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Characterization of the inhibition of breast cancer resistance protein-mediated efflux of mitoxantrone by pharmaceutical excipients

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ABSTRACT

Previously we showed that some excipients can inhibit breast cancer resistance protein (BCRP/ABCG2) in vitro and in vivo. We then evaluated the reversibility and the mode of BCRP inhibition of excipients, such as Tween 20 and Pluronic P85, by the intracellular mitoxantrone uptake study. To evaluate the reversibility of BCRP inhibitory effects, BCRP expressing cells were preincubated with the excipients and the intracellular mitoxantrone uptake was determined after removing or not removing the excipients. To evaluate the mode of BCRP inhibitory effects, the intracellular mitoxantrone uptake at the different mitoxantrone concentrations in the medium with the excipients was determined. Both Tween 20 and Pluronic P85 increased the mitoxantrone uptake in BCRP expressing cells, but these effects were disappeared when the excipients were removed. Moreover, both excipients increased the uptake at low substrate concentrations. However, at high substrate concentrations, Tween 20 increased the uptake to less extent compared with low substrate concentrations, whereas there was no such effect of Pluronic P85. Taken together, Pluronic P85 and Tween 20 appear to inhibit BCRP-mediated efflux of mitoxantrone reversibly and the inhibition mode of Pluronic P85 may be competitive but not that of Tween 20, which may be mixed type.

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Our previous studies showed that some excipients, such as Tween 20 and Pluronic P85, can inhibit breast cancer resistance protein (BCRP/ABCG2) and improve the oral bioavailability of topotecan by inhibiting intestinal BCRP (Yamagata et al., 2007a,b). BCRP is a drug efflux half-transporter of the ABC family (van Hervaarden and Schinkel, 2006). It has a broad substrate specificity and can actively extrude several compounds across the plasma membrane of cells. In the present study, we evaluated the reversibility and the mode of BCRP inhibition of these excipients by measuring the uptake of mitoxantrone, which is transported by BCRP, in BCRP expressing-Mardin-Darby canine kidney (MDCK)-II cells.

Parent MDCK-II cells were seeded in 24-well plates at a density of 0.5×10^5 cells/well and grown for 2 days. Then, they were infected with a recombinant adenovirus harboring the gene for green fluorescent protein (GFP) or BCRP expression vector at 50 MOI. After 2 days, BCRP and GFP expressing cells (BCRP/MDCK-II and GFP/MDCK-II cells, respectively) were preincubated in transport buffer (pH 7.4) for 15 min. Uptake was initiated by adding

30 nM [³H]mitoxantrone (Moravek Biochemicals, Inc., St. Louis, MO, USA) with or without different concentration of unlabeled mitoxantrone (Sigma–Aldrich Co., St. Louis, MO, USA) in the presence or absence of 250 μM Tween 20 (Sigma–Aldrich) and 20 μM Pluronic P85 (BASF Co., Parsippany, NJ, USA). At the designated times, uptake was terminated. To investigate the reversibility of the inhibitory effects of BCRP, uptake was measured using two methods (Fig. 1).

1. Method 1

Cells were incubated with the transport buffer including 250 μM Tween 20 or 20 μM Pluronic P85 at 37 °C for 2 h, then, 30 nM [³H]mitoxantrone was added. After 1 h incubation, uptake was terminated.

2. Method 2

Cells were incubated with the transport buffer including 250 μM Tween 20, or 20 μM Pluronic P85, then, the incubation buffer was removed. Thirty nM [³H]mitoxantrone was added. After 1 h incubation, uptake was terminated.

