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Inhibition of multidrug resistance proteins MRP1 and MRP2 by a series of α,β-unsaturated carbonyl compounds

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Abstract

To study the possible interplay between glutathione metabolism of and MRP inhibition by thiol reactive compounds, the interactions of a series of α,β-unsaturated carbonyl compounds with multidrug resistance proteins 1 and 2 (MRP1/ABCC1 and MRP2/ABCC2) were studied. α,β-Unsaturated carbonyl compounds react with glutathione, and therefore either their parent compound or their intracellularly formed glutathione metabolite(s) can modulate MRP-activity. Inhibition was studied in Madin-Darby canine kidney cells stably expressing MRP1 or MRP2, and isolated *Sf*9-MRP1 or *Sf*9-MRP2 membrane vesicles. In the latter model system metabolism is not an issue. Of the series tested, three distinct groups could be discriminated based on differences in interplay of glutathione metabolism with MRP1 inhibition. Curcumin inhibited MRP1 transport only in the vesicle model pointing at inhibition by the parent compound. The glutathione conjugates of curcumin also inhibit MRP1 mediated transport, but to a much lesser extent than the parent compound curcumin. In the cellular model system, it was demonstrated that glutathione conjugation of curcumin leads to inactivation of its inhibitory potential. Demethoxycurcumin and bisdemethoxycurcumin inhibited MRP1 in both the vesicle and cellular model pointing at inhibitory potency of at least the parent compound and possibly their metabolites. A second group, including caffeic acid phenethyl ester inhibited MRP1-mediated calcein transport only in the MDCKII-MRP1 cells, and not in the vesicle model indicating that metabolism appeared a prerequisite to generate the active inhibitor. Finally cinnamaldehyde, crotonaldehyde, *trans*-2-hexanal, citral, and acrolein did not inhibit MRP1. For MRP2, inhibition was much less in both model systems, with the three curcuminoids being the most effective. The results of this study show the importance to study the complex interplay between MRP-inhibitors and their cellular metabolism, the latter affecting the ultimate potential of a compound for cellular MRP-inhibition.

Keywords: MRP1; MRP2; α,β-Unsaturated carbonyl compounds; Inhibition

1. Introduction

One of the mechanisms underlying multidrug resistance (MDR) in mammalian tumor cells has been assigned to enhanced removal of drugs due to overexpression of efflux transporter proteins, such as P-glycoprotein (Pgp/ABCB1), the multidrug resistance proteins MRP1/ABCC1, and to a lesser extent MRP2/ABCC2 [1,2]. These membrane-embedded proteins all belong to the ATP-binding cassette (ABC) transporter protein family differing and MRP2 have many substrates in common, among them glutathione-, glucuronide- and significantly. Also unconjugated drugs, such as daunomycin, vinca alkaloids, methotrexate, fluorouracil and chlorambucil are transported, in which process the tripeptide glutathione plays an essential role [3]. For vincristine and daunorubicin, it has been suggested that their transport out of the cell is accompanied by co-transport of reduced glutathione (GSH) whereas for other

Abbreviations: ACRO, acrolein; BDCUR, bisdemethoxycurcumin; Calcein-AM, calcein acetoxymethylester; CAPE, caffeic acid phenethyl ester; CINN, cinnamaldehyde; CITR, citral; CROT, crotonaldehyde; CsA, cyclosporin A; CUR, curcumin; DCUR, demethoxycurcumin; EA, ethacrynic acid; EASG, glutathionylconjugate of ethacrynic acid; GST, glutathione *S*-transferase; HBSS, Hanks' balanced salt solution; HEX, *trans*-2-hexenal; MDCK cells, Madin-Darby canine kidney cells; MDR, multidrug resistance protein; Pgp, P-glycoprotein; MRP, multidrug resistance protein; *Sf*9, *Spodoptera frugiperda* insect cells

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unconjugated MRP substrates, it has been suggested that allosteric interactions of glutathione with the transporter protein are required for their transport without the need for transport of GSH itself [3]. Both MRP1 and MRP2 confer in vitro cellular resistance to many anticancer drugs like vincristine [4–6], methotrexate [7] and anthracyclines [4,6].

In the search for inhibitors of this process, several potent MRP inhibitors have been described, such as the leukotriene D4 receptor agonist MK571, glutathione conjugates [8], and tricyclic isoxazole derivatives [9]. Many of these inhibitors however are described to be very effective in vitro, but have often limited effect in vivo due to their low bioavailability or toxic side effects [2,10].

The over-expression of efflux transporters in tumor cells is often accompanied with high levels of GSH and glutathione *S*-transferase (GST), which results in an enhanced detoxification and efflux of anticancer agents. Naturally occurring plant polyphenols available in our normal diet have been reported to interact with GSH and GSH-associated biotransformation enzymes [11–13], but also membrane transport proteins [14–17]. Moreover, it has been shown that several plant polyphenols can sensitize cancer cells to anticancer agents, even at low concentrations [18–20]. Using non-toxic food compounds as inhibitors of GSH-dependent metabolism and MRP efflux in synergy with cancer treatment could be a possible way to decrease drug resistance. In this respect, curcumin is of great interest because it has been shown to deplete GSH, to inhibit GST [11], and to inhibit MRP1 and MRP2 [16]. Curcumin, a spice and a coloring agent derived from the root of the plant *Curcuma longa*, contains two electrophilic α,β-unsaturated carbonyl groups, which can react with GSH. For other compounds with an α,ß-unsaturated moiety in their structure, like the endogenous compounds prostaglandin (PGA1, PGA2) and the diuretic drug ethacrynic acid, it was demonstrated that their GS-metabolites are high affinity substrates for MRP1, whereas the parent compounds PGA1, PGA2 and ethacrynic acid are not substrates nor inhibitors of MRP1 [21,22]. In contrast, for curcumin, it was shown that the parent compound was a better inhibitor of MRP1 and MRP2 than its GSH metabolites, as metabolism in intact cells diminished its inhibitory potential, especially in cells with high GSH levels [16]. Apparently, compounds containing an α,β-unsaturated moiety can modulate MRP1- and MRP2-mediated transport processes via different mechanisms, including (i) formation of GSH conjugates which can competitively inhibit MRP1 and MRP2, (ii) depletion of GSH, or (iii) direct inhibition of the MRP1 and/or MRP2 mediated transport process through interaction of the parent compound with the MRP molecule. The objective of the present study was to obtain insight in the possible interplay between GSH dependent metabolism of possible MRP inhibitors and MRP inhibition by these compounds.

