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Reversal agent inhibition of the multidrug resistance pump in human leukemic lymphoblasts

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Multidrug resistant cancer cells of the MDR-1 phenotype utilize an ATP-dependent pump to excrete toxic drugs. Rhodamine 123 (R123) is a fluorescent substrate of the MDR pump. An assay for the ATP-dependent initial efflux of R123 from CEM/VLB_{100} human leukemic lymphoblasts has been developed. The MDR-1 cells were treated with a reversal agent and preloaded with 40.0 nM R123 in buffer at 30°C that contained sodium azide and 2-deoxyglucose. The cells were rinsed with cold buffer and resuspended in L-glutamine/glucose solution at 23°C. The cell suspension was passed through a filter and R123 in the filtrate was detected at 2-s intervals by fluorescence. Efflux of R123 was inhibited by the reversal agents amiodarone, cyclosporin A, Ro11-2933 (DMDP), quinidine, and the optical isomers of propranolol. The MDR pump is stereospecific for the (*R*)-diastereomer quinidine; however, the (*S*)-diastereomer quinine is a relatively weak inhibitor of the pump. Cyclosporin A was the most potent inhibitor tested against the efflux of R123 by the MDR pump.

Introduction

Reversal of multidrug resistance (MDR) produces remission in some patients with a phenotypic MDR-1 neoplasm [1]. The identification of new resistance reversal (RR) agents with high reversal potency and low toxipathy to the patient will be advantageous to the clinical chemotherapy of cancer [2–4]. Prior research studies of MDR-1 cancer cells indicate that the P-170 glycoprotein, located in the plasma membrane, functions as an ATP-dependent efflux pump for drug substrates such as doxorubicin and vinblastine sulfate (VLB) [5]. Cellular drug efflux catalyzed by the MDR pump is a rapid reaction [6–10]. Although the efflux activity of the pump has been demonstrated, it may be difficult to demonstrate inhibition of efflux with RR agents or other chemotherapy drugs [11].

In this report, MDR-1 phenotypic cells were preloaded with a low concentration of a highly fluorescent substrate. The inhibitory effects of known RR agents on the initial rate of one-way substrate transport through the MDR pump were determined; experiments of this type are required to characterize the inhibition mechanisms of RR agent action [12].

Several known substrates of the MDR efflux pump, including anthracycline drugs [3] and rhodamine dyes [13,14], are fluorescent substances [15–17]. The fluorescence intensity of anthracyclines is relatively weak, however, in comparison with rhodamine 123 (R123) [16]. R123 was selected as the substrate for this study because the high fluorescent yield near 520 nm [17] permits detection of R123 in concentrations down to 0.2 nM. The CEM/VLB_{100} leukemic lymphoblast cell line was selected because these cells have already been well characterized [17–19]. The drug transport characteristics of these human MDR-1 cells were compared with nonresistant CCRF-CEM cells and the effects of 0.01 M NaN_3 on the ATP levels of the CEM/VLB_{100} cells were determined [20].

In the experiments reported here, the CEM/VLB_{100} cells were treated with several different RR agents in succinate buffer to permit uptake of the agent at a relatively low temperature and short time interval. The mild incubation conditions were chosen to minimize potential cell damage by the RR agents. The cells were treated with sodium azide, preloaded with R123, and the effect of each RR agent on the initial ATP-depen-

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Abbreviations: MDR, multidrug resistance; RR, resistance reversal; VLB, vinblastine sulfate; R123, rhodamine 123; AMI, amiodarone; CysA, cyclosporin A; DMDP, Ro11-2933; RQ, quinidine; SQ, quinine; PROP, propranolol; PBS, Dulbecco's phosphate-buffered saline without $MgCl_2$ or $CaCl_2$, from Gibco.

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dent efflux of R123 was determined. The following RR agents were tested: amiodarone (AMI); cyclosporin A (CysA); Ro11-2933 (DMDP); quinidine (RQ); quinine (SQ); and each of the optical isomers of propranolol (PROP). Three potent inhibitors of the pump have been identified for further studies of reversal mechanisms.

