enhanced expression of the c-*myc* oncogenes in human colorectal carcinomas. *Path Res Pract* 1990, **186**, 205–211.
17. Japanese Research Society for Cancer of Colon and Rectum. General rules for clinical and pathological studies on cancer of the colon, rectum and anus. Part I. Clinical classification. *Jpn J Surg* 1983, **13**, 557–573.
18. Dukes CE. The classification of cancer of the rectum. *J Pathol Bact* 1932, **35**, 323–332.
19. Chomczynski P, Sacci N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analyt Biochem* 1987, **162**, 156–159.
20. Watt R, Stanton LW, Marcu KB, *et al.* Nucleotide sequence of cloned cDNA of human c-*myc* oncogene. *Nature* 1983, **303**, 725–820.
21. Kelly K, Cochran HB, Stiles CD, *et al.* Cell-specific regulation of the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factors. *Cell* 1983, **35**, 603–610.
22. Thompson CB, Challoner PB, Neiman PE, *et al.* Levels of c-*myc* oncogene mRNA are invariant throughout the cell cycle. *Nature* 1985, **314**, 363–366.
23. Hann SR, Thompson CB, Eisenman RN. C-*myc* oncogene protein synthesis is independent of the cell cycle in human and avian cells. *Nature* 1985, **314**, 366–369.
24. Westin EH, Gallo RC, Arya SK, *et al.* Differential expression of the amv gene in human hematopoietic cells. *Proc Natl Acad Sci USA* 1982, **79**, 2194–2198.
25. Muller R, Salmon DJ, Tremblay JM, *et al.* Differential expression of cellular oncogenes during pre- and postnatal development of the mouse. *Nature* 1982, **299**, 640–644.
26. Guillem JG, Levy MF, Hsiah LL, *et al.* Increased levels of phorbin, c-*myc*, and ornithine decarboxylase RNAs in human colon cancer. *Molec Carcinogenesis* 1990, **3**, 68–74.
27. Sugio K, Kurata S, Sasaki M, *et al.* Differential expression of c-*myc* gene and c-*fos* gene in premalignant and malignant tissues from patients with familial polyposis coli. *Cancer Res* 1988, **48**, 4855–4861.
28. Viel A, Maestro R, Toffoli G, *et al.* c-*myc* overexpression is a tumor-specific phenomenon in a subset of human colorectal carcinomas. *J Cancer Res Clin Oncol* 1990, **116**, 288–294.

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Influence of Dexniguldipine-HCl on Rhodamine-123 Accumulation in a Multidrug-resistant Leukaemia Cell Line: Comparison With Other Chemosensitisers

R. Boer, S. Haas and A. Schödl

In the clinical therapy of cancer, resistance to many cytostatic drugs is a major cause of treatment failure. Among other mechanisms, the expression and pumping activity of P-glycoprotein (PGP) in the membrane of resistant cancer cells is responsible for the reduced uptake of cytostatics. The blockade or inhibition of PGP activity by chemosensitisers seems to be a tenable way to restore sensitivity to antineoplastic drugs and therapeutic efficacy. In the present work the influence of the new chemosensitiser dexniguldipine on rhodamine-123 accumulation in multidrug-resistant leukaemia cells was investigated. Dexniguldipine increases cellular rhodamine-123 accumulation dose-dependently. pEC_{50} values (−log concentration of drug showing a half maximal effect) in accumulation studies are dependent on pH of the test system and are in the range of 6.5 (pH 7.2) to 7.2 (pH 8.0) for dexniguldipine. In comparison with other chemosensitisers such as SDZ PSC 833, cyclosporin A, verapamil, dipyridamole, quinidine and amiodarone, dexniguldipine is the most potent drug in this test system. In addition to equilibrium measurements of rhodamine-123 accumulation, efflux of rhodamine-123 was analysed in the absence and presence of chemosensitisers. A clear dose-dependency was seen and, moreover, a dramatic decrease in efflux rates was achieved in the presence of chemosensitisers. The described system can be used to investigate PGP-mediated drug transport on a pharmacological and biochemical basis.

Key words: rhodamine-123 uptake, MDR cells, dexniguldipine, SDZ PSC 833, chemosensitiser, resistance reversal, rhodamine-123 efflux

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INTRODUCTION

THE SUCCESSFUL treatment of cancer with cytostatic drugs is often impaired by the development of resistance to these substances. There are multiple mechanisms which can lead to resistant cancer cells. The best studied and probably most frequent mechanism is the appearance and overexpression of P-glycoprotein (PGP), whose pumping activity reduces the intracellular drug concentrations below the effective levels [1, 2]. PGP shows a rather non-specific substrate specificity in removing drugs of different chemical classes with different

modes of action, such as vinca alkaloids, anthracyclines, colchicine and actinomycin-D [3, 4]. This kind of resistance is, therefore, termed multidrug resistance (MDR). Stimulated by the observation of Tsuruo [5] that verapamil can reverse MDR and restore cellular accumulation of anticancer drugs at least in some *in vitro* systems, an intensive search for MDR-reversing compounds was initiated. This resulted in a great number of drugs which could circumvent MDR *in vitro* and *in vivo*. Reversing compounds are of a similar diversity and multiplicity in structure as the transported cytostatics (for review see [6]).

The molecular mechanisms by which transport of the different cytostatic substrates by PGP is influenced by reversing compounds are still a mystery. Different binding sites for vinblastine and azidopine, both substances which are transported by PGP, have been identified [7].

Most work on chemosensitisers is performed *in vitro* using cancer cell lines of different nature and origin. The clinical usefulness of a certain chemosensitiser for anticancer therapy is often limited by serious side-effects, as very high concentrations must be given to achieve a therapeutic effect. Thus, the use of verapamil is restricted by serious cardiovascular side-effects [8–10] and the immunosuppressive action of cyclosporin A is also a matter of concern.

