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Interaction of the breast cancer resistance protein with plant polyphenols☆

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Abstract

Multidrug transporters influence drug distribution in vivo and are often associated with tumour drug resistance. Here we show that plant-derived polyphenols that interact with P-glycoprotein can also modulate the activity of the recently discovered ABC transporter, breast cancer resistance protein (BCRP/ABCG2). In two separate BCRP-overexpressing cell lines, accumulation of the established BCRP substrates mitoxantrone and bodipy-FL-prazosin was significantly increased by the flavonoids silymarin, hesperetin, quercetin, and daidzein, and the stilbene resveratrol (each at 30 μM) as measured by flow cytometry, though there was no corresponding increase in the respective wild-type cell lines. These compounds also stimulated the vanadate-inhibitable ATPase activity in membranes prepared from bacteria (*Lactococcus lactis*) expressing BCRP. Given the high dietary intake of polyphenols, such interactions with BCRP, particularly in the intestines, may have important consequences in vivo for the distribution of these compounds as well as other BCRP substrates.
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Keywords: Multidrug resistance; ABC transporter; Flavonoids; Polyphenols; Resveratrol; Breast cancer resistance protein; ABCG2; ATPase activity; Mitoxantrone

Multidrug transporters of the ATP-binding cassette (ABC) superfamily are able to expel a wide range of substrates from cells at the expense of ATP hydrolysis. Some such as the well-researched member of the ABCB subfamily, P-glycoprotein, transport many hydrophobic, amphipathic compounds. It plays an important role at barrier sites, e.g., colon, blood–brain and blood–testis barriers and placenta in limiting entry of xenobiotics but may also become overexpressed in tumours so contributing to clinical drug resistance. Other ABC transporters associated with multidrug resistance are the multidrug resistance-associated proteins (MRPs), members of the ABCC subfamily. These efflux anionic compounds as well as hydrophobic drugs conjugated with reduced glutathione, glucuronide or sulphate. They are expressed in tissues involved in elimination such as liver and kidney.

A more recently discovered multidrug ABC transporter is the breast cancer resistance protein (BCRP), a member of the ABCG 'half-transporter' subfamily [1]. It has a drug resistance profile similar though not identical to P-glycoprotein and, like P-glycoprotein, is located at barrier sites [2] where it may influence entry of xenobiotic material. These include the intestine, where it confers protection from dietary toxins [3,4]. In addition, it has been suggested that BCRP at the placenta may protect the fetus from maternal steroids such as estradiol-17β-glucuronide [5] whilst at the blood–brain barrier [6] the transporter is ideally placed to prevent unwanted material in the circulation from passing into the brain. Potentially therefore agents that impair BCRP could modify the entry of ingested material to the circulation.

Plant polyphenols are substances that may be taken into the circulation in substantial amounts from the diet. These can be classified into flavonoids, stilbenes (such as resveratrol), phenolic acids, and lignans. Flavonoids are particularly abundant in fruits and vegetables, providing

☆ *Abbreviations:* ABC transporter, ATP-binding cassette transporter; BCRP, breast cancer resistance protein.

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much of the flavour and colour, and also in nuts, stems, flowers, wine, and tea. Several hundred milligrams a day of flavonoids may be consumed as part of the Western diet [7]. Polyphenols occur in nature predominantly as conjugates with sugars (glycosides) and upon ingestion undergo enzymatic cleavage to the free aglycone form in which they are absorbed into the epithelial cells of the gut wall. Ingested flavonoids undergo extensive Phase I and Phase II metabolism and are present in the circulation as a complex mixture of free aglycone with glucuronidated, methylated, and sulphated forms. However, cleavage by glucuronidases at several sites in the body can restore the aglycone form [8] which shows enhanced ability to partition across membranes and access intracellular sites due to its greater lipophilicity [9]. For instance, at the blood–brain barrier, cultured endothelial cells were shown to convert the glucuronides of the citrus flavonoids hesperetin and naringenin to their aglycone forms [10]. In addition, the non-polar aglycone may accumulate in lipophilic tissues [11] while certain conditions such as inflammation can elevate β-glucuronidase activity leading to higher levels of the physiologically active free aglycone in the plasma [8]. Certain flavonoids such as quercetin are capable of inhibiting steroid conjugating enzymes (e.g., sulphotransferases) in the liver [12] which may also lead to elevated levels of aglycones in vivo.