To this end, the interaction of a series of natural thiol reactive α,β-unsaturated carbonyl derivatives (Fig. 1), all available in our normal diet, with MRP1- and MRP2-mediated transport activity was studied. Next to curcumin, its derivatives demethoxycurcumin and bisdemethoxycurcumin, which are present in commercially available curcumin-mixtures, were investigated. Other α,β-unsaturated carbonyl compounds tested in this study are caffeic acid phenethyl ester (CAPE; honey), cinnamaldehyde (cinnamon), crotonaldehyde (red wine), *trans*-2-hexenal (banana), citral (citrus fruits) and acrolein (red wine). These electrophilic derivatives are all thiol reactive compounds and known to interact with GSH. Especially, exposure to crotonaldehyde, curcumin, CAPE, cinnamaldehyde and *trans*-2-hexenal have been shown to decrease intracellular GSH levels with 25–50% [11,23]. The possible interplay between GSH-dependent metabolism of these compounds and MRP-inhibition was investigated in intact Madin-Darby canine kidney (MDCKII) cells stably transfected with human MRP1 and MRP2. In addition, the direct interactions of the α,β-unsaturated carbonyl derivatives with MRP mediated transport and associated ATPase activity was studied in isolated membrane vesicles of either MRP1- or MRP2-overexpressing *Spodoptera frugiperda* (*Sf*9) insect cells – in which metabolism is not an issue.

2. Methods

2.1. Chemicals and cell lines

Curcumin, demethoxycurcumin and bisdemethoxycurcumin (all purity >99%) were obtained from Alexis Biochemicals (Kordia, Leiden, The Netherlands). Caffeic acid phenethyl ester and ethacrynic acid were from Sigma Chemical Co. (St. Louis, MO). Acrolein, citral, crotonaldehyde, *trans*-cinnamaldehyde and *trans*-2-hexenal were obtained from Aldrich Chemie (Zwijndrecht, The Netherlands). Dulbecco's Minimum Essential Medium (DMEM) with GlutaMax, Grace's insect medium, fetal calf serum, penicillin/streptomycin and gentamycin were all from Gibco (Paisley, Scotland). The typical MRP1 inhibitor MK571 was obtained from BioMol (Plymouth Meeting, PA), the Pgp inhibitor PSC833 (Valspodar) was a kind gift from Novartis Pharma AG (Basel, Switzerland). The typical MRP2 inhibitor cyclosporin A was from Fluka (Zwijndrecht, The Netherlands). Calcein acetoxymethylester ester was obtained from Molecular Probes (Eugene, OR). Glutathione reductase and creatine kinase were from Boehringer (Mannheim, Germany). HPLC-grade trifluoroacetic acid was obtained from Baker (Deventer, The Netherlands). HPLC-grade methanol was from Merck (Darmstadt, Germany). [^{14}C]-Ethacrynic acid ([2,3-dichloro-4-(2-methylene-1-oxobutyl)phenoxy] acetic acid) with a specific activity of 555 MBq/mmol was purchased from Amersham Biosciences (Little Chalfont, Buckin-

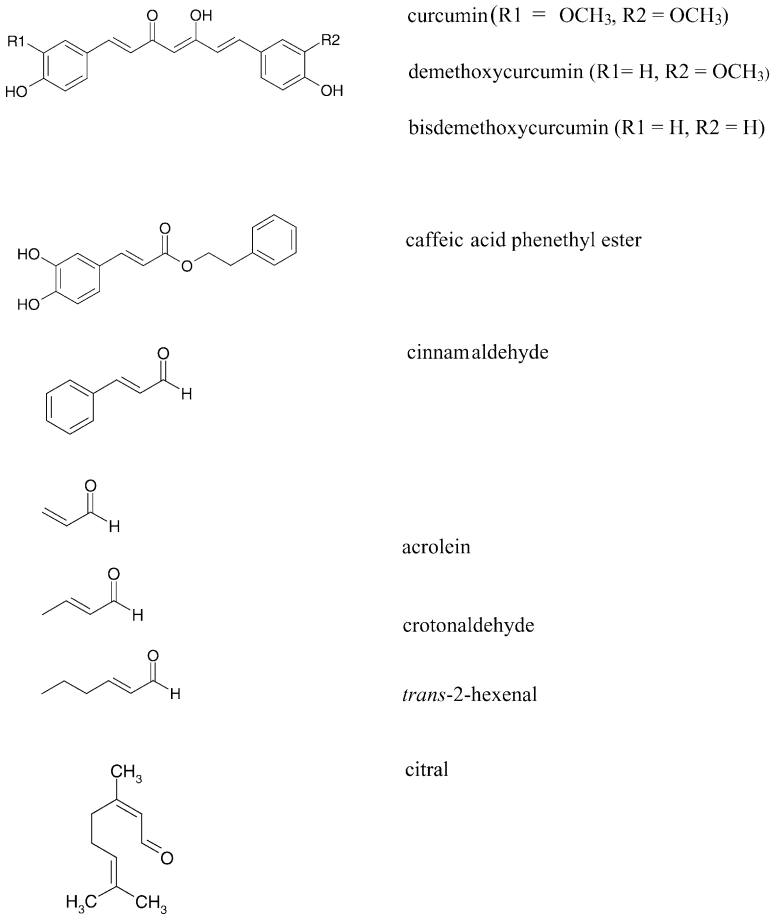

Fig. 1. Structures of α,β-unsaturated carbonyl derivatives used in the present study.

ghamshire, United Kingdom). The radioactive GSH conjugate of ethacrynic acid was synthesized according to Ploemen et al. [24]. Other chemicals were from Sigma Chemical Co. (St. Louis, MO), unless stated otherwise.