The therapeutic effectiveness of a chemosensitiser can be characterised by its potency, which is the concentration necessary to achieve a certain effect and by the availability at the site of action, i.e. the tumour tissue. Potency, in pharmacological terms, is dependent on the 'affinity' of a substance to the target protein or the target process, i.e. efflux of drugs from MDR cells. Availability is, among other factors, dependent on the blood level, lipophilicity of the substance and accumulation in the tumour tissue. Compounds with high potency and low availability will barely be effective and *vice versa*.

To investigate the interaction of chemosensitisers with PGP, a rhodamine-123 accumulation assay was adapted to measure concentration–response curves of chemosensitisers and the EC_{50} was determined as a measure of potency. Rhodamine-123 has been shown to be transported by PGP in a cytostatic-like manner [11, 12] and this efflux can be blocked by known chemosensitisers [12]. Measurement of intracellular rhodamine-123 is most conveniently performed in a fluorescence-activated cell sorter (FACS) without the need to separate intracellular and extracellular rhodamine-123. Another objective of the study was to compare some of the most promising chemosensitisers in the same system. To date, large amounts of data have been obtained with these chemosensitisers in different cell lines and under different assay conditions but a comparative study in one cell line is still missing.

MATERIALS AND METHODS

Cell lines and cell culture

The human T-lymphoblastoid cell line CCRF-CEM was obtained from American Type Culture Collection (Rockville, Maryland, U.S.A.). The selection of MDR CCRF-CEM sublines was published elsewhere [13, 14]. The cell line CCRF VCR 1000 used in this study was selected and cultured in 1000 ng/ml vincristine, and showed distinct PGP expression [15]. Cells were grown in RPMI 1640 (Gibco BRL, Berlin, Germany) supplemented with 10% fetal calf serum (FCS) (Gibco BRL). SDZ PSC 833 was a kind gift from Sandoz Pharma AG (Basel, Switzerland). All other chemicals are available commercially and of greatest purity.

Dexniguldipine (proposed INN, former name: B859-35) and all other chemosensitisers were dissolved in dimethylsulfoxide (DMSO) and diluted 100-fold by addition to the test mixture.

Rhodamine-123 accumulation

Cells were centrifuged at 100 *g* for 10 min and resuspended in RPMI 1640 without FCS in the absence (pH 7.8) or presence of 50 mM HEPES (pH values as indicated in the text). Two hundred thousand cells per sample were incubated in a total volume of 1 ml in the presence of chemosensitiser or solvent (DMSO) for 30 min at 37°C. Glass tubes were used for all experiments. Rhodamine-123 was added to achieve a total concentration of 0.8 mg/l. Incubation was continued for 60 min. Cells were analysed on an Epics Profile II FACS (Coulter, Krefeld, Germany). The excitation wavelength was 488 nm and cell-associated rhodamine-123 fluorescence was measured at 520 nm. Concentration–response curves were constructed for each chemosensitiser. The concentration leading to half maximal increase in cell-associated rhodamine-123 (EC_{50}) was calculated by fitting a sigmoid curve to the data. For the comparison of data obtained in two different experiments, the quotient of the rhodamine-123 value of the sensitive control cells (CCRF-CEM) from two experiments was calculated and data from one experiment were multiplied by that factor. This is in principle the same as setting the rhodamine-123 value of the sensitive control cells to 100% for every experiment and expressing the accumulation data as per cent of control.

For rhodamine-123 efflux experiments, cells were incubated in the presence of different concentrations of chemosensitiser with rhodamine-123 for 1 h. Cells were then centrifuged at 100 *g* for 10 min and resuspended in rhodamine-123-free RPMI 1640 containing chemosensitiser at the appropriate concentration. Cell-associated rhodamine-123 fluorescence was measured at various time points after resuspension as indicated in Figures 1 and 2.

Forward and sideward light scatter parameters of the cells were used as a rough measure of cell viability. Only cells with unchanged light scatter parameters were analysed for their rhodamine-123 content.

RESULTS

MDR CCRF VCR 1000 cells show a drastically reduced intracellular accumulation of rhodamine-123 in comparison to the parental cell line CCRF-CEM. Figure 3 shows the accumulation of rhodamine-123 in sensitive and resistant cells. Sensitive cells accumulate rhodamine-123 time-dependently, and equilibrium is not obtained until after 3 h. Resistant cells show very low accumulation and equilibrium is achieved almost immediately at a low level. In the presence of 0.3 μM dexniguldipine resistant cells accumulated rhodamine-123 in a similar manner as sensitive cells, although the absolute level of cellular fluorescence was somewhat lower. This is probably due to the suboptimal dexniguldipine concentration of 0.3 μM (see below). The uptake of rhodamine-123 into sensitive and resistant cells increases almost linearly with concentration (0.2–1.6 mg/l) under the conditions used (data not shown).

