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Reversal of in vitro cellular MRP1 and MRP2 mediated vincristine resistance by the flavonoid myricetin

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Abstract

In the present study, the effects of myricetin on either MRP1 or MRP2 mediated vincristine resistance in transfected MDCKII cells were examined. The results obtained show that myricetin can inhibit both MRP1 and MRP2 mediated vincristine efflux in a concentration dependent manner. The IC_{50} values for cellular vincristine transport inhibition by myricetin were 30.5 ± 1.7 μM for MRP1 and 24.6 ± 1.3 μM for MRP2 containing MDCKII cells. Cell proliferation analysis showed that the MDCKII control cells are very sensitive towards vincristine toxicity with an IC_{50} value of 1.1 ± 0.1 μM. The MDCKII MRP1 and MRP2 cells are less sensitive towards vincristine toxicity with IC_{50} values of 33.1 ± 1.9 and 22.2 ± 1.4 μM, respectively. In both the MRP1 and MRP2 cells, exposure to 25 μM myricetin enhances the sensitivity of the cells towards vincristine toxicity to IC_{50} values of 7.6 ± 0.5 and 5.8 ± 0.5 μM, respectively. The increase of sensitivity represents a reversal of the resistance towards vincristine as a result of MRP1 and MRP2 inhibition. Thus, the present study demonstrates the ability of the flavonoid myricetin to modulate MRP1 and MRP2 mediated resistance to the anticancer drug vincristine in transfected cells, indicating that flavonoids might be a valuable adjunct to chemotherapy to block MRP mediated resistance.
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Keywords: MRP1; MRP2; Flavonoids; Vincristine; Multidrug resistance; Myricetin

1. Introduction

Resistance to multiple anticancer drugs is a major obstacle for successful chemotherapy in cancer. This phenomenon, known as multidrug resistance (MDR), can be caused by different mechanisms. One such mechanism of MDR is the overexpression of membrane-bound drug efflux pumps like P-glycoprotein and the multidrug resistance proteins (MRPs), including MRP1 and MRP2 [1–3]. The MRPs belong to the ATP-binding cassette (ABC) transporter family and, at present, nine MRP-subfamily transporters have been identified which differ widely in substrate specificity, tissue distribution and intracellular location [3]. MRP1 (ABCC1) is a 190-kDa protein that transports a number of endogenous and exogenous organic anions and a wide variety of compounds conjugated to glutathione (GSH), glucuronate or sulphate [4]. MRP1 confers resistance to a variety of compounds like anthracyclines, epipodophylotoxins and some vinca alkaloids [3]. Although MRP1 is a typical glutathione-*S*-conjugate (GS-X) pump, chemotherapeutic agents that are not metabolized to a glutathione conjugate such as daunomycin, methotrexate, fluorouracil, chlorambucil and vinca alkaloids including vincristine are also substrates for MRP1 [5,6]. MRP2 (ABCC2) was originally identified as the canalicular multispecific organic anion transporter (cMOAT). Despite limited amino acid identity (49%) to MRP1, the spectrum of substrates transported by MRP1

Abbreviations: GSH, glutathione; GS-X, glutathione conjugate; IC_{50}, 50% inhibition concentration; MDR, multidrug resistance; MRP, multidrug resistance protein

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and MRP2 overlap to a large extent. Indeed, like MRP1, MRP2 confers in vitro cellular resistance to many anticancer drugs like vincristine [7–9], methotrexate [10] and anthracyclines [7,8], despite differences in cellular localization and kinetic properties.

In the present study, the possible reversal of cellular MRP1 or MRP2 mediated anticancer drug resistance was investigated using vincristine as the model anticancer drug. Vincristine has been demonstrated to be a suitable substrate for MRP1 and MRP2 mediated efflux studies and MRP1 and MRP2 have been reported to confer resistance towards this drug [7–9]. Vincristine is a member of the group of vinca alkaloids and has been used in anticancer chemotherapy since the 1960s [11]. Vincristine is a weak organic base and does not conjugate with GSH. However, GSH is required for vincristine resistance, as depletion of cellular GSH abolished MRP1 mediated resistance against vincristine [12,13]. Moreover, in vesicular transport experiments, transport of vincristine occurred only in the presence of reduced GSH [14,15].

One possible strategy for reversal of MRP mediated multidrug resistance is inhibition of the activity of these transport proteins. Several inhibitors of MRP1 and MRP2 have been described in the literature. Compounds such as sulphinpyrazone, benzbromarone and probenecid are relatively non-specific inhibitors of organic anion transporters [3,16]. Furthermore, some MRP1 inhibitors, like certain tricyclic isoxazoles, do inhibit MRP1 in intact cells at micromolar concentrations but are much less active against MRP2 [17].

