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# Cellular Drug Efflux and Reversal Therapy of Cancer

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A prevalent form of multidrug resistance (MDR) in cancer cells is caused by an ATP-dependent drug efflux pump; this pump catalyzes the rapid exit of cytotoxic chemotherapy drugs from the cells. The Michaelis equation can be used to describe drug efflux through the MDR pump at a low drug substrate concentration [*S*]. The inhibition mechanism of an MDR reversal agent can be characterized when two different values of [*S*] are used to determine two values for the half-inhibition of efflux through the pump ($I_{50}$). The reaction is noncompetitive when the two values of $I_{50}$ are identical; the reaction is competitive when an increase in [*S*] produces a significant increase in the value of $I_{50}$. The $I_{50}$ has been determined for several different reversal agents with the substrate rhodamine 123. The inhibition potency observed is: cyclosporin A > DMDP > amiodarone > verapamil > quinidine > quinine > propranolol. Chemotherapy drugs that are potent inhibitors of the MDR pump could be used for the treatment of MDR neoplasia.

**KEY WORDS:** Efflux inhibition; resistance reversal; multidrug resistance; cancer chemotherapy; efflux pump; active transport.

## INTRODUCTION: INHIBITION OF THE MDR PUMP

Every year in this country 500,000 patients require systemic therapy for disseminated cancer; more than 450,000 of the patients treated with chemotherapy drugs become short-term survivors. To a great extent, this poor prognosis is due to the drug resistance exhibited by metastatic tumors. Drug resistance may be intrinsic or the tumor may initially respond to chemotherapy but recur later as a neoplasm with acquired chemotherapy resistance (Gottesman and Pastan, 1993). Cancer cells with a pattern of resistance to a variety of different chemotherapy drugs are called multidrug-resistant (MDR) cells. In the most prevalent form of drug-resistant cancer, a decreased intracellular accumulation of drug is observed (Danø, 1973). The low drug accumulation is due to the presence of an ATP-dependent drug efflux pump, the P-170 glycoprotein or MDR pump. Many reviews of research on the P-170 glycoprotein have been published (Shapiro and Ling, 1995; Wigler and Patterson, 1993).

In the presence of a resistance-reversal (RR) agent, MDR-1 cancer cells[2] accumulate daunomycin (DAUNO) at intracellular levels that are close to the levels observed with nonresistant cells (Kessel and Wilberding, 1985; Boesch *et al.*, 1991). Although RR agents have been used in clinical chemotherapy protocols, the high agent concentrations usually needed for reversal may be toxic to the patients (Pennock *et al.*, 1991). The purpose of this article is to discuss the effect of inhibitors of the MDR pump on the kinetics of drug efflux. Drug efflux from MDR-1 cells has a profound effect on cell survival (Shapiro and Ling, 1995).

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[2] Abbreviations: MDR-1 cancer cells, multidrug-resistant cancer cells with the MDR pump; RR, resistance reversal; DAUNO, daunomycin; VCR, vincristine; R123, rhodamine 123; Cys A, cyclosporin A; PBS, phosphate-buffered saline; DMDP, *N*-(3,4-dimethoxyphenethyl)- *N*-methyl- 2- (2- naphthyl)- *m*- dithiane- 2- propylamine hydrochloride; $I_{50}$, the concentration of RR agent inhibitor that produces a 50% inhibition of R123 efflux through the MDR pump.



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## THE EFFECT OF SUBSTRATE CONCENTRATION ON EFFLUX

When nonresistant CCRF-CEM human leukemia cells are treated with 1.0 nM vincristine (VCR) for 48 h, cell proliferation is reduced by 50% (Cass *et al.*, 1989; Häußermann *et al.*, 1991). Lipophilic vinca alkaloids are ordinarily concentrated in cells; based on the drug-retention studies of Kessel *et al.*, (1991), the total intracellular VCR concentration would increase about 20-fold. To produce a survival benefit for MDR-1 cells, the MDR pump must decrease the intracellular concentration of VCR to a nontoxic level. The MDR-1 phenotypic cell line CEM/VLB$_{100}$ is capable of proliferation in VCR at 900 nM (Cass *et al.*, 1989), and at a higher substrate concentration [S] the cells may develop additional forms of drug resistance.