Chemosensitisers increase the intracellular rhodamine-123 content of MDR CCRF VCR 1000 cells in a concentration-dependent manner. If rhodamine-123 accumulation is measured

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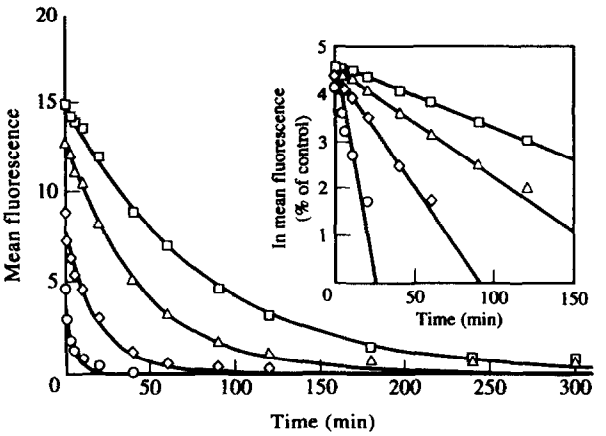

Figure 1. Influence of dexniguldipine on rhodamine-123 efflux from CCRF VCR 1000 cells. Cells were loaded in the presence of 0.3 μM (□), 0.1 μM (△), 0.03 μM (◇) and 10 nM (○) dexniguldipine. After removal of extracellular rhodamine-123, cellular rhodamine-123 content was measured at the times indicated. The inset shows linearised plots of the data. The values at the times indicated were calculated as % of the value at $t = 0$. The percentage of remaining fluorescence is expressed as the natural logarithm and was plotted versus time. The slope of the lines will give the K_{eff} constants.

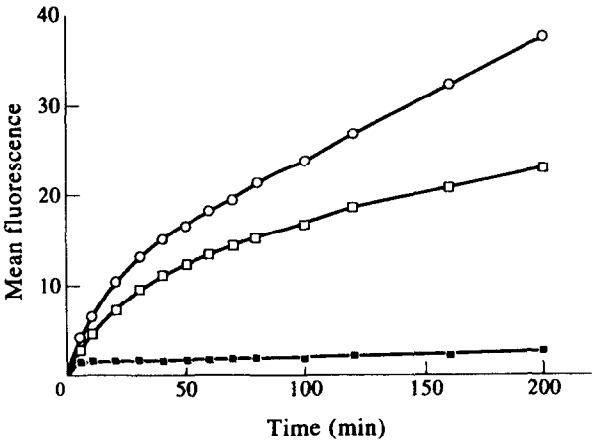

Figure 3. Rhodamine-123 accumulation in resistant CCRF VCR 1000 cells (□,■) and sensitive CCRF-CEM cells (○) in the absence (■) and presence (□) of 0.3 μM dexniguldipine. Fluorescence is displayed in arbitrary units. Rhodamine-123 concentration in the assay was 0.8 mg/l.

in the presence of increasing concentrations of chemosensitiser, complete dose–response curves are obtained and a maximal effect is achieved if the chemosensitiser concentration is raised to sufficiently high levels. Figure 4 shows typical dose–response curves for the new chemosensitiser dexniguldipine at different pH values. A clear pH dependence of the compound's modulating potency is seen. Curve fitting using a sigmoid model resulted in pEC_{50} values of 6.45 (pH 7.2), 6.52 (pH 7.4), 6.79 (pH 7.6), 6.97 (pH 7.8) and 7.20 (pH 8.0). The rhodamine-123 accumulation of the sensitive cell line in the absence of chemosensitiser is shown at the left upper corner of the figure. A small but insignificant increase in rhodamine-123 content is seen in sensitive cells at higher pH values. The accumulation deficit of resistant cells is reversed almost to the level of the sensitive cells.

In order to compare different chemosensitisers, dose–response measurements were performed for the various chemosensitisers, as described for dexniguldipine. Having demonstrated a clear pH dependence for the modulating activity of dexniguldipine, measurements were performed at pH 7.8 and 7.3 to assess whether other chemosensitisers show a similar pH dependence.

Figure 5 shows the respective concentration–response curves at pH 7.8 for quinidine, cyclosporin A and SDZ PSC 833 and in Figure 4 the data for amiodarone, dipyridamole and verapamil are presented. To compare concentration–response curves from different experiments, the data were normalised to the rhodamine-123 content of the sensitive cells. At pH 7.8, cyclosporin A and SDZ PSC 833 both show a similar potency with pEC_{50} values of 6.19 and 6.48, respectively, although the maximal achievable accumulation effect seems somewhat lower for cyclosporin A. No clear pH dependence is seen for the two compounds. Verapamil shows low potency and the concentration–response curve with verapamil is shallow compared to the curves obtained with the cyclosporins. The rhodamine-123 level of the sensitive cell line is probably achieved at

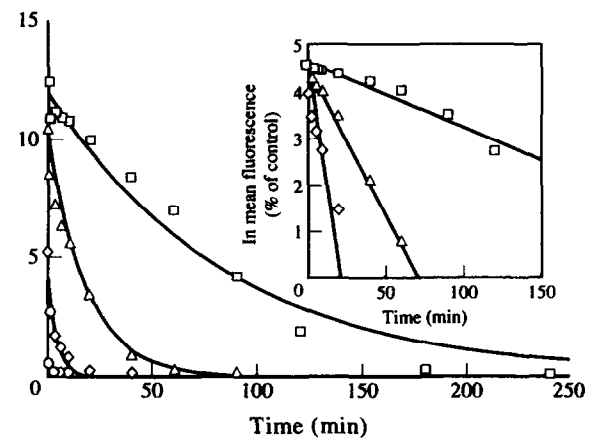

Figure 2. Influence of SDZ PSC 833 on rhodamine-123 efflux from CCRF VCR 1000 cells. Cells were loaded in the presence of 1 μM (□), 0.3 μM (△), 0.1 μM (◇) and 30 nM (○) SDZ PSC 833. After removal of extracellular rhodamine-123, cellular rhodamine-123 content was measured at the times indicated. The inset shows linearised plots of the data (see Figure 5).

