Jpn. J. Cancer Res. **93**, 209–215, February 2002

Bromocriptine Reverses P-Glycoprotein-mediated Multidrug Resistance in Tumor Cells

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One of the most important causes of anticancer treatment failure is the development of multidrug resistance (MDR). The main characteristics of tumor cells displaying the MDR phenomena are cross-resistance to structurally unrelated cytotoxic drugs having different mechanisms of action and the overexpression of the *MDR1* gene, which encodes a transmembrane glycoprotein named P-glycoprotein (P-gp). This study evaluated whether bromocriptine, a D_2 dopaminergic receptor agonist, influenced anticancer drug cytotoxicity and P-gp activity in a P-gp-expressing cell line compared to a non-expressing subline. The K_i values for P-gp of cyclosporine and verapamil were 1.09 and 540 μM, respectively, and that of bromocriptine was 6.52 μM in a calcein-AM efflux assay using porcine kidney epithelial LLC-PK1 and L-MDR1 cells, overexpressing human P-gp. Bromocriptine at 10 μM reduced the IC_{50} of doxorubicin (DXR) in K562-DXR from 9000 to 270 ng/ml and that of vincristine (VCR) in K562-VCR from 700 to 0.30 ng/ml, whereas the IC_{50} values of DXR and VCR in the K562 subline were only marginally affected by these drugs. Bromocriptine restored the anticancer effect of DXR, VCR, vinblastine, vinorelbine and etoposide on MDR-tumor cells overexpressing P-gp. These observations suggest that bromocriptine has the potential to reverse tumor MDR involving the efflux protein P-gp in the clinical situation.

Key words: P-Glycoprotein — Bromocriptine — Multidrug resistance

One of the most important causes of anticancer treatment failure is the development of multidrug resistance (MDR) in the body. The main characteristics of tumor cells displaying the MDR phenomena are cross-resistance to structurally unrelated cytotoxic drugs having different mechanisms of action and the overexpression of the *mdr-1* gene, mapping to chromosome 7 (q21–31), which encodes a transmembrane glycoprotein named P-glycoprotein (P-gp).[1–3] P-gp, which is a 170- to 180-kDa membrane glycoprotein, has been extensively investigated with regard to the MDR phenomenon in tumor cells.[4, 5] The sequence and domain organization of P-gp are characteristic of the ABC (ATP-binding cassette) superfamily of active transporters, which include the related MDR associated protein (MRP).[6] P-gp functions as an ATP-dependent drug-efflux pump, actively excreting a variety of structurally unrelated anticancer drugs, such as anthracyclines, vinca alkaloids, epipodophyllotoxin, actinomycin D, mitomycin C and taxol, from cells before the drugs can exert their cytotoxic effects, thus producing resistance.[7, 8]

In contrast, MDR modulators, substrates capable of blocking P-gp-mediated drug efflux, have been suggested to reverse P-gp-mediated drug resistance, and to improve the outcome of cancer chemotherapy. MDR modulators include the antiarrhythmic verapamil and the immunosuppressant cyclosporine, and PSC 833 and MS 209 have been developed as second-generation MDR modulators. However, clinical application of these drugs is limited by side-effects and the possibility that they may inhibit other transporters which are not related to MDR.

Bromocriptine (Fig. 1) has previously been reported to inhibit the ATPase activity and the function of P-gp.[9] Bromocriptine is a hydrophobic polycyclic molecule, classically described as D_2 dopaminergic receptor agonist. Bromocriptine has been used clinically to treat hyperprolactinemia, acromegaly and Parkinson's disease for more two decades and has slight side-effects in clinical use.[10] However, there are no available data on the MDR-reversing effect of bromocriptine, and the effect of bromocriptine on anticancer agents, such as P-gp substrates, in acquired MDR cells has not been investigated.

Previously, we reported an assay system for evaluation of P-gp-mediated transcellular transport using human P-gp-overexpressing cells, L-MDR1 cells.[11, 12] Based on data from this assay system, we predicted that bromocriptine might be clinically effective, with weaker side-effects than cyclosporine and verapamil, for cancer chemotherapy and for overcoming P-gp-mediated drug interaction.