*Sf*9 cells were obtained from Invitrogen (Groningen, The Netherlands). Recombinant baculoviruses containing either the human *MRP1* cDNA or the human *MRP2* cDNA were a kind gift from Prof. Dr. B. Sarkadi, National Institute of Haematology and Immunology, Research Group of the Hungarian Academy of Sciences, Budapest, Hungary. The Madin-Darby Canine Kidney cell lines, stably expressing either human *MRP1* cDNA (hereafter called MRP1 cells) or *MRP2* cDNA (hereafter called MRP2 cells), or transfected with an empty vector (hereafter called MII cells) were kindly provided by Prof. P. Borst (NKI, Amsterdam). Cells were previously described and characterized [16,25,26]. In these studies, Western blotting revealed very limited background levels of endogenous canine Pgp, and canine Mrp2, and no background of endogenous canine Mrp1. Cells were cultured in Dulbecco's minimum essential medium (DMEM) with GlutaMax (4.5 g glucose per liter), 10% fetal calf serum and 1% penicillin/streptomycin (each 10,000 units/ml), and grown in a humidified atmosphere of 5% CO_2 at 37 °C. It was shown that in these polarized cell lines MRP1 routes to the basolateral side, whereas MRP2 routes to the apical side [26].

Cytotoxic effects of α,β-unsaturated carbonyl compounds were determined using the neutral red uptake assay, which is based on the ability of viable cells to incorporate neutral red in the lysosomes [27]. Cells (4×10^5 cells/well) were exposed to test compounds for 90 min. No cytotoxicity was detected for curcumin, demethoxycurcumin, bisdemethoxycurcumin, caffeic acid phenethyl ester, cinnamaldehyde, crotonaldehyde, citral, *trans*-2-hexenal, MK571, and cyclosporin A (all tested up to 50 μM).

2.2. Transport studies in transfected MDCKII cells: accumulation and efflux assays

Interactions of α,β-unsaturated carbonyl compounds with MRP1- and MRP2-mediated transport were studied using two different model systems in which either the accumulation or the efflux of calcein, which is a good substrate for both MRP1 and MRP2 [16], was determined. The non-fluorescent ester of calcein, calcein-acetoxymethyl ester (calcein-AM), equilibrates very rapidly over the cellular plasma membrane [28]. Once inside the cells, cleavage of this non-fluorescent calcein-AM ester by intracellular esterases leads to formation of the fluorescent

derivative calcein. The non-fluorescent calcein-AM, is a good substrate for both P-glycoprotein (Pgp) and MRP1 [29]. Therefore, PSC833 (0.1 μM) was added to inhibit possible efflux by endogenous Pgp. The PSC833 concentration was chosen based on the results obtained by Evers et al. [25]. In this study, the Pgp-dependent efflux of various substrates was tested in mock, MRP1 and MRP2 transfected MDCKII cell lines. A concentration of 0.1 μM PSC833 inhibited endogenous Pgp transport, but did not affect the efflux by MRP1 or MRP2 in the transfected cell lines. The leukotriene D4 receptor antagonist MK571 was used as a typical MRP1 inhibitor [30] and cyclosporin A was used as a typical MRP2 inhibitor [5].

2.2.1. Calcein accumulation studies

The interactions of α,β-unsaturated carbonyl compounds with MRP1 and MRP2 were evaluated by measuring their ability to reverse the reduced accumulation of calcein in MRP1 and MRP2 transfected cells. The accumulation of calcein was determined by incubating MII, MRP1 and MRP2 cells in 6.4 mm diameter wells (96 wells Costar Corp.) with calcein-AM. Cells (4×10^5 cells/well) were exposed to α,β-unsaturated carbonyl compounds (50 μM) for 10 min in DMEM without phenol red in the presence of 0.1 μM PSC833. Thereafter, a concentrated stock solution of 25 μM calcein-AM in DMEM without phenol red was added, yielding a final calcein-AM concentration of 2.5 μM, and cells were incubated at 37 °C for 40 min. Cells were washed three times with ice-cold PBS and fluorescence of intracellular calcein was determined using a Cytofluor 2300 (Millipore, Ettenleur, The Netherlands) with excitation at 485 nm and emission at 530 nm.

2.2.2. Calcein efflux studies

To exclude possible side-effects of the test compound on the uptake of calcein, calcein efflux studies were performed as described [16]. In brief, 10^5 cells/cm^2 were seeded on microporous polycarbonate filters (0.4 μm pore size, 24.5 mm, Costar Corp. Cambridge, MA) and grown to confluence in 3 days. First, cells were loaded with calcein by culturing the cells in DMEM without phenol red containing calcein-AM (1 μM) and PSC833 (0.1 μM) for 2 h at 7 °C. At this temperature premature MRP-dependent efflux of calcein could be reduced without the use of a MRP inhibitor during loading time. For the kinetic characterization of calcein efflux inhibition by the α,β-unsaturated carbonyl compounds, several calcein-AM concentrations were used (0.2–8 μM). At these calcein-AM concentrations the efflux of calcein by MRPs is not saturated since the calcein efflux was a linear function of the calcein-AM concentration. For inhibition studies it is important that the substrate concentrations do not reach the V_{max} of the transporter proteins. Analysis of the calcein mass-balance before and after the efflux experiments showed that during the efflux experiments no significant increase in total calcein amounts were observed (data not shown). Apparently, the cleavage-step by intracellular esterases is not saturated and the intracellular calcein concentrations were approximately the same as the extracellular calcein-AM concentration in the experiments. Loaded cells were washed with DMEM without phenol red and exposed to fresh medium (37 °C) containing 0.1 μM PSC833 and 25 μM of the test compound. Control cells received fresh medium (37 °C) with 0.1 μM PSC833 and vehicle only (0.5% DMSO, v/v). The efflux of calcein was measured in media samples from either the basolateral (MRP1) or the apical (MRP2) compartment after 25 and 45 min. Fluorescence of calcein was determined using a Cytofluor 2300 (Millipore) with excitation at 485 nm and emission at 530 nm. The fluorescence of the samples was corrected for the minor changes in background fluorescence caused by the test compounds. Dose-dependent effects of the natural α,β-unsaturated carbonyl compounds (0–50 μM), on either apical (MRP2) or basolateral (MRP1) calcein efflux were determined. IC_{50} values were determined with non-linear regression curve fit using the sigmoid dose response curve (variable slope) with Prism® software from Graph Pad Software, Inc. (San Diego, CA).

