recently employed a panel of only 10 cell lines representing defined mechanisms of resistance for prediction of mechanism of action of cytotoxic drugs (Dhar et al., 1996). The differential activity of drugs across these 10 cell lines provided a pattern which was used to describe the degree of similarity among standard drugs using correlation analysis (Dhar et al., 1996). In this study, high correlation coefficients were obtained for drugs sharing a common mechanism of action and allowing reasonable discrimination between mechanistically different classes of drug. An important question is as to how various drug resistance mechanisms of resistance influence the outcome of the test. High expression of drug efflux pumps not linked to the target for cytotoxicity in the cell line panel may, thus, lead to erroneous mechanistic classifications.

Both Pgp (P-glycoprotein) and MRP (multidrug resistance associated protein) are membrane bound molecules believed to confer cellular resistance to a broad range of

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compounds by a high capacity energy dependent drug efflux (Azzaria et al., 1989; Cole et al., 1992; Grant et al., 1994). Our cell line panel contains both Pgp (myeloma RPMI 8226/Dox_{40}; Dalton et al., 1986) and MRP (H69AR; Mirski et al., 1987; Slovak et al., 1993) expressing cell lines. In the present study we investigated the role of these cell lines for the detection of mechanism of action and resistance of cytotoxic drugs.

Calcein/AM [4′5′-bis(*N*,*N*-bis (carboxymethyl) aminomethyl) fluorescein acetoxymethyl ester] was employed as a functional probe for Pgp and MRP mediated resistance. Calcein/AM is a non-fluorescent fluorescein analog which enters the cell rapidly where it is hydrolyzed to free, strongly fluorescent calcein by cytoplasmic esterases. Due to its high negative charge, de-esterified calcein is well retained by viable cells with intact plasma membranes (Morris, 1990; Weston and Parish, 1990). Calcein/AM ester is probably a substrate for both Pgp and MRP and may consequently be used as an easily operated functional fluorescent probe for these drug efflux proteins (Bozyczko-Coyne et al., 1993; Liminga et al., 1994; Hollo, 1994; Feller et al., 1995a,b; Hollo et al., 1996; Versantvoort et al., 1995).

2. Materials and methods

2.1. Cell line panel

The cell line panel consisted of four sensitive parental cell lines, five drug-resistant sublines and one cell line with primary drug resistance. The cell lines included were the leukemia cell line CCRF-CEM and its subline CEM/VM-1 (kind gifts from WT Beck, Department of Pharmacology, College of Medicine, University of Tennessee, Memphis, TN, USA), the lymphoma cell line U-937-GTB and its subline U-937-vcr, the small cell lung carcinoma (SCLC) cell line NCI-H69 and its subline H69AR (American Type Culture Collection; ATCC, Rockville, MD, USA), the myeloma cell line RPMI 8226/S and its sublines 8226/Dox_{40} and 8226/LR5 (kind gifts from WS Dalton, Department of Medicine, Arizona Cancer Center, University of Arizona, Tucson, AZ, USA) and the renal adenocarcinoma cell line ACHN (ATCC).

The 8226/Dox_{40} was selected for doxorubicin resistance and shows the classical MDR phenotype with overexpression of Pgp (Dalton et al., 1986, 1989). The 8226/LR5 was selected for melphalan resistance, proposed to be associated with increased levels of glutathione (Bellamy et al., 1991; Mulcahy et al., 1994). The U-937-vcr was selected for vincristine resistance, proposed to be tubulin associated (Botling et al., 1994). The H69AR, selected for doxorubicin resistance, expresses a multidrug resistance phenotype proposed to be mediated by MRP (Mirski et al., 1987; Slovak et al., 1993). The CEM/VM-1, selected for teniposide resistance, expresses the atypical MDR phenotype, which is proposed to be topoisomerase II associated Danks et al., 1987, 1988). The drug resistance of the primary resistant ACHN cell line is probably multifactorial Nygren and Larsson, 1991).

The cell lines were grown in culture medium RPMI-1640 (HyClone, Cramlington, UK), supplemented with 10% heat-inactivated fetal calf serum (HyClone), 2 mM glutamine, 50 μg/ml of streptomycin and 60 μg/ml of penicillin (HyClone). The RPMI 8226/Dox_{40} cell line was treated once a month with 0.24 μg/ml of doxorubicin and the 8226/LR5 at each change of medium with 1.53 μg/ml melphalan. U-937-Vcr was continuously exposed to 10 ng/ml of vincristine and H69AR was alternately fed with drug-free medium and medium containing 0.46 μg/ml doxorubicin. The CEM/VM-1 cell line could be grown in the absence of drug for 3–4 months without loss of resistance. Every 2–3 months, the cell lines were tested for maintained drug resistance. Growth and morphology were monitored on a weekly basis.