We report here interactions of bromocriptine in L-MDR1 and K562-DXR cells, in addition to the parental cell line LLC-PK1 and K562 cells, and the MDR-reversing effect of bromocriptine on human MDR-tumor cells.

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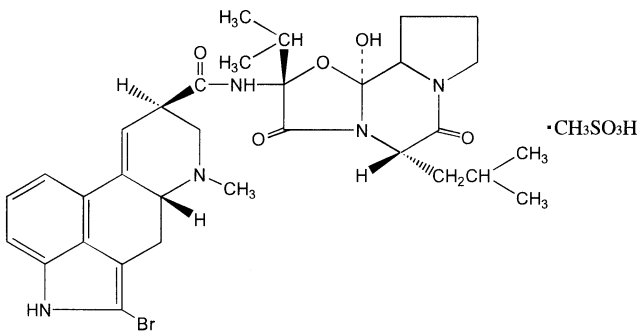

Fig. 1. Chemical structure of bromocriptine methanesulfonate (MW: 750.71).

MATERIALS AND METHODS

Drugs and chemicals Vincristine (VCR) and vinblastine were gifts from Eli Lilly Japan (Kobe). Doxorubicin (DXR) and vinorelbine were gifts from Kyowa Hakko Kogyo Co., Ltd. (Tokyo). Etoposide was a gift from Nippon Kayaku Co., Ltd. (Tokyo). Cyclosporine and bromocriptine were gifts from Novartis Pharma (Basel, Switzerland). Calcein acetoxymethyl ester (calcein-AM) was purchased from Molecular Probes Co. (Eugene, Oregon). Verapamil hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

Cell culture Porcine kidney epithelial LLC-PK1 and L-MDR1 cells transfected with human *MDR1* cDNA were generously given by St. Jude Children's Research Hospital, Memphis, TN. Cells were maintained in a complete medium consisting of Medium 199 supplemented with 10% fetal bovine serum, 1% penicillin and streptomycin, and 640 n*M* VCR for L-MDR1 cells. DXR and VCR-sensitive and resistant erythroleukemia K562, K562-DXR and K562-VCR cells were provided by Dr. T. Tsuruo, Institute of Molecular and Cellular Biosciences, University of Tokyo. K562-DXR and K562-VCR cells had previously been shown to exhibit a high level of P-gp expression as determined by northern blot analysis.[13] Non-small-cell lung carcinoma A549 cell was provided by Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University. A549 cells had previously been shown to exhibit a high level of MRP expression as determined by RT/PCR and flow cytometry analysis.[14] The cells were maintained in a RPMI-1640 medium supplemented with 10% fetal bovine serum. All cells were grown under an atmosphere of 5% CO_2-95% air at 37°C.

Cellular influx and efflux assay To study the function of the P-gp pump on K562-DXR cells, rhodamine 123 or DXR was used in an efflux assay.[12] Cells (5.0×10^5) were washed and resuspended in 1 ml of serum-free RPMI-1640. Cells were incubated with 1 μM rhodamine 123 or DXR and P-gp inhibitors for 1 h at 37°C. After 1 h, the cells were washed twice with serum-free medium to remove excess rhodamine 123, DXR or P-gp modulators, resuspended in 1 ml of serum-free medium, and recultured at 37°C for 2 h. After 2 h of culture, cells were washed and analyzed on FACScaliber by accumulating events in the FL1 (rhodamine 123) and FL2 (DXR) channel. To quantify the effect of inhibitor treatment on rhodamine 123 and DXR efflux from K562-DXR cells, we measured the shift of the histogram to the right as compared with the control (rhodamine 123 or DXR alone).

