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 mdr-1 gene, which encodes a transmembrane glycoprotein named P-glycoprotein (P-gp). This study evaluated whether bromocriptine, a D2 dopaminergic receptor agonist, influenced anticancer cytotoxicity and P-gp activity in a P-gp-expressing cell line compared to a non-expressing subline. The K 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 IC50 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 IC50 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
cells were grown under an atmosphere of 5% CO2-95% air medium supplemented with 10% fetal bovine serum. All other chemicals were of reagent grade. Chemicals Co. (St. Louis, MO). All other chemicals were of reagent grade. Cyclosporine and streptomycin 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.

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 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 nM VCR for L-MDR1 cells. DXR and VCR-sensitive and resistant erythroleukemia K562, K562-DXR and K562-VC were provided by Dr. T. Tsuruo, Institute of Molecular and Cellular Biosciences, University of Tokyo. K562-DXR and K562-VC cells had previously been shown to exhibit a high level of MRP expression as determined by northern blot analysis. 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. The cells were maintained in a RPMI-1640 medium supplemented with 10% fetal bovine serum. All cells were grown under an atmosphere of 5% CO2-95% air at 37°C.

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 nM VCR for L-MDR1 cells. DXR and VCR-sensitive and resistant erythroleukemia K562, K562-DXR and K562-VC cells were provided by Dr. T. Tsuruo, Institute of Molecular and Cellular Biosciences, University of Tokyo. K562-DXR and K562-VC cells had previously been shown to exhibit a high level of P-gp expression as determined by northern blot analysis. 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. The cells were maintained in a RPMI-1640 medium supplemented with 10% fetal bovine serum. All cells were grown under an atmosphere of 5% CO2-95% 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. Cells (5.0×10⁶) 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 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 1×10⁵ 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% CO2-95% 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 Ascent (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, Finland). The inhibitory effect on P-gp was calculated based on the calcein fluorescence at 30 min by using the following equation:

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\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}]}
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and the \( K_i \) value for P-gp was calculated using a modified form of the Michaelis-Menten equation. Alamar Blue cytotoxicity assay To quantify the effects of bromocriptine on anticancer drug cytotoxicity, Alamar Blue was used in a semiautomated fluorometric assay. Alamar Blue is a non-fluorescent substrate that is cleaved to a fluorescent product by living cells. This activity is measured at 0 to 30 min by using the following equation:

\[
\text{Inhibitory effect on Alamar Blue} = \frac{[\text{L-MDR with drug}] - [\text{L-MDR without drug}]}{[\text{L-MDR without drug}] - [\text{LLC-PK1 without drug}]}
\]

and the \( K_i \) value for P-gp was calculated using a modified form of the Michaelis-Menten equation.
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 \( \mu M \) rhodamine 123 or DXR alone, or in combination with 1, 5 and 10 \( \mu M \) bromocriptine. Then the cells were washed twice, resuspended in FBS-free medium, and incubated for 2 h at 37°C with 1 \( \mu M \) inhibitor.

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 \( \mu M \) rhodamine 123 alone or in combination with 1 \( \mu M \) inhibitor. Then the cells were washed twice, resuspended in FBS-free medium, and incubated for 2 h at 37°C with 1 \( \mu M \) 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 \( \mu M \) inhibitor for 30 min. Calcein-AM at the concentration of 2 \( \mu M \) 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 \( \mu M \), and this effect was dose-dependent. However, it was difficult to examine the inhibitory effect of bromocriptine concentrations over 10 \( \mu M \) on P-gp because of its low solubility. The efflux of DXR was completely inhibited by only 1 \( \mu M \) 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 is 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 \( \mu M \) cyclosporine, bromocriptine and verapamil, respectively. Cyclosporine, bromocriptine and verapamil inhibited P-gp function with \( K_i \) values of 1.09, 6.52 and 540 \( \mu M \), 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 \( \mu M \) bromocriptine, and the MDR-reversing indexes of bromocriptine. Bromocriptine

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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 \( \mu M \), ▲ 5 \( \mu M \), ■ 10 \( \mu M \) bromocriptine.
Effect of Bromocriptine on P-gp

(10 µM) reduced IC₅₀ 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 IC₅₀ of cells cultured without bromocriptine by IC₅₀ 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 Kᵢ for P-gp inhibition was calculated from calcein accumulation. Cyclosporine, bromocriptine and verapamil inhibited P-gp function with Kᵢ 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 efflux assay. This difference might be caused by the differences in the methods used, and in the affinities of rhodamine 123 and calcein-AM efflux assays. 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.

Table I. MDR-reversing Effect of Bromocriptine

|                | Doxorubicin | Vinorelbine | Vinblastine | 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 indexᵃ |           |             |             |           |
| 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 = IC₅₀ without bromocriptine / IC₅₀ with bromocriptine.
The results of this study demonstrated that bromocriptine inhibits the efflux of rhodamine 123 and calcine-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, since bromocriptine is extensively metabolized by cytochrome P450 in the liver and about 96% of orally administered bromocriptine is converted to metabolites. To examine the feasibility of using bromocriptine 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, m ergocriptine, showed a P-gp-inhibitory effect, and a more detailed investigation of this analog is also warranted.

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