2.2. Drugs and exposure

The 10 cell lines were tested against a total of 20 different cytotoxic drugs (Table 2), using the fluorometric microculture cytotoxicity assay (FMCA; Larsson and Nygren, 1989, 1990). Each drug was tested at five different drug concentrations, obtained by 10-fold serial dilution, using 100 μg/ml as the maximum concentration for all drugs. Five drugs from each of the groups, tubulin-active agents, topoisomerase II inhibitors, alkylating agents and antimetabolites were included. All drugs were acquired from commercial sources. V-shaped 96-well microtiter plates (Nunc, Roskilde, Denmark) were prepared with 20 μl of drug solution at 10 times the desired final concentration, using a pipetting robot (Pro/Pette; Perkin Elmer, Norwalk, CT, USA). The plates were kept frozen up to 2 months at −70°C until further use (Larsson et al., 1992). A continuous drug exposure of 72 h was used.

2.3. Measurement of drug activity

The FMCA is based on measurement of fluorescence generated from hydrolysis of fluorescein diacetate to fluorescein by cells with intact plasma membranes (Larsson and Nygren, 1989, 1990). The seeding cell density per well for each cell line was selected to give optimal signal while still being in log phase at the time of measurement. The cell number ranging between $5–20 \times 10^3$ cells per 180 μl medium per well were seeded into experimental microtiter plates, prepared with drugs. Each drug concentration was tested in triplicate. Six wells with cells but without drugs served as control and six wells with only culture medium as blank. The plates were incubated at 37°C and 5% CO_2 for 72 h without change of medium. At the end of the incubation period, the medium and drugs were removed

and the cells were washed once with phosphate buffered saline (PBS). Fluorescein diacetate, dissolved in dimethyl sulfoxide (DMSO) and diluted in PBS to 10 μg/ml, was added to each well making the final volume 100 μl. The plates were incubated for 30 min and the fluorescence generated from each well was then measured at ex 485 nm and em 538 nm in the microtiter plate fluorometer, Fluoroscan II (Labsystems Oy, Helsinki, Finland). The fluorescence is proportional to the number of living cells and cell survival is presented as survival index, defined as the fluorescence in experimental wells in percent of that in control wells, with blank values subtracted.

Quality criteria for a successful assay included $>90\%$ cell viability prior to assay incubation as judged by standard a trypan blue exclusion test, a fluorescence signal in control cultures of more than 10 times mean blank values and a coefficient of variation in test and control cultures of $<30\%$. A successful assay according to these criteria was required for inclusion into the drug database. For most drugs the results were confirmed by repeated testing.

2.4. Calcein / AM uptake

Cells were washed and resuspended in PBS containing 5 mM glucose and the cell number was adjusted to the concentration of 350 000/ml. A total of 180 μl of each cell line suspension was subsequently seeded in 96-well microtiter plates in quadruplicate. Finally, 20 μl of 50 μg/ml calcein/AM (diluted, with PBS from stock solution) was dispensed into each well. The plates were incubated at 37°C for 30 min, and then centrifuged for 5 min at $200 \times g$. The plates were then washed in a microtiter-plate washer (Dynatech Laboratories, Chantilly, VA) and fluorescence was subsequently measured in the Fluoroscan II, with excitation and emission wavelengths set at 485 and 538 nm, respectively. The calcein/AM accumulation was presented as the mean of absolute fluorescence signal values (quadruplicates) subtracted from the blank and the data was then imported to the Macintosh personal computer (Apple Computer, Cupertino, CA) using Microsoft Excel (Microsoft, Redmond, WA). In separate experiments, addition of genistein (Sigma Chemical, St. Louis, MO) and SDZ 833 (Sandoz) was used to inhibit MRP and Pgp, respectively (Feller et al., 1995a,b; Tsuruo and Tomida, 1995).

2.5. Immunochemistry

The monoclonal antibodies C219 and QCRL-1 (Centocor, Malvern, PA) were used for Pgp and MRP staining, respectively. RPMI 8226/S (negative) and RPMI $8226/Dox_{40}$ (positive) were used as controls for Pgp expression and NCI-H69 (negative) and H69AR (positive) were used as controls for MRP staining. The cytocentrifuge cell preparations were fixed in acetone and 70% methanol (Cordell et al., 1984) and subsequently incubated