Calcein-AM efflux assay A kinetic fluorometric assay[11, 15, 16] was used to study the interaction of bromocriptine with P-gp. For the calcein-AM efflux assay, L-MDR1 and LLC-PK1 cells were seeded on 96-well tissue culture plates at a cell density of 1×10^5 cells/well. Cells were cultured in 200 μl per well of Medium 199 supplemented with 10% fetal bovine serum, 1% benzylpenicillin and streptomycin in an atmosphere of 5% CO_2-95% air at 37°C for 1 day. Cells were plated in 96-well tissue culture plates in Medium 199 containing various P-gp modulators. After 30-min incubation, calcein-AM was added to a final concentration of 1 μM and the plates were placed in a Fluoroscan Asent (Labsystems, Finland). Fluorescence was measured at 0 to 30 min with 485 nm excitation and 530 nm emission filters. The rate of calcein accumulation in the presence and absence of modulators was calculated by linear regression analysis using the Asent software (Labsystems). The inhibitory effect on P-gp was calculated based on the calcein fluorescence at 30 min by using the following equation

$$\text{Inhibitory effect on P-gp (\%)} = \frac{\text{[L-MDR with drug]} - \text{[L-MDR without drug]}}{\text{[LLC-PK1 without drug]} - \text{[L-MDR without drug]}}$$

and the K_i value for P-gp was calculated using a modified form of the Michaelis-Menten equation.[17]

Alamar Blue cytotoxicity assay To quantify the effects of bromocriptine on anticancer drug cytotoxicity, Alamar Blue was used in a semiautomated fluorometric assay.[12, 18] Alamar Blue is non-fluorescent substrate that is cleaved to a fluorescent product by living cells. This activity is dependent on the cell viability. The amount of fluorescence thus correlates with the number of living cells. Cells were harvested and plated at 5.0×10^3 cells/well in a volume of 80 μl in 96-well microtiter plates. Twenty microliter aliquots of drugs in the culture medium at the desired drug concentration were added and the cells were cultured for 48 h under the conditions described above. Ten microliter of Alamar Blue was added to each well of the plates, which were then incubated under the culture conditions for an additional 4 h. Measurement of fluorescence was done

by a plate reader, Fluoroscan Asent (Labsystems), with excitation at 544 nm and emission at 590 nm.

Statistical analysis Student's *t* test was used to evaluate the significance of differences. A *P* value of 0.05 or less was considered to be significant.

RESULTS

Effect of bromocriptine on the efflux of rhodamine 123 and DXR We measured rhodamine 123 and DXR efflux as a functional test to evaluate the effects of bromocriptine