Flavonoids have many beneficial effects including antioxidant, antibacterial, antiviral, antiinflammatory, antiallergic, and anticarcinogenic actions [7,13] though whether these effects can be attributed to the aglycone forms or their metabolites is not entirely clear. Resveratrol, found in high concentrations in red wine, is a stilbene polyphenol with antioxidative, antiatherogenic, antiproliferative, and cardioprotective properties [14]. It has a structure similar to diethylstilbestrol, a known inhibitor of BCRP function [15].

Several studies have shown that flavonoids can modulate the activities of both P-glycoprotein and multidrug resistance-associated protein 1 (MRP1) [16,17] affecting drug accumulation, cell viability following cytotoxic drug exposure, and the ATPase activity of P-glycoprotein [16]. However, there are as yet no reports of interactions of the polyphenols with BCRP. Modulation of BCRP activity in vivo is known to influence bioavailability, distribution, hepatic clearance, and fetal entry of at least some of its substrates [3,4,18]. Given that the daily dietary intake of polyphenols could result in relatively high concentrations of the aglycone in the gut as well as to a lesser extent in the plasma, it is of value to establish whether these ubiquitous compounds in our diet could influence BCRP activity.

Here we investigate the effects of five different polyphenolic compounds on BCRP-mediated efflux activity, i.e., the flavonoids quercetin, hesperetin, silymarin and daidzein, and the stilbene resveratrol. We show that each of these compounds is able to increase accumulation of two separate BCRP substrates, mitoxantrone and bodipy-FL-prazosin, in two different BCRP-overexpressing cell types. Using inside-out vesicles prepared from BCRP-transformed *Lactococcus lactis* bacterial cells, we further demonstrate that these compounds stimulate the BCRP-associated ATPase activity. Taken together, these results provide compelling evidence that polyphenols interact with BCRP.

Materials and methods

Materials. Bodipy-FL-prazosin was from Molecular Probes. All other materials were obtained from Sigma unless otherwise stated. Silymarin consists of three flavonolignans, the most abundant being silybin [19]. The molecular weight of silybin was used to calculate molar concentrations. The mitoxantrone selected BCRP-overexpressing epithelial breast cancer cell line, MCF/MR [20], and its parent cell line MCF7/WT were kindly provided by Prof. Rik Scheper (Free University, the Netherlands) while the BCRP transfected human myelogenous leukemia cell line, K562/BCRP [15], and its parental cell line K562 were gifts from Prof. Yoshikazu Sugimoto (Japanese Foundation for Cancer Research). The specific BCRP inhibitor KO143 [21] was kindly provided by Prof. Alfred Schinkel (the Netherlands Cancer Institute). Cells were cultured in an atmosphere of 5% CO_2 in air at 37 °C in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco-BRL). MCF7/MR cells were maintained in 80 nM mitoxantrone. *L. lactis* strains NZ9000 and NZ9700 [22] were grown at 30 °C in M17 medium (Difco) supplemented with 0.5% glucose. Chloramphenicol (5 μg/ml) was added where appropriate.

Western blotting. Fifty micrograms of total cell lysate from K562, K562/BCRP, MCF7/WT, and MCF7/MR cells was subjected to SDS–PAGE, transferred to 0.45 μm nitrocellulose membranes (Amersham), and blocked as previously described [6]. Blots were probed overnight with a 1:100 solution of primary antibodies, either anti-BCRP BXP-21 (Signet, USA) or anti-P-glycoprotein C219 (ID Labs, Glasgow, UK) and then with peroxidase-conjugated anti-mouse secondary antibody (1:2000, Amersham) for 30 min. Proteins were detected using enhanced chemiluminescence (Amersham). All blots were stained post-transfer with Ponceau-S solution (Sigma) to verify equal protein loading in each well prior to probing.

Preparation of inside-out membrane vesicles and ATPase assay. Amplification of the BCRP gene from pcDNA3-BCRP by PCR and cloning into the lactococcal pNZ8048 expression vector to give pNZ-BCRP were as described previously [22]. BCRP-containing and control inside-out membrane vesicles were isolated from *L. lactis* NZ9000 cells harbouring pNZ-BCRP or pNZ8048, respectively. Measurement of the ATPase activity of BCRP in vesicles was based on the colorimetric reaction between ammonium molybdate and liberated P_i in the presence of ascorbic acid [22].