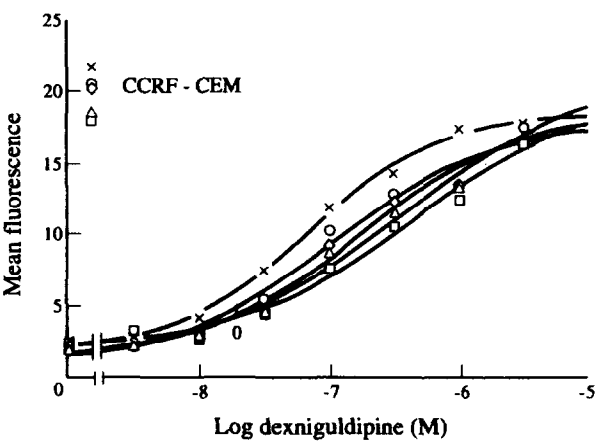

Figure 4. Dose–response curves of dexniguldipine for increase in cellular rhodamine-123 fluorescence at pH 7.2 (□), pH 7.4 (△), pH 7.6 (◇), pH 7.8 (○) and pH 8.0 (×). The symbols at the left upper corner represent the rhodamine-123 fluorescence of the sensitive control cell line CCRF-CEM. Cells were analysed after 60 min incubation at 37°C.

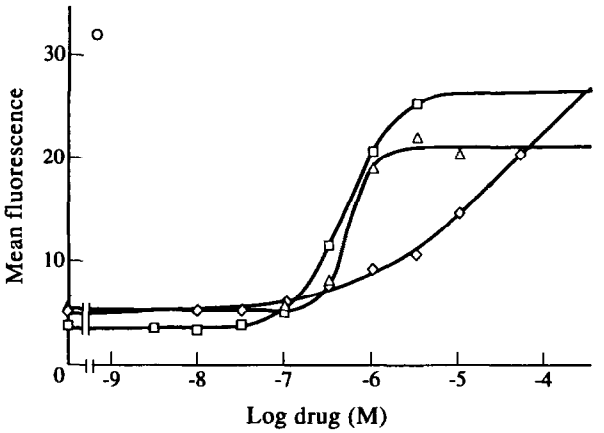

Figure 5. Dose–response curves of SDZ PSC 833 (□), cyclosporin A (△) and quinidine (◇) for increases in cellular rhodamine-123 fluorescence at pH 7.8. Data from different experiments were pooled and normalised to the value of the sensitive cell line. The open circle represents the normalised fluorescence of the sensitive cell line CCRF-CEM.

Table 1. Potency (pEC_{50} value) of chemosensitisers to reverse multidrug resistance-induced rhodamine-123 accumulation deficits in CCRF VCR 1000 cells at pH 7.8 and 7.3 (mean ± S.D.)

Chemosensitiser	pEC_{50} value (M) pH 7.8	pH 7.3
Verapamil	5.17 ± 0.15*	4.59 ± 0.36*
Dipyridamole	4.82 ± 0.15*	4.59 ± 0.07*
Quinidine	4.46 ± 0.36*	
Amiodarone	7.07 ± 0.11	6.52 ± 0.08
Cyclosporin A	6.19 ± 0.17	n.c.
SDZ PSC 833	6.48 ± 0.16	6.66 ± 0.12
Dexniguldipine	7.35 ± 0.25	6.71 ± 0.26

Dose–response curves were measured for each chemosensitiser, as described in Materials and Methods. Number of experiments vary from four to 10 for each substance. pEC_{50} values are given ± S.D. n.e., not evaluable. * Apparent pEC_{50} value.

high concentrations of verapamil. Due to technical reasons, higher concentrations than 10^{-4} M could not be used in this assay system. Amiodarone (Figure 6) is a potent chemosensitiser, but the maximally achievable accumulation level is clearly below the rhodamine-123 level of the sensitive line. Quinidine and dipyridamole are two compounds with very low potency. Due to the low potency, a saturating effect could not be obtained with these two compounds. Therefore, the pEC_{50} values for these compounds should be considered as apparent pEC_{50} values. The dose–response curve for quinidine is as shallow as the curve for verapamil. Table 1 summarises the pEC_{50} values for all chemosensitisers, tested at pH 7.8 and 7.3. A similar pH dependency as for dexniguldipine is seen for verapamil, amiodarone and quinidine. All of these compounds show a lower pEC_{50} value at pH 7.3. In contrast SDZ-PSC 833 is slightly more potent at pH 7.3. The same tendency is found for cyclosporin A, although the curves obtained at pH 7.3 were not evaluable due to the fact that a large increase in rhodamine-123 fluorescence was seen at high concentrations (10 μM), which exceeded the value of the sensitive cell line by 2–3-fold. The reason for this dramatic increase of fluorescence at high cyclosporin A concentration at pH 7.3 is not clear.

The influence of chemosensitisers on sensitive CCRF-CEM cells was investigated at pH 7.3. Verapamil (up to 100 μM), dipyridamole (up to 100 μM), quinidine (up to 100 μM), amiodarone (up to 3 μM), SDZ PSC 833 (up to 10 μM) and dexniguldipine (up to 3 μM) did not change the rhodamine-123 content of sensitive cells (data not shown). Cyclosporin A had no influence on rhodamine-123 content of sensitive cells up to a concentration of 1 μM. At a 10-fold higher concentration of 10 μM, a similar increase in rhodamine-123 content was seen as in resistant cells (data not shown). Therefore, the effect of 10 μM cyclosporin A seems not to be due to blockade of PGP.