Materials and Methods

Chemicals

The Ro11-2933: *N*-(3,4-dimethoxyphenethyl)-*N*-methyl-2-(2-naphthyl)-*m*-dithiane-2-propylamine hydrochloride (DMDP) was a gift from F. Hoffmann-LaRoche, Basel, Switzerland. The VLB was a gift from Lilly Research Laboratories, Indianapolis, IN, and CysA was obtained from Sandoz Pharmaceuticals, East Hanover, NJ. Additional chemicals were obtained from Sigma, St. Louis, MO.

Cell cultures

The CEM/VLB_{100} human MDR-1 cells were a gift from Dr. William T. Beck of St. Jude Children's Research Hospital, Memphis, TN. The nonresistant cells originally isolated (CCRF-CEM) were from a patient with leukemia [21]. The MDR-1 cells were grown in RPMI 1640 medium (Gibco) with 10% fetal calf serum (Gibco), 0.004 M L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5% CO_2. The MDR-1 cells were grown in culture tubes with 100 nM VLB in complete medium at 37°C for 3 days, and alternately grown without VLB for 4 days. The cells were suspended in 40 ml of complete medium without VLB and grown for 7 to 10 days in 200-ml flasks.

Measurement of pump activity

The initial (zero-trans) efflux of R123 was determined by a modification of the method of Wigler and Shah [22]. The SLM 8225 fluorescence spectrophotometer was set for an 8 nm slit width, excitation at 498 nm, and emission at 520 nm. The spectrophotometer was equipped with a 450 W xenon lamp, an SLM fluorescence enhancer, a cooled 9635QA PMT, a data acquisition processor, a digital plotter, and a dot matrix printer. The voltage control was set at an emission/excitation ratio of 0.5 with a 0.4 nM solution of R123 in PBS.

The R123, RQ, SQ, and the isomers of PROP (HCl salts) were dissolved in DMSO and PBS. Samples of AMI, DMDP, CysA, and VLB were dissolved in warm 95% ethanol; the ethanol solutions were subsequently diluted into PBS. After the stock solutions of RR agents in PBS were diluted into the cell suspension, the final ethanol concentrations were from 0.1% to 0.5%. The ethanol content of the controls was adjusted to correspond with the cell suspensions with RR agents in PBS. Samples of CEM/VLB_{100} cells ($2.0 \cdot 10^7$) were suspended in 0.5% ethanol in 10 ml PBS and incubated with gentle stirring for 3 h at 30°C. Nonviable cells were detected with a Becton Dickinson FACScan flow cytometer by the propidium iodide method of Krishan [23]. The ethanol had no significant effect on the number of nonviable cells when ethanol-treated cells were compared with cells incubated with no ethanol.

The MDR-1 cells ($2.0 \cdot 10^7$ viable cells) were suspended in 5.0 ml of RR agent (from 1.0 μM to 125 μM) and 200 μM sodium succinate in PBS and incubated at 30°C with gentle stirring for 60 min. The cells were preloaded with R123 by a modification of the method of Thimmaiah et al. [18]. A solution was added to the cell suspension to provide a final concentration of 40.0 nM R123, 0.004 M 2-deoxyglucose, 0.01 M sodium azide, and an RR agent in 10.0 ml PBS. The RR agent concentration was equal to the level in the first incubation. The cell suspension was incubated at 30°C for 60 min or 120 min. (The incubation times and the concentration of each RR agent are listed in Tables I and II.) The cell suspension was chilled in ice for 15 min.