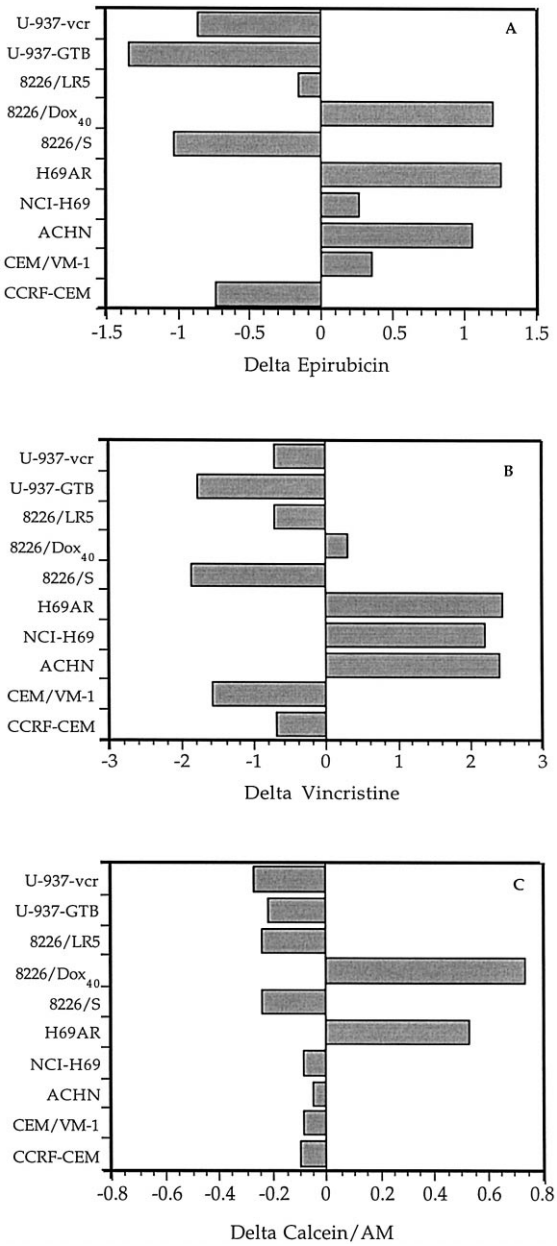

Fig. 1. From the concentration–response curves, mean $^{10}\log IC_{50}$ was determined defined as the mean of the $^{10}\log$ values of all 10 individual IC_{50} values obtained for Epirubicin and Vincristine. The variable denoted as delta was calculated by subtracting the mean $^{10}\log IC_{50}$ from the individual $^{10}\log IC_{50}$ of each cell line. A mean graph panel consisting of the drug specific deltas (along *X*-axis) across the cell line panel could then be constructed to visualise differential cytotoxicity patterns of drugs, Epirubicin (A) and Vincristine (B). Thus, the bars projecting to the left (negative values) indicate cell lines more sensitive than the average and the bars projecting to the right (positive values) indicate drugs more resistant than the average. $^{10}\log$ values of calcein/AM uptake were calculated for obtaining the mean graph for the 10 cell lines (C). The bars deflecting to the right (positive values) show reduced calcein/AM accumulation and the bars deflecting to the left (negative values) denote higher accumulation of calcein/AM in the cell lines compared with the overall mean value.

Table 1
Cell line characteristics and markers of immunohistochemical expression for Pgp and MRP

Cell line	Mechanism of resistance	CAM uptake[a]	Immunochemistry[b]	
			Pgp	MRP
CCRF-CEM	Parental	161	−	−
CEM/VM-1	Topo II-associated	155	−	−
ACHN	Primary resistant	142	+	−
NCI-H69	Parental	154	−	−
H69AR	MRP-associated	37	−	+ + +
RPMI 8226/S	Parental	222	−	−
RPMI 8226/Dox_{40}	Pgp-associated	23	+ + +	−
RPMI 8226/LR5	GSH-associated	220	−	−
U-937-GTB	Parental	209	+	−
U-937-vcr	Tubulin-associated	236	+	−

[a]Calcein/AM (CAM) uptake was measured as described in Section 2 and data points represent one out of three typical experiments.
[b]The specimen were judged as negative (−), weakly (+), moderately (+ +) or strongly positive (+ + +) using RPMI 8226/S and 8226/Dox_{40} as negative and positive controls for Pgp and NCI-H69 and H69AR for MRP staining, respectively.

with the primary antibodies for 2 h at room temperature followed by washing and application of a secondary rabbit antimouse antibody (Dako, Copenhagen, Denmark) for 30 min. After washing, the soluble complex of alkaline phosphatase and a mouse monoclonal anti-alkaline phosphatase (APAAP, Dako) was added for 30 min. The slides were developed using 10 mg/ml Fast red (Sigma) dissolved in a 0.5 M Tris buffer containing 2 mg/ml Napthol-As-Mx-phosphate and 2.4 mg/ml levamisole (Sigma). The specimens were counterstained with Mayers hematoxylin and mounted. The entire preparation was microscopically examined and the fraction of tumor cells with positive C219 and QCRL expression was indicated as follows: negative ($<1\%$ positive cells); +, 1–25% positive tumor cells; + + 26–50% positive cells and + + +, $>51\%$ positive cells. The specimens were evaluated without any prior information. Only the number of positive cells were accounted for and the intensity was not taken into account due to the reason that it may vary in different cytospin preparations.

