Cancer Chemother Pharmacol (1993) 32: 151–155

Cancer Chemotherapy and Pharmacology

Influence of sequential exposure to R-verapamil or B8509-035 on rhodamine 123 accumulation in human lymphoblastoid cell lines*

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Received 7 October 1992/Accepted 23 December 1992

Abstract. Modulators for the reversal of multidrug resistance such as R-verapamil and B8509-035, a dihydropyridine, effectively overcome multidrug resistance in vitro and are currently undergoing clinical trial. One problem with their use is the application protocol; the question as to whether they should be given by continuous administration or in sequential doses in combination with the cytotoxic drugs has to be addressed. Therefore, we examined the influence of the exposure time and the sequence of modulator administration on the active transport of the fluorescent dye rhodamine 123 (R123), a substrate for the P-glycoprotein, in the resistant lymphoblastid cell line VCR1000 and the parental nonresistant cell line CCRF-CEM. Our results demonstrate the importance of coadministration of R-verapamil and the cytotoxic agent for the modulation of multidrug resistance, whereas the exposure sequence does not seem to be such an essential parameter in the case of B8509-035. This observation should be considered for the further design of clinical studies.

Introduction

Innate or acquired resistance of tumor cells to structurally unrelated cytotoxic agents is a major problem in cancer chemotherapy. The multidrug resistance phenotype is often due to the increased expression of a membrane glycoprotein with a molecular weight of 170 kDa that is encoded by the *mdr1* gene [11]. P-glycoprotein functions as an energy-dependent drug efflux pump [12, 18, 30], causing a decrease in intracellular drug accumulation. P-glycoprotein expression has been identified in certain normal tissues with diverse physiological functions, including excretory and secretory cells [4]. High levels of P-glycoprotein in tumor cells have been associated with clinical resistance to chemotherapy at the point of diagnosis or in the course of therapy [22].

Agents for the reversal of P-170-mediated multidrug resistance (MDR) are capable of restoring the drug sensitivity of cells via inhibition of the multidrug-efflux system [8, 29]. One prominent group of chemosensitizers structurally belongs to the class of calcium-channel blockers. However, the clinical use of these modulating agents is limited by toxic side effects occurring at clinically optimal doses. R-verapamil and B8509-035 have been shown to be potent agents in the circumvention of multidrug resistance independent of calcium-antagonistic activity [10, 13, 14, 17, 26]. Due to their slight cardiovascular side effects, both substances are currently under study in clinical phase I trials [1, 3].

We examined the effect of various exposure protocols for the two resistance modifiers on rhodamine 123 accumulation in the sensitive lymphoblastoid cell line CCRF-CEM and the resistant subline VCR1000. For a functional assay we used the fluorescent dye rhodamine 123 (R123). It has previously been shown that R123 is a sensitive probe for the identification of cells with the MDR phenotype based on an enhanced outward transport [21, 24]. The positive charge of R123 at physiological pH seems to be essential, since no differential sensitivity to zwitterionic R123 analogs was found between anthracycline-sensitive and -resistant Friend leukemia cells, whereas a marked differential toxicity between these cell types was shown for the positively charged R123 [23]. R123 shares several chemical characteristics such as planar aromatic rings, a nitrogen atom, and lipophilicity with the substances transported by the P-170 glycoprotein.

Materials and methods

Drugs and chemicals. R-verapamil, 5-[(3,4-dimethoxyphenethyl)-methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropyl-valeronitrile hydrochloride, was obtained from Knoll AG, Ludwigshafen, Germany.

* This research was supported by the Robert-Bosch-Foundation, Stuttgart, Germany

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Table 1. Degree of resistance of CCRF-CEM and VCR1000 cells to different cytostatic drugs as determined by the MTT test

Drugs	IC_{50} CCRF-CEM[a] (ng/ml)	IC_{50} VCR1000[a] (ng/ml)	RF
Vincristine	6	1,750	292
Etoposide	182	31,800	177
Vinblastine	4	413	96
Doxorubicin	74	6,209	84
Mitoxantrone	13	950	73
Daunomycin	46	2,430	52
Colchicine	4	191	48
Mitomycin C	29	690	24
Amsacrine	33	668	20
Actinomycin D	12	227	19
Idarubicin	11	122	11

[a] Data represent mean values for 3–5 independent experiments
IC_{50}, drug concentration required for 50% growth inhibition; RF, resistance factor (IC_{50} of VCR1000 cells/IC_{50} of CCRF-CEM cells)

