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Experimental report

Ranking of P-glycoprotein substrates and inhibitors by a calcein-AM fluorometry screening assay

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In order to compare the capacities of a variety of compounds to interfere with P-glycoprotein (Pgp) function, a novel assay was set up to work on a large screening scale. The model assay measures the capacity of parental sensitive (Par) and multidrug-resistant (MDR) cells to efflux a small fixed amount of acetoxymethyl calcein (calcein-AM) after their pretreatment with concentration ranges of known Pgp modulators. This microplate cytometry-based assay was performed with two different pairs of cell lines, the human lymphocytic leukemia CEM cells and the murine monocytic leukemia P388 cells. For a given Pgp-expressing MDR cell line, a Pgp modulator EC_{50} was defined as the concentration required to restore half of the calcein retention shown by similarly treated Par cells. With both MDR-P388 and MDR-CEM cells, EC_{50} comparisons ranked five reference Pgp modulators as follows: SDZ 280-446 > SDZ PSC 833 > cyclosporin A > verapamil > vinblastine. Further use of the MDR-CEM cells could rank 15 Pgp modulators for their capacity to interfere with calcein-AM efflux as follows: SDZ 280-446 1.9 × > SDZ PSC 833 8.3 × > cyclosporin A 3.8 × > amiodarone 1.1 × > quinacrine 1.6 × > verapamil 1.4 × > quinidine 1.1 × > vinblastine 11 × > vincristine 2 × > chloroquine > β-lumicolchicine ≥ γ-lumicolchicine ≥ colchicine > etoposide ≥ doxorubicin. This calcein-AM assay should open the way for ranking large numbers of novel structures for their potential Pgp modulator properties, particularly for an efficient screening of Pgp function antagonists, but it does not allow defining whether their inhibition may be competitive or not.

Key words: Calcein-AM, multidrug resistance, P-glycoprotein, SDZ PSC 833.

Introduction

Overexpression of the class 1 *MDR* gene products, the P-glycoprotein (Pgp) molecules, by tumor cells may confer multidrug resistance (MDR) on them. However, Pgp molecules are also expressed on a variety of normal cells in the body. They occur in locations which suggest they may be involved in the excretion of endogenously generated toxic compounds or in the reduced uptake of xenobiotics of exogeneous origins.[1] A thorough analysis of natural compounds, particularly toxic compounds of various dietary regimens, should discriminate those which are Pgp substrates and those which are not. Several drugs with a variety of structural features may also be substrates or inhibitors of the Pgp molecules. Therefore, it would be useful to monitor the Pgp substrate or inhibitor character of a variety of natural and synthetic compounds.

The principle of such an assay might be based on a competition between the tested compound and a known labeled substrate of Pgp molecules for being effluxed by the latter. Since there is no qualitative difference between the Pgp molecules expressed by human cells of either tumoral or normal origins, any human Pgp-expressing tumoral cell line could be used for evaluating whether a novel compound is or is not a Pgp substrate. In fact, the assay could be designed like a search for resistance-modifying agents (modulators), such as the capacity to increase the sensitivity of MDR cells to a cytostatic agent (i.e. to decrease the IC_{50} of vinblastine, taxol, colchicine, doxorubicin, etoposide, etc.), using Pgp-lacking parental (Par) cells as control cells.[2,3] In the past, competition-type assays were performed with non-cytostatic analogs of anti-cancer drugs and other mitotic inhibitors, such as analogs of anthracyclines,[4] Vinca alkaloids[5–8] and colchicine.[2] However, such assays required cell culture and were not suitable for large-scale screening studies.

This competition methodological principle could be adapted to a Pgp-probe retention assay, such as a retention of radiolabeled anti-cancer drugs (doxorubicin, vinblastine), but the large-scale use of radioactivity was a disadvantage.[9] This assay could

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also be performed by using the retention of fluorescent probes (daunomycin, rhodamine-123), both by single cell analyses and flow cytometry and by bulk cell cultures in microplates and multireader spectrofluorimetery.[10–14] The latter screening method allowed the definite discrimination of Pgp-expressing cells and of Pgp-lacking cells as well as the restoration of Pgp probe retention by chemosensitizers in the MDR cells. Nevertheless, the results were much more satisfactory by use of the membrane permeable acetoxymethyl ester of calcein (calcein-AM) as a probe of Pgp effluxing activity.[15–17] Calcein-AM is highly lipid soluble, rapidly penetrates the plasma membrane of cells and is practically non-fluorescent. By cleavage of the ester bonds, cytosolic esterases quickly and irreversibly convert calcein-AM into the hydrophilic, non-permeable and intensively fluorescent free acid form calcein. While traversing the cell plasma membrane, calcein-AM may be effluxed by Pgp molecules present in the membrane of MDR cells and inhibition of Pgp function may restore calcein-AM uptake in MDR cells and emergence of calcein-specific fluorescence.