2.6. Data analysis and quantification

The IC_{50} values, i.e., the concentration giving a survival index of 50% were calculated using a custom-made program in Excel (Microsoft) based on linear interpolation between data points. For drugs not producing an IC_{50} in more than four cell lines, IC_{30} values were used as substitutes. This was the case for cytarabine. For each drug, the

Table 2
Rank-order of drugs using calcein/AM uptake (CAM-uptake) pattern as reference and the comparison with resistance factors (RF) for Pgp and MRP

Drugs	Class	CAM-uptake (R)[a]	RF[b] Pgp	RF MRP	RF Pgp × MRP[c]
Epirubicin	Topo II inhibitor	0.77	100	10	1000
Doxorubicin	Topo II inhibitor	0.68	38	7	266
Amsacrine	Topo II inhibitor	0.63	12	1	12
Idarubicin	Topo II inhibitor	0.59	12	8	96
Vindesine	Tubulin active	0.51	102	3	306
Vinorelbine	Tubulin active	0.51	35	5	175
Vinblastine	Tubulin active	0.50	64	2	128
Etoposide	Topo II inhibitor	0.49	54	11	594
Vincristine	Tubulin active	0.48	148	2	296
Melphalan	Alkylating agent	0.43	1	4	4
Cladribine	Antimetabolite	0.41	1	1	1
Cytarabine	Antimetabolite	0.39	1	1	1
Taxotore	Tubulin active	0.38	6	6	36
Chlorambucil	Alkylating agent	0.30	0.74	1.1	0.81
Busulfan	Alkylating agent	0.25	0.77	1	0.77
Cisplatin	Alkylating agent	0.25	1	0.15	0.15
5-Fluorouracil	Antimetabolite	0.20	0.80	1	0.80
Mechlorethamine	Alkylating agent	0.12	1.08	0.90	0.07
5-Azacytidine	Antimetabolite	0.09	1	0.55	0.55
6-Thioguanine	Antimetabolite	0.05	1	0.83	0.83

[a]Calcein/AM (CAM) uptake pattern across the cell line panel compared to the drug response patterns.
[b]Resistance factor (RF) = IC_{50} in resistant cell line (RPMI 8226/Dox_{40} and H69AR)/IC_{50} in parental cell line (RPMI 8226/S and NCI-I 169).
[c]Product of RF Pgp and RF MRP.

Table 3
Rank list of the 10 highest correlations (R) among the drugs tested in the cell line panel using Vincristine and Epirubicin as reference compounds (a, d) and when the IC_{50} for the Pgp and MRP expressing cell lines were set to those of the parental lines (b, c) and when the Pgp and MRP expressing cell lines were deleted from the analysis (c, f)

(a)	Vincristine (complete panel)	R	(b)	Vincristine (IC_{50} equalised)	R	(c)	Vincristine (Pgp, MRP deleted)	R	(d)	Epirubicin (complete panel)	R	(e)	Epirubicin (IC_{50} equalised)	R	(f)	Epirubicin (Pgp, MRP deleted)	R
1	Vincristine	1.0	1	Vincristine	1.0	1	Vincristine	1.0	1	Epirubicin	1.0	1	Epirubicin	1.0	1	Epirubicin	1.0
2	Vinblastine	0.97	2	Taxotere	0.94	2	Vinorelbine	0.96	2	Doxorubicin	0.97	2	Etoposide	0.83	2	Doxorubicin	0.97
3	Vinorelbine	0.96	3	Vinblastine	0.90	3	Vinblastine	0.95	3	Idarubicin	0.93	3	Idarubicin	0.80	3	Idarubicin	0.95
4	Taxotere	0.95	4	Vinorelbine	0.89	4	Taxotere	0.95	4	Amsacrine	0.90	4	Doxorubicin	0.72	4	Etoposide	0.91
5	Vindesine	0.93	5	Cladribine	0.83	5	Vindesine	0.91	5	Etoposide	0.87	5	Vindesine	0.72	5	Amsacrine	0.85
6	Cladribine	0.88	6	Vindesine	0.82	6	Cladribine	0.85	6	Vindesine	0.84	6	Taxotere	0.71	6	Vindesine	0.83
7	Doxorubicin	0.84	7	Idarubicin	0.71	7	Doxorubicin	0.81	7	Vinblastine	0.77	7	Vinblastine	0.71	7	Vinblastine	0.77
8	Idarubicin	0.82	8	5-azacytidine	0.70	8	Idarubicin	0.77	8	Vincristine	0.76	8	Cladribine	0.70	8	Vincristine	0.74
9	Epirubicin	0.76	9	5-fluorouracil	0.70	9	Epirubicin	0.74	9	Cladribine	0.73	9	Vincristine	0.69	9	Cladribine	0.74
10	Amsacrine	0.73	10	Doxorubicin	0.68	10	Cisplatin	0.70	10	Vinorelbine	0.73	10	Amsacrine	0.68	10	Taxotere	0.72