Flow cytometry. The effects of the polyphenols on intracellular accumulation of 5 μM mitoxantrone or 250 nM bodipy-FL-prazosin were determined by flow cytometry [23,24]. Cells (1×10^5) were preincubated with polyphenol (30 μM) or vehicle (0.5% DMSO) or the BCRP specific inhibitor KO143 (10 μM [21]) for 30 min in serum-free medium at 37 °C in a humidified atmosphere of 5% CO_2 in air, after which the substrate drug was added for a further 40 min incubation. The tubes were then placed on ice and the cells were washed once with cold PBS and re-suspended in cold PBS before analysis. Measurements of cellular fluorescence were made on a FacSCAN flow cytometer (Becton–Dickinson) with excitation at 488 nm and the emission recorded via 530/30 nm band pass (FL1, bodipy-FL-prazosin), and 670 long pass (FL3, mitoxantrone) filters. A total of 5000 cells were

analysed and non-viable cells were gated out based on forward and side scatter characteristics. The median fluorescences of cells were analysed by a repeated-measures ANOVA with Dunnet's post hoc test (PRISM 3, GraphPad Software).

Results and discussion

We have looked at the effects of a cross section of polyphenols on BCRP function using two well-characterized assays: flow cytometric measurement of mitoxantrone and bodipy-FL-prazosin accumulation [23,24] in two cell lines expressing high levels of BCRP, and stimulation of BCRP-associated ATPase activity in inside-out membrane vesicles derived from BCRP-transformed *L. lactis* bacterial cells [22].

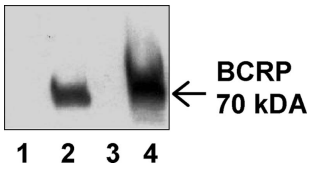

Fig. 1. Western blot of the expression of BCRP in K562 (lane 1), K562/BCRP (lane 2), MCF7/WT (lane 3), and MCF7/MR (lane 4) cells.

Western blotting analysis confirmed expression of high levels of BCRP in MCF7/MR and K562/BCRP cell lines while the respective wild-type cell lines had no detectable expression (Fig. 1). Higher levels of BCRP were evident in MCF7/MR cells than in K562/BCRP cells. No expression of P-glycoprotein was seen in any of the cell lines (data not shown).

Flow cytometry revealed that all the polyphenols increased accumulation of mitoxantrone and bodipy-FL-prazosin in both the BCRP-overexpressing cell lines but not in the wild-type cell lines. Initial experiments established that over the course of 90 min, the median fluorescence values for the two resistant cell lines