In addition to the steady state measurements, the effects of different concentrations of dexniguldipine and SDZ PSC 833 on the efflux of rhodamine-123 from CCRF VCR 1000 cells were investigated. CCRF VCR 1000 cells were loaded with rhodamine-123 in the presence of different concentrations of dexniguldipine or SDZ PSC 833 at pH 7.8. This results in different levels of intracellular rhodamine-123, depending on the concentration of chemosensitiser. After 1 h the cells were centrifuged to remove extracellular rhodamine-123 and resuspended in an equal volume of RPMI 1640 medium containing the chemosensitiser in the respective concentration. Then the cells were analysed by FACS at different time intervals for remaining intracellular rhodamine-123. Figure 1 shows the decrease in intracellular rhodamine-123 in the presence of dexniguldipine. The inset in Figure 1 shows a linear plot of the efflux data after normalising the data to the different starting rhodamine-123 levels (ln fluorescence versus time). Efflux of rhodamine-123 is reduced dose-dependently and data are best described by a monoexponential decay function. Rhodamine-123 half-life times increase dose-dependently from 3 min at 10 nM dexniguldipine to 89 min at 1 μM dexniguldipine (Table 2). Figure 2 shows the respective efflux data for SDZ PSC 833. The kinetic efflux constants describing the above data and the corresponding half-life times at various concentrations of dexniguldipine and SDZ PSC 833 are compiled in Table 2. With respect to efflux inhibition, dexniguldipine seems to be slightly more potent than SDZ PSC 833. Maximal retardation of rhodamine-123 efflux by

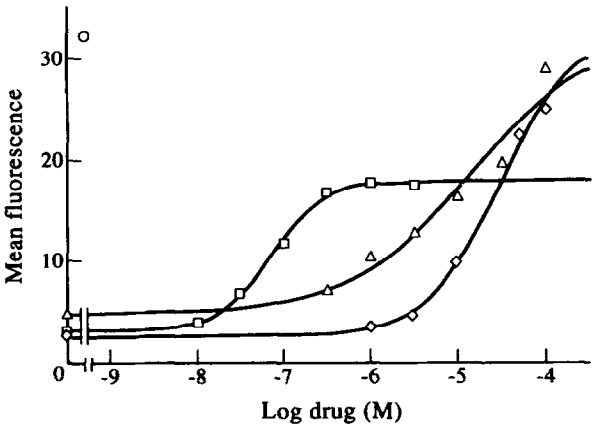

Figure 6. Dose–response curves of amiodarone (□), verapamil (△) and dipyridamole (◇) for increases in cellular rhodamine-123 fluorescence. Data from different experiments were pooled and normalised to the value of the sensitive cell line. The open circle represents the normalised fluorescence of the sensitive cell line CCRF-CEM.

Table 2. Efflux constants K_{eff} and half-life times $t_{1/2}$ of intracellular rhodamine-123 in CCRF VCR 1000 cells in the presence of chemosensitisers

Chemosensitiser log drug (M)	Dexniguldipine K_{eff} (min^{-1})	Dexniguldipine $t_{1/2}$ (min)	SDZ PSC 833 K_{eff} (min^{-1})	SDZ PSC 833 $t_{1/2}$ (min)
−5.5	n.d.	n.d.	0.0088	78.5
−6.0	0.008	89.0	0.0095	73.1
−6.5	0.011	62.0	0.016	43.2
−7	0.026	26.5	0.064	10.9
−7.5	0.045	15.5	0.17	4.1
−8	0.20	3.4	n.d.	n.d.

Data were analysed by the use of the non-linear least square method using a monoexponential decay function. Half-life times were calculated from the fitted efflux constants K_{eff}. n.d., not determined.

SDZ PSC 833 is achieved at concentrations of 1–3 μM ($t_{1/2}$ = 73 and 78 min). Dexniguldipine (1 μM) resulted in rhodamine-123 half-life times of 89 min. A slightly reduced effect was seen with dexniguldipine at a concentration of 0.3 μM ($t_{1/2}$ = 62 min). Sensitive CCRF-CEM cells show an efflux constant of 0.0035 and a corresponding half-life time of 195 min (data not shown). As can be seen from Figures 1 and 2, the intracellular rhodamine-123 concentration at the beginning of the efflux experiment is different due to the presence of different chemosensitiser concentrations. Although the difference is maximally 3-fold, this may complicate interpretation of the results. Therefore, we increased the rhodamine-123 concentration during the loading phase, when efflux in the presence of low dexniguldipine concentrations was measured. This led to almost identical intracellular rhodamine-123 levels at the start of the efflux phase (difference smaller than 20%). Efflux constants K_{eff} for dexniguldipine were 0.010 at 0.3 μM, 0.016 at 0.1 μM, 0.047 at 30 nM and 0.16 at 10 nM. These constants are very similar to the one given in Table 2 for dexniguldipine and show that variation of the intracellular rhodamine-123 concentration by a factor of 3 is not critical for the outcome of the experiment.

DISCUSSION

The present work was performed in order to study the new chemosensitiser dexniguldipine and to compare different chemosensitisers within the same assay system. The human T-lymphoblastoid cell line chosen for our study, was selected from the drug-sensitive parent cell line CCRF-CEM [14] and shows the typical MDR profile with high resistance to vincristine and moderate resistance to doxorubicin and actinomycin-D, together with amplification and distinct overexpression of the *mdr*-1 gene [14, 15]. In humans, only a single gene (*mdr*-1) codes for MDR-related PGP (PGP-1). The *mdr*-3 gene product seems not to be involved in MDR. It has been shown that a mutation from glycine to valine at amino acid position 185 of the human *mdr*-1 gene product changes substrate specificity of PGP. The cell line used in this study was shown to contain a glycine at this position and seems to be representative for classical MDR [16]. Therefore, one can expect that the data obtained with our cell line have relevance, not only for other PGP-positive cells, but also for human tumours expressing PGP.