overall mean $^{10}\log IC_{50}$ was determined defined as the mean of the $^{10}\log$ values for all cell lines. Then, the $^{10}\log$ of each cell line was subtracted from the mean $^{10}\log IC_{50}$ to yield a variable defined as delta. Thus, negative values indicate cell lines more sensitive than the average and positive values indicate drugs more resistant than the average for a particular drug. A mean graph with deltas consisting of $^{10}\log$ values for calcein/AM uptake across the cell line panel was also calculated. In this graph, signs were set so as to indicate reduced uptake with deflections to the right (positive values). The mean graphs consisting of the deltas for each cell line and drug could then be constructed to visualize differential cytotoxicity patterns of drugs or the calcein/AM uptake pattern (Paull et al., 1989; Boyd and Paull, 1995; Fig. 1). A procedure similar to the COMPARE analysis (Paull et al., 1989), using Pearson's correlation coefficient, was employed for comparing the mean graph (deltas) of any particular compound with those of the remaining drugs. $^{10}\log IC_{50}$ values were used in the correlation analysis as these were found to produce identical correlations in comparison to delta values. Because of the setting of calcein/AM mean graph signs (see above), a relationship between high drug IC_{50} and low calcein/AM uptake is consequently described by a positive correlation coefficient.

Resistance factors, defined as the values obtained from IC_{50} from parental cell line divided by IC_{50} from resistant sublines were calculated. IC_{50} values for RPMI 8226/S and RPMI 8226/Dox_{40} were used for calculation of the Resistance factor for Pgp mediated resistance, whereas the values from NCI-H69 and H69AR were used for calculation of the Resistance factor for MRP mediated resistance.

3. Results

In Fig. 1, the differential response pattern of epirubicin and vincristine is shown together with the mean graph profile of calcein/AM uptake. From visual inspection it is apparent that the major similarity among these three graphs is the rightward deflection of the bars of the RPMI 8226/Dox_{40} and H69AR (Pgp and MRP expressing) sublines as compared to their parental cell lines. The patterns clearly differ for other pairs of cell lines.

There was an apparent inverse relationship between basal calcein/AM uptake and immunohistochemically measured expression of Pgp and MRP (Table 1). When drugs were ranked according to degree of correlation with the calcein/AM uptake mean graph pattern (positive values indicating reduced uptake), topoisomerase II inhibitors and tubulin interactive agents were at the top of the list (Table 2), although the correlations obtained were of lower magnitude as compared to drug response pattern correlations used for mechanistic classification (Table 3). There was also an apparent relationship between rank-order of drugs based on calcein/AM accumulation and the resistance factors (Resistance factor; IC_{50} parental cell line/resistant cell line $\times$ 100) suggesting that compounds being substrates for these pumps were being identified (Table 2). A similar rank-order was also obtained when genistein (100 μg/ml) and PSC ([3-keto-Bmt^1]-[Val^2]-cyclosporin); 1 μg/ml) stimulated calcein/AM uptake was used as the measure of combined Pgp and MRP function (data not shown). High correlation coefficients were obtained when the values from the drug-specific correlations to the calcein/AM uptake pattern were compared with those of Resistance factors for P-glycoprotein ($R = 0.75$; $P < 0.001$), MRP ($R = 0.76$; $P < 0.001$) and the product of Pgp and MRP ($R = 0.75$–0.86; $P < 0.001$).

In order to test the potential influence of the Pgp and MRP expressing cell lines on the ability of predicting the mechanism of action, drug-response profiles for epirubicin and vincristine were simulated by equalizing the IC_{50}s for parental and the MRP/Pgp expressing cell lines (Table 3). A very similar rank-order was obtained for the simulated vincristine profile as compared to the original vincristine profile (Table 3). However, for the simulated epirubicin profile, the discrimination of topoisomerse II inhibitors from tubulin active agents was less clear. Excluding the MRP and Pgp expressing cell line from the analysis produced nearly identical correlations and rank-orders compared to the original drug response pattern using all 10 cell lines (Table 3).

4. Discussion

We have previously demonstrated the feasibility of using drug–response curves from a limited number of cell lines representing defined types of drug resistance to provide preliminary information on mechanism of action of cytotoxic drugs (Dhar et al., 1996). The inclusion of cell lines with different resistance mechanisms, rates of proliferation and histologic origin in the panel may contribute to this capability by increasing the heterogeneity of drug sensitivity across the panel. Consequently, the expression of molecular drug targets may be used to identify drugs interfering with a particular biochemical pathway. Indeed, other investigators have demonstrated the feasibility of such approach for identification of substrates for P-glycoprotein using patterns of Pgp expression and function across the NCI cell line panel as a reference probe (Wu et al., 1992; Lee et al., 1994; Alvarez et al., 1995).