B8509-035 (–)-3-methyl-5-[3-(4,4-diphenyl-1-piperidinyl)-propyl]-1,4-dihydro-2,6-dimethyl-4-(3-nitro-phenyl)-pyridine-3,5-dicarboxylate hydrochloride was provided by Byk Gulden Lomberg, Konstanz, Germany. For stock solutions (10^{-2} *M*), modulators were prepared as follows: (+)-R-verapamil, in 0.01 *N* HCl; and B8509-035, in 0.5 ml PEG 400 supplemented with 0.5 ml 0.01 *N* HCl. All solutions were stored in glassware and light-protected. The stock solution of R-verapamil was stored frozen and that of B8509-035 was stored at 4° C for a maximum of 4 weeks. At the concentrations used, the solvents were nontoxic to the cells. R123 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemicals Deisenhofen, Germany.

Cell lines. The human lymphoblastoid cell line CCRF-CEM and its drug-resistant subline VCR 1000 were kindly donated by Dr. V. Gekeler, Tübingen, Germany. VCR 1000 cells were generated as described elsewhere [9] and exhibited the classic MDR phenotype (Table 1). Cells were cultured in CG medium (Vitromex Vilshofen, Germany) supplemented with 100 units penicillin-streptomycin/ml. Cells were incubated at 37° C in a humidified incubator containing 5% CO_2. The resistant cell line was grown in the presence of 1 µg vincristine/ml (Sigma Deisenhofen, Germany). The presence of mycoplasma was excluded by the rapid mycoplasma detection system (Boehringer Mannheim, Germany).

Flow cytometry. Flow-cytometric measurements were performed on a fluorescence-activated cell sorter (FACScan; Becton Dickinson, Mountain View, Calif.) equipped with an argon-ion laser tuned to 488 nm.

Determination of P-glycoprotein expression. For flow-cytometric determination of P-glycoprotein expression we used monoclonal antibody (mAb) MRK16, which recognizes an external surface membrane epitope of the P-glycoprotein [15]. After two washing steps with medium, cells were resuspended in medium to a concentration of 10^5 cells/ml, and an aliquot of 100 µl was incubated with 3 µg mAb MRK16 [13] for 30 min at 4° C. In parallel, an identical aliquot was incubated with an isotypic control to allow quantification of nonspecific binding. After two washing steps with phosphate-buffered saline (PBS), cells were resuspended in 100 µl medium containing 6 µg of a phycoerythrin (PE)-labeled goat anti-mouse IgG fraction (Dianova Hamburg, Germany). The suspension was incubated for 20 min at 4° C under protection from light. Thereafter, the specimens were washed twice with PBS and analyzed by flow cytometry using an emission wavelength of 566 nm.

Measurement of R123 accumulation by flow cytometry. Confluently growing cells were washed with medium, and 10^6 cells/ml medium were stained for 30–180 min at 37° C with R123 (R123, 300 ng/ml), and then washed twice with ice-cold PBS. For competition with P-170-mediated transport, R-verapamil or B8509-035 was added at the concentrations described below. Cells were kept on ice until analysis. Fluorescence was detected by FACScan flow cytometry by its emission at 530 nm (530/30-nm filter) following excitation at 488 nm as described by Ludescher et al. [24], with slight modifications. Nonviable cells were excluded using propidium iodide (Sigma Chemicals Deisenhofen, Germany) according to the medhod described by Sasaki et al. [28].

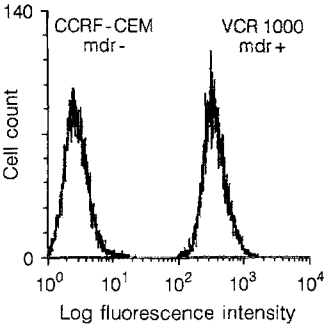

Fig. 1. P-170 expression in the human lymphoblastoid CCRF-CEM (mdr–) and VCR1000 (mdr+) cell lines as determined by flow cytometry using mAb MRK16.