Chemosensitisers are often characterised by calculating the shift in cytostatic concentration–response curves between resistant cells in the absence and resistant cells in the presence of a single concentration of chemosensitiser. The data obtained in these assays are only valid for the one concentration tested. If a clinically achievable concentration is used these data may have some predictive value for the successful use of the respective chemosensitiser in patients. To characterise the different chemosensitisers in more pharmacological terms, rhodamine-123 accumulation in MDR cells was measured in the absence or presence of chemosensitisers. Dose–response curves were analysed for each chemosensitiser. The concentration yielding half maximal sensitisation (EC_{50}) is calculated and represents the potency of each substance with respect to chemosensitisation.

None of the compounds tested except cyclosporin A had an influence on rhodamine-123 accumulation in sensitive CCRF-CEM cells. Cyclosporin A (10 μM) increased rhodamine-123 fluorescence of sensitive cells to a similar value as in resistant cells. Therefore, the effect seen at 10 μmol/l is probably not PGP-mediated. A similar chemosensitisation of sensitive parental cell lines in the presence of cyclosporin A was observed by others [17, 18]. From their work it seems clear that the immunosuppressive activity of cyclosporin A and derivatives is not responsible for the sensitising activity of these compounds.

There is one clear difference between cyclosporin A and SDZ PSC 833. While cyclosporin A also sensitises the sensitive cell line, SDZ PSC 833 does not show this effect in the concentration range tested, although both compounds are very similar in structure. The reason for this is unknown.

The high potency of dexniguldipine in modulating rhodamine-123 accumulation in the resistant cells is clearly pH-dependent. An almost linear reduction in potency is found for dexniguldipine with decreasing pH, resulting in pEC_{50} values between 6.4 (pH 7.2) and 7.2 (pH 8.0). A similar relationship between pH value and potency is displayed by verapamil, quinidine and amiodarone. One possible explanation could be the involvement of a basic amine function, which is present in dexniguldipine and is also found in verapamil, amiodarone and quinidine. An increased pH may lead to deprotonation of these compounds, resulting in increased lipophilicity and an enhanced ability to cross the lipophilic membrane compartment of the cell. This could result in higher intracellular or plasma membrane drug concentrations and enhanced effectiveness. On the other hand, the pumping activity of PGP could show pH-dependency with an optimum at basic pH values. However, the data with both cyclosporins, which show no pH-dependence, make this rather improbable.

Our work compares the chemosensitising activity of dexniguldipine and other chemosensitisers. Dexniguldipine has been shown to be a potent chemosensitiser in the above assay system [19] and increases the accumulation of [^{3}H]daunorubicin into murine friend leukaemia cells displaying the MDR phenotype (F4/6RADR cells) [20]. The pEC_{50} value for dexniguldipine with respect to [^{3}H]daunorubicin accumulation is 6.16 and only slightly lower than the value of 6.7 reported in our work. This may indicate that daunomycin and rhodamine-123 uptake are modulated in a similar way by dexniguldipine, although different cell lines were used.

From the great number of chemosensitisers, those which were chosen have already entered clinical trials or are expected to do so in the near future. Verapamil is in clinical trials and shows some effectiveness in myeloma patients [21] and in patients with non-Hodgkin's lymphoma [22]. Cyclosporin A is being intensively tested for its MDR-reversing properties *in vitro* [18, 23] and clinical phase II studies have been initiated [24]. The new non-immunosuppressive cyclosporin derivative SDZ

PSC 833 has been shown to be effective *in vitro* [25] and *in vivo* [26]. Dipyridamole has chemosensitising properties *in vitro* [27]. In addition to the modulation of classical MDR, the compound is being evaluated for its modulation of non-MDR cytostatics [28]. Amiodarone [29] and quinidine [30] are two further compounds with some efficacy in MDR models. A lot of literature data are available for each substance, but the use of different assay protocols or different cell lines renders a comparison between different laboratories difficult. In the above assay system dexniguldipine was the most potent compound followed by amiodarone, SDZ PSC 833 and cyclosporin A. All other compounds were of minor potency.

The maximally achievable level of sensitisation can also be analysed in this system. There are clear differences in the absolute increase in rhodamine-123 accumulation between different substances. Dexniguldipine sensitises the resistant cells with highest potency almost to the level of the sensitive control cell line. Verapamil, SDZ PSC 833 and dipyridamole show a similar maximal effect as dexniguldipine but display lower potency. The lowest level of maximal accumulation is achieved with amiodarone, although this compound shows high potency. The reason for the different maximal levels of sensitisation is unclear, but it may be related to different modes of action, i.e. competitive or allosteric interaction with the transported substrates. A competitive interaction between cyclosporin A and [^{3}H]vincristine uptake has been demonstrated in inside-out vesicles of DC-3F/VCRd-5L Chinese hamster cells [31]. On the other hand, a non-competitive interaction of dihydropyridines with vinblastine has been shown [7, 32].

Our data show the influence of chemosensitisers on rhodamine-123 efflux. Rhodamine-123 has been shown to be transported by PGP and, though not a classical cytostatic, this assay system proved to be a valuable model for PGP-mediated drug efflux. In this system the pump function of PGP or related proteins can be measured directly while in the frequently used proliferation assays, additional resistance mechanisms (altered topoisomerase I or II, altered glutathione metabolism and others), which may be modulated as well, are measured. The efflux measurements are in rough agreement to the steady state measurements in that dexniguldipine is more potent than SDZ PSC 833. Both compounds lead to a dose-dependent increase in rhodamine-123 half-life times. The rhodamine-123 efflux can be described by a monoexponential decay function. This indicates a single mechanism responsible for efflux and an apparently homogeneous population of PGP molecules in these cell lines.