The treatment of MDR-1 phenotypic cells with 10 mM sodium azide plus 10 mM 2-deoxyglucose inhibits ATP synthesis, and the uptake and intracellular concentration of a vinca alkaloid can be demonstrated (Thimmaiah *et al.*, 1990). This observation indicates that transport of vinca alkaloids occurs via a reversible passive pathway. There are three potential mechanisms for passive VCR flux: diffusion through the lipids of the cell membrane; transport through a membrane channel; or passive flux through a gated transporter. Flux through the passive pathway follows the substrate gradient and is readily reversible with a change in the gradient; high medium [S] causes substrate influx and high intracellular [S] causes substrate efflux.

The foregoing comments provide sufficient information to show the effect of preloading MDR-1 cancer cells with different [S] values, on the rate of cell exit of VCR (Fig. 1). Cell efflux of VCR is the sum of the active efflux through the MDR pump and passive efflux through the cell membrane. At low intracellular concentrations of VCR the predominant pathway for VCR exit is through the MDR pump; however, the substrate binding site of the pump is saturated at elevated [S]. At high VCR concentrations passive efflux may be the predominant exit pathway. Spoelstra *et al.* (1992) have demonstrated that a high DAUNO concentration in MDR-1 phenotypic cells produces a rapid passive efflux of the substrate. The passive pathway may be a nonsaturable reaction, or the binding site for the passive pathway may be saturable at a much higher concentration than the [S] that saturates the MDR pump. These authors also observed two substrate binding sites in the MDR pump that exhibit positive cooperativity at high [S].

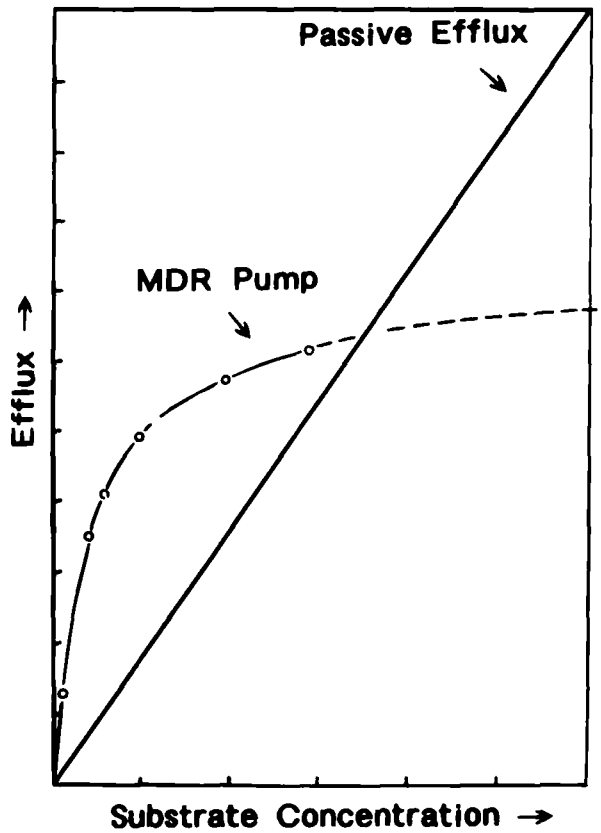


**Fig. 1.** The effect of preloading substrate concentration on passive efflux of the substrate and on substrate efflux through the ATP-dependent MDR pump.

Cytotoxicity studies with VCR or DAUNO indicate that the MDR pump is an effective catalyst for cell exit of these substrates at low [S]. Estimates of the rate of drug exit from MDR-1 cells are also available. For example, Kessel and Wilberding (1985) reported that the half-time for efflux of intracellular loosely bound DAUNO from P388/ADR cells is 1.6 min at 37°C. With another line of MDR-1 phenotypic cells (CEM/VLB$_{100}$), the apparent half-time for efflux of loosely bound rhodamine 123 (R123) is approximately 2.7 min at 23°C (Wigler and Patterson, 1994). Based on this observation, the initial velocity period for efflux through the MDR pump may be less than 0.5 min. When these cells are preloaded with 40 nM R123, flux through the MDR pump is 2.7-fold faster than the passive efflux of R123. The passive pathway is a significant component of substrate efflux through the membrane of MDR-1 cancer cells (Spoelstra *et al.*, 1992).