The search for MRP inhibitors showed that many natural constituents, including plant polyphenols like flavonoids were promising candidates for both MRP1 and MRP2 inhibition [18–23]. Recently, we described structure activity relationship studies on inhibition of MRP1 and MRP2 mediated calcein efflux by flavonoids in MRP transfected MDCKII cells [23]. It was demonstrated that many flavonoids, including myricetin, robinetin and quercetin, are able to inhibit MRP1 activity. Regarding inhibition of MRP2 activity, myricetin and robinetin appeared to be particularly good inhibitors [23].

Therefore, in the present study, the effects of myricetin (Fig. 1) on either MRP1 or MRP2 mediated vincristine resistance in transfected MDCKII cells are examined. The possible use of myricetin for reversal of MRP mediated multidrug resistance is discussed.

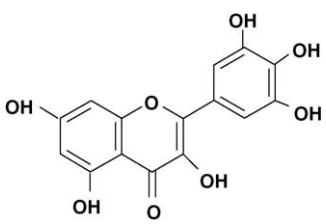

Fig. 1. Structural formula of myricetin.

2. Materials and methods

2.1. Materials

The Madin–Darby Canine Kidney (MDCKII) cell lines, stably expressing either a control vector (hereafter, called control cells), human *MRP1* cDNA (hereafter, called MRP1 cells) or *MRP2* cDNA (hereafter, called MRP2 cells) were kindly provided by Prof. P. Borst (NKI, Amsterdam).

Dulbecco's Minimum Eagle Medium (DMEM) with GlutaMax, fetal bovine serum, penicillin/streptomycin and gentamycin were all purchased from Gibco (Paisley, Scotland). MK571 was obtained from BioMol (Plymouth Meeting, PA); PSC833 was a kind gift from Novartis Pharma AG (Basel, Switzerland). Cyclosporin A was obtained from Fluka (Zwijndrecht, The Netherlands). [^{3}H]-vincristine sulphate (4.9 Ci/mmol) was obtained from Amersham Biosciences (Buckinghamshire, UK). Flo-Scint scintillation cocktail was purchased from Packard (Groningen, The Netherlands). Myricetin, was purchased from Sigma Chemical Co. (Zwijndrecht, The Netherlands). The cell proliferation ELISA BrdU-kit was purchased from Roche Molecular Biochemicals (Mannheim, Germany). DMSO and HPLC-grade methanol were obtained from Acros Organics (Geel, Belgium).

2.2. MDCKII cell culture

The Madin–Darby Canine Kidney cell lines (control and MRP1 or MRP2 transfected) were cultured in Dulbecco's Minimum Essential Medium with GlutaMax (4.5 g glucose/l), 10% fetal calf serum and 0.01% penicillin/streptomycin, and were grown in a humidified atmosphere in 5% CO_2 at 37 °C.

2.3. Vincristine transport by MRP1 and MRP2

For transport experiments 4×10^5 cells/cm^2 were grown on microporous polycarbonate filters ((0.4 μm pore size, 1 cm^2) Costar Corp. Cambridge, MA). It was shown earlier [24] that in these polarized cells MRP1 routes to the basolateral plasma membrane, whereas MRP2 routes to the apical plasma membrane. Culturing MDCKII cells on a filter in transwells provides the opportunity to study the MRP mediated efflux of a compound to either the apical or basolateral side of intact cells. Although these cell lines do contain low endogenous MRP-like and/or other efflux transporters, the levels of the introduced MRPs are very profound which makes these cell lines ideal for comparative studies on the role of MRP1 or MRP2 in drug resistance [25,26]. The volumes of media in the basolateral and apical compartments were 1.8 and 0.5 ml, respectively. Cells were cultured to confluency for three days and medium was replaced every 24 h. Confluency of the monolayers was checked by transepithelial electric resistance

(TEER) measurement, validated by determination of the paracellular flux of inulin[^{14}C]carboxylic acid (185 kBq/mol, 4.2 μM) [27]. TEER-values of each monolayer were measured using a Millicell-ERS epithelial volt/ohm meter (Millipore, Bedford). The corrected TEER-value of a confluent monolayer of MDCKII cells ranged between 120 and 140 Ω cm^2 as reported before [26]. Three days post-seeding, the cells were loaded with 0.5 μM [^{3}H]-vincristine (2.8 μCi/well) in DMEM without phenol red containing 0.1 μM PSC833 (to exclude any possible effects of P-glycoprotein), for 2 h at 37 °C. The use of the cyclosporin derivative PSC833 as a Pgp inhibitor was used based on studies by Evers et al. [24]. A relatively low concentration of PSC833 (0.1 μM) was shown to completely inhibit apical efflux of vinblastine from the control cells, whereas the vinblastine efflux by MRP2 in the MRP2 cells appeared not to be affected by this dose [24]. Furthermore, experiments were performed to test this concentration (0.1 μM) of PSC833 on MRP1 and MRP2 vincristine transport by the transfected cells, which showed no changes in respectively basolateral or apical efflux characteristics (data not shown). These findings support the use of 0.1 μM PSC833 as a rather specific inhibitor of Pgp that does not affect MRP1 and MRP2 mediated transport in the MRP1 and MRP2 transfected cell lines. Experiments to determine optimal loading conditions revealed that for the MRP transfected cell lines, upon exposure to vincristine for 2 h at 37 °C, the intracellular amount of vincristine reached up to 2.4% of the total amount of vincristine present. For the control cells, the intracellular amount of vincristine reached up to 3.3% of the total amount of vincristine present. After loading of the cells at 37 °C, efflux of vincristine was determined as follows. Loaded cells were washed twice with DMEM without phenol red and exposed to fresh medium (DMEM without serum at 37 °C) containing 0.1 μM PSC833 and different concentrations of myricetin (0–50 μM), 30 μM MK571 (as a typical MRP1 inhibitor) or 30 μM cyclosporin A (as a typical MRP2 inhibitor) [28]. Cells receiving vehicle only (0.5% DMSO, v/v) served as control (blank). Efflux of vincristine at various time-points was measured in media samples from both the apical and basolateral compartment upon 60 min exposure to myricetin, MK571 or cyclosporin A. After 60 min, the efflux medium was removed and cells were washed three times in ice-cold PBS. The filters with cells were placed in KOH solution containing 20% methanol for 24 h to disrupt the cells for measurement of the intracellular radioactivity. Radioactivity was counted using a Packard 1600 Liquid Scintilator with Packard Ultima Gold as scintillation cocktail (Packard, Groningen, The Netherlands).