Cytotoxicity assays. For determination of chemosensitivity the MTT assay was used [25]. The test is based on the generation of a purple formazan product after addition of the yellow substrate to living cells. Nonadherent, exponentially growing cells were harvested by centrifugation and resuspended in fresh medium. They were plated in an end volume of 100 µl medium at a density of 5×10^4 cells/well in 96-multiwell flat-bottomed plates (Costar, Berlin, Germany). Cytostatic drugs were immediately added. Two wells were used for each concentration. Controls consisted of untreated cells. The plates were incubated at 37° C in a humidified incubator containing 5% CO_2 für 3 days. At the 4th day, 10 µl MTT solution (5 mg/ml in PBS) was added to each well and the plate was incubated for an additional 4 h at 37° C in a dark environment. The MTT-formazan crystals were dissolved with 100 µl 1 *N* HCl: isopropanol (1 : 24, v/v) per well under thorough mixing with a multichannel pipette. The absorbance at 570 nm was determined with an enzyme-linked immunosorbent assay (ELISA) plate reader (Dynatech, Denkendorf, Germany).

Results

Determination of resistance pattern

Table 1 shows the 50% growth-inhibitory (IC_{50}) values for various cytostatic drugs in the parental CCRF-CEM cell line and the resistant subline VCR1000 as obtained by growth-inhibition assays (MTT test). The cross-resistance pattern is consistent with the classification of VCR1000 cells as a classic MDR cell line.

Correlation of P-glycoprotein expression and drug resistance

Expression of P-glycoprotein in the cell lines was examined by FACScan analysis using mAb MRK16 [15]. Cells were stained indirectly by a secondary PE-labeled antibody. The drug-sensitive cell line CCRF-CEM (mdr–) showed no reactivity with mAb MRK16, whereas VCR1000 cells (mdr+) were strongly positive, indicating a high level of P-glycoprotein expression (Fig. 1).

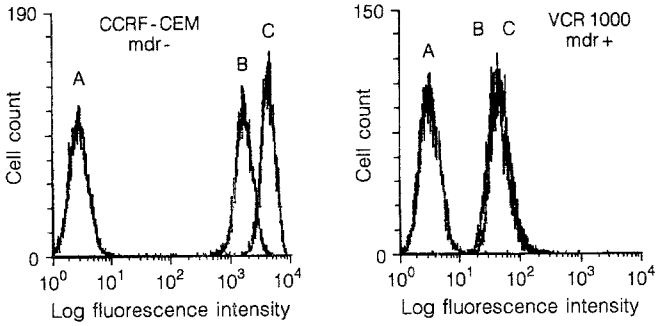

Fig. 2 Time-dependent increase in intracellular R123 fluorescence in CCRF-CEM and VCR1000 cells exposed to R123 dye (300 ng/ml). *A*, Control (cells without dye); *B*, R123 uptake after 30 min incubation; *C*, R123 uptake after 120–180 min incubation

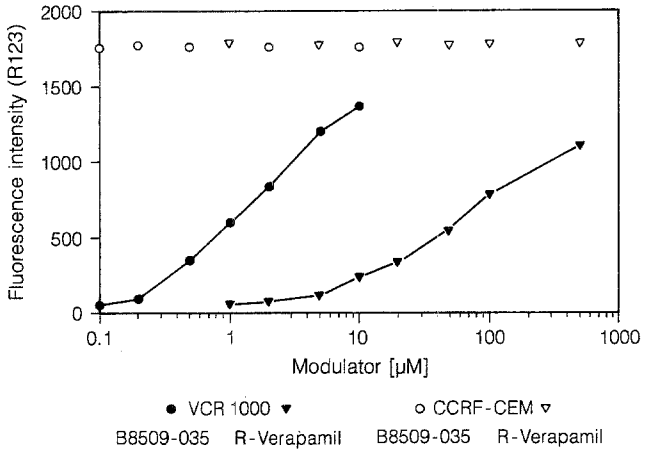

Fig. 3 Fluorescence intensities of CCRF-CEM and VCR1000 cells incubated with various concentrations of R-verapamil or B8509-035 and R123 (300 ng/ml) for 90 min