Calcein-AM but not free calcein was shown to be an excellent activator of the MDR1-ATPase in isolated membranes ($K_a \geq 1$ μM) and calcein accumulation was prevented in MDR1-expressing cells. The intracellularly formed and trapped calcein is well retained by the cells ($T_{50\%}$ of calcein leakage is about 3 h at 37°C) and is no longer a substrate of Pgp.[15,16] Measurement of calcein accumulation was thus used to test for the functional presence of the multidrug transporter. Indeed, over short periods of incubation where calcein leakage can be neglected, the fluorescence of the cells should regularly increase with their time of incubation and as a function of the calcein-AM concentration in the culture medium.[15,16] The fluorescence increase would be slowed down by the calcein-AM efflux by Pgp molecules; the level of effluxing activity per cell would itself depend on the low or high membrane density of Pgp molecules, the presence or absence of Pgp inhibitors, the decrease or increase of Pgp-ATPase activity. This was indeed shown to be the case using spectrophotometry and flow cytometry.[15–17]

Restoration of calcein-specific fluorescence of MDR cells by exposure to the low calcein-AM concentrations sufficient to give similar fluorescence levels was obtained by MDR cell treatment with various Pgp substrates such as vinblastine[16] or vincristine,[17] and inhibitors of the Pgp function, either directed at its ATPase moiety level such as oligomycin[16] and KCN,[17] or working at undefined competitive or non-competitive target sites, such as verapamil,[16,17] cyclosporin A,[16,17] SDZ PSC 833[17] and anti-Pgp monoclonal antibodies.[16] In this paper, we describe our adaptation of the calcein-AM method to a screening scale with fluorometry in microtiter plates, and we show some typical examples obtained with well-known Pgp substrates and inhibitors.

Materials and methods

Tumor cell lines

Two pairs of parental (Par) and multidrug-resistant (MDR) tumor cell lines were used: the murine monocytic leukemia Par-P388 and MDR–P388 (obtained through doxorubicin-resistance selection), and the human T leukemia Par-CEM and MDR-CEM (obtained through vinblastine-resistance selection); those cell line pairs were kindly provided by, respectively, Dr M Grandi (Farmitalia, C Erba Research Center, Milano, Italy) and Professor M Cianfriglia (Istituto di Sanita, Roma, Italy). Further characterization of these cell line pairs was published earlier as well as their culture conditions.[14] Both MDR tumor lines were continuously grown in the presence of the drug used for their selection: 0.25 μg/ml doxorubicin for MDR-P388 cells and 0.1 μg/ml vinblastine for MDR-CEM cells.

Drugs

Calcein-AM (Molecular Probes Europe, Leiden, The Netherlands) was prepared as a stock solution at 1 mg/ml in DMSO; since calcein-AM has a MW of 994.87, this is nearly equal to a 1 mM solution. As calcein-AM was used on the cells at 0.25 μM, there is a 4000-fold dilution of the DMSO. The reference Pgp substrates and/or inhibitors checked for interference with the Pgp-mediated efflux of calcein-AM were the following: amiodarone (Sigma, St Louis, MO; MW = 681.8; 1.0 μg/ml = ± 1.5 μM), chloroquine (Sigma; MW = 515.9; 1.0 μg/ml = ± 2μM), colchicine (Sandoz Pharma, Basel, Switzerland; MW = 399.4; 1.0 μg/ml = ± 2.5 μM), cyclosporin A (Sandoz; MW = 1206.6; 1.0 μg/ml = ± 0.8 μM), doxorubicin (Sigma; MW = 580; 1.0 μg/ml = ± 1.7 μM), etoposide (Sandoz; MW = 588.6; 1.0 μg/ml = ± 1.7 μM), β-lumicolchicine (Sigma; MW = 399.4; 1.0 μg/ml = ± 2.5 μM), γ-lumicolchicine (Sigma; MW = 399.4; 1.0 μg/ml = ± 2.5 μM),

quinacrine (Sigma; MW = 472.9; 1.0 μg/ml = ± 2.1 μM), quinidine (Sandoz; MW = 324.4; 1.0 μg/ml = ± 3.1 μM), SDZ PSC 833 (Sandoz; MW = 1214.65; 1.0 μg/ml = ± 0.8 μM) and SDZ 280-446 (Sandoz; MW = 1182.6; 1.0 μg/ml = ± 0.8 μM), verapamil (Sigma; MW = 491.1; 1.0 μg/ml = ± 2 μM), vinblastine (Janssen Chimica, Geel, Belgium; MW = 909.1; 1.0 μg/ml = ± 1.1μM) and vincristine (Sigma; MW = 923.1; 1.0 μg/ml = ± 1.1 μM). The Pgp substrates or inhibitors were prepared as stock solutions at 10 mg/ml in absolute ethanol for all except doxorubicin, vinblastine and vincristine (stock solution in NaCl 0.9%). The ranges of final concentrations of the Pgp substrates/inhibitors in the assays were 0, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10, 30, 100 and 300 μg/ml.

Bulk culture microplate fluorometry with calcein-AM

The method was directly derived from our earlier procedures which used fluorometry assays of bulk microcultures for monitoring the retention of two classical Pgp probes,[14] daunomycin and rhodamine-123, or of a novel one, a fluorescent cyclosporin derivative (NBDL-CsA).[18]

Fifty microliters of a range of concentrations of potential Pgp substrates and/or inhibitors were distributed in 96-well microtiter plates (3799; Costar, Cambridge, MA). Most tested compounds were highly hydrophobic and required solvent for the preparation of the stock solutions, but all assays contained the same amount of solvent. The Par cells and MDR cells were then added at 5×10^5 cells/well in 100 μl culture medium and the [cell + compound] mixtures were incubated for 15 min at 37°C in the water bath. To reveal this steady-state of activity of Pgp-modulator-treated cells in comparison with naive cells, calcein-AM (50 μl of a 1 μM solution in culture medium) was then added to reach the final 0.25 μM concentration in the microcultures and the cells were further incubated for 15 min at 37°C in the water bath.