2.4. Vincristine detection

Samples taken from the efflux medium after 20, 40 or 60 min were analyzed for vincristine metabolites using reversed-phase HPLC according to a method developed by Tikhomiroff and Jolicoeur to detect the major indole alkaloids of *Catharanthus roseus* (e.g. vincristine) and their metabolites [29]. In short, analysis was carried out using a Merck Hitachi HPLC system equipped with a L6200 pump and a L4200 UV–vis detector combined with a Packard Flo-One on-line radioactivity detector using Flo-Scint as scintillation cocktail. For a typical run, 50 μl of the samples were injected onto a Zorbax Eclipse XDB-C_{18} 250 mm × 4.6 mm column. The column was eluted at a flow rate of 0.8 ml/min by isocratic elution at 95% A (5 mM Na_2HPO_4 pH 6) and 5% B (acetonitrile) for 20 min followed by a linear gradient to 15% B in 10 min, and finally a linear gradient to 80% B in 5 min.

2.5. Measurement of intracellular glutathione

To determine the effect of myricetin exposure on the intracellular GSH levels the three MDCKII cell lines were treated similar as for the efflux experiments described above. After 1 h exposure to 0 or 25 μM myricetin, the cellular fractions were analyzed using the DTNB-GSSG reductase recycling assay as described by Baker et al. [30].

2.6. Cell proliferation

The effect of myricetin on the inhibition of cell proliferation by the anticancer drug vincristine was determined for the control and the MRP1 or MRP2 transfected MDCKII cells using the BrdU cell proliferation assay, adapted for transwells, using the Cell Proliferation ELISA, BrdU (colorimetric) kit from Roche Diagnostics (Mannheim, Germany). For the assay 0.5×10^5 cells/cm^2 were grown on microporous polycarbonate filters ((0.4 μm pore size, 0.33 cm^2) Costar Corp. Cambridge, MA). The volume of media in the basolateral and apical compartments was 0.6 and 0.1 ml, respectively. After 24 h, the cells were exposed to a range of vincristine concentrations (0.01–100 μM) in DMEM without phenol red containing 0.1 μM PSC833 (to exclude any possible effects of P-glycoprotein), for 2 h at 37 °C. After this exposure, cells were rinsed twice with DMEM without phenol red and cultured in normal culture medium for 24 h in the presence or absence of inhibitors. Final concentrations of the inhibitors used were 25 μM myricetin, 30 μM MK571 or 30 μM cyclosporin A. Higher myricetin concentrations showed inhibitory effects on the cell proliferation scores upon 24 h exposure and could therefore not be tested. After 24 h culturing, cell proliferation was determined by labeling the cells with BrdU for 2 h at 37 °C. Absorbance of the converted substrate tetramethyl-benzidine was measured at 370 nm using a Thermomax microplate reader (Molecular Devices Corp., Menlo Park, CA, USA). Results were expressed as percentage cell proliferation compared to the blank (vehicle only).

2.7. Cytotoxicity

Toxicity of different concentrations of myricetin was measured using the LDH-leakage method for cell viability [31] with some minor adaptations for transwell plates. Cells were grown to confluent monolayers as described for the efflux assays. The cells were exposed to different concentrations myricetin in both compartments for 24 h. Before measurements of the LDH activity in the medium, the samples from the apical (0.6 ml) and basolateral (1.0 ml) compartments were pooled. The filter membranes containing the cells were washed twice with cold PBS and removed from the inserts. Cells were sonicated in 1 ml PBS and all samples were stored at −20 °C until analysis of the LDH activity.

2.8. Data analysis

A one-way analysis of variance test was used for all data analysis ($P < 0.05$) using SPSS 10.1.0 software from SPSS Inc.