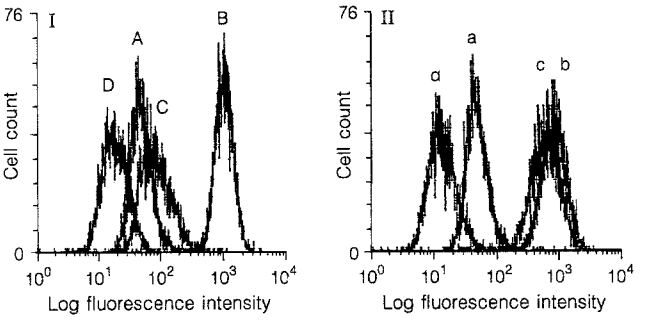

Fig. 4 Fluorescence histograms of VCR1000 cells exposed to 50 μ*M* R-verapamil (*I*) or 1 μ*M* B8509-035 (*II*). Influence of simultaneous and sequential exposures on R123 accumulation. *Left panel* (*I*), Incubation shedule: *A*, medium (90 min) – washing step – medium with R123 (90 min); *B*, medium (90 min) – washing step – coadministration of R123 and R-verapamil (90 min); *C*, medium with R-verapamil (90 min) – washing step – medium with R123; *D*, medium with R123 (90 min) – washing step – medium with R-verapamil (90 min). *Right panel* (*II*), Incubation shedule: *a*, medium (90 min) – washing step – medium with R123; *b*, medium (90 min) – washing step – coadministration of R123 and B8509-035 (90 min); *c*, medium with B8509-035 (90 min) – washing step – medium with R123 (90 min); *d*, medium with R123 (90 min) – washing step – medium with B8509-035 (90 min)

Analysis of multidrug transport with the dye R123

First of all we determined the time dependence of intracellular R123 uptake in the cell lines used (Fig. 2). Our uptake kinetics showed significant differences in R123 extrusion depending on P-glycoprotein expression. The total amount of R123 taken up by the CCRF-CEM cells was about 94-fold that accumulated by the resistant cell line. Maximal intracellular R123 accumulation was attained in VCR1000 cells after 30 min exposure and in CCRF-CEM cells after 120 min incubation.

Dose-dependent influence of the modulators R-verapamil and B8509-035 on R123 accumulation

For detection of enhanced R123 accumulation mediated by inhibition of the drug-efflux pump, modulator and R123 were simultaneously applied for 90 min. Continuous exposure to R-verapamil and B8509-035 increased the intracellular amount of R123 in the VCR1000 cells in a dose-dependent manner. B8509-035 was approximately 50 times more effective than R-verapamil in restoring R123 accumulation in the resistant cells (Fig. 3). In P-glycoprotein-deficient CCRF-CEM cells, no enhanced uptake of R123 was observed after the addition of an optimal modulator concentration. In subsequent combination experiments we added 10 μ*M* R-verapamil and 5 μ*M* B8509-035, as preliminary experiments had shown that these concentrations resulted in optimal modulation without producing cytotoxic effects themselves. Moreover, higher modulator concentrations would have been outside the clinical range.

Influence of the application schedule on the MDR-reversing potency of the modulators

To mimic clinically relevant exposure protocols, cells were exposed sequentially or simultaneously to modulator and R123. First VCR1000 cells were preincubated for 90 min with either R-verapamil (10 μ*M*) or B8509-35 (5 μ*M*), then R123 was added to the culture medium for 90 min. This situation was compared with coadministration of modulator and R123 for 90 min. Both application protocols led to the same result, producing a significant reduction in R123 extrusion. Preincubation with R123 for 90 min followed by the addition of modulator failed to influence R123 retention.

A striking difference, however, was seen when cells were washed with medium following their exposure to R-verapamil and then resuspended in medium containing R123 in the absence of R-verapamil; no significant retention of R123 was achieved in this case (Fig. 4, panel I). This observation was totally different from the results obtained with B8509-035 (Fig. 4, panel II). No difference was found in the inhibition of drug extrusion when cells were washed with medium following B8509-035 exposure and then transferred to medium containing R123 only (Fig. 5, B); even after an incubation period of 3 h in fresh medium, no measurable reduction in inhibitory potential

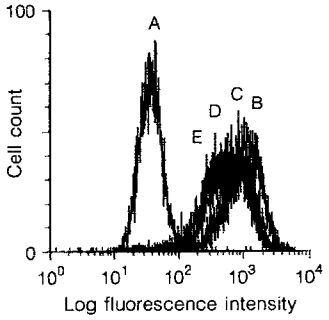

Fig. 5. Fluorescence histogram of VCR1000 cells. *A*, Medium with R123 (90 min); *B*, medium with B8509-035 (90 min) – washing step – medium, with R123 (90 min); *C–E*, medium with B8509-035 (90 min) – washing step – incubation in modulator-free medium for 1 h (*C*), 2 h (*D*), and 3 h (*E*) – medium with R123 (90 min)

was seen (Fig. 5, E). As a control, cells were exposed to R123 for 90 min (Fig. 5, A). In another experiment, R123-loaded cells were washed prior to their incubation with modulator, and this led to a slightly reduced accumulation as compared with the control value (data not shown).