The radioactivity and protein concentration of the cell lysate were measured. Intracellular uptake was estimated by dividing

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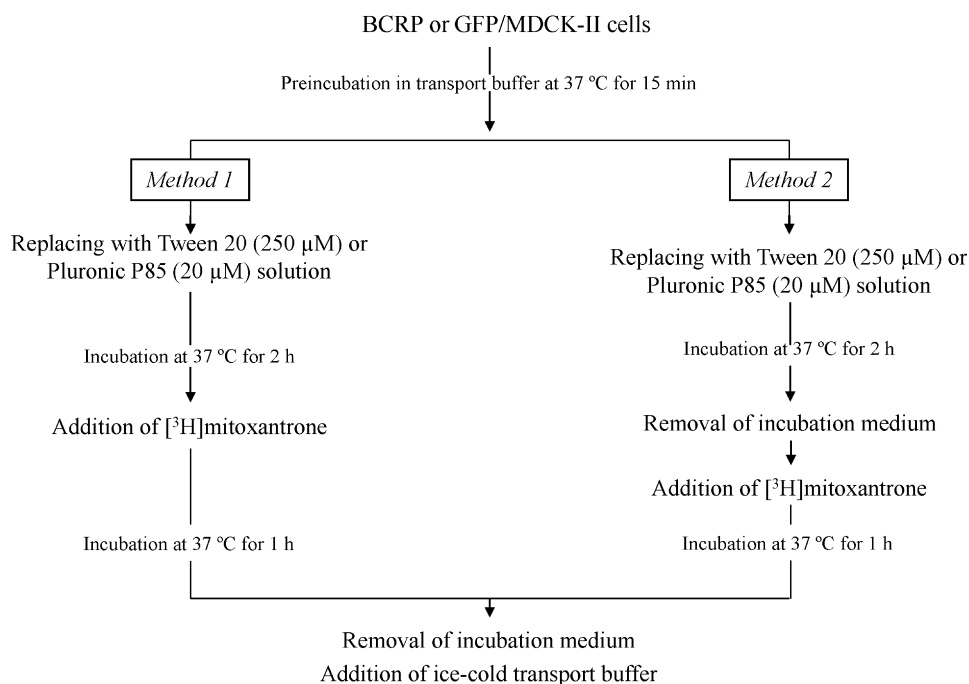


Fig. 1. Flow chart of the two methods of the mitoxantrone uptake study to investigate the reversibility of BCRP inhibitory effects of excipients.

the intracellular content by the concentration in the incubating medium. To inhibit endogenous P-gp in MDCK-II cells, 5 μ M PSC833 (Novartis Pharma AG, Basel, Switzerland), a P-gp specific inhibitor, was added during incubation. Inhibition ratios (IRs) were obtained by dividing the ratio of increase in the intracellular

uptake by excipients in BCRP/MDCK-II cells by that in GFP/MDCK-II cells.

First, we investigated the reversibility of the inhibitory effects on BCRP function by excipients. Using method 1, there was a significant increase in the uptake of mitoxantrone in BCRP/MDCK-II