2.3. Transport studies in isolated Sf9 membrane vesicles

Membrane vesicles were prepared from *Spodoptera frugiperda* (*Sf*9) insect cells infected with recombinant baculoviruses containing either human MRP1 cDNA or human MRP2 cDNA as described [16]. A full characterisation of the MRP1 and MRP2 expression and there activities in the S*f*9 vesicles is given in Wortelboer et al. [16]. ATP-dependent uptake of the radioactive GSH conjugate of ethacrynic acid ([^{14}C]-EASG) in isolated *Sf*9 cell membrane vesicles was determined according to Zaman et al. [21]. In brief, vesicles were rapidly thawed and pre-incubated for 1 min at 37 °C in Tris–sucrose buffer (TS buffer: 10 mM Tris, 250 mM sucrose, pH 7.4) containing 4 mM ATP, 10 mM $MgCl_2$, 10 mM creatine phosphate, 100 μg/ml creatine kinase, and 50 μM test compound. The reaction was started by addition of [^{14}C]-EASG to a final concentration of 4 μM (555 MBq/mmol). After 1 min incubation at 37 °C, the reaction mixture was diluted with 1 ml of ice-cold TS buffer and rapidly filtered through pre-soaked nitrocellulose filters (0.45 μm pore size) (Schleicher and Schuell, Dassel, Germany) in a 1225 sampling Manifold filtration unit (Millipore, Ettenleur, The Netherlands). Filters were rinsed three times with 5 ml TS buffer and radioactivity associated with the filters was measured by liquid scintillation counting. In control experiments, ATP was replaced by 4 mM AMP-PCP (α,β-methylene adenosine 5′-triphosphate). ATP-dependent [^{14}C]-EASG uptake was calculated by subtracting the values obtained in the presence of AMP-PCP from those in the presence of ATP.

2.4. Sf9-membrane ATPase activity assay

The basal ATPase activity of MRP1 and MRP2 was measured in principle according to Sarkadi et al. [31] by the colorimetric detection of the liberation of inorganic phosphate from ATP. Either MRP1 or MRP2 containing *Sf*9-membranes (40 μg protein) were incubated at 37 °C in a 50 mM MOPS-Tris/KCl buffer containing 5 mM Na-azide, 2 mM DTT, 0.1 mM EGTA, and 1 mM ouabain for 30 min in the absence or presence of 50 μM of the test compound. The reaction was started with the addition of 5 mM Mg-ATP. Parallel incubations containing also 1 mM Na-orthovanadate (ATPase inhibitor) were used to determine the vanadate sensitive fraction.

2.5. Statistical analysis

Data are presented as mean ± S.D. where appropriate. Statistical differences between group means were determined by Student's two-tailed *t*-test, for unpaired samples ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). The data of calcein accumulation were analyzed with one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test ($P < 0.05$).

3. Results

3.1. Interactions of α,β-unsaturated carbonyl compounds with MRP1- and MRP2-transport of calcein in MDCKII cells

3.1.1. Accumulation studies

The intracellular levels of calcein in MII, MRP1 and MRP2 cells were determined after 40 min exposure to calcein-AM at 37 °C. In the MII cells, calcein reached values up to 5408 ± 268 fmol calcein/monolayer, whereas in the MRP1 and MRP2 cells calcein accumulated up to 479 ± 40 and 205 ± 23 fmol calcein/monolayer, respectively, due to active efflux of calcein by MRP. Subsequently, the interactions of a series of α,β-unsaturated carbonyl compounds with MRP1 and MRP2 were evaluated by measuring their ability to reverse the reduced accumulation of calcein in MRP1 and MRP2 transfected cells. In MRP1 cells, bisdemethoxycurcumin (BDCUR) > demethoxycurcumin (DCUR) > caffeic acid phenethyl ester (CAPE) > curcumin (CUR), had the strongest inhibitory effect, as calcein levels significantly increased in the MRP1 cells to 508, 493, 460, and 222% of the values observed for DMSO treated cells, respectively (Fig. 2). The other tested α,β-unsaturated carbonyl compounds showed no statistically significant effect. As expected, a dose of 50 μM MK571 significantly inhibited calcein efflux from the MRP1 cells, as intracellular calcein reached levels up to 818 ± 63% of vehicle-treated MRP1 cells (i.e. 3918 fmol calcein/monolayer). In the parental MII cells, with the high intracellularly calcein level of 5408 fmol, the α,β-unsaturated carbonyl compounds had a marginal effect, that appeared to reach statistical significance for some compounds.