It is not known whether a certain chemosensitiser is equally effective in reversing the resistance towards different types of cytostatics affected by PGP-associated MDR or if different cytostatics are modulated differently. Hunter and coworkers measured the influence of verapamil on transepithelial transport of vinblastine and found a pEC_{50} value of 5.5 [33], which slightly higher than our value of 5.17. This suggests a similar modulation of rhodamine-123 and vinblastine transport at least by verapamil. Clearly, more investigations are needed to elucidate the biochemical mechanisms of drug transport by PGP.

Dexniguldipine at concentrations between 0.1 and 1 μM was shown to effectively modulate doxorubicin and vincristine cytotoxicity in the CCRF VCR 1000 cell line [34]. These data are in good agreement with the rhodamine-123 accumulation data presented above, where a half maximal increase in rhodamine-123 accumulation was seen at a dexniguldipine concentration of 0.2 μM.

Cytotoxicity of compounds in relation to their chemosensitising potency is an important factor for their clinical usefulness. In the CCRF VCR-1000 cell line cytotoxicity of dexniguldipine, as measured in a 3-day MTT proliferation assay, is seen in the micromolar range. Half-maximal inhibition of cell growth, as measured by inhibition of [^{3}H]thymidine incorporation into DNA and [^{3}H]uridine into RNA of CCRF-CEM cells, is found at concentrations between 5 and 10 μM, which are almost one order of magnitude higher than concentrations shown to be effective in our rhodamine-123 accumulation assay [35].

In vivo reversibility of MDR by dexniguldipine was shown elegantly in a murine F4-6 tumour model. While doxorubicin alone was ineffective in resistant and PGP-positive F4-6ADR cells, combinations of doxorubicin with dexniguldipine reduced tumour size and the number of viable tumour cells to 33% of controls [36].

In the clinical situation the cellular steady state level of a certain cytostatic is an important parameter in achieving cell kill. Therefore, steady-state measurements as described here can be helpful in assessing the potential of chemosensitisers. The rhodamine-123 efflux system does not represent the clinical situation as, among others, the measurements are performed in the absence of serum proteins. Lipophilicity and bioavailability are important factors which contribute to the effectiveness of therapy, and may lead to a different outcome as expected from such a simple assay system. The above measurements were performed in the absence of serum, which is an undefined mixture of proteins, lipoproteins and lipids. It is, therefore, quite possible that some components of serum will modify PGP activity and distort results. Modulating compounds of unknown structure were found in human serum [37]. Nevertheless, the influence of serum on the activity of PGP and on the modulating potency of chemosensitisers poses important questions and further studies are needed to answer these questions.

In the meanwhile, clinical phase I/II studies with dexniguldipine as the chemosensitiser have been initiated.

Summarising our data, the above assay system is a reliable and fast method for the comparison of different chemosensitisers describing the potency of the compounds by giving EC_{50} values for chemosensitisation. In addition to steady state measurements, efflux of fluorescent compounds like rhodamine-123 or doxorubicin can be monitored directly. The method may also be useful for the fast screening of new compounds.

1. Biedler JL, Riehm H. Cellular resistance to actinomycin D in Chinese hamster cells *in vitro*: cross resistance, radioautographic, and cytogenetic studies. *Cancer Res* 1970, **30**, 1174–1184.
2. Juliano RL, Ling V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim Biophys Acta* 1976, **455**, 152–162.
3. Gupta RS, Murray W, Gupta R. Cross resistance pattern towards anticancer drugs of a human carcinoma multidrug-resistant cell line. *Br J Cancer* 1988, **58**, 441–447.
4. Selassie CD, Hansch C, Khwaja TA. Structure-activity relationships of antineoplastic agents in multidrug resistance. *J Med Chem* 1990, **33**, 1914–1919.
5. Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y. Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. *Cancer Res* 1981, **41**, 1967–1972.
6. Ford JM, Hait WN. Pharmacology of drugs that alter multidrug resistance in cancer. *Pharmacol Rev* 1990, **42**, 155–199.
7. Tamai I, Safa AR. Azidopine noncompetitively interacts with vinblastine and cyclosporin A binding to P-glycoprotein in multidrug resistant cells. *J Biol Chem* 1991, **266**, 16796–16800.
8. DeFaire U, Lundman T. Attempted suicide with verapamil. *J Cardiol* 1977, **6**, 195–198.