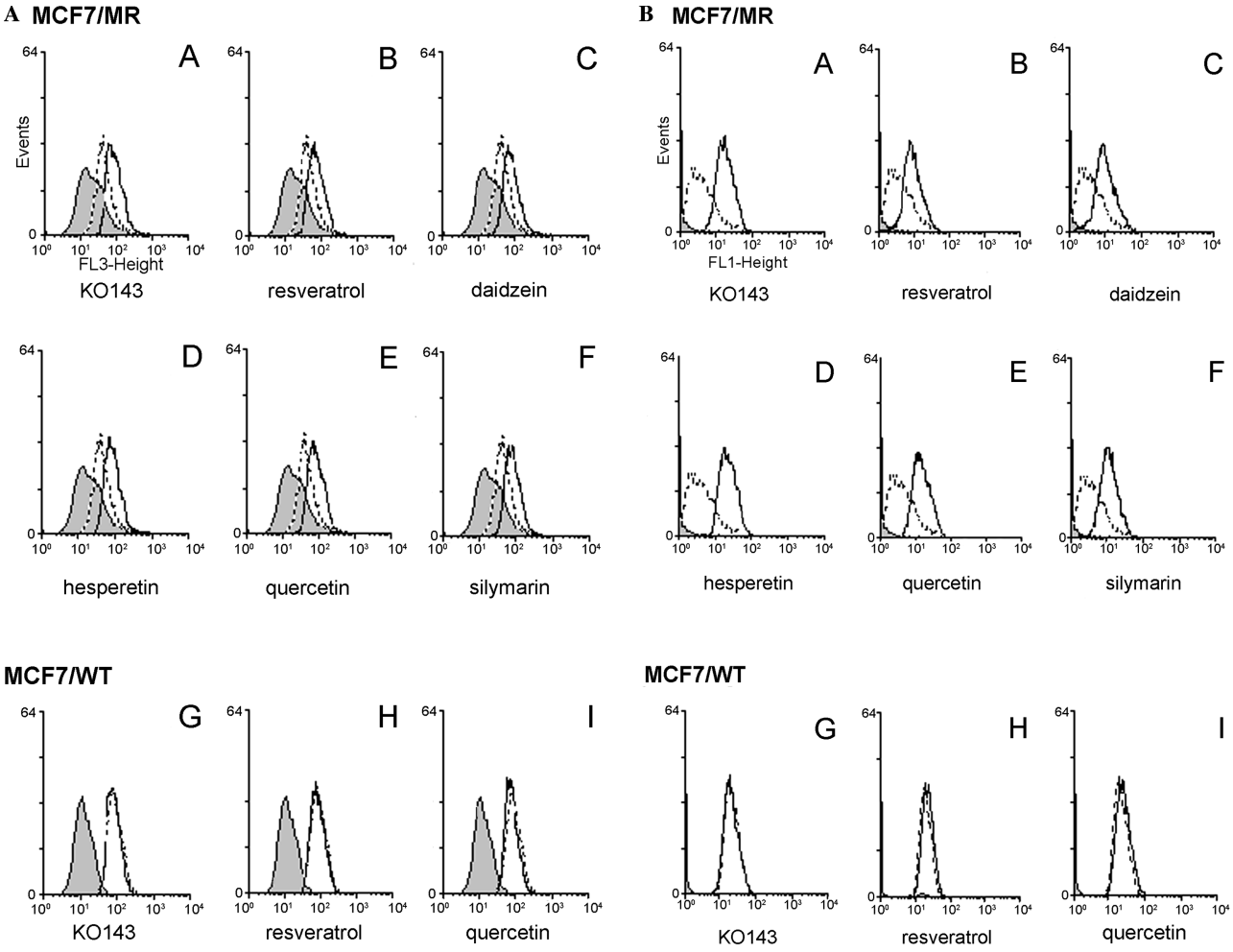

Fig. 2. Effects of plant polyphenols on the intracellular accumulation of 5 μM mitoxantrone (A) and 250 nM bodipy-FL-prazosin (B) in MCF7/MR (A–F) and MCF7/WT (G–I) cells. Cellular content of fluorescent substrate was analysed by flow cytometry after incubating cells with or without substrate in the presence or absence of test compounds (10 μM KO143 or 30 μM polyphenols). Shaded histogram indicates autofluorescence of cells; dotted line, cells with substrate alone; and solid line, cells with substrate and test compound. In MCF7/MR cells, a fluorescence peak shift of the control (dotted line) to the right (solid line) indicates increased accumulation of substrate in the presence of a test compound; no equivalent peak shift was seen with MCF7/WT cells. Results are representative of three independent experiments in each case.

increased to a plateau by 40 min following exposure to either substrate. In all subsequent experiments, a single time point of 40 min was chosen. In the MCF7/MR cell line, all five polyphenols at 30 μM as well as KO143 at 10 μM caused a right shift in the fluorescence peaks associated with mitoxantrone (Fig. 2A, histograms A–F) and bodipy-FL-prazosin (Fig. 2B, histograms A–F) though there were no comparable effects in the corresponding wild-type cells (Fig. 2, Table 1). Similar results were obtained for the K562/BCRP and K562 cell lines (Table 2). Used at 30 μM, the flavonoids alone did not contribute any intracellular fluorescence of their own nor did they alter cell shape or size as evidenced from the forward and side scatter profiles. The choice of 30 μM concentration was based on the previous report [15] that estrone, diethylstilbestrol or tamoxifen at 30 μM inhibits BCRP activity. These are compounds with close structural similarities with polyphenols. A concentration of 30 μM also falls midway between the extremes of polyphenol concentrations used in previous in vitro studies, ranging from nanomolar to micromolar and millimolar [25].

In three independent experiments, median fluorescence of cells treated with both substrate and polyphenol was significantly greater than that of cells treated with substrate alone for the two BCRP-overexpressing cell lines but not for the wild-type cell lines (Tables 1 and 2) implying inhibition of BCRP by the polyphenols. At 30 μM the citrus flavonoid hesperetin produced the largest inhibition of BCRP activity, similar to that produced by KO143 at 10 μM. The polyphenols produced greater increases in accumulation of substrate in MCF7/MR cells than in K562/BCRP cells, consistent with the greater expression of BCRP in the MCR7/MR cells (Fig. 1) and hence presumably a greater degree of efflux that can be inhibited.