The microplates were then centrifuged 5 min at 200 *g* and the cells resuspended in cold culture medium (= one wash); after two further washes, the cells were resuspended in 200 μl culture medium at 4°C and immediately transferred in flat bottom microplates (NUNC Maxisorp). The calcein retention was measured as calcein-specific fluorescence (excitation and emission maxima of 496 and 517 nm, respectively). The microplates were analyzed with a fluorescence reader (Cytofluor™ 2350; Millipore, St Quentin, France), using an excitation at 484 ± 20 nm and reading emission at 530 ± 25 nm).

Assay of a Pgp modulator for interference with calcein-AM efflux was performed in at least three independent experiments (duplicated assays). The data were not shown as absolute fluorescence units but as relative retention of calcein. The measured ratios of specific calcein fluorescence (*Y*-axes) were expressed as function of the modulator concentrations (*X*-axes) as dose–response curves as follows. For Par cells, the calcein retention in modulator-treated Par cells (*Y*-axes) was expressed as percentage of the retention shown by the Par cells in the absence of modulator exposure. For the MDR cells, the calcein retention in modulator-treated MDR cells (*X*-axes) was expressed as percentage of the retention shown by Par cells exposed to the same modulator concentration.

The modulator concentrations required to achieve in the MDR cells 10, 20 and 50% of the calcein-specific fluorescence shown by similarly treated Par cells could be determined from the dose–response curves and were defined as EC_{10}, EC_{20} and EC_{50}.

Results

Comparison of two pairs of Par and MDR cell lines for calcein-AM efflux studies

The two pairs of murine Par-P388 and MDR-P388 monocytic leukemia cells and of human Par-CEM and MDR-CEM lymphocytic leukemia cells were compared for their calcein-specific fluorescence levels following exposure to 0.25 μM calcein-AM. In the absence of modulator, the MDR cells displayed low fluorescence levels in comparison to their Par cell controls: 5.7 ± 2.6% in the MDR-P388 cell case and 6.3 ± 1.4% in the MDR-CEM cell case. While the Par-CEM cells were little affected by the Pgp modulators within the concentration range tested, there was a large modulator dose-dependent increase of the calcein retention in Par-P388 cells treated with cyclosporin A, verapamil and vinblastine, and a moderate one at the highest concentrations of the other two modulators (Figure 1). However, the increased calcein retention was much larger in both lines of MDR cells than in their Par cell controls, when they were exposed to calcein-AM in the presence of increased concentrations of the five tested modulators. When such modulator-dependent calcein retention in the MDR