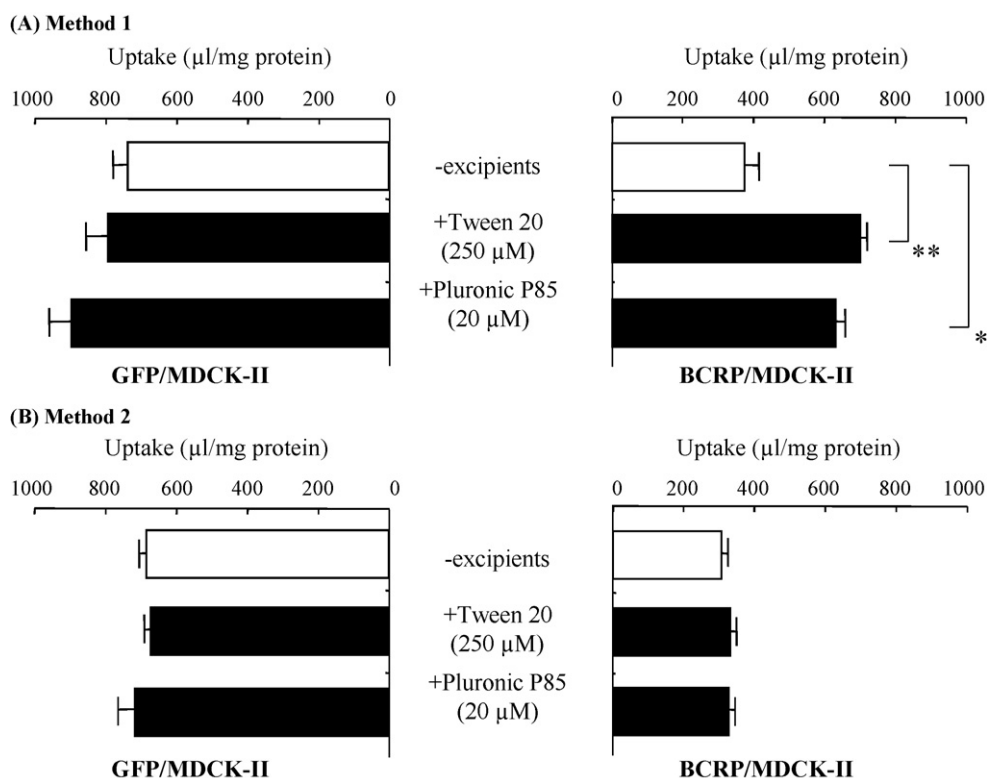


Fig. 2. Reversibility of BCRP inhibitory effects of Tween 20 and Pluronic P85. Uptake of mitoxantrone was measured by method 1 (A) and method 2 (B). Each column represents the mean \pm S.E. ($n = 3$). * $p < 0.05$; ** $p < 0.01$, significant difference by Dunnett's multiple comparison test.

cells in the presence of 250 μM Tween 20 and 20 μM Pluronic P85 and the IRs were 1.7 and 1.4, respectively (Fig. 2(A)), indicating that the exposure of cells to the excipients can inhibit BCRP function. On the other hand, in method 2, there was no increase in the uptake in either cell line and the IRs for Tween 20 and Pluronic P85 were 1.1 and 1.0, respectively (Fig. 2(B)), which revealed that the function of BCRP recovered when the excipients were removed. These results suggest that the inhibitory effects of Tween 20 and Pluronic P85 on BCRP are transient and reversible. Obviously, for clinical application, a transient effect on BCRP is preferable to persistent effects. Because BCRP mediates the secretion of several carcinogens or dietary toxins, a transient effect could minimize any serious side effects caused by inhibiting BCRP.

To evaluate the mode of BCRP inhibition by Tween 20 and Pluronic P85, the intracellular uptake of mitoxantrone was measured at different concentrations of mitoxantrone in the incubating medium with or without excipients. The time profiles for the uptake of mitoxantrone with or without 20 μM Pluronic P85 in BCRP and GFP/MDCK-II cells are shown in Fig. 3. It is noteworthy that at each individual experimental set, the uptake of mitoxantrone increased linearly rather than reached a steady-state, which indicates that each uptake velocity should be constant from 10 to 120 min (Fig. 3). In theory, when the substrate concentration is low, the inhibitory effect of inhibitors will be independent of their inhibition modes. In contrast, when the substrate concentration is much high, the degree of inhibition by competitive inhibitors will be minimal, whereas inhibition by noncompetitive inhibitors is unaffected by substrate concentration. For Pluronic P85, at low substrate concentrations, the uptake of mitoxantrone in BCRP/MDCK-II cells was increased significantly (the IRs at 0.03 and 0.1 μM were 1.4 and 1.4, respectively; Fig. 4(A) and (B)). However, the effect of Pluronic P85 completely disappeared at high substrate concentrations (the