It has been shown previously that BCRP expressed in the bacterium *L. lactis* [22] exhibits a high capacity, vanadate-inhibitable ATPase activity which can be stimulated by daunorubicin, a known BCRP substrate, as well as by a number of sterols. This activity is inhibited by the specific BCRP inhibitor fumitremorgin-C [22]. The present study shows that the established BCRP substrate mitoxantrone stimulates ATPase activity (Fig. 3A) and that this stimulation is blocked by the BCRP specific inhibitor KO143 (Fig. 3B). This system has been used to explore possible interactions of polyphenols with BCRP. All polyphenols tested showed a

Table 1
Effects of polyphenols or KO143 on the accumulation of mitoxantrone and bodipy-FL-prazosin in MCF7/WT and MCF7/MR cells

	5 μM mitoxantrone		250 nM bodipy-FL-prazosin	
	MCF7/WT	*MCF7/MR*	*MCF7/WT*	*MCF7/MR*
Control (substrate alone)	73 ± 6	26 ± 1	15.4 ± 3.4	2.5 ± 0.1
10 μM KO143	71 ± 3	78 ± 10	14.8 ± 3.2	16.6 ± 0.6
30 μM resveratrol	67 ± 3	72 ± 14	11.1 ± 3.1	6.8 ± 0.7
30 μM deidzein	66 ± 1	72 ± 11	13.9 ± 3.2	10.4 ± 1.6
30 μM hesperetin	68 ± 6	90 ± 21	21.3 ± 1.4	20.2 ± 0.7
30 μM quercetin	59 ± 4	84 ± 15	13.9 ± 1.6	14.6 ± 1.6
30 μM silymarin	71 ± 3	78 ± 13	13.7 ± 2.5	11.5 ± 0.8

Values correspond to the median fluorescence of 5000 cells as measured by flow cytometry after exposure to 5 μM mitoxantrone or to 250 nM bodipy-FL-prazosin. p values (repeated measures ANOVA, relative to control) were 0.19 (mitoxantrone) and 0.32 (bodipy-FL-prazosin) for the MCF7/WT cells and 0.0004 (mitoxantrone) and <0.0001 (bodipy-FL-prazosin) for the MCF7/MR cells. Dunnet's post hoc test for the resistant cells showed all treatments differed from control ($p < 0.05$); no differences were seen for the wild-type cells.

Table 2
Effects of polyphenols or KO143 on the accumulation of mitoxantrone and bodipy-FL-prazosin in K562 and K562/BCRP cells

	5 μM mitoxantrone		250 nM bodipy-FL-prazosin	
	K562	*K562/BCRP*	*K562*	*K562/BCRP*
Control (substrate alone)	80 ± 20	36 ± 8	117 ± 33	43 ± 8
10 μM KO	77 ± 22	79 ± 16	106 ± 27	152 ± 1
30 μM resveratrol	72 ± 18	62 ± 13	113 ± 27	83 ± 3
30 μM daidzein	73 ± 16	69 ± 16	110 ± 38	84 ± 3
30 μM hesperetin	77 ± 23	67 ± 16	122 ± 31	145 ± 3
30 μM quercetin	66 ± 18	60 ± 13	117 ± 28	103 ± 2
30 μM silymarin	81 ± 23	63 ± 19	111 ± 24	90 ± 9

Values correspond to the median fluorescence of 5000 cells as measured by flow cytometry after exposure to 5 μM mitoxantrone or to 250 nM bodipy-FL-prazosin. p values (repeated measures ANOVA, relative to control) were 0.31 (mitoxantrone) and 0.65 (bodipy-FL-prazosin) for the K562 cells and 0.0007 (mitoxantrone) and <0.0001 (bodipy-FL-prazosin) for the K562/BCRP cells. Dunnet's post hoc test for the resistant cells showed all treatments differed from control ($p < 0.05$); no differences were seen for the wild-type cells.