3. Results

3.1. Vincristine transport inhibition in MDCKII MRP1 and MRP2 cells

To study the efflux of vincristine by MDCKII MRP1 and MRP2 cells, the cells were loaded with 0.5 μM [^{3}H]-vincristine (2.8 μCi/well) for 2 h at 37 °C. Table 1 shows the total amounts of intracellular vincristine upon loading. Upon loading, the two MRP transfected cell lines reached comparable total accumulated vincristine quantities that amounted to a maximum of 11.8 ± 0.6 pmol. For the control cell line, the total accumulated amount of vincristine reached to a maximum of 16.2 ± 1.0 pmol, a value that was significantly higher ($P < 0.05$) than the amount of vincristine reached in the MRP1 or MRP2 cells. After loading, the cells were exposed to fresh efflux medium with or without inhibitor for 60 min at 37 °C. After 60 min, a maximum of ± 20% (2.2 pmol) of the total amount of vincristine present after loading of the cells appeared to be excreted to the medium (Table 1).

Fig. 2 shows the efflux of vincristine to the basolateral and apical side from vincristine loaded cells (A) MDCKII control, (B) MDCKII MRP1 and (C) MDCKII MRP2 cells upon exposure to 0 or 25 μM myricetin. In the control cells, only limited vincristine efflux took place. In MRP1 cells, vincristine was predominantly excreted to the basolateral side (12 times higher than apical efflux), whereas in MRP2 cells, the efflux of vincristine was predominantly to the apical side (4 times higher than basolateral efflux). Furthermore, the basolateral efflux of vincristine by MRP1 cells after 60 min (2.0 ± 0.2 pmol/monolayer) appeared to be almost twice the apical efflux of vincristine by MRP2 cells after 60 min (1.1 ± 0.1 pmol/monolayer). In the presence of 25 μM myricetin the basolateral MRP1 mediated efflux of vincristine was reduced by 52 ± 8% to 1.0 ± 0.3 pmol/monolayer. For MRP2, the presence of 25 μM myricetin reduced the apical MRP2 mediated vincristine efflux by 41 ± 5% to 0.7 ± 0.2 pmol.

3.2. Intracellular vincristine concentrations

One of the goals that should be reached to reverse MRP mediated multidrug resistance is preservation of the intracellular drug concentration through inhibition of MRP

Table 1
Total accumulated vincristine amounts upon 2 h loading and the distribution of vincristine after 60 min efflux at the apical, basolateral and the intracellular compartment from the vincristine loaded control, MRP1 or MRP2 cells in the absence or presence of various MRP inhibitors

	Total amount of VCR at $t = 0$[a] (pmol)	Amount of VCR at apical compartment at $t = 60$ (pmol)	Amount of VCR at basolateral compartment at $t = 60$ (pmol)	Amount of VCR at intracellular compartment at $t = 60$ (pmol)	% VCR intracellular of total
Control					
Blank	16.2 ± 1.0	0.1 ± 0.1	0.3 ± 0.1	15.8 ± 1.1	98
Myricetin		0.1 ± 0.1	0.3 ± 0.1	15.8 ± 0.9	98
MRP1					
Blank	11.8 ± 0.6[‡]	0.2 ± 0.1	2.0 ± 0.2	9.3 ± 0.7	79
Myricetin		0.1 ± 0.1	1.0 ± 0.3[*]	10.2 ± 0.5	87
MK571		0.1 ± 0.1	0.2 ± 0.1[*]	11.5 ± 0.6[*]	97
MRP2					
Blank	10.7 ± 0.7[‡]	1.1 ± 0.1	0.3 ± 0.1	8.8 ± 0.4	83
Myricetin		0.7 ± 0.2[*]	0.3 ± 0.1	9.7 ± 0.4[*]	91
CsA		0.4 ± 0.2[*]	0.4 ± 0.1	9.9 ± 0.5[*]	93

Concentrations used were 25 μM for myricetin, 30 μM for MK571 and 30 μM for cyclosporin A (CsA). Results represent average ± S.D. from triplicate measurements.

[a] The total amount of vincristine was measured after 2 h loading time without inhibitors.

[*] Statistically significant different from corresponding cells exposed to vehicle control (blank) ($P < 0.05$).

[‡] Statistically significant different from control cells ($P < 0.05$).