In the present study, we show that drugs known to be associated with Pgp and MRP mediated resistance could be separated from those not associated with these mechanisms of resistance using the uptake pattern of calcein/AM as a reference pattern in the 10 cell lines. The ranking of drugs resulting from this analysis correlated well to the estimated specific resistance factors for Pgp and MRP mediated resistance confirming the involvement of these resistance mechanisms in determining calcein/AM uptake.

Relatively low resistance factors were obtained for tubulin active drugs in the subline of NCI-H69 (data not shown). This seems to be related to the low rate of proliferation, and hence high IC_{50} values, of these cell lines under current assay conditions. Many of the tubulin actives do not produce 'true' IC_{50} values in H69AR, which means that the highest concentration tested (normally 100 μg/ml) will be the IC_{50} recorded in the database. Although there is an apparent advantage for the drug response heterogeneity of the panel to have a large variability in rate of proliferation, the lack of true IC_{50} values for all drugs may also distort predictions of mechanisms of action (Boyd, 1993). In this case, one obvious solution would be to extend dose–response curves for such drugs or use another measure of potency (for example, IC_{30}). However, despite this potential drawback, the ability to detect mechanism of action based on the present data appears good and there was a reasonably high correlation between Calcein/AM uptake and resistance factors for both Pgp and MRP mediated resistance. The known Pgp and MRP substrates could also be distinguished by the resistance factors alone.

The detection of drugs interfering with other molecular pathways than those introduced by the specific resistance mechanisms will depend on the variability of expression in the panel of the particular target of interest. Undoubtedly, this approach might benefit from larger number of cell lines of different histologic origins like that of the NCI screening panel (Boyd, 1993). However, preliminary results of immunocytochemical P53 and Bcl_2 staining also show considerable variability among the 10 cell lines of the present panel (data not shown). Whether this variability can be demonstrated for other potential drug targets as well, and if it would be of sufficient magnitude for correlation with response patterns of drugs specifically interfering with these targets, remains to be elucidated.

The above approach might give important information on the cellular pharmacology of drugs interacting with known target molecules. However, at a future stage there is a possibility of developing a database on differential expression of unknown molecules using quantitative 2-D SDS (Sodium Dodecyl Sulfate) protein electrophoresis (Anderson et al., 1991) or detection of differential mRNA expression using the differential display PCR (Polymerase chain reaction) technique (Liang and Pardee, 1992). Although the feasibility of such an approach is uncertain, research in this direction has begun at the NCI (Weinstein et al., 1997; Myers et al., 1997).

Would the present 10-cell line panel be capable of detecting drugs not being substrates for the membrane pumps but having the same mechanism of action, e.g., a putative agent sharing all tubulin interactive properties with the vinca alkaloids, but not being a substrate for Pgp or MRP? This is an important question since the presence of Pgp and MRP expressing cell lines in the panel might give an erroneous mechanistic classification. In the present study, we simulated two such drugs by setting the IC_{50} for the of Pgp and MRP expressing cell lines to that of the parental cell line using the data for vincristine and epirubicin. For a simulated profile of epirubicin, but not of vincristine, the discrimination of topoisomerase II inhibitors from tubulin active agents diminished to some extent. However, exclusion of the MRP and Pgp expressing cell line from the analysis produced nearly identical correlations and rank-orders compared to the original drug response analysis from all 10 cell lines. It should also be noted that overall, the correlation obtained by using the calcein/AM uptake pattern as reference pattern was of lesser magnitude compared to drugs sharing a common mechanism of action. Thus, the influence of the overexpression of Pgp and MRP on mechanistic predictions appear moderate and may be eliminated by excluding them in a confirmatory analysis.

5. Conclusion

The results indicate that data on differential molecular function/expression in a cell line panel of limited size may be used to identify drugs interacting with specific biochemical pathways. Furthermore, the presence of cell lines overexpressing membrane drug transport proteins do not seem to affect, to any major extent, the mechanistic classification, at least not for the classes of drugs presently used.

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References

Alley, M., Scudiero, D., Monks, A., Hursey, M., Czerwinski, M., Fine, D., Abbott, B., Mayo, J., Shoemaker, R., Boyd, M., 1988. Feasibility of drug screening with panels of human tumor cell lines using a microculture tetrazolium assay. Cancer Res. 48, 589–601.

Alvarez, M., Paull, K., Monks, A., Hose, C., Lee, J., Weinstein, J., Grever, M., Bates, S., Fojo, T., 1995. Generation of a drug resistance profile by quantitation of mdr-1/P-glycoprotein in the cell lines of the National Cancer Institute anticancer drug screen. J. Clin. Invest. 95, 2205–2214.