The cell suspension was centrifuged at 1500 rpm for 2 min at 4°C. The cells were rinsed 3 times with an ice-cold solution of 490 μM $MgCl_2$ and an RR agent in PBS at the prior concentration. The cells were rinsed once without an RR agent. The cold cell pellet was resuspended in 10.0 ml PBS at 23°C that contained 490 μM $MgCl_2$, 0.01 M glucose, and 0.006 M L-glutamine in PBS. The cell suspension was quickly poured into a flow apparatus with a 0.45-μm polysulfone filter (Gelman Sciences) and the flow rate was regulated at 1.0 ml/min with a polystaltic pump. The filtrate was drained through a 10×4.0 mm quartz flow cell located upstream from the pump. Fluorescence intensity values were determined at 2-s intervals and stored in the memory of the data processor. After a 120-s delay the R123 was detected in the flow cell. The initial steady state efflux was determined from an apparent zero order plot from 10 to 30 s. The percent inhibition was calculated from the ratio of the efflux for RR agent-treated cells divided by the rate for control cells (not treated with an RR agent). The cells were counted before each experiment in a Neubauer hemacytometer.

Negative controls were performed by preloading MDR-1 cells with AMI and R123 in the usual way. After four cold rinses, the cells were resuspended in 0.004 M 2-deoxyglucose and 0.01 M sodium azide in PBS at 23°C. The cell suspension was passed through the filtration-flow apparatus and the rate of R123 efflux was determined.

Uptake and 90 min release of unbound R123 from cells

Experiments were performed to estimate the uptake of R123 into CEM/VLB_{100} cells in the presence of

metabolic inhibitors and CysA. Cell samples ($2.2 \cdot 10^7$ cells) were incubated with gentle stirring in 5.0 ml 200 μM sodium succinate and 1.0 μM CysA in PBS for 60 min at 30°C. The cells were then incubated in 10.0 ml 40.0 nM R123, 1.0 μM CysA, 0.01 M NaN_3, and 0.004 M 2-deoxyglucose for 120 min at 30°C. The cells were chilled in ice for 15 min and centrifuged for 2 min at 1500 rpm and 4°C. The cells were rinsed three times with ice-cold 1.0 μM CysA and 490 μM $MgCl_2$ in PBS. For the controls, the CysA was omitted during the incubations and cold rinses. The cells were rinsed once with cold PBS without CysA. The cell pellets were then resuspended in 10.0 ml 0.01 M glucose, 0.006 M L-glutamine, and 490 μM $MgCl_2$ in PBS and incubated with gentle stirring for 90 min at 30°C. The cell suspensions were centrifuged for 5 min at 5000 rpm and the supernatant was decanted. The concentration of R123 in the supernatant (10×10 mm cell) was determined by fluorescence. The spectrophotometer was set at an emission/excitation ratio of 0.54 with 5.0 nM R123.

Results

The efflux of R123 from CEM/VLB_{100} cells is shown in Fig. 1. In this experiment, the cell pellet was suspended in L-glutamine/glucose buffer (23°C) at zero time and R123 was detected in the flow cell after 120 s. The inhibitory effect of a prior incubation with 3.0 μM AMI is also shown in Fig. 1. The release of substrate from control cells after 300 s was 0.3 nM R123.