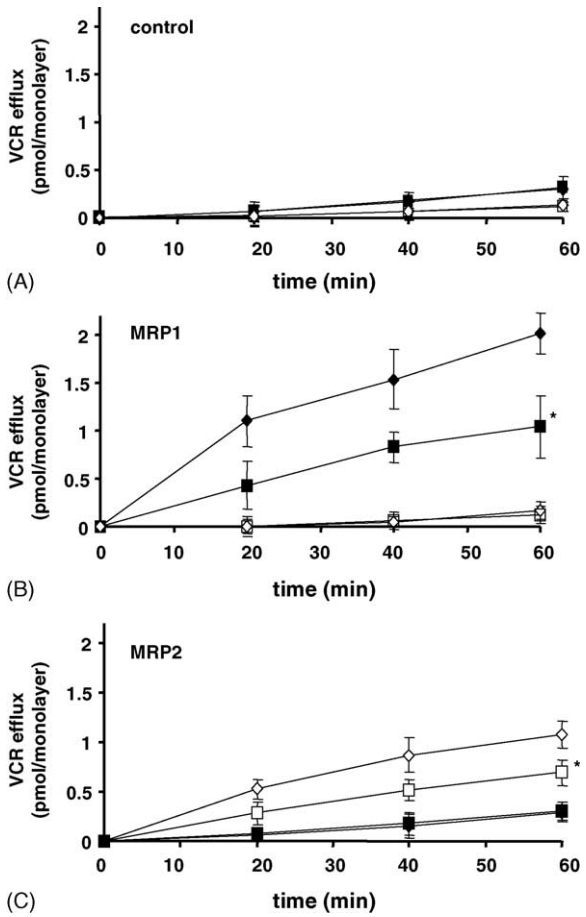

Fig. 2. Vincristine efflux to the basolateral (closed symbols) and apical (open symbols) side by (A) MDCKII control, (B) MDCKII MRP1 and (C) MDCKII MRP2 cells upon exposure to 0 μM (◆, ◇) or 25 μM (■, □) myricetin for 60 min. Data points represent the mean ± S.D. from triplicate measurements Asterisks (*) represents statistically significant differences from the cells exposed to vehicle only (blank) ($P < 0.05$).

mediated drug efflux. Table 1 summarizes the effects of 25 μM myricetin and two typical MRP inhibitors, 30 μM MK571 for MRP1 and 30 μM cyclosporin A for MRP2, on the efflux of vincristine to the apical and basolateral compartments of the three transfected MDCKII cell lines. Furthermore, the accompanying effects of efflux inhibition on the intracellular vincristine concentration in MDCKII control, MRP1 and MRP2 cells are shown in Table 1. From these results, it can be derived that inhibition of MRP1 mediated vincristine efflux by 25 μM myricetin at the end of the 1 h efflux period resulted in a reduced decrease of the intracellular vincristine amount from 79% in the absence, up to 87% of the original quantity after loading of the cells in the presence of myricetin. Exposure to the typical MRP1 inhibitor MK571 results in a residual intracellular vincristine amount of 97% of the original quantity after loading. Thus, the effect of 30 μM MK571 on maintenance of the intracellular vincristine amount in MRP1 cells is almost maximal whereas 25 μM myricetin significantly, but not fully, prevents vincristine efflux from the cells. For MRP2, it can be seen that in the presence of 25 μM myricetin the intracellular vincristine amounts up to 91% of the original quantity after loading of the cells, instead of the 83% observed in the absence of myricetin. Exposure to 30 μM of the typical MRP2 inhibitor cyclosporin A results in intracellular vincristine amounts at the end of the 1 h efflux that amounts to 93% of the original amount present after loading of the cells. This effect was almost similar to the effect of 25 μM myricetin.

As observed, the effects of 25 μM myricetin on vincristine efflux inhibition and intracellular vincristine quantities are not optimal, since only partial inhibition of the vincristine efflux was observed. To determine whether the inhibition of MRP mediated vincristine efflux by myricetin was concentration dependent, experiments with myricetin concentrations up to 50 μM myricetin were performed. Fig. 3 shows the effects of increasing myricetin concentrations on the relative increase of the residual intracellular vincristine amount after 60 min efflux for MDCKII MRP1 and MRP2 cells. The results obtained reveal that myricetin inhibits vincristine efflux, thereby increasing the intracellular vincristine concentration, in a concentration dependent manner. Interestingly, 50 μM myricetin almost completely inhibits vincristine efflux in both cell lines to give an effect identical to the typical MRP inhibitors MK571 and cyclosporin A (Table 1). Based on the data displayed in Fig. 3, the estimated IC_{50} values, representing the concentrations at which 50% of the vincristine efflux is inhibited, are 30.5 ± 1.7 μM for MRP1 and 24.6 ± 1.3 μM for MRP2. Additionally, the figure displays the effects of increasing myricetin concentrations on the cell viability of the MDCKII MRP1 and MRP2 cells upon 24 h incubation. The cell lines showed comparable sensitivity towards myricetin with 50 μM myricetin being slightly cytotoxic to the cells. However, upon incubation of the cells with increasing concentrations myricetin for only 1 h, identical to the efflux assay performed in this study, no significant toxicity was observed (data not shown).