## HALF-INHIBITION AND MECHANISMS OF REVERSAL AGENTS

It is convenient to use a simple four-step mechanism to represent substrate efflux through the MDR pump at low [S] (Wigler and Patterson, 1993). The drug substrate (or the RR agent inhibitor) is bound to the transporter at the inside surface of the cell membrane. The transporter–substrate complex reacts with ATP; the transporter then produces inorganic phosphate and undergoes a protein conformational change that releases substrate into the medium outside the cell. In the last step, a spontaneous recovery occurs to return the transporter to the original conformation. The mechanism is shown in Fig. 2.

In Fig. 2, $S_i$ represents the intracellular substrate, $S_o$ represents the substrate outside the cell, T represents the transporter, P represents phosphate, and I represents the RR agent inhibitor. The lower case letters are rate constants; S-T, T-S, and I-T are the transporter-substrate and transporter-inhibitor complexes. When cancer cells are used for efflux studies, the cells are usually preloaded with the substrate and the inhibitor in a warm buffer. The cells are chilled and rinsed several times with cold buffer to remove the extracellular substrate. The cells are then resuspended in warm buffer to initiate efflux (Wigler and Patterson, 1994).

The $[S_i]$ at the inside surface of the cell membrane would be needed to determine the Michaelis constant ($K_s$) for the drug substrate and the MDR pump. Since it is difficult to determine the localized concentrations of substances inside a living cell, the $K_s$ may be determined from studies with reconstituted vesicles that contain the purified P-170 glycoprotein (Shapiro and Ling, 1995). An apparent $K_s$ value may be determined by an indirect procedure, from efflux experiments with a competitive inhibitor. During the early part of the efflux reaction when $S_o$ is almost zero, the steady-state rate equation for initial velocity in the absence of inhibitor is the Michaelis equation.

$$v = V_s[T_t]\,[S_i]/([S_i] + K_s) \qquad (1)$$

The $V_s[T_t]$ is the maximum velocity and the $v$ is the initial efflux through the MDR pump with no inhibitor present. When the RR agent is a noncompetitive inhibitor of the pump, the inhibitor (I) does not displace the substrate (S) from the substrate binding site (Segal, 1959). An inhibition constant based on the concentration of I at the inside surface of the cell membrane would be difficult to determine. Consequently, the apparent inhibition constant ($K_I'$) will be based on the I concentration [I] used to preload the cells. If the steady-state approximation is used to derive a rate equation for initial velocity, we obtain

$$v_I = V_S[T_t]\,[S_i]/([S_i] + K_S) \times (1 + [I]/K_I') \qquad (2)$$

The initial efflux through the MDR pump in the presence of I is represented by $v_I$. The [I] that is required for a value of $v_I$ equal to half the value of $v$ (controls with no I treatment) is represented by $I_{50}$. When the reaction is noncompetitive, the [I] for half-inhibition ($I_{N50}$) is equal to $K_I'$. The simultaneous solution of Eqs. (1) and (2) gives an equation for $I_{N50}$ or $K_I'$.

$$I_{N50} = [I]v_I/(v - v_I) = K_I' \qquad (3)$$

Equation (3) shows that the $I_{N50}$ value for flux through the MDR pump can be determined at a single [I], and for a noncompetitive reaction the $I_{N50}$ is not influenced by the value of $[S_i]$. The application of Eq. (3) will follow. When the I is a competitive inhibitor of the pump, the I displaces the S from the substrate binding site. More detailed discussions of competitive inhibition can be found in Segal (1959) or Wigler and Alberty (1960). The initial velocity equation for competitive inhibition and the mechanism in Fig. 2 is:

$$v_I = V_S[T_t]\,[S_i]/([S_i] + K_S + K_S[I]/K_I') \qquad (4)$$

The simultaneous solution of Eqs. (1) and (4) gives Eq. (5). When the reaction is competitive, the [I] for half-inhibition of efflux through the pump is represented by $I_{C50}$. Equation (5) shows that an increase in $[S_i]$ produces a higher $I_{C50}$ value:

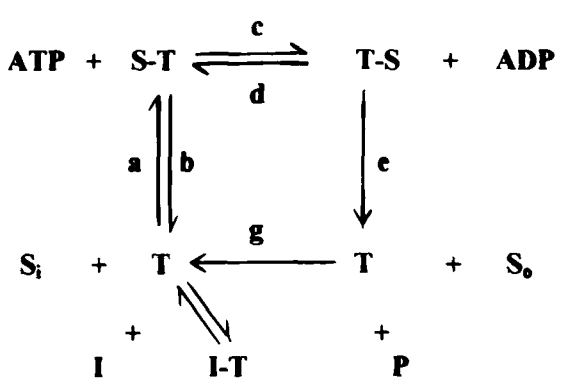


**Fig. 2.** Mechanism of the multidrug resistance pump with intracellular inhibition by a reversal agent. The efflux of a cytotoxic substrate ($S_i$), the change in conformation of the transporter, and the recovery of the unloaded transporter are shown. The inhibitory effect of a reversal agent is also indicated.

$$I_{C50} = [I]v_I/(v - v_I) = K_I'(1 + [S_i]/K_S) \quad (5)$$

An inspection of Eqs. (3) and (5) shows that it may be simple to discriminate between a competitive reaction and a noncompetitive reaction. Changes in $[S_i]$ have no effect on the $I_{N50}$ for a noncompetitive reaction. For a competitive reaction, however, an increase in $[S_i]$ produces an elevation in the $I_{C50}$ value. Equation (5) shows that when two $I_{C50}$ values are determined at two different values of $[S_i]$, an apparent $K_s$ value can be estimated. Furthermore, $I_{50}$ values can be used for mechanism studies of any enzyme reaction or transport system that is consistent with Eq. (1).

Equations (3) and (5) can be used to design an experiment to determine the mechanism of RR agent inhibition of the MDR pump. Initial efflux values with CEM/VLB$_{100}$ cells, preloaded with 10–30 nM R123, provide an apparent $K_s$ value of 34 nM (Wigler and Patterson, 1993). The mechanism experiment should be performed by preloading the cells with 10 or 40 nM R123. The preloading concentration of the inhibitor, cyclosporin A (CysA), should be high enough to produce an 80% inhibition of the MDR pump when the cells are preloaded with 10 nM R123. Based on preliminary experiments, this is a preloading concentration of approximately 500 nM CysA. Several controls would be needed to determine $v$ for efflux of R123 through the MDR pump at each $[S_i]$ and to determine the rates of the passive exit of R123.

## QUANTITATIVE COMPARISON OF REVERSAL AGENTS

The determination of reversal potency by quantitative *in vitro* procedures, performed before the RR agent is administered to cancer patients, will be a useful adjunct to a chemotherapy program. Although the kinetic characteristics of the MDR pump should be the same if the MDR-1 cells are located in a culture tube or located in a cancer patient, observations made in the clinic are often incompatible with data obtained in the laboratory. This contradiction may be due in part to the inactivation of the RR agent by binding to serum proteins; however, an *in vitro* assay of RR agent potency can be performed with a buffer that contains serum proteins (Genne *et al.*, 1992, 1994).

Fluorescent substrates, such as hydroxyrubicin, can be used to study substrate efflux from MDR-1 cells (Borrel *et al.*, 1994). In this laboratory, we have determined $I_{50}$ values for inhibition of R123 efflux through the MDR pump by several known RR agents (see Table I). A preloading concentration of 40 nM R123 was used in all of these experiments. Reversal potency data obtained with one $[S_i]$ value can be useful even though it is not known if the inhibition mechanism is noncompetitive or competitive for these agents. Except for the data on verapamil, these experiments were reported earlier (Wigler and Patterson, 1994).