Anderson, L.A., Esquer-Blasko, R., Hofman, J.P., Anderson, M., 1991. A two-dimensional gel database of rat liver proteins useful in gene regulation and drug effect studies. Electrophoresis 12, 907–912.

Azzaria, M., Schurr, E., Gros, P., 1989. Discrete mutations introduced into the predicted nucleotide binding sites of the *mdr*1 gene abolish its ability to confer multidrug resistance. Mol. Cell. Biol. 9, 5289–5297.

Bellamy, W., Dalton, W., Gleason, M., Grogan, T., Trent, J., 1991. Development and characterisation of a melphalan-resistant human multiple myeloma cell line. Cancer Res., pp. 995–1002.

Botling, J., Liminga, G., Larsson, R., Nygren, P., Nilsson, K., 1994. Development of vincristine resistant and increased sensitivity to cyclosporin A and verapmil in the human U-937 lymphoma cell line without overexpression of the 170 kDa P-glycoprotein. Int. J. Cancer 58, 269–274.

Boyd, M., 1993. The future of new drug development. In: Neiderhuber, J. (Ed.), Current Therapy In Oncology. B.C. Decker, Philadelphia, pp. 11–22.

Boyd, M.R., Paull, K.D., 1995. Some practical considerations and applications of the National Cancer Institute in vitro anticancer drug discovery screen. Drug Dev. Res. 34, 91–109.

Bozyczko-Coyne, D., Mckenna, B.W., Connors, T.J., Neff, N.T., 1993. A rapid fluorometric assay to measure neuronal survival in vitro. J. Neurosci. Methods 50, 205–216.

Cole, S., Bhardwaj, G., Gerlach, J., 1992. Overexpression of a transport gene in a multidrug-resistance human lung cell line. Science 258, 1650–1654.

Cordell, J., Falini, B., Erber, W., Ghosh, A., Abdulaziz, Z., Macdonald, S., Pulford, K., Stein, H., Mason, D., 1984. Immunoenzymatic labelling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP Complexes). J. Histochem. Cytochem. 32, 219–229.

Dalton, W., Durie, B., Alberts, D., Gerlach, J., Cress, A., 1986. Characterisation of a new drug-resistant human myeloma cell line that expresses P-glycoprotein. Cancer Res. 46, 5125–5130.

Dalton, W.S., Grogan, T.M., Meltzer, P.S., Scheper, R.J., Durie, B.G.M., Taylor, C.W., Miller, T.P., Salmon, S.E., 1989. Drug-resistance in multiple myeloma and non-Hodgkin's lymphoma: detection of P-glycoprotein and potential circumvention by addition of verapamil to chemotherapy. J. Clin. Oncol. 7, 415–424.

Danks, M., Yalowich, J., Beck, W., 1987. Atypical multidrug resistance in a human leukemic cell line selected for resistance to teniposide (VM-26). Cancer Res. 47, 1297–1301.

Danks, M., Schmidt, C., Cirtain, M., Suttle, D., Beck, W.T., 1988. Altered catalytic activity of and DNA cleavage by DNA topoisomeras II from human leukemic cells selected for resistance to VM-26. Biochemistry 27, 8861–8869.

Dhar, S., Nygren, P., Csoka, K., Botling, J., Nilsson, K., Larsson, R., 1996. Anti-cancer drug characterisation using a human cell line panel representing defined types of drug resistance. Br. J. Cancer 74, 888–896.

Feller, N., Broxterman, H., Wahrer, D., Pinedo, H., 1995a. ATP-dependent efflux of calcein by the multidrug resistance protein (MRP): no inhibition by intracellular glutathione depletion. FEBS Lett. 386, 385–388.

Feller, N., Kuiper, C., Lankelma, J., Ruhdal, J., Scheper, R., Pinedo, H., Broxterman, H., 1995b. Functional detection of MDR1/P190-mediated multidrug resistance in tumor cells by flow cytometry. Br. J. Cancer 72, 543–549.

Grant, C., Valdimarsson, G., Hipfner, D., Almquist, K., Cole, S., Deeley, R., 1994. Overexpression of multidrug resistance-associated protein (MRP) increases to natural product drugs. Cancer Res. 54, 357–361.

Hollo, Z., 1994. Calcein accumulation as a fluorometric functional assay of the multidrug transporter. Biochim. Biophys. Acta 384, 1191–1198.

Hollo, Z., Homolya, L., Hegedus, T., Sarkadi, B., 1996. Transport properties of the multidrug resistance-associated protein (MRP) in human tumor cells. FEBS Lett. 383, 99–104.

Larsson, R., Nygren, P., 1989. A rapid fluorometric method for semiautomated determination of cytotoxicity and cellular proliferation of human tumor cell lines in microculture. Anticancer Res. 9, 1111–1120.