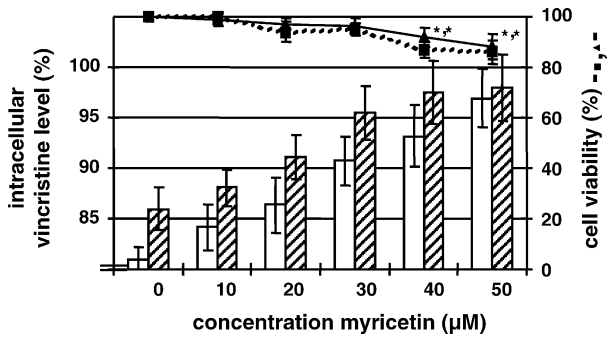

Fig. 3. Relative intracellular vincristine levels compared to the vincristine levels present at $t = 0$ in MDCKII MRP1 cells (blank bars) and MDCKII MRP2 cells (striped bars) after 60 min efflux in the presence of varying concentrations of myricetin. The *y*-axis at the right displays the effects of increasing myricetin concentrations on MDCKII MRP1 (▲) or MRP2 (■) cell viability upon 24 h incubation. Asterisks (*) represent statistically significant differences from the cells exposed to vehicle control (blank) ($P < 0.05$). Data points represent the mean ± S.D. from duplicate measurements.

3.3. Stability of vincristine

To determine whether vincristine does not become metabolized during the efflux experiments, HPLC radioactivity analysis was performed. The analyses showed that in all samples analyzed, only one major peak (>95% of the total radioactivity) was observed which was identified as vincristine itself. The presence of only one major peak in the HPLC-radioactivity chromatogram confirms that vincristine does not become metabolized during the time course of the efflux experiments.

3.4. Effects of myricetin on intracellular GSH levels

The intracellular GSH levels differs for the three cell lines tested. Whereas the control cells contains 31.3 ± 0.8 nmol/monolayer and the MRP2 cells contain 27.4 ± 2.0 nmol/monolayer, the MRP1 cells contain approximately 10 times less GSH (3.6 ± 0.6 nmol/monolayer). Upon exposure to 25 μM myricetin, neither the GSH levels in the control cells nor in the MRP2 cells are affected. In contrast, in the MRP1 cells the GSH levels decrease with approximately 65% to 1.3 ± 01 nmol/monolayer when exposed to 25 μM myricetin for 1 h.

3.5. Effects of myricetin on vincristine toxicity in transfected MDCKII cells

The ability of myricetin to sensitize MRP1, or MRP2 transfected MDCKII cells to the cytotoxic effects of vincristine was tested by measuring cell proliferation.

Fig. 4 shows the effects of 25 μM myricetin on vincristine sensitivity of control (A and B) and MRP1 (A) or MRP2 (B) transfected MDCKII cells. Fig. 4A reveals that the MDCKII MRP1 cells are less sensitive to vincristine toxicity than the MDCKII control cells, a phenomenon ascribed to the presence of MRP1. When the vincristine loaded MDCKII MRP1 cells are exposed to 25 μM myricetin the curve shifts to the left, demonstrating an increase of the sensitivity towards vincristine toxicity. Fig. 4B shows that upon comparison of the chemosensitivity of the MDCKII control cells and the MDCKII MRP2 cells, the MDCKII MRP2 cells also are less sensitive to vincristine toxicity, an observation ascribed to the presence of MRP2. However, this effect is less profound than for MRP1. When the vincristine loaded MDCKII MRP2 cells are exposed to 25 μM myricetin the curve also shifts to the left, again demonstrating an increase in sensitivity to vincristine toxicity. Addition of myricetin or the typical MRP inhibitors MK571 and cyclosporin A to vincristine loaded MDCKII control cells does not affect the vincristine chemosensitivity of these cells. Table 2 lists the IC_{50} values and the relative resistance factors, calculated as the relative ratios between IC_{50} values, derived from the various curves. These results show that MDCKII cells become 30-fold less sensitive to vincristine due to the presence of MRP1 and 20-fold less sensitive due to the presence of MRP2. Inhibition of MRP mediated vincristine efflux by 25 μM myricetin significantly reduces the resistance factor for both MRP1 and MRP2 to seven- and five-fold, respectively.