We have observed that the incubation of cancer cells at 37°C with toxic drugs in PBS produces extensive cell damage and cell lysis. Apparently, cells are sensitive to toxic drugs during cell division at 37°C. The incubation of cells at 23°C is unsatisfactory because of the slow drug uptake into the cells. Thus, the CEM/VLB$_{100}$ cells were incubated at 30°C with an RR agent inhibitor and succinate in PBS. The MDR-1 cells were then incubated at 30°C with the RR agent, R123, sodium azide, and 2-deoxyglucose. The cells were chilled and rinsed four times with cold PBS. The cell pellet was resuspended in PBS (at 23°C) that contained L-glutamine and glucose. The cell suspen-

**Table I.** Inhibition of R123 Efflux Through the MDR Pump[a]

| Inhibitor | [I] (μM) | Cell efflux[b] (% control) | Pump efflux[c] (%) | $I_{50}$[d] (μM) |
|---|---|---|---|---|
| DMDP[e] | 6.0 | 32.0 | 5.0 | 0.32 |
| Amiodarone | 3.0 | 44.0 | 17.0 | 0.61 |
| S-verapamil[f] | 15.0 | 46.0 | 19.0 | 3.5 |
| R-verapamil[f] | 15.0 | 48.0 | 21.0 | 4.0 |
| Quinidine | 30.0 | 54.0 | 27.0 | 11.0 |
| Quinine | 60.0 | 70.0 | 43.0 | 45.0 |
| S-propranolol | 125.0 | 55.0 | 28.0 | 49.0 |
| R-propranolol | 125.0 | 70.0 | 43.0 | 94.0 |
| Cyclosporin A[g] | 1.0 | 27.0 | 0 | — |

[a] CEM/VLB$_{100}$ cells ($2.0 \times 10^7$) were treated with I in 5.0 ml 200 μM succinate in PBS at 30°C for 1 h. The cells were incubated with 40 nM R123 and I in $NaN_3$ and 2-deoxyglucose in 10 ml PBS at 30°C for 1 h. The cells were rinsed four times in cold buffer, resuspended in 10 ml warm L-glutamine-glucose in PBS, and the initial efflux was determined at 23°C (performed in duplicate).

[b] Cell Efflux (%) = 100 × (rate for inhibitor treated cells/rate for controls). Control cells were not treated with inhibitor.

[c] Pump efflux = cell efflux − passive efflux.

[d] $I_{50} = [I]\, v_I/(v - v_I)$. The initial efflux of R123 through the MDR pump in the presence of I is $v_I$. Pump efflux for controls is $v$.

[e] DMDP: The *N*-(3,4-dimethoxyphenethyl)-*N*-methyl-2-(2-naphthyl)-*m*-dithiane-2-propylamine hydrochloride was a gift from F. Hoffmann-LaRoche Ltd., Basel.

[f] Unpublished experiments. The enantiomers of verapamil were gifts of BASF Bioresearch Corp., Cambridge, Massachusetts.

[g] Passive efflux was determined from cell efflux when the cells were preloaded with 1.0 μM cyclosporin A.

sion was placed in a flow system and the appearance of R123 in the extracellular buffer was detected by fluorescence. The MDR pump appears to be completely blocked when CEM/VLB$_{100}$ cells are preloaded with 1.0 μM CysA (Wigler and Patterson, 1994); thus, a cellular efflux value of 27% is due to passive efflux. Cell efflux values from 32 to 70% of the control were used for the determination of $I_{50}$ values.

Efflux through the MDR pump is determined by subtracting the passive flux (27%) from the cell efflux of R123; the $I_{50}$ is then calculated from [I],$v_I$, and $v$. The most striking feature of the data of Table I is the wide range of inhibitory potency for the different RR agents tested. For example, both amiodarone and quinine have been used as RR agents in clinical trials (van der Graaf *et al.*, 1991; Solary *et al.*, 1992). Based on the $I_{50}$ data of Table I, quinine inhibition of the MDR pump requires an unbound serum quinine concentration 74-fold higher than the free amiodarone required for inhibition of the pump. The data listed in Table I may be useful for the design of chemotherapy protocols. Empirical observation is not enough; research must be quantitative for science to progress.