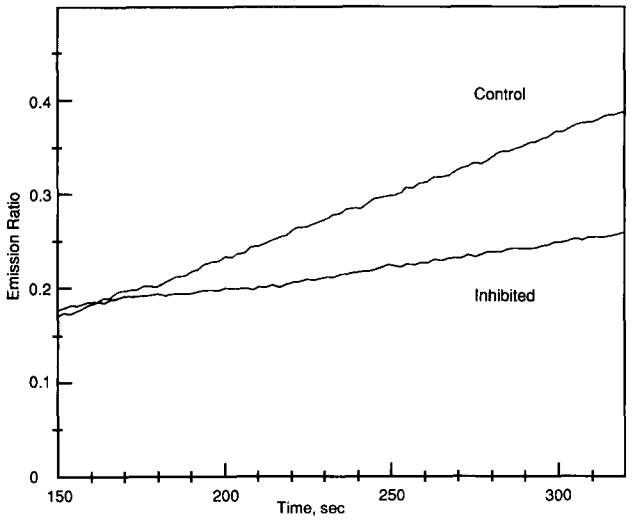

Fig. 1. Efflux of R123 from CEM/VLB_{100} cells after incubation with 3.0 μM amiodarone. The cells ($2.0 \cdot 10^7$) were incubated with AMI in 5.0 ml 200 μM succinate in PBS for 60 min at 30°C. The cells were incubated with 40.0 nM R123, AMI, NaN_3, and 2-deoxyglucose for 60 min at 30°C. The cells were rinsed cold 4 times, resuspended in 10 ml L-glutamine/glucose at 23°C, and the suspension was poured into the filtration-flow apparatus at time zero. The emission ratio was determined at 2-s intervals at 498 nm and 520 nm. (A ratio of 0.5 is equal to 0.4 nM R123.) The controls were not treated with AMI.

TABLE I

Inhibition of R123 efflux through the MDR pump

$2.0 \cdot 10^7$ CEM/VLB_{100} cells were treated with RR agent in 5.0 ml 200 μM succinate in PBS for 60 min at 30°C. The cells were incubated with 40.0 nM R123 (and RR agent) in NaN_3 and 2-deoxyglucose in 10 ml PBS for 60 (or 120) min at 30°C. The cells were rinsed cold 4 times, resuspended in 10 ml warm L-glutamine/glucose in PBS, and the initial efflux was determined at 23°C (performed in duplicate).

Reversal agent	Concn. (μM)	Incubation (min)	Percent inhibition [a]	S.D.
CysA	1.0	120	72.7	3.9
CysA	1.0	60	83.6	3.5
AMI	3.0	60	55.6	1.0
AMI	3.0	120	65.1	3.3
DMDP	6.0	60	78.3	6.5
DMDP	6.0	60	67.7	6.1
VLB [b]	25.0	60	75.0	11.2
(N.C.)AMI [c]	5.0	60	70.5	7.4
(N.C.)AMI	3.0	60	71.4	9.0

[a] 100–100 (rate for RR agent treated cells/rate of controls). Control cells were not treated with RR agent.

[b] Cells treated with VLB but no succinate.

[c] Negative controls. Cells were treated with AMI in succinate buffer and loaded with R123 in NaN_3 and 2-deoxyglucose in PBS. The N.C. values were determined with NaN_3 and 2-deoxyglucose in the absence of glucose at 23°C.

The inhibitory effects of several RR agents on the initial efflux of R123 from MDR-1 cells are shown in Table I. The negative controls are of interest because a finite efflux of R123 is observed even when the cellular ATP is limiting and the MDR pump is blocked with AMI. This observation indicates an additional passive, slow pathway for the efflux of R123. CysA is the most effective inhibitor of the ATP-dependent efflux of R123 tested. Inhibition with 1.0 μM CysA is comparable to the inhibition observed with the negative controls. Apparently, the time selected for preloading the cells with R123 does not influence the inhibitory effects of CysA or AMI. Fig. 1 shows that treatment with 3.0 μM AMI produces a strong inhibition of R123 efflux, but the inhibition is less than the effect with the negative controls (Table I). Inhibition of the MDR pump by 6.0 μM DMDP is close to the values observed for the negative controls.

Inhibition of the MDR pump by intracellular VLB is expected because VLB is a known substrate of the pump [5,18]. A relatively high concentration of VLB was required for inhibition of R123 efflux in comparison with the RR agents listed in Table I.

The RR agents listed in Table II are less potent flux inhibitors than the agents listed in Table I. The inhibitory effect of RQ (the (*R*)-diastereomer of SQ) on the MDR pump at 30 or 40 μM is less than 50%.

TABLE II

Inhibitory effect of optical isomers on R123 efflux through the MDR pump

$2.0 \cdot 10^7$ CEM/VLB_{100} cells were treated with RR agent in 5.0 ml succinate buffer for 60 min at 30°C (see Table I). The cells were incubated with 40.0 nM R123, RR agent, NaN_3, and 2-deoxyglucose in 10 ml PBS for 60 min at 30°C. The cells were rinsed cold four times, resuspended in 10 ml warm L-glutamine/glucose in PBS, and the initial efflux was determined at 23°C (performed in duplicate).