Larsson, R., Nygren, P., 1990. Pharmacological modification of multi-drug resistance (MDR) in vitro detected by a novel fluorometric microculture cytotoxicity assay. Reversal of resistance and selective cytotoxic actions of cyclosporin A and verapamil on MDR leukemia T-cells. Int. J. Cancer 46, 67–72.

Larsson, R., Kristensen, J., Sandberg, C., Nygren, P., 1992. Laboratory determination of chemotherapeutic drug resistance in tumor cells from patients with leukemia using a fluorometric microculture cytotoxicity assay (FMCA). Int. J. Cancer 50, 177–185.

Lee, J., Paull, K., Alvarez, M., Hose, C., Monks, M., Grever, M., Fojo, A., Bates, S., 1994. Rhodamine efflux patterns predict P-glycoprotein substrates in the National Cancer Institute drug screen. Mol. Pharmacol. 46, 627–638.

Liang, P., Pardee, A.B., 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257, 967–971.

Liminga, G., Nygren, P., Larsson, R., 1994. Microfluorometric evaluation of calcein acetoxymethyl ester as a probe for P-glycoprotein-mediated resistance: effect of cyclosporin A and its non-immunosuppressive analogue SDZ PSC 833. Exp. Cell Res. 212, 291–296.

Mirski, S., Gerlach, J., Cole, S.P., 1987. Multidrug resistance in a human small cell lung cancer cell line selected in adriamycin. Cancer Res. 47, 2594–2598.

Monks, A., Scudiero, D., Skehan, P., Shoemaker, R., Paull, K., Vistica, D., Hose, C., Langley, J., Cronise, P., Vaigro-Wolff, A., Gray-Goodrich, M., Campbell, H., Mayo, J., Boyd, M., 1991. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. J. Natl. Cancer Inst. 83, 757–765.

Morris, S., 1990. Real-time multi-wavelength fluorescence imaging of living cells. BioTechniques 8, 296–312.

Mulcahy, R., Bailey, H., Gipp, J., 1994. Up-regulation of gamma-glutamylcyateine synthetase activity in melphalan-resistant human multiple myeloma cells expressing increased glutathione levels. Cancer Chemother. Pharmacol. 34, 67–71.

Myers, T.G., Anderson, N.L., Waltham, M., Li, G., Buolamwini, J.K., Scudiero, D.A., Paull, K.D., Sausville, E.A., Weinstein, J.N., 1997. A protein expression database for the molecular pharmacology of cancer. Electrophoresis 18, 1–7.

Nygren, P., Larsson, R., 1991. Differential in vitro sensitivity of human tumor and normal cells to chemotherapeutic agents and resistance modulators. Int. J. Cancer 48, 598–604.

Paull, K., Shoemaker, R., Hodes, L., Monks, A., Scudiero, D., Rubinstein, L., Plowman, J., Boyd, M., 1989. Display and analysis of patterns of differential activity of drugs against human tumor cell lines: development of mean graph and COMPARE algorithm. J. Natl. Cancer Inst. 81, 1088–1092.

Slovak, M., Ho, J., Bhardwaj, G., Kurz, E., Deeley, R., Cole, S., 1993. Localization of a novel multidrug resistance-associated gene in the HT1080/DR4 and H69AR human tumor cell lines. Cancer Res. 53, 3221–3225.

Tsuruo, T., Tomida, T., 1995. Multidrug resistance. Anticancer Drugs 6, 213–218.

Versantvoort, C., Bagrij, T., Wright, K., Twentyman, P., 1995. On the relationship between the probenecid-sensitive transport of daunorubicin or calcein and the glutathione status of cells overexpressing the multidrug resistance-associated protein (MRP). Int. J. Cancer 63, 855–862.

Weinstein, J.N., Myers, T.G., O'Connor, P.M., Friend, S.H., Fornace, A.J. Jr., Kohn, K.W., Fojo, T., Bates, S.E., Rubinstein, L.V., Anderson, N.L., Buolamwini, J.K., Van Osdol, W.W., Monks, A.P., Scudiero, D.A., Sausville, E.A., Zaharevitz, D.W., Bunow, B., Viswanadhan, V.N., Johnson, G.S., Wittes, R.E., Paull, K.D., 1997. An information-intensive approach to the molecular pharmacology of cancer. Science 275, 343–349.

Weston, S., Parish, C., 1990. New fluorescent dyes for lymphocyte migration studies. J. Immunol. Methods 133, 87–97.

Wu, L., Symthe, A.M., Stinson, S.F., Mullendore, L.A., Monks, A., Scudiero, D.A., Paull, K.D., Koutsoukos, A.D., Rubinstein, L.V., Boyd, M.R., Shoemaker, R.H., 1992. Multidrug-resistant phenotype of disease-oriented panels of human tumor cell lines used for anticancer drug screening. Cancer Res. 52, 3029–3034.