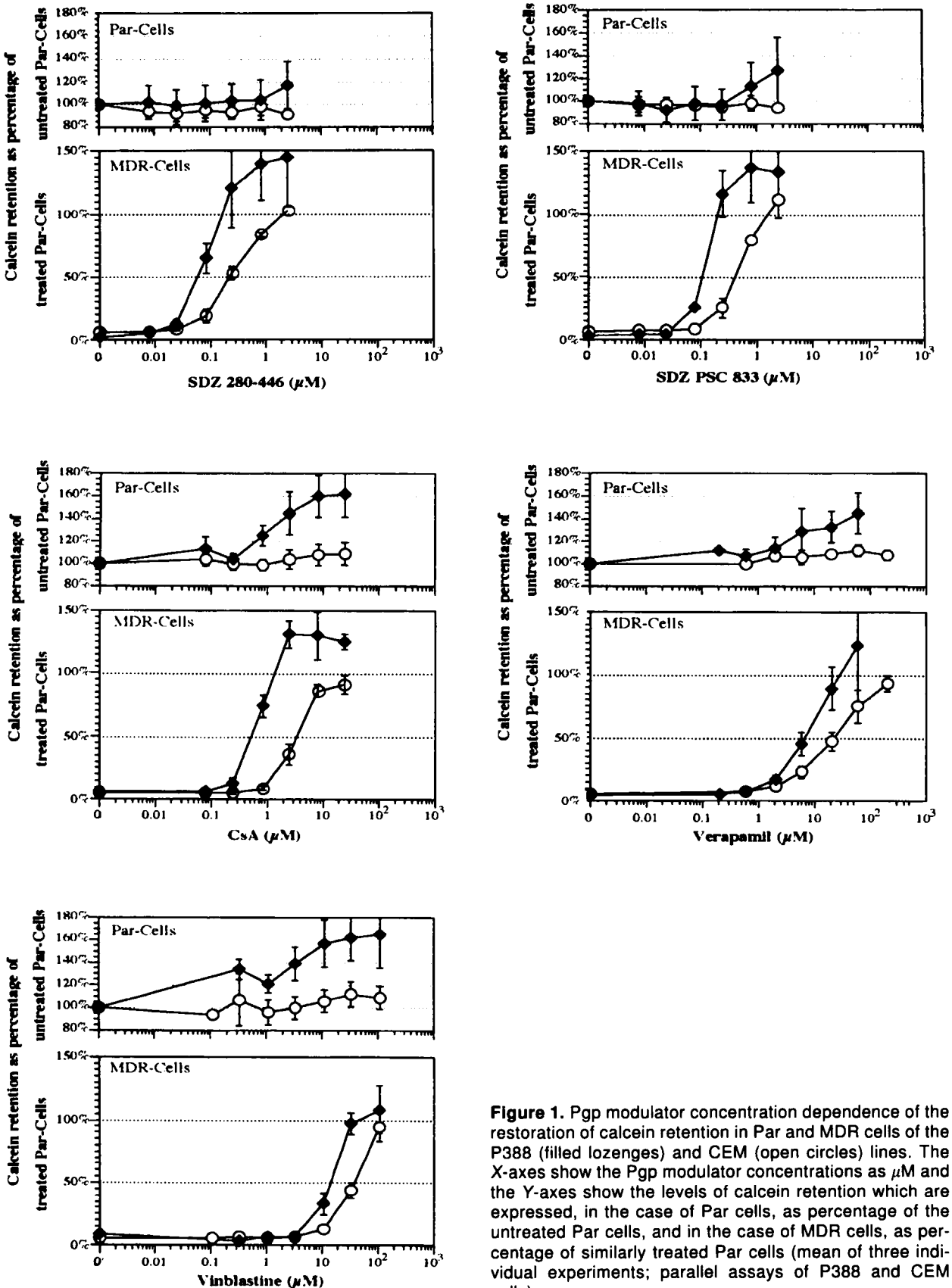


Figure 1. Pgp modulator concentration dependence of the restoration of calcein retention in Par and MDR cells of the P388 (filled lozenges) and CEM (open circles) lines. The *X*-axes show the Pgp modulator concentrations as μM and the *Y*-axes show the levels of calcein retention which are expressed, in the case of Par cells, as percentage of the untreated Par cells, and in the case of MDR cells, as percentage of similarly treated Par cells (mean of three individual experiments; parallel assays of P388 and CEM cells).

Table 1. Comparisons of five reference inhibitors and/or substrates of Pgp for their EC_{50} for the restoration of calcein retention in MDR-P388 and MDR-CEM cells

Pgp substrate	EC_{50} (mean $\mu M \pm SD$) (*n*)	
	MDR-CEM cells	MDR-P388 cells
SDZ 280-446	0.22 ± 0.04 (3)	0.06 ± 0.01 (3)
SDZ PSC 833	0.41 ± 0.06 (3)	0.11 ± 0.01 (3)
Cyclosporin A	3.4 ± 0.55 (5)	0.51 ± 0.07 (3)
Verapamil	22.5 ± 11 (4)	7.2 ± 2.2 (3)
Vinblastine	34 ± 4.6 (3)	14.6 ± 2.3 (3)

cells was expressed as a percentage of its retention in the similarly treated Par cells, all five modulators could restore Par cell levels of retention. The increase of calcein retention in the MDR-P388 cells occurred at lower modulator concentrations than in MDR-CEM cells and the maximal retention may be higher in MDR-P388 cells than in their Par-P388 controls.

Comparisons of the EC_{50} of the five tested modulators revealed that they showed the same ranking in the case of CEM and P388 cells, with similar factors of differential potency (Table 1). Nevertheless, the different Pgp-mediated pumping capacities of MDR-CEM and MDR-P388 cells were obvious when the MDR cells were exposed to their Pgp modulators: higher concentrations of modulator were required with MDR-CEM cells than with MDR-P388 cells to achieve the same level of restoration of calcein retention. This correlates with the higher resistance levels found for the MDR-CEM cells in earlier chemosensitization assays, e.g. the vinblastine IC_{50} values were 0.5 ng/ml (Par-CEM) versus 490 ng/ml (MDR-CEM) and 1.6 ng/ml (Par-P388) versus 105 ng/ml (MDR-P388). Since both cell line pairs gave a similar ranking of the modulators, but that the Par cell controls were more satisfactory in the CEM cell line case, further assays were performed with the CEM cell line pair, with control assays only with the P388 cell line pair.

Ranking of 15 compounds for their capacity to interfere with calcein-AM efflux

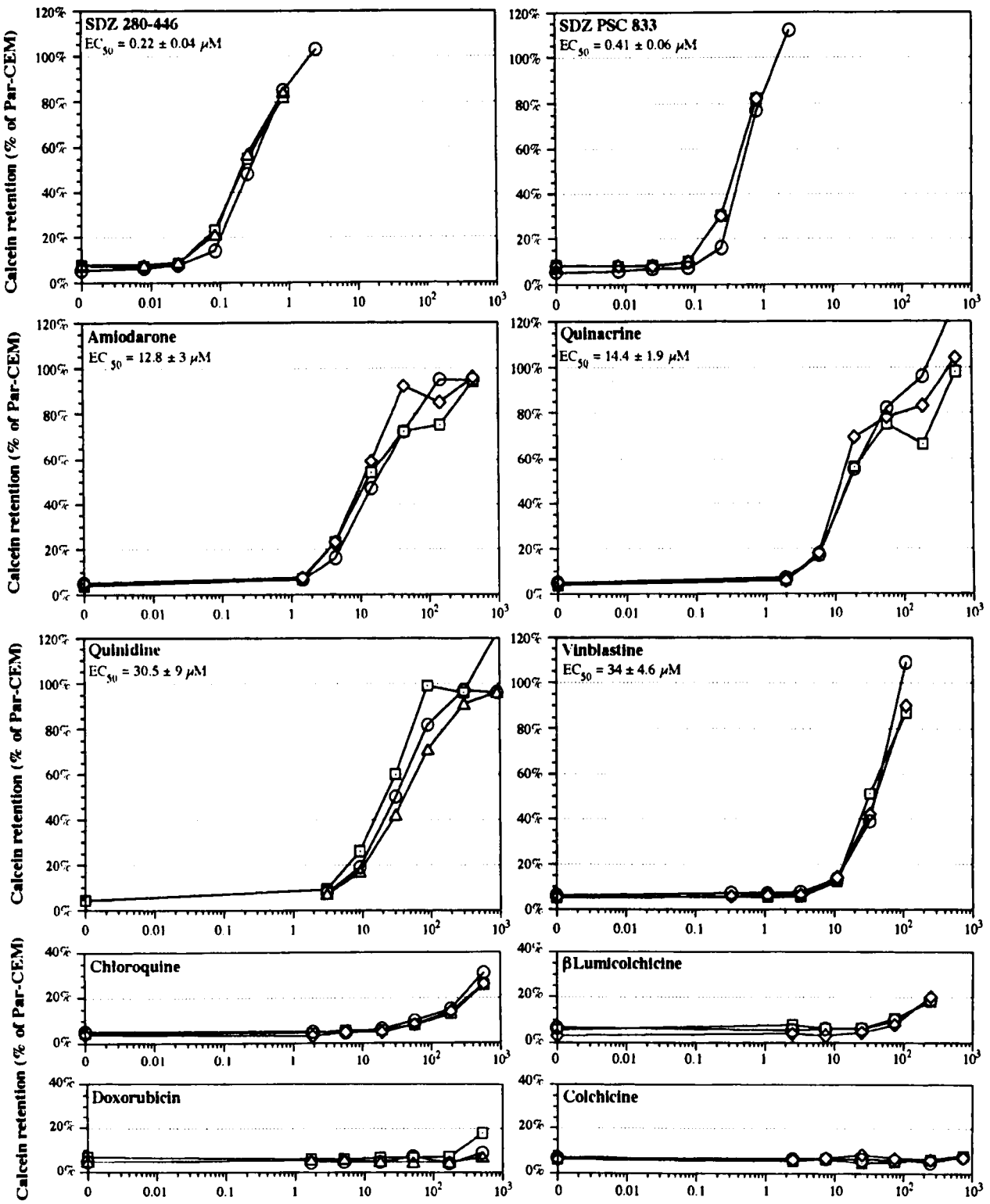
When the MDR-CEM cells were pretreated with various compounds known to be substrates and/or inhibitors of Pgp function, their capacity to interfere with the efflux of calcein-AM could be ranked from very potent inhibitors to compounds giving no detectable inhibition (Figure 2).