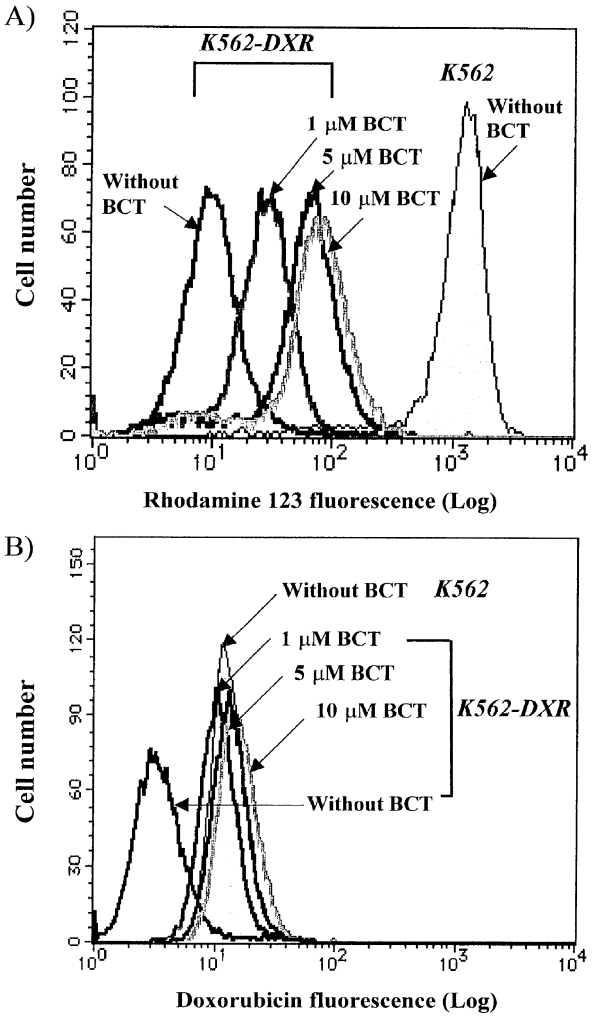

Fig. 2. Effects of bromocriptine on rhodamine 123 (A) and doxorubicin (DXR) (B) efflux as a functional test for the P-gp pump in K562 and K562-DXR cells. The cells (1×10^6 cells per ml) were incubated for 30 min at 37°C with 1 μM rhodamine 123 or DXR alone, or in combination with 1, 5 and 10 μM bromocriptine. Then the cells were washed twice, resuspended in fetal bovine serum (FBS)-free medium, and incubated for 2 h at 37°C with 1, 5 and 10 μM bromocriptine.

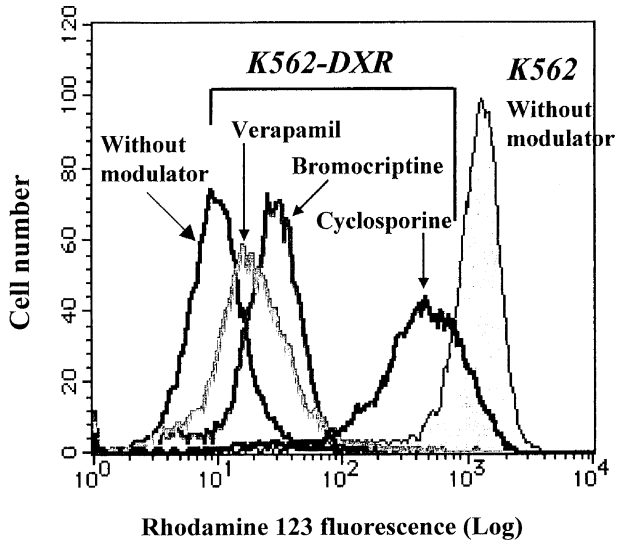

Fig. 3. The rhodamine 123 efflux as a functional test for the P-gp pump in K562 and K562-DXR cells. The cells (1×10^6 cells per ml) were incubated for 30 min at 37°C with 1 μM rhodamine 123 alone or in combination with 1 μM inhibitor. Then the cells were washed twice, resuspended in FBS-free medium, and incubated for 2 h at 37°C with 1 μM inhibitor.

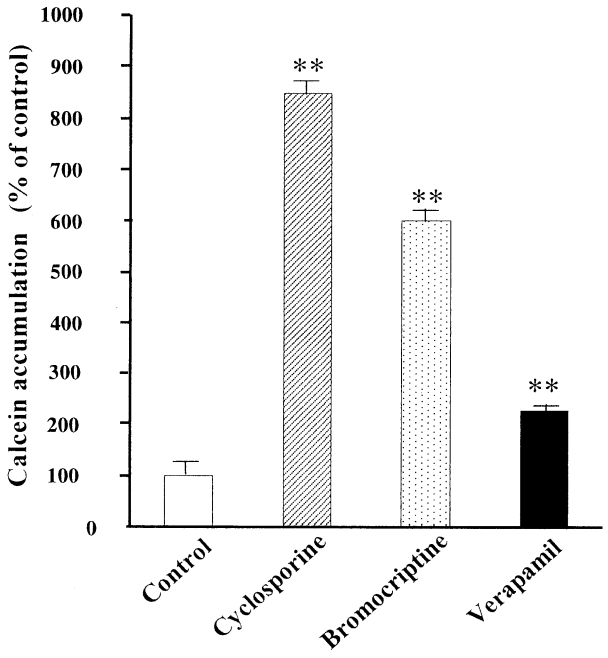

Fig. 4. Effects of cyclosporine, bromocriptine and verapamil on efflux of calcein-AM in L-MDR1 cell monolayers. The height of each column indicates the extent of accumulation of calcein in L-MDR1 cells incubated with 10 μM inhibitor for 30 min. Calcein-AM at the concentration of 2 μM was added at time 0 and fluorescence was measured at 30 min. Each value represents mean±SEM of six independent measurements. A significant difference from the control is indicated by ** $P<0.01$.