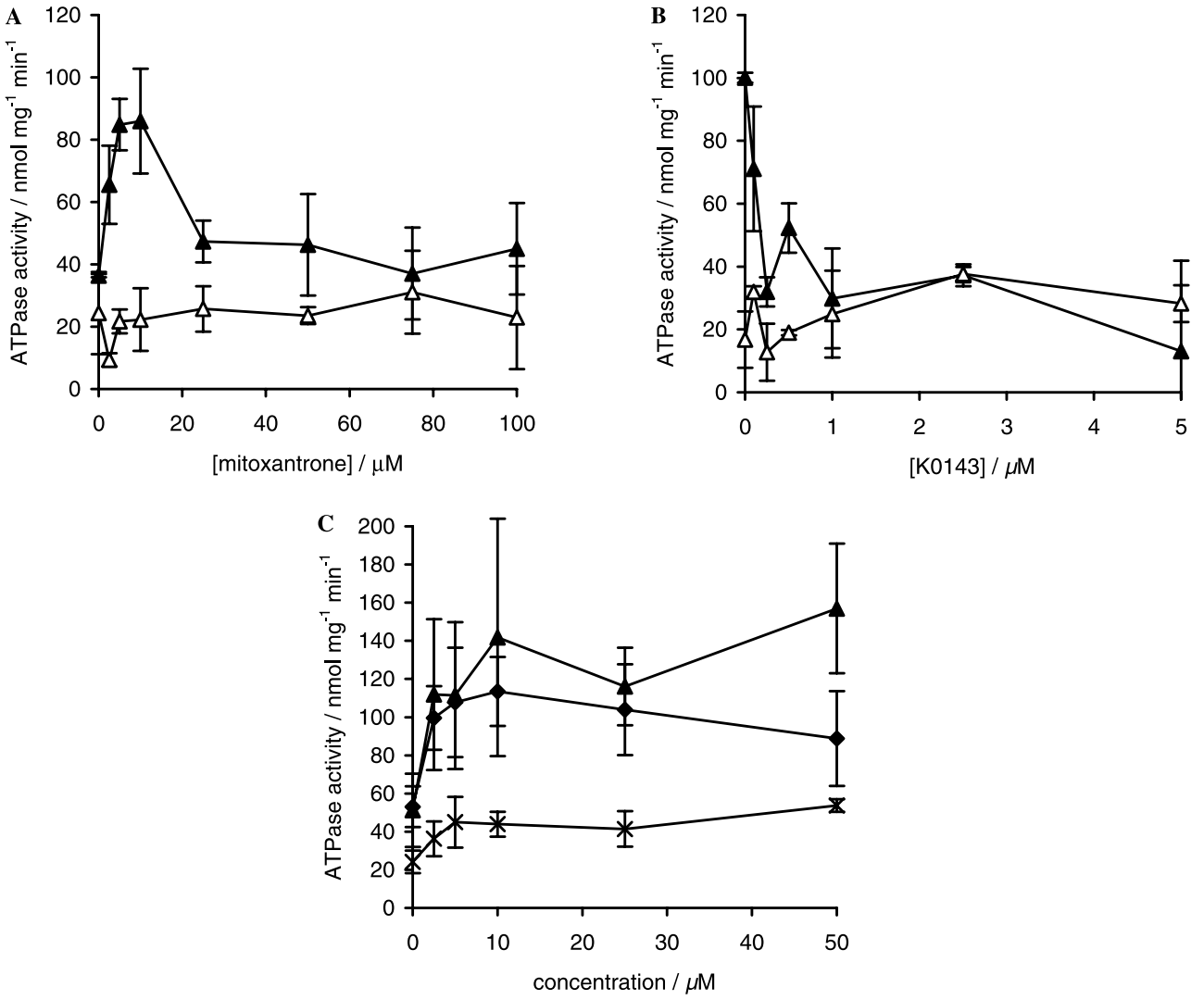

Fig. 3. BCRP-associated ATPase activity in inside-out membrane vesicles of *L. lactis*. (A) Vanadate-inhibitable ATPase activity of inside-out membrane vesicles containing BCRP (▲) and control membrane vesicles (△) in the presence of increasing concentrations of mitoxantrone. (B) Effect of increasing concentrations of the BCRP inhibitor KO143 on the mitoxantrone-stimulated vanadate-inhibitable ATPase activity of BCRP-containing membrane vesicles (▲) and control membrane vesicles (△). The mitoxantrone concentration was 10 μM. (C) Vanadate-inhibitable ATPase activity of inside-out membrane vesicles containing BCRP in the presence of increasing concentrations of quercetin (▲), daidzein (◆) or resveratrol (×). Stimulation of ATPase activity was observed for all of the polyphenols tested. Ratios of the activity at 25 μM to that of paired controls were daidzein 2.1 ± 0.6, hesperetin 1.3 ± 0.2, quercetin 4.4 ± 2.2, resveratrol 2.6 ± 1.4, and silymarin 6.8 ± 3.2. Hesperetin produced a larger stimulation at 75 μM, 3.9 ± 1.0. Data points represent the means ± SE of duplicate determinations (except mitoxantrone) from two independent experiments using different membrane vesicle batches.

stimulation of BCRP associated ATPase activity (Fig. 3C). No stimulation was evident in control vesicles derived from cells expressing the empty vector.

The ATPase activity detected here for BCRP expressed in a bacterial system compares well with that observed for BCRP overexpressed in Sf9 insect cells [26]. In that system, BCRP exhibits a high capacity, vanadate-inhibitable ATPase activity which can be stimulated by the BCRP substrates, mitoxantrone and prazosin, and inhibited by the BCRP specific inhibitor, fumitremorgin-C. Interpretation of ATPase activity data, however, has to be done with care as stimulation of the BCRP-associated ATPase activity in a previous study could not be directly correlated with actual transport of certain substrates [27]. Similarly, verapamil, a well-known P-gp inhibitor, is a very good stimulator of P-gp-associated ATPase activity [28]. Yet, no net transport of verapamil could be demonstrated. A plausible explanation is that being highly hydrophobic, verapamil may act as a 'fast-diffusing' substrate, partitioning rapidly back into the cell membrane as fast as it is pumped out and so creating a futile cycle of ATP hydrolysis without observable net translocation [28]. Given the hydrophobicity of the polyphenol aglycones employed in our study, it is tempting to speculate that they may also be 'fast-diffusing' substrates for BCRP, competitively inhibiting transport of

mitoxantrone and bodipy-FL-prazosin while stimulating its ATPase activity.