While known Pgp inhibitors could give substantial Pgp inhibition levels, providing a measurable EC_{50}, within the range of tested concentrations several well-known Pgp substrates did not detectably interfere with calcein-AM efflux even at the highest tested concentrations. In the case of modulators which could give 50% inhibition of calcein-AM influx, the observed ranking was SDZ 280-446 > SDZ PSC 833 > cyclosporin A > amiodarone > quinacrine > verapamil > quinidine > vinblastine (Table 2). For this specific assay of a capacity to inhibit Pgp function, i.e. within the limits of the highly resistant MDR-CEM cell line used, the tested range of modulator concentrations, the standard 0.25 μM calcein-AM assayed, the timings of each incubation and a read-out performed on bulk cell populations (rather than on single cells), a number of known Pgp substrates could not lead to 50% inhibition of Pgp function. Some of these, however, could give 20 or 10% inhibition of the measured MDR-CEM Pgp activity without significantly affecting the calcein retention shown by the control Par-CEM cells, thus excluding unspecific membranous (or other) effects possibly leading to higher intracellular calcein contents. Such modulators could also be compared on the basis of the highest achievable restoration of calcein fluorescence which was Pgp-specific (i.e. occurring at concentrations devoid of effects on the Par-CEM cells), providing the following ranking: vincristine > chloroquine > β-lumicolchicine $\geq$ γ-lumicolchicine $\geq$ colchicine $\geq$ etoposide $\geq$ doxorubicin. The significance of the latter comparisons is limited, as the maximal modulator concentrations which could be used for comparisons differed because of their different direct cytotoxicity.

Discussion

The described microplate cytometry-based assay was actually set up to measure, on a large screening scale, the capacities of Par and MDR cells to efflux a small fixed amount of a Pgp substrate (calcein-AM) after their pretreatment with a range of concentrations of various compounds which may be globally termed Pgp modulators.

As already reported elsewhere,[15,16] in comparison with two other commonly used fluorescent probes (the fluorescent anthracycline daunomycin and the mitochondrial dye rhodamine-123), an advantage of calcein-AM is a high molar emission coefficient with a fluorescence essentially insensitive to changes in pH, Ca^{2+} and Mg^{2+}. At variance,


SDZ 280-446
EC50 = 0.22 ± 0.04 μM
SDZ PSC 833
EC50 = 0.41 ± 0.06 μM
Amiodarone
EC50 = 12.8 ± 3 μM
Quinacrine
EC50 = 14.4 ± 1.9 μM
Quinidine
EC50 = 30.5 ± 9 μM
Vinblastine
EC50 = 34 ± 4.6 μM
Chloroquine
βLumicolchicine
Doxorubicin
Colchicine
Calcein retention (% of Par-CEM)