In MRP2 cells, the interaction of α,β-unsaturated carbonyl derivatives with MRP2 appeared to be much less as demethoxycurcumin (DCUR) > bisdemethoxycurcumin (BDCUR) > curcumin (CUR) > caffeic acid phenethyl ester (CAPE) > crotonaldehyde (CROT) increased calcein accumulation to a maximum of 233% as compared to the DMSO treated MRP2 cells (Fig. 2). The MRP1 inhibitor MK571 enhanced the intracellular calcein level in MRP2 cells almost three-fold (291 ± 13%), pointing at inhibition of MRP2 by MK571. The known MRP2 inhibitor cyclosporin A (CsA), however, did not increase but decrease calcein levels in all three cell lines, resulting in 49% (MII), 48% (MRP1) and 56% (MRP2) of the calcein levels in DMSO-treated cells, indicating that cyclosporin A most likely interferes with either the uptake of calcein-AM or hydrolysis of the calcein-AM ester to calcein.

3.1.1.1. Efflux studies. To circumvent possible side-effects of the test compounds on the uptake or hydrolysis of calcein-AM in the above mentioned calcein accumulation assay, interactions of α,β-unsaturated derivatives with MRP1 and MRP2 were also tested using calcein efflux studies. Confluent monolayers of polarized MDCKII cells, cultured on transwells, were first loaded with calcein at a temperature of 7 °C to inhibit premature ATP-dependent efflux of calcein. Under these conditions intracellular calcein levels reached comparable levels in all three cell lines within a time span of 3 h (Fig. 3). For the efflux studies a 2 h loading with calcein was chosen, whereafter efflux was determined at 37 °C. A clear polarized efflux of calcein – in MRP1 cells to the basolateral side, and in MRP2 cells to the apical side – was observed as described before [16].

The interactions of the α,β-unsaturated carbonyl compounds with MRP-mediated efflux of calcein was investigated. As shown in Fig. 4, the efflux of calcein from MRP1 cells is significantly inhibited by bisdemethoxycurcumin (BDCUR) > caffeic acid phenethyl ester (CAPE) > demethoxycurcumin (DCUR) to 52, 57 and 68% of the value for DMSO-treated calcein loaded MRP1 cells. Dose-response curves for the inhibition of MRP1-mediated calcein efflux by curcumin (CUR), demethoxycurcumin (DCUR), bisdemethoxycurcumin (BDCUR), and caffeic acid phenethyl ester (CAPE), resulted in IC_{50} values of 31.7 ± 1.1, 24.7 ± 1.0, 22.1 ± 1.1 and 16.4 ± 1.2 μM, respectively. All other α,β-unsaturated carbonyl derivatives tested did not significantly affect the efflux of calcein in MRP1 cells. When cells were loaded with calcein-AM in the presence of the test compound, the inhibitory effect of almost all test compounds on MRP1 transport was enhanced, except for cinnamaldehyde (CINN). Exposure of MRP1 cells to

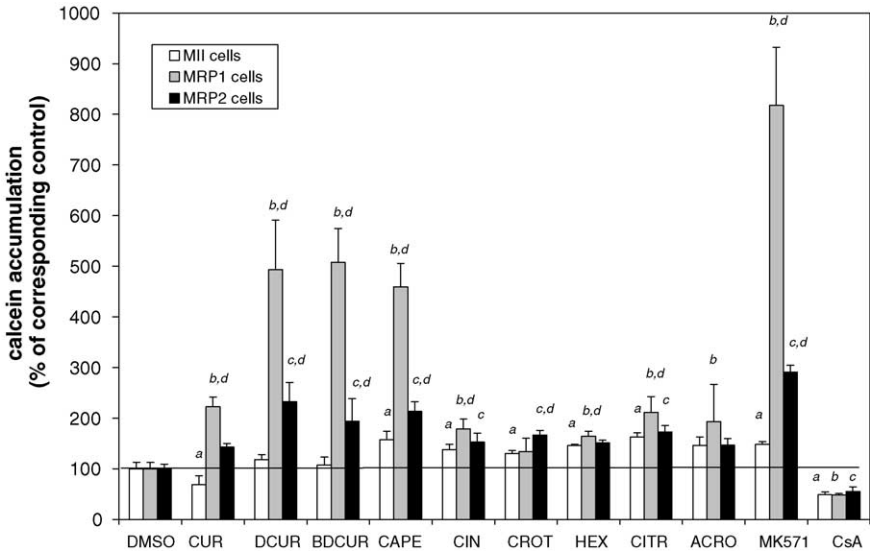

Fig. 2. Calcein accumulation in parent MII (white bars), MRP1- (grey bars) and MRP2- (black bars) MDCKII cells in the absence (DMSO) or presence of 50 μM α,β-unsaturated carbonyl derivatives. Data are presented as percentage of treated with vehicle alone (0.5% DMSO). Control values are 5408 ± 268, 479 ± 40, and 205 ± 23 fmol calcein/monolayer for the MII, MRP1, and MRP2 cells, respectively. Each point represents the average ± S.D. of a typical experiment performed ($n = 6$). Those marked with an italic letter differ significantly (ANOVA + Dunnett's *t*-test) from DMSO treated cells of the corresponding cell line (*a*, MII, *b*, MRP1 and *c*, MRP2), or from the MII cells with the same treatment (*d*). CUR, curcumin; DCUR, demethoxycurcumin; BDCUR, bisdemethoxycurcumin; CAPE, caffeic acid phenethyl ester; CINN, cinnamaldehyde; CROT, crotonaldehyde, HEX, *trans*-2-hexenal; CITR, citral; ACRO, acrolein; CsA, cyclosporin A.

trans-2-hexenal (HEX), especially when cells were pre-incubated with this compound, resulted in an enhanced efflux of calcein.

In MRP2 cells, the interactions of α,β-unsaturated carbonyl compounds with calcein efflux was limited (Fig. 5). When cells were loaded with both calcein-AM and the test compound, demethoxycurcumin (DCUR) and bisdemethoxycurcumin (BDCUR) significantly inhibited MRP2-mediated calcein transport. No inhibition of MRP2-mediated calcein efflux by any of the other tested α,β-unsaturated carbonyl compounds could be detected. In this cellular efflux assay, cyclosporin A (CsA) clearly inhibited MRP2-mediated transport of calcein.