on the P-gp pump. As shown in Fig. 2A, rhodamine 123 was accumulated in K562 cells but not in K562-DXR cells, although the efflux of rhodamine 123 from K562-DXR cells could be blocked by bromocriptine at the concentration of 10 μM, and this effect was dose-dependent. However, it was difficult to examine the inhibitory effect of bromocriptine concentrations over 10 μM on P-gp because of its low solubility. The efflux of DXR was completely inhibited by only 1 μM bromocriptine (Fig. 2B). Fig. 3 shows that the inhibitory effects of P-gp modulators were in the order; cyclosporine > bromocriptine > verapamil.

Effect of bromocriptine on calcein-AM efflux in L-MDR1 cells Fig. 4 shows calcein-AM efflux in L-MDR1 cells in the presence of cyclosporine, verapamil and bromocriptine, which are potent P-gp modulators. The calcein fluorescence in L-MDR1 cells without drugs was significantly lower than that of LLC-PK1 cells, the parental cells.[11] Cyclosporine, verapamil and bromocriptine significantly increased calcein fluorescence in L-MDR1 cells ($P<0.05$). These P-gp modulators did not affect calcein accumulation in LLC-PK1 cells (data not shown).

Fig. 5 summarizes the effects of cyclosporine, bromocriptine and verapamil on the extent of calcein accumulation in L-MDR1 cells compared to LLC-PK1 cells. Inhibitory effect on P-gp modulators was calculated from the calcein fluorescence at 30 min, as described in "Materials and Methods." The inhibitory effects on P-gp were 75%, 58% and 10% at the concentration of 10 μM cyclosporine, bromocriptine and verapamil, respectively. Cyclosporine, bromocriptine and verapamil inhibited P-gp function with K_i values of 1.09, 6.52 and 540 μM, respectively. In this analysis, bromocriptine inhibited P-gp activity with affinities in the order cyclosporine>bromocriptine>verapamil, the same order as that observed in flow cytometry analysis using rhodamine 123.

Effect of bromocriptine on MDR in human tumor cells The K562 cell line was approximately 90 times as sensitive to DXR as K562-DXR, and 700 times as sensitive to VCR as K562-VCR. The IC_{50} values for DXR and VCR were approximately 9000 and 700 ng/ml for K562-DXR and K562-VCR (Fig. 6), respectively. Table I shows the IC_{50} values of anticancer drugs for various cancer cell lines with and without 10 μM bromocriptine, and the MDR-reversing indexes of bromocriptine. Bromocriptine

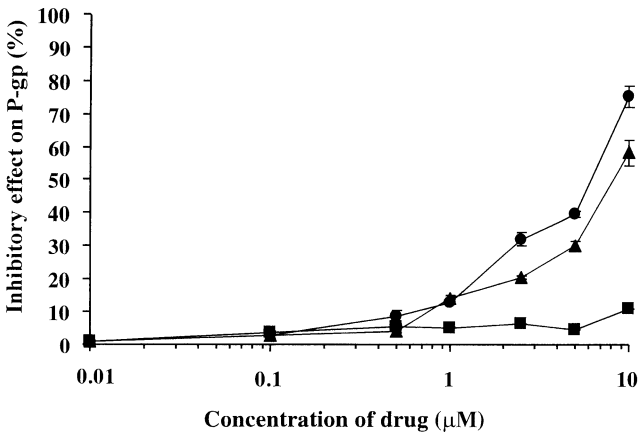

Fig. 5. Effects of cyclosporine, bromocriptine and verapamil on calcein accumulation in L-MDR1 cells compared to LLC-PK1 cells. The inhibitory effect on P-gp was measured after 30-min incubation with various concentrations of cyclosporine (●), bromocriptine (▲) and verapamil (■) at 37°C. Each point represents the mean±SEM of six independent measurements.