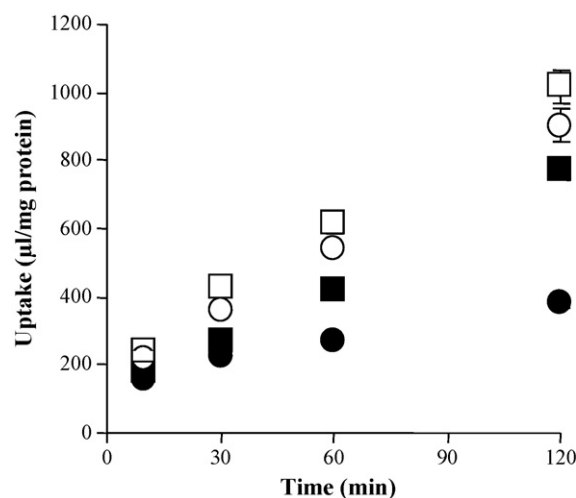


Fig. 3. Time-dependent uptake of mitoxantrone in BCRP and GFP expressing MDCK-II cells. Uptake of mitoxantrone was measured for 10, 30, 60, 120 min with (square) or without (circle) 20 μM Pluronic P85 using BCRP (closed) and GFP expressing cells (open). Each point and bar represents the mean \pm S.E. ($n = 3$). Data of uptake without excipient in BCRP and GFP expressing cells were quoted from Yamagata et al., 2007a.

IRs at 100 and 500 μM were 1.2 and 1.1, respectively; Fig. 4(C) and (D)). Thus, Pluronic P85 may inhibit BCRP competitively. On the other hand, the uptake of mitoxantrone in BCRP/MDCK-II cells was increased significantly by Tween 20 at both low and high substrate concentrations (the IRs at 0.03, 0.1, 100 and 500 μM were 1.6, 1.6, 1.3 and 1.3, respectively; Fig. 4). Therefore, the inhibitory effect of Tween 20 was observed even at high substrate concentration,

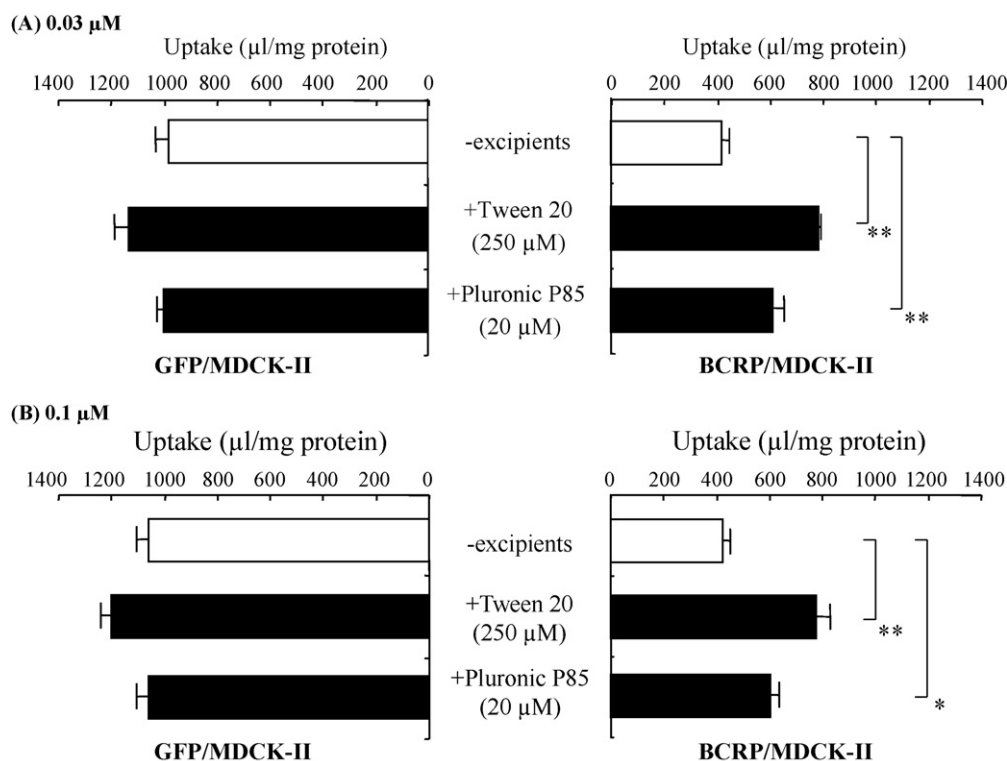


Fig. 4. Effect of mitoxantrone concentration on BCRP inhibitory effects of Tween 20 and Pluronic P85. Uptake of mitoxantrone was measured for 2 h in the presence of different concentrations of mitoxantrone: 0.03 μM (A); 0.1 μM (B); 100 μM (C) and 500 μM (D). These were applied with or without 250 μM Tween 20 or 20 μM Pluronic P85. Panels A and B represent uptake values up to 1400 $\mu\text{l/mg}$ protein and panels C and D represent uptake values up to 600 $\mu\text{l/mg}$ protein, respectively. Each column represents the mean \pm S.E. ($n = 3$). * $p < 0.05$; ** $p < 0.01$, significant difference by Dunnett's multiple comparison test.