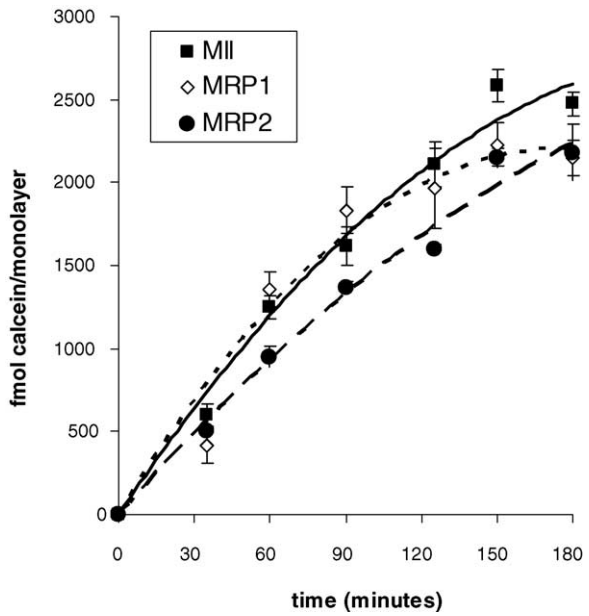

Fig. 3. Time-dependent calcein accumulation in MDCKII cells, parent MII (■), MRP1 (◇) and MRP2 (●) transfected cells. MDCKII cells were loaded in the presence of 0.1 μM PSC833 and at a temperature of 7 °C to inhibit premature calcein efflux. The results are mean ± S.D. of duplicate measurements.

3.1.2. Non-competitive inhibition of MRP1 by curcuminoids and CAPE

To study the characteristics of inhibition of MRP1 by demethoxycurcumin, bisdemethoxycurcumin, and CAPE, Lineweaver Burk plots were obtained with cells loaded with different concentrations of calcein-AM. Apparent $K_{m,calcein\text{-}AM}$-values as calculated for MRP1 and MRP2 in this cellular efflux were essentially identical for both transporters (1.45 ± 0.11 and 1.54 ± 0.07 μM, respectively). Inhibition plots revealed a typical non-competitive inhibition pattern for all three compounds (Fig. 6). Apparent K_i-values for demethoxycurcumin (DCUR), bisdemethoxycurcumin (BDCUR) and caffeic acid phenethyl ester (CAPE) on MRP1-mediated efflux of calcein were 11.1 ± 0.7, 18.6 ± 1.3, and 13.4 ± 0.9 μM, respectively.

3.1.3. Interactions of α,β-unsaturated derivatives with MRP1 and MRP2 in isolated Sf9 membrane vesicles

Subsequently, to study whether the parent compound can inhibit MRP transport, the interactions of the α,β-

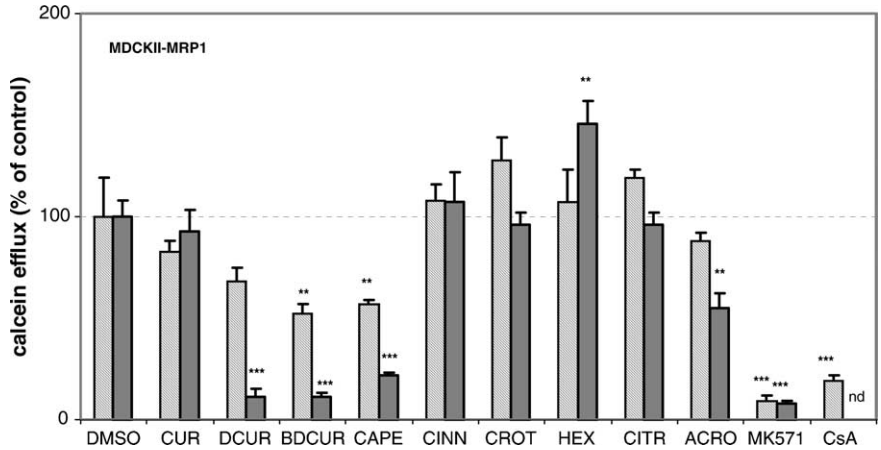

Fig. 4. The effect of α,β-unsaturated carbonyl derivatives (25 μM) on the calcein efflux from MDCKII-MRP1 cells. Cells were loaded with calcein-AM for 2 h at 7 °C without (hatched grey bars) and with (grey bars) test compound, after which calcein efflux in the presence of test compound was measured at 37 °C. Each point represents mean ± S.D. of a typical experiment performed in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$ significant different (Student's *t*-test) from vehicle-treated cells. nd = not determined.

unsaturated derivatives with MRP1 and MRP2 were determined in isolated *Sf*9-MRP1- and *Sf*9-MRP2-membrane vesicles, in which no metabolism takes place. In agreement with our previous study [16] curcumin (CUR) clearly inhibited the ATP-dependent [^{14}C]-EASG uptake in both MRP1- and MRP2-containing *Sf*9 vesicles (Fig. 7). The demethoxy-derivatives of curcumin appeared to be even better inhibitors of MRP1. Interestingly, caffeic acid phenethyl ester (CAPE) which inhibited MRP1, and to a lesser extent MRP2 in intact cells, did not affect either MRP1 or MRP2 transport in isolated membrane vesicles, which indicates that not the parent compound but rather a metabolite is responsible for the inhibition of MRP-transport in cells. Of the other α,β-unsaturated carbonyl compounds tested, a significant inhibition was observed for citral (CITR) for both MRP1 and MRP2, whereas crotonaldehyde (CROT) inhibited MRP2 (Fig. 7).