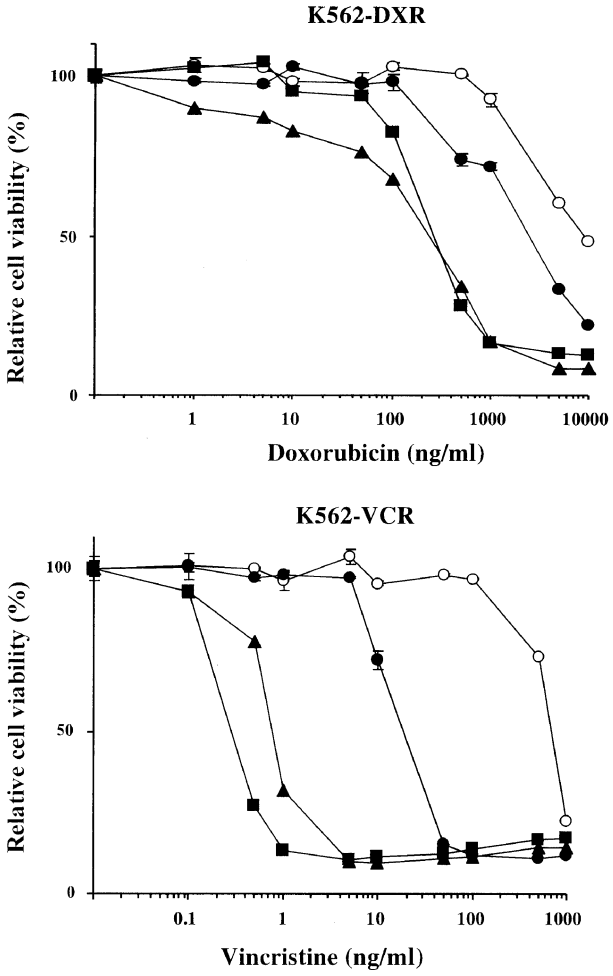

Fig. 6. Cell viability curves of K562-DXR and K562-VCR cells with DXR and vincristine (VCR) in the presence or absence of bromocriptine. Cytotoxicity was quantified by Alamar Blue assay. Each point represents the mean±SEM of three independent measurements. ○ no bromocriptine, ● 1 μM, ▲ 5 μM, ■ 10 μM bromocriptine.

Table I. MDR-reversing Effect of Bromocriptine

IC_{50} (ng/ml)	Doxorubicin	Vincristine	Vinblastine	Vinorelbine	Etoposide
Without bromocriptine					
K562	110	1.3	0.65	2.2	1 800
K562-DXR	9 000	1 000	200	660	50 000
K562-VCR	10 000	700	30	280	50 000
A549	550	90	9.0	1.0	10 000
With 10 μM bromocriptine					
K562	70	0.27	0.10	0.68	180
K562-DXR	270	0.83	0.045	1.5	3 000
K562-VCR	150	0.30	0.031	0.65	3 500
A549	170	18	4.0	0.20	6 000
MDR-reversing index[a)]					
K562	1.6	4.8	6.5	3.2	10
K562-DXR	33	1 200	490	440	17
K562-VCR	67	430	1 000	2 300	14
A549	3.2	5.0	2.3	5.0	1.6

a) MDR-reversing index $= \frac{IC_{50} \text{ without bromocriptine}}{IC_{50} \text{ with bromocriptine}}$.

(10 μM) reduced IC_{50} for DXR to 270 ng/ml for K562-DXR and that for VCR to 0.30 ng/ml for K562-VCR. On the other hand, bromocriptine had no effect on DXR and VCR cytotoxicity in K562, the parental line (data not shown). The MDR-reversing indexes were determined by dividing IC_{50} of cells cultured without bromocriptine by IC_{50} of the cells cultured with bromocriptine. Bromocriptine restored the anticancer effect of these drugs (DXR, VCR, vinblastine, vinorelbine and etoposide) on these MDR-tumor cells overexpressing P-gp. In particular, bromocriptine reversed the cytotoxic effect of vinca alkaloids (VCR, vinblastine and vinorelbine) on K562-DXR and K562-VCR by as much as 400 to 2000-fold. In contrast, in the study of A549 cells, which overexpress MRP, bromocriptine did not affect the cytotoxicity of any of the anticancer drugs we used.