Experiment	Reversal agent	Concn. (μM)	Percent inhibition [a]	S.D.
1	RQ	30.0	46.2	11.8
2	RQ	40.0	45.3	9.7
3	SQ	60.0	29.6	4.7
2	SQ	80.0	39.5	9.6
4	SQ	90.0	51.8	7.6
5	(*R*)-PROP	125.0	30.3	3.6
5	(*S*)-PROP	125.0	45.5	6.3

[a] 100–100 (rate for RR agent treated cells/rate of controls). Control cells were not treated with RR agent.

Higher concentrations of SQ are required to produce inhibition of the pump. From 60 to 90 μM SQ, the percent inhibition increases with the level of the RR agent. It is apparent that SQ is a weak inhibitor of the pump compared to RQ. (The RQ and SQ concentrations of pH 7.4 test solutions were rechecked by measurement of the fluorescence emission intensity at 392 nm.)

The data of Table II indicate that (*S*)-PROP may be more potent than (*R*)-PROP as an inhibitor of the MDR pump. Relatively high concentrations of each PROP isomer are required to observe inhibitory activity.

Control cells were preloaded with 40.0 nM R123, rinsed cold 4 times, and incubated at 30°C for 90 min; an R123 concentration of 5.5 nM was observed in the extracellular buffer (see Table III). The prior incubations with 1.0 μM CysA enhanced the R123 concentration slightly to 5.8 nM, but this difference may not be significant. The ATP-dependent release of R123 from the CEM/VLB_{100} cells at 23°C for 300 s gives an extracellular R123 of 0.3 nM (see Fig. 1). Thus, the 300 s efflux of R123 represents approximately the first 5% of the unbound intracellular R123.

TABLE III

Uptake and 90-min release of unbound R123 from cells

$2.2 \cdot 10^7$ CEM/VLB_{100} cells were incubated with 1.0 μM CysA and succinate as in Table I. The cells were incubated again with 40.0 nM R123, CysA, NaN_3, and 2-deoxyglucose for 120 min. After 4 cold rinses, the cells were incubated in 10 ml L-glutamine/glucose for 90 min at 30°C. Control cells were not treated with CysA.

Treatment	R123 concn. [a] (nM)	Cellular R123 (nmol/10^9 cells)
CysA	5.8 ± 0.07	2.7
Control	5.5 ± 0.02	2.6

[a] The R123 concentration observed for the 10.0 ml supernatant. S.D. is calculated for $N = 2$.

The intracellular/extracellular distribution of R123 can be estimated from the data of Table III. Based on the ^3H_2O method of Beck et al. [20], the average volume of CEM/VLB_{100} cells is approximately $1.64 \cdot 10^{-12}$ liter per cell. From the data of Table III, a distribution ratio of 40 was obtained.

Discussion

The uptake of anthracycline or Vinca alkaloid drugs by MDR-1 cells is markedly enhanced by depletion of ATP with NaN_3 [18]. On the other hand, Sehested et al. [24] have shown that there is little uptake of the RR agent verapamil by ATP-depleted cells. These authors suggested, therefore, that the uptake of verapamil is an ATP-dependent process. To enhance the uptake of the RR agents in these experiments, the CEM/VLB_{100} cells were incubated with an RR agent in succinate buffer before treatment with NaN_3 and 2-deoxyglucose. The MDR-1 cells were kept in contact with a constant level of RR agent throughout the R123 preloading and for three cell rinsing procedures to retain RR agent in the cells.

The MDR-1 cells were incubated with an RR agent and R123 for 2–3 h at 30°C. Cultured cells are relatively resistant to the cytotoxic effects of RR agents at this temperature because cell division is very slow. After four cold rinses a single inside-outside flux of the R123 is detected. To observe RR agent inhibition of R123 efflux through the MDR pump, it may be necessary to use a low substrate concentration. If the binding site in the pump is saturated with substrate, the RR agent is displaced unless very high RR agent concentrations are present.