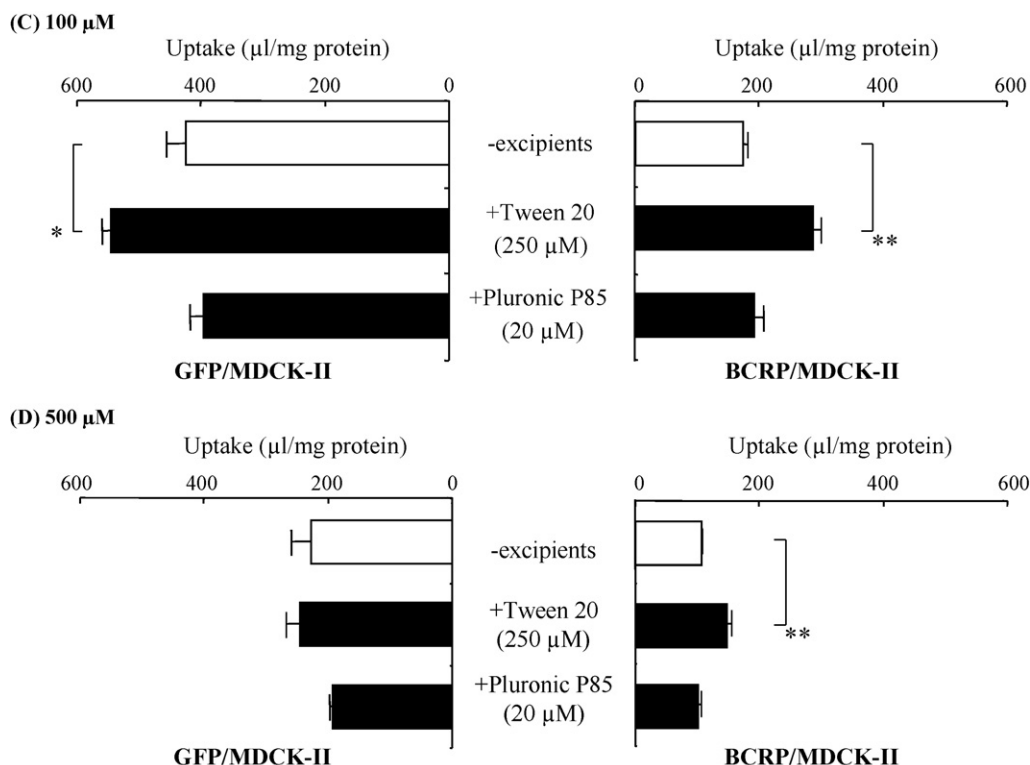


Fig. 4. (Continued).

suggesting that the inhibition by Tween 20 may not be competitive. Moreover, the degree of increase in mitoxantrone uptake into BCRP/MDCK-II cells by Tween 20 decreased slightly at high substrate concentrations (Fig. 4), indicating that the inhibition mode by Tween 20 may be a mixed type (competitive and noncompetitive). The uptake of mitoxantrone decreased markedly in both cell lines at high substrate concentrations (Fig. 4). It would be due to the saturation of some influx transporters and this result is consistent with the previous finding (Pan and Elmquist, 2007). To determine the mode of inhibition, the directly additional experiments would be necessary which are difficult to perform due to the limited sensitivity of the assays and the complexity of the experimental system.

Taken together, these results indicate Pluronic P85 and Tween 20 appear to inhibit BCRP-mediate efflux of [3 H]mitoxantrone reversibly and the mode of inhibition of Pluronic P85 may be competitive but not that of Tween 20, which may be mixed type.

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