DISCUSSION

Resistance to anticancer drug therapy is a major clinical problem leading to treatment failure. Therefore, investigations aimed at circumvention of intrinsic tumor-mediated drug resistance are warranted. The major cellular mechanism of drug resistance is overexpression of P-gp, which is an ATP-dependent drug efflux pump. In these experiments, we compared the ability of bromocriptine to inhibit P-gp function by using L-MDR1 cells transfected with human *MDR1* cDNA, and MDR-tumor cells, which overexpress P-gp.

We demonstrated that K562-DXR cells were 90-fold less sensitive to DXR than K562 cells, and that rhodamine 123 was not accumulated in K562-DXR cells. Cyclosporine and verapamil, classical inhibitors of the P-gp pump, inhibited the efflux of rhodamine 123. Using this model, we investigated the ability of bromocriptine to alter the efflux of rhodamine 123 and DXR from P-gp-overexpressing K562-DXR cells. The order of affinity for P-gp was cyclosporine>bromocriptine>verapamil (Figs. 3 and 4). As it was reported that MRP and lung resistance-related protein are not expressed on DXR-resistant K562 cells,[19] we consider that the inhibitory effects of the agents on rhodamine 123 and DXR efflux reflect the affinities of these drugs for P-gp. To access the effects of bromocriptine on P-gp activity, calcein-AM efflux was examined in LLC-PK1 and L-MDR1 cells and K_i for P-gp inhibition was calculated from calcein accumulation. Cyclosporine, bromocriptine and verapamil inhibited P-gp function with K_i values of 1.09, 6.52 and 540 μM, respectively (Fig. 5). There was some difference in the inhibitory effect of bromocriptine on P-gp as assessed by flow cytometry analysis and by calcein-AM efflux assay. This difference might be caused by the differences in the methods used, and in the affinities of rhodamine 123 and calcein-AM for P-gp. These findings indicate that bromocriptine interacts with P-gp to inhibit the P-gp-mediated efflux of drugs.

Our results showed that bromocriptine blocked the P-gp-mediated drug resistance in K562-DXR and K562-VCR. We have obtained similar results with mouse MDR-cancer cells, P388-DXR and P388-VCR.[20] On the other hand, in the case of A549, MRP-overexpressing cells, bromocriptine had little effect on the cytotoxicity of anticancer drugs. These data suggested that bromocriptine inhibits P-gp, but has little effect on MRP. Our preliminary data confirmed that the effect of bromocriptine on MRP-related efflux was slight, as judged from fluorescein accumulation assay in A549 cells.

The results of this study demonstrated that bromocriptine inhibits the efflux of rhodamine 123 and calcein-AM from P-gp-overexpressing cells, and reverses P-gp-mediated drug resistance in MDR-tumor cells. As bromocriptine is better tolerated and causes fewer adverse reactions compared to other MDR modulators, especially verapamil, we believe that bromocriptine is a promising candidate for a new MDR-reversing agent. However, the plasma concentration of bromocriptine is expected to be very low in clinical use,[21] since bromocriptine is extensively metabolized by cytochrome P450 3A in the liver and about 96% of orally administered bromocriptine is converted to metabolites.[22, 23] To examine the feasibility of using bromocryptine *in vivo*, we administered vinorelbine in combination with bromocriptine to VCR-resistant P388 cell-bearing CDF1 mice in a single dosing schedule. Preliminary data indicate that bromocriptine or cyclosporine increased life span by about 20%. Further work is in progress. We further found that a bromocriptine analog, mergocriptine, showed a P-gp-inhibitory effect, and a more detailed investigation of this analog is also warranted.

(Received August 11, 2001/Revised November 16, 2001/ Accepted November 26, 2001)

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