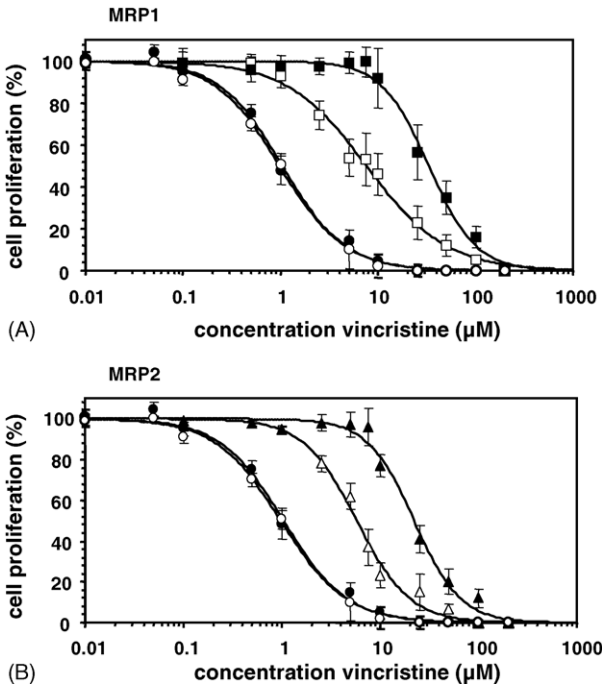

Fig. 4. Effects of myricetin on vincristine sensitivity in (A) control and MRP1 or (B) control and MRP2 transfected MDCKII cells. Vector control transfected MDCKII cells (●, ○), MRP1 transfected MDCKII cells (■, □) and MRP2 transfected MDCKII cells (▲, △) were incubated in the presence (○, □, △) or absence (●, ■, ▲) of 25 μM myricetin. Data points represent the mean ± S.D. from triplicate measurements.

Table 2
IC_{50} values and relative resistance factors for vincristine toxicity in MDCKII control cells (control vector transfected), MDCKII MRP1 cells and MDCKII MRP2 cells in the absence (blank) or presence of 25 μM myricetin, 30 μM MK571 or 30 μM cyclosporin A (CsA)

	IC_{50} (μM)	Relative resistance factor[a]
Control		
Blank	1.1 ± 0.1	–
Myricetin	1.0 ± 0.2	0.9
MRP1		
Blank	33.1 ± 1.9	30
Myricetin	7.6 ± 0.5*	7
MK571	5.1 ± 1.0*	5
MRP2		
Blank	22.2 ± 1.4	20
Myricetin	5.8 ± 0.5*	5
CsA	5.2 ± 0.8*	5

Results are mean ± S.D. from triplicate measurements.
[a] The relative resistance factor was calculated by dividing the IC_{50} value of cells transfected with MRP1 or MRP2 expression vectors by the IC_{50} values of cells transfected with the control vector (control cells) and exposed to vehicle control (blank).
* Statistically significant difference from IC_{50} value for the control cells exposed to vehicle control (blank) ($P < 0.05$).

4. Discussion

Inhibitors of drug transporters, like MRP1 and MRP2, are potentially useful tools to reverse transporter mediated cellular resistance to anticancer drugs and, eventually, to enhance the effectiveness of the treatment of patients with drug-resistant cancer. In this study, we have tested the ability of the flavonoid myricetin to inhibit the efflux of the anticancer drug vincristine by two drug transporters, MRP1 and MRP2, in transfected MDCKII cells. In previous studies, it was shown that myricetin is a suitable inhibitor of MRP1 and MRP2 activity [20,22,23]. However, in these studies it was also shown that the magnitude of MRP1 or MRP2 inhibition by myricetin might vary with the type of substrate as well as with the substrate and inhibitor concentrations and the in vitro test system used. Therefore, the in vivo effects of myricetin on MRP mediated vincristine resistance are not easily extrapolated from these in vitro studies. The present study examines the use of myricetin to reverse MRP mediated vincristine resistance in an in vitro model. The results obtained show concentration dependent modulation of MRP1 and MRP2 mediated vincristine efflux in MDCKII cells by myricetin, reaching almost complete inhibition of the efflux at 50 μM concentration. The IC_{50} values, representing the concentrations of myricetin at which 50% of the vincristine efflux is inhibited, are 30.5 ± 1.7 μM for MRP1 MDCKII and 24.6 ± 1.3 μM for MRP2 MDCKII cells. These values are in the same order of magnitude as the IC_{50} values previously described for the inhibition of MRP1 and MRP2 mediated calcein efflux by myricetin of 20.2 ± 4.3 and 22.2 ± 3.9 μM, respectively [23]. The mechanism involved in MRP mediated vincristine efflux inhibition by flavonoids is not known although for calcein it was shown that the flavonoid robinetin inhibits calcein efflux by both MRP1 and MRP2 in a competitive way.

The observed decrease of the intracellular GSH amounts in the MRP1 cells upon exposure to 25 μM myricetin is most likely the result of increased GSH efflux by MRP1 rather than by a decrease of GSH synthesis since GSH levels in the other two cell lines were unaffected by myricetin. Increased efflux of GSH in the MRP1 cells upon exposure to myricetin is in accordance with a study by Leslie et al., who demonstrated that some flavonoids stimulate MRP1 mediated GSH transport by increasing the apparent affinity of the transporter for GSH, although no evidence was found that a co-transport mechanism is involved [32]. However, current opinion suggests that an increase of GSH efflux could be accompanied by an increase, not a decrease, in the efflux of vincristine, since vincristine efflux is under allosteric regulation by GSH [33]. The finding that the MRP1 cells contain approximately 10 times less GSH (3.6 ± 0.6 nmol/monolayer) than the control cells (31.3 ± 0.8 nmol/monolayer) and the MRP2 cells (27.4 ± 2.0 nmol/monolayer) are in accordance to previous findings by Wortelboer et al. for the same cells. The decrease of intracellular GSH levels in the MRP1 cells, upon exposure to myricetin was only observed for the MRP1 cells and not for the MRP2 cells or the control cells. Because the effects of myricetin on MRP1 and MRP2 mediated efflux of vincristine and the corresponding chemosensitizing effects are comparable for both cell lines, it is unlikely that enhanced GSH efflux is an explanatory mechanism for the results obtained. Apparently another, inhibitory mechanism, exists in which flavonoids most likely compete with the substrate at the substrate-binding site. The inhibitory effects of flavonoids on the ATPase activity of MRPs might also be one of the mechanisms by which flavonoids inhibit MRP activity [20,34]. The diverse effects of flavonoids on MRPs are confirmed by a study of Trompier et al. in which it was shown that the flavonoid dehydrosilybin and its derivatives interact with multiple-binding sites of MRP1, located in both cytosolic and transmembrane domains of MRP1 [35].

