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

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

<table>
<thead>
<tr>
<th>Drugs</th>
<th>IC50 CCRF-CEM (ng/ml)</th>
<th>IC50 VCR1000 (ng/ml)</th>
<th>RF</th>
</tr>
</thead>
<tbody>
<tr>
<td>vincristine</td>
<td>6</td>
<td>1,750</td>
<td>292</td>
</tr>
</tbody>
</table>
etoposide          | 182                   | 31,800               | 177  |
vinblastine        | 4                     | 413                  | 96   |
doxorubicin        | 74                    | 6,209                | 84   |
mitoxantrone       | 13                    | 950                  | 73   |
dactomycin         | 46                    | 2,430                | 52   |
colchicine         | 4                     | 191                  | 48   |
imidicin-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.

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 (+)-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 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% CO2. 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) MKR16, 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 10⁶ cells/ml, and an aliquot of 100 μl was incubated with 3 μg mAb MKR16 [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 10⁶ 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 method described by Sasaki et al. [28].

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 x 10⁶ 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% CO2 for 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 (IC50) 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 MKR16 [15]. Cells were stained indirectly by a secondary PE-labeled antibody. The drug-sensitive cell line CCRF-CEM (mdr−) showed no reactivity with mAb MKR16, whereas VCR1000 cells (mdr+) were strongly positive, indicating a high level of P-glycoprotein expression (Fig. 1).