The demonstrable effects of the polyphenols on BCRP activity shown here should have some bearing on the actions of this multidrug transporter in vivo, particularly within the gut. High concentrations of the aglycone forms of polyphenols would be present here and so have the potential to modify efflux activity of BCRP. BCRP is known to influence the oral bioavailability of the cytotoxic drug, topotecan, in both P-glycoprotein knockout mice and in humans following treatment with BCRP inhibitors [18].

BCRP, like Pgp, has been localized to the luminal surface of the blood–brain barrier in humans [6]. Though there is as yet no evidence from in vivo transport studies to show that BCRP can modify entry of any of its substrates to the brain [18], this has been well documented for Pgp [29]. Results of a study using cultured brain endothelial cells suggested that the porcine paralogue of BCRP has a greater influence than P-glycoprotein on daunorubicin transport from the basolateral to the apical surface [30]. Direct extrapolation to the in vivo situation is difficult since species differences as well as changes in expression during endothelial cell culture [31] may account for these observations. Thus, the extent to which BCRP can influence transfer of material across the blood–brain barrier is still unclear. Polyphenols are thought to exert important neuroprotective effects, particularly under conditions of oxidative stress, due to their strong anti-oxidant properties [32]. They themselves may be effluxed by BCRP if indeed the polyphenols shown here to interact with BCRP are in fact BCRP substrates. It may thus be that the presence of BCRP at the blood–brain barrier might be influential in limiting entry of these potentially useful compounds to the brain.

The recent studies on the transport of steroid conjugates such as estrone sulphate [33] and estradiol 17β-glucuronide [5] by BCRP are of interest in addressing the possibility that this transporter may interact with polyphenol conjugates. If this were to be the case, BCRP expression at various sites would have profound global influences on the distribution of not only the aglycone but also the conjugate forms of polyphenols and their access to barrier sites such as the brain and placenta.

In conclusion, we have shown here that polyphenols interact directly with BCRP, modulating both its transport function and ATPase activity. It is possible that the tested polyphenols are directly transported by BCRP, though conclusive proof can only be provided by direct transport assays. Given that polyphenols form a significant portion of our diet, and that BCRP is expressed at several barrier sites in the body, further advances in our knowledge of the interactions of these compounds with this transporter will be of utmost significance.

Acknowledgments

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