## CONCLUSIONS: DESIGN OF CHEMOTHERAPY PROTOCOLS

Inhibition of efflux through the pump can be demonstrated with RR agents at a physiological low preloading [S] (Wigler *et al.*, 1994). Furthermore, Eq. (1) is valid for the analysis of data on initial cell efflux (Haldane, 1930; Hankin *et al.*, 1972). An inspection of Eqs. (3) and (5) shows that at least two concentrations of substrate are required to distinguish between a competitive and a noncompetitive inhibition mechanism. Equation (5) for a competitive reaction shows that the $I_{50}$ increases when [S$_i$] increases. If two or more [S$_i$] values are used to determine $I_{50}$, however, a single [I] value is sufficient to distinguish between a competitive and a noncompetitive reaction. Equation (5) shows that a change in [I] has no effect on the $I_{50}$ value.

There are numerous reports of the broad substrate and RR agent specificity of the MDR pump (Raderer and Scheithauer, 1993); however, the data of Table I shows that inhibition of the pump can be very specific. Based on the $I_{50}$ value, quinidine inhibition of the MDR pump is 4-fold greater than the diastereomer (quinine). This observation shows that a binding site in the pump is stereospecific with respect to interaction with diastereomeric inhibitors. The stereospecific effect of quinidine on MDR was first reported by Genne *et al.* (1992).

The reversal activity of propranolol has been reported (Hofsli and Nissen-Meyer, 1990). The data of Table I show that DMDP is almost 300-fold more potent than R-propranolol and approximately 150-fold more potent than S-propranolol as an inhibitor of the MDR pump. It seems clear that propranolol is not an appropriate candidate agent for reversal chemotherapy because high blood levels would be required to inhibit the MDR pump. Although many compounds appear to be RR agents, the data of Table I indicate that a wide range in potency is observed when these agents are tested as inhibitors of the MDR pump.

The values of $I_{50}$, determined *in vitro* with MDR-1 cells at various [S] levels, do not provide sufficient information for clinical application. There are three characteristics of an RR agent that must be considered before the agent is used to treat a patient. The RR agent may block the kidney excretion of a chemotherapeutic drug; this phenomenon enhances the *in vivo* toxicity of the drug to essential MDR-1 tissue cells as well as to drug-resistant tumor cells (Lum *et al.*, 1992, 1993). The treatment of patients with high serum levels of an RR agent can produce severe toxic reactions (Pennock *et al.*, 1991). The reversal potency of an RR agent may be reduced by binding of the agent to blood serum proteins (Genne *et al.*, 1992). Additional information will be needed to cope with the problem of serum inactivation of RR agents. For example, the *in vitro* flux inhibition experiments could include suspension of the MDR-1 cells in a buffer that contains serum proteins. The RR agent can be dissolved in blood serum and the fraction of free-to-bound RR agent determined. The results of these experiments will be useful before a clinical trial is performed with a new RR agent.

Further information on the inhibitory mechanisms of RR agents or chemotherapy drugs may facilitate the design of clinical reversal therapy protocols. Although Eqs. (3) and (5) are derived for initial velocity, these relationships may provide an approximation of RR agent inhibitory effects on efflux after the initial velocity period has ended. For example, consider the treatment of a patient with VCR in combination with the RR agent CysA. For purposes of calculation, suppose the $K_s$ for VCR and the MDR pump is 20 nM. The physician decides to increase the serum concentration of unbound VCR 8-fold, from 5.0 to 40 nM VCR. If pump inhibition is competitive, Eq. (5) shows that $I_{50}$ increases from $1.25K_I$ to $3.0K_I$. Thus, the concentration

of unbound serum CysA should be increased about 2.4 times to maintain the same inhibitory effect on the MDR pump. If CysA is a noncompetitive reversal agent [see Eq. (3)], the serum concentration of unbound VCR can be modified and reversal maintained with no change in the level of the CysA. Another approach that will be useful is to identify a chemotherapy drug that is also a potent inhibitor of the MDR pump.

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