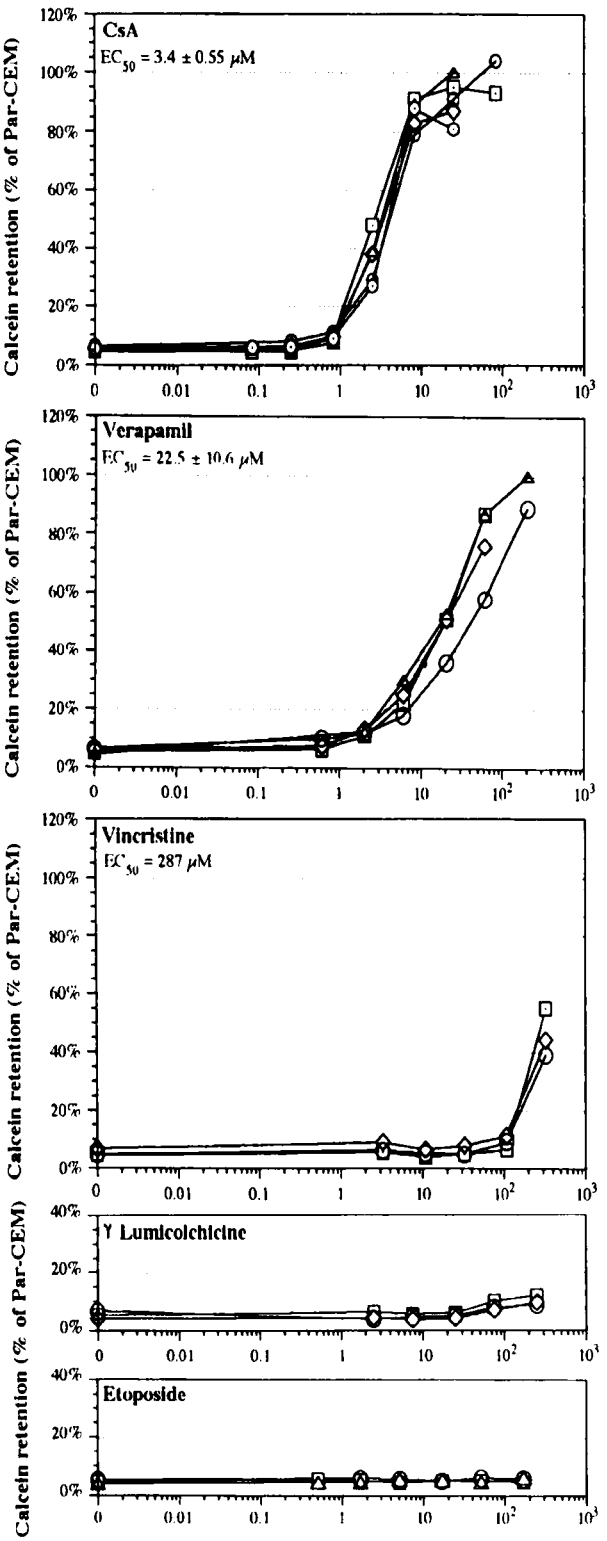


Figure 2. (b)

daunomycin fluorescence is quenched upon binding to DNA, while rhodamine-123 easily leaks out of the cells, accumulates in hydrophobic compartments and/or intracellular organelles, and shows a spectral shift and fluorescence intensity change, inside the cells, due to its interaction with various cellular components. Thus, calcein-AM looks to be most suitable for measuring instantaneous levels of Pgp pumping activity as can be done by exposing Pgp-expressing cells to a short pulse of that Pgp probe.

However, the major advantage of calcein-AM is that it allows us to study, at the whole cell level, the direct Pgp-mediated interference with drug influx (from cytosol to medium), without the contribution of a significant efflux component (from cytosol to medium). This is because, at the plasma membrane level, the only Pgp substrate source for Pgp-mediated efflux comes from the extracellularly added calcein-AM. Indeed, once calcein-AM has reached the cytosol, it is quickly converted into the non-permeant calcein. Therefore, any calcein-AM that leaks in the cytosol because of too low Pgp function, either because of too few Pgp molecules per cell or because of inhibited Pgp function, cannot be pumped back outside the cell: as calcein cannot partition from the cytosol to the plasma membrane, it is no longer a Pgp substrate.

This feature of the calcein-AM assay makes its use particularly relevant for studies, on the interference with Pgp function, of cytostatic drugs which remain sequestered within the cell and are no longer exchangeable with the cell plasma membrane, either because they enter some sub-cellular compartments or because they display a high affinity (low K_d) for their cytosolic target, such as Vinca alkaloids, taxol or colchicine for tubulin, or cyclosporin A for cyclophilin. When overloading of the available Pgp effluxing activity allows their leakage into the cytosol, such drugs become irreversibly trapped in the cell, however strong the Pgp activity

Figure 2. Pgp modulator concentration dependence of the restoration of calcein retention in MDR-CEM cells. The X-axes show the Pgp modulator concentrations as μM and the Y-axes show the levels of calcein retention expressed as percentage of the similarly treated Par-CEM cells. The different diagrams show the results of three individual experiments (five for cyclosporin A and four for verapamil) and are ranked from left to right and from top to bottom from the strongest modulators to the weakest ones. The calculated EC_{50} is given in the top of each diagram; in the case of vincristine, it was achievable in one out of three experiments.

Table 2. Comparisons of 15 reference inhibitors and/or substrates of Pgp for their capacity to restore calcein retention in MDR-CEM cells: Pgp ligand molarity (μM) giving 50, 20 or 10% calcein retention restoration[a]

Pgp ligands	*n*	Mean EC_{50} ± SD	Mean EC_{20}	Mean EC_{10}	Maximum	
					%	at μM
SDZ 280-446	3	0.22 ± 0.04	0.08	0.03	103	2.6
SDZ PSC 833	3	0.41 ± 0.06	0.17	0.08	112	2.5
Cyclosporin A	5	3.4 ± 0.55	1.2	0.83	91	24
Amiodarone	3	12.8 ± 3.0	3.9	1.8	85	150
Quinacrine	3	14.4 ± 1.9	6.2	2.8	82	200
Verapamil	4	22.5 ± 11	3.7	1.1	89	200
Quinidine	3	30.5 ± 9.5	8.7	3.7	95	308
Vinblastine	3	34 ± 4.6	13.2	6.2	95	110
Vincristine	3	NA	145	108	46	325
Chloroquine	3	NA	308	74	27	580
β-Lumicolchicine	3	NA	NA	78	19	250
γ-Lumicolchicine	3	NA	NA	250	10	250
Colchicine	3	NA	NA	NA	7	750
Etoposide	3	NA	NA	NA	5.2	51
Doxorubicin	3	NA	NA	NA	5.5	170