Discussion

Efforts to enhance sensitivity to chemotherapy by the addition of P-glycoprotein-blocking agents to cytotoxic agents have not been very rewarding in initial clinical trials as compared with the high resistance-reversing potency of the modulators in vitro [5, 6]. In general, insufficient plasma concentrations of chemosensitizers are thought to be the main factor leading to therapeutic failure of such combination therapy, but another possible explanation for the differing clinical results may be the sequence of administration. We tested whether modifications in the protocol design would lead to results better than those obtained following cotreatment with modulators such as B8509-035 and R-verapamil.

In our present work we examined the influence of the sequence of exposure to the modulators B8509-035 or R-verapamil in combination with chemotherapeutic agents on MDR-reversing capacity in human lymphoblastoid cell lines. The MDR phenotype was assessed using mAb MRK16, which recognizes a surface epitope of P-glycoprotein. A high level of expression of P-glycoprotein was shown for the resistant VCR1000 cell line. By means of flow-cytometric analysis, P-glycoprotein-mediated transport was measured using the dye R123. We observed a high total uptake of R123 in CCRF-CEM cells and a low level of accumulation in CCRF-CEM cells, correlating with the degree of resistance depending on the expression of the MDR phenotype. As previously reported, both substances exhibit strong potency in circumventing MDR without causing excessive cardiotoxicity [1]. Cotreatment of resistant cells with modulator markedly enhanced intracellular accumulation of R123. Our results demonstrate the dose-dependent abilities of R-verapamil and of B8509-035 to reverse MDR, and these findings correlate well with data presented by other investigators, showing that B8509-035 is the more effective modulator in vitro [16]. In our assay system using R123 as the substrate for the drug-efflux pump, B8509-035 was about 50 times more potent than R-verapamil in increasing intracellular R123 accumulation in the resistant cell line VCR1000.

The present study also demonstrates that the chemosensitizing capacity of R-verapamil is lost if the drug is not present during the administration of the cytostatic agent. These data are comparable with the observations of Cass and co-workers [2], who found the greatest enhancement of vincristine activity when verapamil was present in the culture medium both during and after a 4-h vincristine exposure.

In contrast to R-verapamil, the highly lipophilic modulator B8509-035 showed a totally different behavior concerning cellular retention time. Even a short duration of exposure to B8509-035 (90 min) terminated by washing steps and an additional incubation in modulator-free medium for several hours had no significant influence on its modulating activity.

It has previously been shown that verapamil and dihydropyridine photolabel the P-glycoprotein [19, 31]. Our findings suggest that B8509-035 is not actively transported across the plasma membrane, and these observations are in good agreement with previous reports. Data describing transport studies carried out in the P388 murine leukemia cell line and the Adriamycin-resistant subline P388/ADR indicate that verapamil and nitrendipine exert their effects by different classes of membrane interactions because nitrendipine does not appear to be a substrate for the outward transport mediated by the P-glycoprotein [20]. In a recent report of a study using surface membranes from a P-glycoprotein-expressing human lung-cancer cell line, it is speculated that both the 1,4-dihydropyridine-selective and the vinca alkaloid-selective binding domains are topographically distinctly located on P-glycoprotein, but they must be allosterically coupled [7]. The data presented herein suggest that R-verapamil competitively inhibits drug transport and is also a substrate for this transport mechanism. Direct binding to P-glycoprotein and energy-dependent transport across the plasma membrane has previously been reported for verapamil [27, 31]. Therefore, it is improbable that minor retention of R-verapamil depends only on its low affinity for the P-glycoprotein leading to diffusion across the cell membrane in modulator-free medium. These studies imply a difference in the reversal mechanisms of these substances, even though both exhibit remarkable MDR-reversing capacities. No enhancement of R123 accumulation was obtained by exposure of the parental CCRF-CEM cell line to modulator, consistent with the lack of P-glycoprotein expression.

Hence, it can be concluded that for optimal inhibition of drug extrusion, R-verapamil has to be applied simultaneously with the cytotoxic agent. As the administration schedule does not seem to be so crucial for B8509-035, perhaps after saturation of the efflux pump the doses can be reduced to minimize clinical complications. These results are of significant value for the design of future studies aimed at overcoming MDR using combination chemotherapy with anticancer drugs and modulators to block drug efflux.

Acknowledgement. We thank Dr. V. Gekeler for generously providing the CCRF-CEM and VCR1000 cell lines.

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