From comparison of the vincristine sensitivity of the MDCKII control cells and the MRP1 or MRP2 transfected cells, it is concluded that these MRPs both decrease the cellular sensitivity to vincristine. This decrease in sensitivity represents the MRP mediated resistance towards vincristine. Incubation in the presence of myricetin resulted in an increase in the vincristine sensitivity of the MRP1 and MRP2 transfected MDCKII cells, although vincristine sensitivity is not increased to the level seen for the MDCKII control cells. The myricetin concentration used (25 μM) was shown not to completely inhibit vincristine efflux by MRP1 and MRP2, and this may in part explain the partial instead of full reversal of the vincristine sensitivity. Inhibition of MRP mediated vincristine efflux by 25 μM myricetin significantly reduces the resistance factor for both MRP1 and MRP2 to seven- and five-fold, respectively. However, these changes in chemosensitivity of the MRP1 and MRP2 cells do not result in a sensitivity similar to that of the control cells. Interestingly, upon exposure to the typical MRP inhibitors MK571 or cyclosporin A that were shown to inhibit vincristine efflux almost to the maximal extent, the reduction of the resistance factor also does not reach the level observed for the control cells, reflecting that also in these MK571 or cyclosporin A exposed MRP cells the vincristine sensitivity does not reach the level of the control cells.

The data presented in Table 1 provide an explanation for this observation. Upon 2 h loading of the cells, the amount of vincristine accumulated in the control cells appeared to be more than 40% higher than the vincristine levels accumulated in the two MRP transfected cell lines. Since the loading conditions in the cell proliferation assay were identical to the loading conditions used in the efflux assay, it can be concluded that in the cell proliferation assay a comparable loading difference between the control cells and the MRP cells has been present. This higher vincristine loading level in the control cells is likely to be due to the absence of vincristine efflux during the loading period. As

a result, IC_{50} values in the subsequent cell proliferation assay are lower for the control cells because at lower vincristine concentrations in the medium, higher intracellular vincristine levels are achieved. Since, due to the presence of the MRP protein, loading levels in the MRP cells are lower than in the control cells, intracellular vincristine levels in the MRP cells at a specific medium vincristine concentration will never be as high as those in the control cells. As a result, IC_{50} values obtained for the MRP cells will never be as low as those observed in the control cells, not even in the presence of more efficient inhibitors.

Flavonoids are substances that can interact with several different physiological pathways. Not only are they considered as good antioxidants, they can also exhibit anti-inflammatory, anti-tumor, anti-thrombogenic and anti-viral effects [36]. In theory, flavonoids might serve as potent inhibitors of MRP1 and MRP2 for potential clinical use to reverse multidrug resistance since they are generally regarded as safe and relatively non-toxic [37]. It can be argued that flavonoid usage in clinical settings might show negative side effects as a result of the inhibitory effects on other enzymes such as topoisomerases, cytochromes P450, protein kinases and other transporters like P-glycoprotein and ABCG2 (BCRP) [19,38–40]. However, these possible effects need to be examined in vivo in further detail taking into account that increased plasma dosages as a result of supplementation of the diet are considered safe and relatively non-toxic [37]. An important factor that needs to be considered upon extrapolation of the results of the present study to the clinical situation is the fate and concentrations of myricetin in the human body. Elevation of plasma levels to levels approaching the 20–30 μM myricetin, observed in the present study to be active in modulating cellular vincristine sensitivity, might prove difficult if not impossible via oral supplementation due to the low oral bioavailability of flavonoids and the high first pass effect. A more promising option to increase myricetin plasma levels is via intravenous bolus injection. Human pharmacokinetic studies have demonstrated serum concentrations of the related flavonoid quercetin to range from 1 to 400 μM after a non-toxic i.v. dose of quercetin with a half-life of 1–2 h [37,41]. Therefore, repeated i.v. doses or infusion seem to be the preferred way to administer myricetin as an adjunct to chemotherapy.

In conclusion, this study demonstrates the ability of the flavonoid myricetin to modulate MRP1 and MRP2 mediated resistance to the anticancer drug vincristine in transfected cells indicating that this flavonoid might be a valuable adjunct to chemotherapy to decrease MRP mediated resistance.

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