[a] The EC_{50}, EC_{20}, EC_{10} and maximum % values were measured as concentrations giving in the MDR-CEM cells 50, 20, 10 or maximal % of the calcein-specific fluorescence of Par-CEM cells, in conditions suggesting Pgp-specific effects, i.e. which did not alter the calcein retention by the Par cells. In the absence of Pgp modulator treatment, the retention of calcein by MDR-CEM cells reached 3.8–5.9% of the Par-CEM cell retention. *n*, number of individual experiments; NA, not achievable

may then become, because, just like calcein, they remain unavailable for Pgp-mediated pumping.

Our calcein-AM method was designed for screening a variety of compounds for Pgp modulatory properties. Our rationale was that the MDR cells should be first exposed to the compound, their resulting level of Pgp activity being then revealed with calcein-AM. Thus, our cells were pre-incubated for 15 min at 37°C with a range of concentrations of the potential modulator before adding 0.25 μM calcein-AM. After we had set up the microplate fluorometry assay and used it for studying a large variety of potential modulators, another screening method using calcein-AM was described.[17] Although Pgp inhibitor discovery could be done by that method as well as ours, the two types of assays show substantial differences, which may not be important so long as strong Pgp-antagonists are searched for, but which might lead to different conclusions in other searches such as for Pgp-agonists. Two major differences must be taken into account: first, the use of 5 μM calcein-AM[17] instead of 0.25 μM (this paper); second, the simultaneous addition of the cells and the calcein-AM together to the microculture wells containing the various modulator concentrations,[17] instead of the MDR cell pre-exposure to the potential modulators with a *delayed* calcein-AM addition (this paper). In our method, at the time of calcein-AM addition, the MDR cell Pgp molecules should have acquired a Pgp-effluxing activity level which is a function of the modulator properties, calcein-AM then revealing that activated, unchanged or inhibited status. The ATPase-dependent effluxing function of the cell-expressed Pgp molecules should be functioning at a steady-state level which was established as a function of the intrinsic capacity of the tested compound: 15 min exposure to the potential modulator was enough to get this steady-state level, since, according to the published curves of free calcein accumulation, this occurred as a linear function of time starting immediately after cell exposure to the modulator.[15,16] Thus, at the time of calcein-AM addition, we should already be dealing with a steady-state Pgp activity and the added calcein-AM might essentially function as an indicator of Pgp activity, although without excluding its own contribution to activation of the ATPase moiety of Pgp. In contrast, in the other assay,[17] the cells were simultaneously exposed to the modulator and to a much larger calcein-AM concentration. The latter might be the leading factor in activating the ATPase-dependent pumping activity of Pgp, thus actually controlling the level of Pgp activity, other modulators remaining anyhow detectable but only if they are strong Pgp inhibitors.

For a given Pgp-expressing MDR cell line, a modulator EC_{50} might be defined as the concentration

required to reach half of the calcein uptake shown by similarly treated Par cells. Modulator EC_{50} values should obviously differ from one MDR cell line to another, particularly depending on their resistance level, in this case on their Pgp expression level (see below). All tested modulators were known to interact with Pgp molecules, either by interfering with their function (Pgp inhibitors) or by being effluxed by Pgp (Pgp substrates), or by sharing both properties as could occur for slow Pgp substrates causing competitive inhibition for the Pgp-mediated efflux of other substrates.

To restore in the MDR cells 50% of the calcein fluorescence of the Par cells (EC_{50}), about three to seven times higher concentrations of Pgp modulators were required in the CEM cell case than in the P388 cell case: within the same experiments using this calcein-AM efflux as read-out, the SDZ 280-446 EC_{50} values were 0.06 μM for MDR-P388 cells and 0.22 μM for MDR-CEM cells, the SDZ PSC 833 EC_{50} values were 0.11 μM for MDR-P388 cells and 0.41 μM for MDR-CEM cells, and the cyclosporin A EC_{50} values were 0.51 μM for MDR-P388 cells and 3.4 μM for MDR-CEM cells. Although this generally correlates with the roughly 5- to 6-fold larger relative resistance level of the MDR-CEM cells (1000-fold resistance versus Par CEM cells) than that of MDR-P388 cells (150- to 200-fold resistance versus Par-P388 cells), a number of other factors may play a role, among which are potential differences of plasma membrane structure and/or composition (altering the rate of free passive influx of calcein-AM and/or passive efflux of calcein) and of cytosolic esterase levels (altering the rate of calcein production). Thus, the present correlation may be just coincidental, because besides differences of Pgp activity levels, there might be a number of other factors that may play a role as well. In any case, with both MDR cell lines, SDZ 280-446 and SDZ PSC 833 gave stronger inhibition of the Pgp-mediated calcein-AM efflux than cyclosporin A, itself being more potent than verapamil, itself more potent than vinblastine. Therefore, these reference modulators suggest that the calcein-AM assay can be used with high confidence for the identification of novel compounds which may influence the activity of Pgp molecules. For some unknown reasons, the calcein retention by the control Par-P388 cells was often altered by compound concentrations usually assayed for Pgp-modulatory studies, whereas no such effects were found with the Par-CEM (or other) cell lines. Moreover, the identity of the *class 1 mdr* gene (*1a* and/or *1b*?) expressed in P388 cells was unknown, whereas there is a unique *class 1 MDR* gene in human. Therefore, for our large-scale screening for Pgp modulators (manuscripts in preparation), we preferred the use of the human cell line, in spite of their higher resistance level.