9. van der Bliek A, Borst P. Multidrug resistance. *Adv Cancer Res* 1989, **52**, 165–203.
10. Benson AB, Trump DL, Koeller JM, *et al.* Phase I study of vinblastine and verapamil given by concurrent i.v. infusion. *Cancer Treat Rep* 1985, **69**, 795–799.
11. Efferth T, Löhrke H, Volm M. Reciprocal correlation between expression of P-glycoprotein and accumulation of rhodamine 123 in human tumors. *Anticancer Res* 1989, **9**, 1633–1638.
12. Lampidis TJ, Munck JN, Krishan A, Tapiero H. Reversal of resistance to rhodamine-123 in adriamycin-resistant friend leukemia cells. *Cancer Res* 1985, **45**, 2626–2631.
13. Gekeler V, Frese G, Diddens H, Probst H. Expression of a P-glycoprotein gene is inducible in a multidrug-resistant human leukemia cell line. *Biochem Biophys Res Commun* 1988, **155**, 754–760.
14. Niethammer D, Diddens H, Gekeler V, *et al.* Resistance to methotrexate and multidrug resistance in childhood malignancies. In Weber G, ed. *Advances in Enzyme Regulation*. New York, Pergamon Press, 1989, 231–245.
15. Gekeler V, Frese G, Noller A, *et al.* Mdr1/P-glycoprotein, topoisomerase, and glutathione-S-transferase gene expression in primary and relapsed state adult and childhood leukaemias. *Br J Cancer* 1992, **66**, 507–517.
16. Gekeler V, Weger S, Probst H. MDR1/P-glycoprotein gene segments analysed from various human leukemic cell lines exhibiting different multidrug resistance profiles. *Biochem Biophys Res Commun* 1990, **169**, 796–802.
17. Chambers SK, Hait WN, Kacinski BM, Keyes SR, Handschumacher RE. Enhancement of anthracycline growth inhibition in parent and multidrug-resistant chinese hamster ovary cells by cyclosporin A and its analogues. *Cancer Res* 1989, **49**, 6275–6279.
18. Gaveriaux C, Boesch D, Boelsterli JJ, *et al.* Overcoming multidrug resistance in chinese hamster ovary cells *in vitro* by cyclosporin A (Sandimmune) and non-immunosuppressive derivatives. *Br J Cancer* 1989, **60**, 867–871.
19. Hofmann J, Wolf A, Spitaler M, *et al.* Reversal of multidrug resistance by B859-35, a metabolite of B859-35, niguldipine, verapamil and nitrendipine. *J Cancer Res Clin Oncol* 1992, **118**, 361–366.
20. Reymann A, Looft G, Woermann C, Dietel M, Erttmann R. Reversal of multidrug resistance in friend leukemia cells by dexniguldipine-HCl. *Cancer Chemother Pharmacol* 1993, **32**, 25–30.
21. Salmon BE, Dalton WS, Grogan TM, *et al.* Multidrug-resistant myeloma: laboratory and clinical effects of verapamil as a chemosensitizer. *Blood* 1991, **78**, 44–50.
22. Dalton WS, Grogan TM, Meltzer PS, *et al.* 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* 1989, **7**, 415–424.
23. Hu XF, Matin TJ, Bell DR, deLuise M, Zalcberg JR. Combined use of cyclosporin A and verapamil in modulating multidrug resistance in human leukemia cell lines. *Cancer Res* 1990, **50**, 2953–2957.
24. Rodenburg CJ, Nooter K, Herweijer H, *et al.* Phase II study of combining vinblastine and cyclosporin-A to circumvent multidrug resistance in renal cell cancer. *Anal Oncol* 1991, **2**, 305–306.
25. Boesch D, Gaveriaux C, Jachez B, Pourtier-Manzanedo A, Bollinger P, Loor F. *In vivo* circumvention of P-glycoprotein-mediated multidrug resistance to tumor cells with SDZ PSC 833. *Cancer Res* 1991, **51**, 4226–4233.
26. Keller RP, Altermatt HJ, Nooter K, *et al.* SDZ PSC 833, a non-immunosuppressive cyclosporine: its potency in overcoming P-glycoprotein-mediated multidrug resistance of murine leukemia. *Int J Cancer* 1992, **50**, 593–597.
27. Furusawa S, Fujimura T, Kawauchi H, Sasaki K, Takayanaga Y. Potentiation of pirarubicin cytotoxicity by dipyridamole in doxorubicin-resistant mouse P388 leukemia cells. *Res Commun Chem Pathol Pharmacol* 1991, **71**, 321–336.
28. Schmoll HJ, Harstrick A, Köhne-Wömpner CH, Schöber C, Wilke H, Poliwoda H. Modulation of cytotoxic drug activity by dipyridamole. *Cancer Treat Rev* 1990, **17** (suppl. A), 57–65.
29. Chauffert B, Rey D, Coudert B, Dumas M, Martin F. Amiodarone is more efficient than verapamil in reversing resistance to anthracyclines in tumour cells. *Br J Cancer* 1987, **56**, 119–122.
30. Tsuruo T, Iida H, Kitatani Y, Yokota K, Tsukagoshi S, Yakurai Y. Effects of quinidine and related compounds on cytotoxicity and cellular accumulation of vincristine and adriamycin in drug-resistant tumor cells. *Cancer Res* 1984, **44**, 4303–4307.
31. Tamai I, Safa AR. Competitive interaction of cyclosporins with the vinca alkaloid-binding site of P-glycoprotein in multidrug resistant cells. *J Biol Chem* 1990, **265**, 16509–16513.
32. Ferry DR, Russell MA, Cullen MH. P-glycoprotein possesses a 1,4-dihydropyridine-selective drug acceptor site which is allosterically coupled to a vinca-alkaloid-selective binding site. *Biochem Biophys Res Commun* 1992, **188**, 440–445.
33. Hunter J, Hirst BH, Simmons NL. Transepithelial vinblastine secretion mediated by P-glycoprotein is inhibited by forskolin derivatives. *Biochem Biophys Res Commun* 1991, **181**, 671–676.
34. Ise W, Gekeler V, Sanders KH. Use of dexniguldipine-HCl (B8509-035) to circumvent multidrug resistance (MDR) mediated by P-170 glycoprotein. *Clin Exp Metastasis* 1992, **10** (Suppl. 1).
35. Noller A, Wilisch A, Häußermann K, Gekeler V. MDR modulating and antineoplastic effects of B859-35, and its metabolite. *Ann Oncol* 1992, **3** (Suppl. 1).
36. Dietel M, Nicopoulos T, Reymann A, Pest S, Bunge A, Seidel A. *In vivo* reversibility of MDR by the niguldipine derivative B859-35. *Proc Am Assoc Cancer Res* 1993, **34**, 323.
37. Ichikawa M, Yoshimura A, Furukawa T, Sumizawa T, Akiyama S. Modulators of the multidrug-transporter, P-glycoprotein, exist in the human plasma. *Biochem Biophys Res Commun* 1990, **166**, 74–80.

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