The potential uptake of R123 into the mitochondria of nonresistant or MDR-1 cells should also be considered [17]. When CEM/VLB_{100} cells were treated with 3.0 μM R123 a 2.5-fold enhancement in the intracellular drug concentration was observed; nevertheless, Kessel et al. [17] reported no mitochondrial localization of R123 under these conditions. In the experiments reported here, an extracellular concentration of 40.0 nM R123 was used. The low R123 concentrations may minimize drug uptake into the mitochondria. If treatment with CysA influences the mitochondrial localization of R123, a difference in the total cellular R123 level would be expected. As can be seen in Table III, CysA has a relatively small effect on the cellular R123 content.

Numerous reports of reversal activity by a variety of substances suggest a broad specificity for the MDR pump. Reversal can be demonstrated by cytotoxicity or

drug retention when MDR-1 cells are exposed to Vinca alkaloid or anthracycline drugs in combination with an RR agent. Potent reversal activity has been reported for CysA with several different MDR-1 cell lines [3,25–29]. There are relatively few reports of RR activity by AMI [2,3] or DMDP [30,31].

A strong inhibition of R123 efflux was observed with 1.0 μM CysA when the cells were preloaded with 40 nM R123 (see Table I). Efflux inhibition was also demonstrated with 3.0 μM AMI or 6.0 μM DMDP; apparently, these RR agents interfere with the binding of R123 inside the carrier. The efflux of R123 is also inhibited by VLB, although in this case higher concentrations of the drug are required.

The results of Table II indicate that flux inhibition by RQ is approximately 2-fold greater than the (*S*)-diastereomer, SQ. This observation is consistent with the results of Akiyama et al. [32]; these investigators reported that RQ is more potent than SQ when these RR agents were tested as inhibitors of the photoaffinity labeling of P-170 glycoprotein by a VLB analog. Furthermore, Genne et al. [33] have reported that the RR potency of RQ is greater than SQ. These findings are also consistent with the VLB-cytotoxicity experiments of Lehnert et al. [34] that show SQ is a weak RR agent when tested with an MDR-1 cell line.

Boesch et al. [3] reported the reversal of resistance of an MDR-1 murine leukemia cell line, based on cytotoxicity assays and determinations of cellular drug retention. The potency of several different RR agents was compared. These authors reported that the RR potency of CysA is greater than AMI and the potency of AMI is greater than RQ. These observations appear to be consistent with the potency as determined by the inhibition of efflux of R123 (see Tables I and II).

The effects of RR agents on the accumulation of actinomycin D by MDR-1 cells have been studied by Hofsli and Nissen-Meyer [35]. In these studies, racemic PROP was found to be a more potent RR agent than verapamil. The (*S*)-PROP is a potent antagonist of β-adrenergic receptors; whereas, the (*R*)-PROP is relatively inactive [36]. The results of Table II indicate that (*S*)-PROP is slightly more potent as an inhibitor of the MDR pump than the corresponding (*R*)-isomer. Inhibition of R123 efflux was observed at relatively high concentrations of (*R*)- (or (*S*)-) PROP. From these observations, PROP does not seem promising as an RR agent for clinical trials.

The most important observation reported here shows the inhibitory effect by known RR agents on the initial efflux of R123 through the MDR pump. CysA is the most potent inhibitor of the MDR pump observed in this study. Recent studies have shown that the RR potency of the (*R*)-enantiomer of verapamil is equal to the potency of (*S*)-verapamil [37,38]. By contrast, Genne et al. [33] have shown that the (*R*)-diastereomer cinchonine is 2-fold more potent as an RR agent than the (*S*)-diastereomer, cinchonidine. We now report that the (*R*)-diastereomer RQ is approximately 2-fold more potent as an inhibitor of the MDR pump than the (*S*)-diastereomer, SQ.

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