3.1.4. ATPase activity in isolated membrane vesicles

Multidrug resistance ABC transporters such as MRP1 and MRP2 utilize the energy of ATP for their drug transport activity. A clear increase in ATPase activity is often used as an indicator for substrate transport, whereas a decrease in either basal or substrate-induced ATPase activity clearly indicates inhibition of MRP. In the present study the effect on basal MRP1-associated ATPase activity was determined. The data presented in Fig. 8 clearly show the potent inhibition of this basal MRP1-associated ATPase activity in *Sf*9-MRP1 vesicles by all three curcuminoids. Crotonaldehyde (CROT) and acrolein (ACRO) slightly enhanced MRP1-associated ATPase activity whereas all other tested compounds were not significantly different from the controls. MRP2-associated ATPase activity appeared to be inhibited by most of the α,β-unsaturated carbonyl compounds indicating that these compounds may possibly interact with the ATPase site of MRP2.

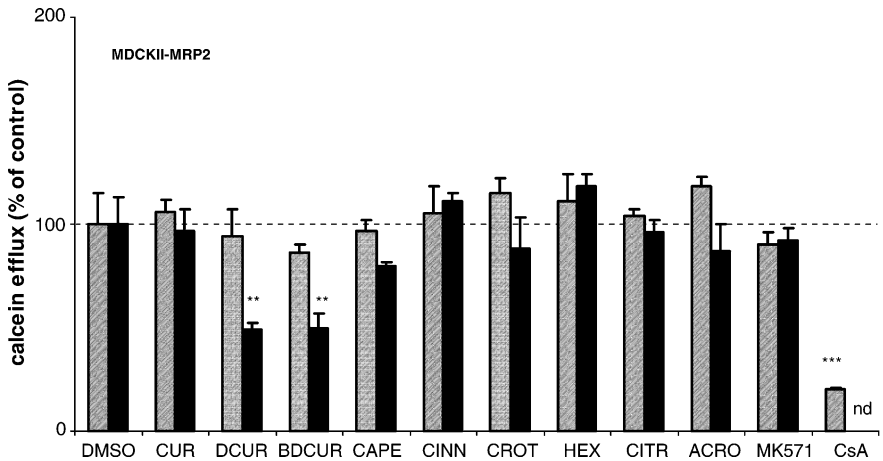

Fig. 5. The effect of α,β-unsaturated carbonyl derivatives (25 μM) on the calcein efflux from MDCKII-MRP2 cells. Cells were loaded with calcein-AM for 2 h at 7 °C without (hatched black bars) and with (black bars) test compound, after which calcein efflux in the presence of the test compound was measured at 37 °C. Each point represents mean ± S.D. of a typical experiment performed in triplicate. $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$ significant different (Student's *t*-test) from vehicle-treated cells. nd = not determined.

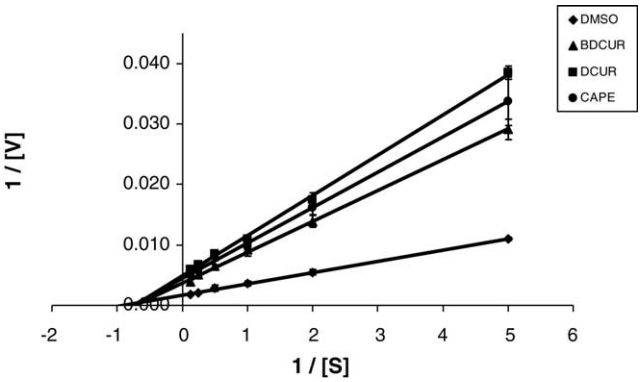

Fig. 6. Lineweaver-Burk of inhibition of MRP1-mediated calcein efflux in MRP1 cells by demethoxycurcumin (■), bisdemethoxycurcumin (▲) and caffeic acid phenethyl ester (●) (25 μM) in comparison with cells exposed to vehicle alone (0.5% DMSO; (◆)). Data represent mean ± S.D. of experiments performed in triplicate.

4. Discussion

In intact cells, inhibition of membrane transporters was studied either by the calcein accumulation assay in which increased cellular acumulation points at inhibition of transport proteins, as well as by the active efflux of calcein after pre-loading the cells with calcein (calcein efflux assay). The known MRP1 and MRP2 inhibitors MK571 and cyclosporin A were used as positive controls. In the calcein accumulation study, however, cyclosporin A did not increase but decrease the intracellular calcein level in all three cell lines, indicating that cyclosporin A affected either the uptake of calcein-AM or the hydrolysis to fluorescent calcein, but not the efflux by either MRP1 or MRP2. The latter was confirmed in the calcein efflux study as a clear inhibitory effect of cyclosporin A on MRP1 and MRP2 mediated efflux of calcein was observed. For cyclosporin A, it is known that it can also inhibit other transporter proteins, such as Pgp [32] but also uptake transporters such as the organic anion transporter proteins [33]. As it is expected that many inhibitors do not specifically inhibit one single cellular process – which has also been observed for MK571 – data obtained with transport protein inhibitors in intact cells containing several transport proteins should be interpreted keeping this in mind.

Reversal of drug resistance in vitro has been described for several inhibitors of MRP, but their use in vivo is often limited due to low bioavailability or toxic side effects [2,10]. In order to obtain insight in the possible interplay between GSH dependent metabolism of and MRP inhibition by thiol-reactive compounds, the interactions of a series of naturally occurring α,β-unsaturated carbonyl phytochemicals, available in our normal diet, on MRP1 and MRP2-mediated transport were studied. Two transport models were used, either intact MRP1- or MRP2-transfected MDCKII cells and isolated membrane vesicles of MRP1 or MRP2 containing *Sf*9 insect cells. The results are summarized in Table 1. It is clear that the α,β-unsaturated carbonyl derivatives tested in the present study (three curcuminoids, caffeic acid phenethyl ester, cinnamaldehyde, crotonaldehyde, citral, *trans*-2-hexenal, and acrolein) varied significantly not only between the compounds but also between the outcomes in the two model systems for MRP1- and MRP2-mediated transport.