Interestingly, SDZ 280-446 ranked as the strongest Pgp inhibitor not only with MDR-CEM cells but also with MDR-P388 cells. Although SDZ PSC 833 and SDZ 280-446 were found to be roughly equipotent when compared on several cell lines, the one was a slightly stronger chemosensitizer for some MDR cell lines and the other for other MDR cell lines.[19] With the MDR-P388 cell line, SDZ 280-446 was always found weaker than SDZ PSC 833 in common tests which measured the restoration of either the cell growth sensitivity to anti-cancer drugs (chemosensitization to colchicine,[19] vincristine,[19] daunomycin,[19] doxorubicin,[19] etoposide[19] and taxol[20]) or the restoration of the retention of Pgp probes (daunomycin[12,19] and rhodamine-123[11]), whereas by use of the calcein-AM assay, SDZ 280-446 emerged as the strongest Pgp inhibitor. How this difference should be related with specific features of the calcein-AM assay remains speculative.

By comparing the EC_{50}, EC_{20} and EC_{10} data from Table 2, the following ranking from strong to weak Pgp function inhibitors seems to emerge: SDZ 280-446 > SDZ PSC 833 > cyclosporin A > amiodarone > quinacrine > verapamil > quinidine > vinblastine > vincristine > chloroquine > β-lumicolchicine > γ-lumicolchicine $\geq$ colchicine $\geq$ etoposide doxorubicin. In a straight competitive inhibition hypothesis, this might simply reflect a ranking from slow to fast Pgp pump substrates.

Modulators which completely restored calcein retention in the MDR-CEM cells to the Par-CEM cell levels were well established Pgp inhibitors, with the exception of vinblastine. Inversely, several known Pgp substrates, which only partly reverse the calcein retention deficiency shown by the MDR-CEM cells, were cytostatic agents, with the exceptions of chloroquine and the β- and γ-lumicolchicines. Obviously, in its present form and with a highly resistant Pgp-expressing MDR-CEM cell line, the method did not allow us to detect some well-known Pgp substrates such as doxorubicin, colchicine and etoposide.

Nevertheless, besides the classical modulators SDZ 280-446, SDZ PSC 833, cyclosporin A and verapamil, a substantial restoration of calcein retention was conferred by hydrophobic cationic compounds with known chemosensitizing activities, amiodarone[21] and quinidine.[22] Out of two

lysosomotropic agents reported to sensitize MDR cells,[23–27] quinacrine strongly restored calcein retention, but choroquine did not.

Although Vinca alkaloids were rather known as Pgp substrates, they were also reported to increase the toxicity of some other anti-cancer drugs such as etoposide.[5,6] In the past, non-antitumor analogs of anthracycline[4] or Vinca alkaloid[5,8,27] were shown to reverse multidrug-resistance in P388 cells. Non-cytostatic analogs of colchicine[2] were also found to have such properties:[2] β-lumicolchicine, a UV-light-induced, membrane-active analog of colchicine (which lacks the tubulin-binding activity of colchicine and its effects on microtubule but shows its membrane effects), could efficiently restore the sensitivity of MDR Chinese hamster ovary cells to colchicine. The interpretation was that while the pumping capacity of the available Pgp molecules was saturated with β-lumicolchicine, these pumps were not available for effluxing colchicine; while partitioning of β-lumicolchicine from the membrane into the cytosol had no effect on cell proliferation, access of colchicine in the cytosol allowed its binding to tubulin and its inhibition of cell division.[2]

The calcein-AM assay did not give the same ranking of the Pgp inhibitors as the one found by other assays such as the restoration of colchicine sensitivity in long-term (3–6 days) *in vitro* culture.[2,3] For instance, quinacrine, although looking to be a stronger chemosensitizer than verapamil when used at low concentrations, did not allow a complete sensitization of MDR Chinese hamster ovary cells because it became cytotoxic by itself at micromolar concentrations.[2] Obviously, this may be due to the different Pgp molecules on hamster and human MDR cell lines. However, the differences of ranking found with the two methods may also come from the quite different experimental conditions: in the calcein-AM assay, comparisons of Pgp inhibition can be performed even with cytostatic or cytotoxic compounds, which cannot be compared over a whole range of concentrations in chemosensitization assays involving cell growth and division; furthermore, the problem of the half-life of the various compounds in the culture medium is of lower concern as the assays are rather short, like the calcein-AM one.

Conclusions

In its present form, the calcein-AM method allowed a ranking of reference compounds from strong Pgp inhibitors to fast Pgp substrates, which fits previously known data. Obviously, when used alone, the calcein-AM assay cannot give any insight on whether or not inhibition of a Pgp function is actually competitive. It could be speculated that if a cell must engage most of its Pgp molecules to pump a Pgp substrate, less Pgp molecules will be free for pumping calcein-AM. Thus, by providing to Pgp-expressing cells other Pgp substrates than calcein-AM to pump, a competition-type situation for Pgp pumping might be created when the steady-state pumping activity will be reached. If the potential Pgp substrate can substantially overload the Pgp molecules, increased leakage of calcein-AM to the cytosol should result in an increased fluorescence signal. Several other alternative interpretations must, however, be considered, particularly to avoid concluding on properties of a Pgp modulator from its behavior in the calcein-AM assay. In spite of the limitations of the calcein-AM assay in its present form, it offers a good potential for the study of the Pgp modulatory properties of novel compounds.

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