Transport by MRP1 and MRP2 can be affected by α,β-unsaturated carbonyl compounds in several ways, either indirectly via interaction with GSH (GSH depletion, metabolism to GSH conjugates) or through interaction of the inhibitor with various sites on the MRP molecule (covalent binding of the parent compound or metabolite, ATPase inhibition). High affinity substrates for MRP1 and MRP2 are conjugated compounds, and as such GSH conjugates of the tested compounds may act as competitive inhibitors for MRP1 and MRP2 transport, as was reported for the glutathionyl conjugates of ethacrynic acid in the vesicle model [8,21]. In addition, for both MRP1 and MRP2 it is

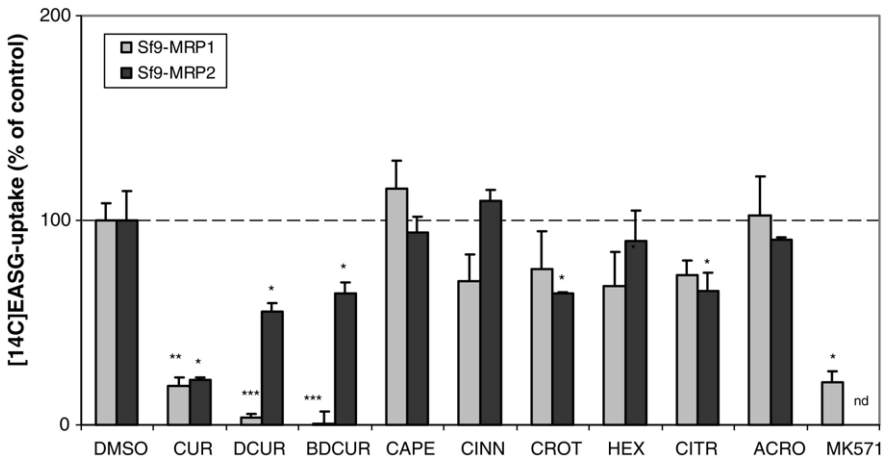

Fig. 7. The effect of α,β-unsaturated carbonyl derivatives (50 μM) on the ATP-dependent uptake of [^{14}C]-EASG in isolated membrane vesicles of *Sf*9 insect cells overexpressing either MRP1 or MRP2. Data are represented as percentage of the corresponding control vesicles, which were incubated in the presence of vehicle alone (DMSO). Data represent mean ± S.D. of three to four measurements. $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$ significant different (Student's *t*-test) from corresponding vehicle-treated *Sf*9 vesicles. nd = not determined.

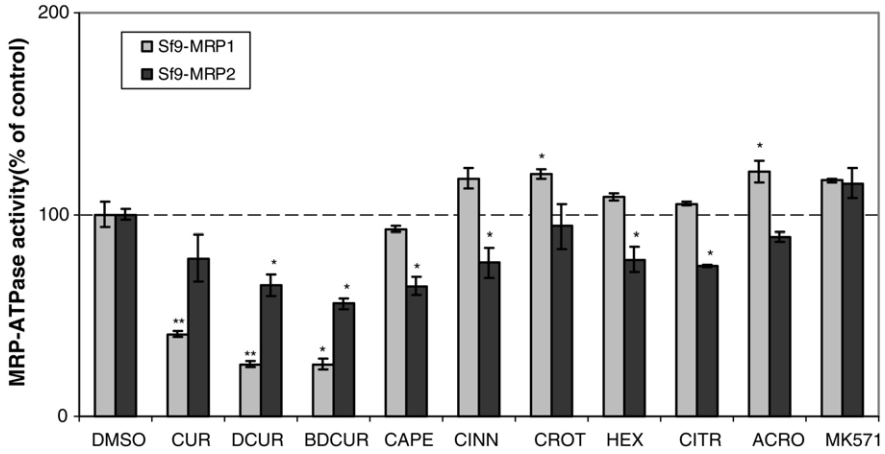

Fig. 8. The effect of α,β-unsaturated carbonyl derivatives (50 μM) on basal ATP-ase activity as measured in *Sf*9-MRP1 and *Sf*9-MRP2 vesicles. Data represent mean ± S.D. of three to four measurements. $^{*}P < 0.05$; $^{**}P < 0.01$ significant different (Student's *t*-test) from corresponding vehicle-treated *Sf*9 vesicles.

known that GSH plays an important role in the transport of both unconjugated but also conjugated compounds, and that depletion of intracellular GSH levels can decrease the transport of several substrates [26,34,35]. Curcumin, crotonaldehyde, cinnamaldehyde, *trans*-2-hexenal, and ethacrynic acid are known to deplete intracellular GSH levels in human melanoma cells, whereas citral and acrolein had only minor effect [11]. Also for CAPE, depletion of GSH in human leukemic cells was described [23]. Next to interaction on MRP via GSH, a direct interaction with the MRP protein is possible as was recently reported for curcumin [16]. In cells, the inhibition of MRP1 and MRP2 by curcumin was diminished due to the metabolism to glutathionyl conjugates, thereby lowering the level of the parent compound. Although the glutathionyl conjugates of curcumin appeared to be substrates for MRP1 and MRP2 their competitive inhibition was much lower than the non-competitive inhibition by the parent compound curcumin [16].

In the present study, both demethoxycurcumin and bisdemethoxycurcumin appeared to be even better MRP1 inhibitors than curcumin, as potent inhibition

Table 1
Effect of α,β-unsaturated carbonyl derivatives on MRP1-and MRP2-mediated transport

Compound	*Sf*9-hMRP1 vesicles		Calcein efflux from MDCKII-MRP1 cells	
	[^{14}C]EASG transport	ATPase activity	2 h pre-incubation	No pre-incubation
Curcumin	+++	+++	O	O
Demethoxycurcumin	+++	+++	+++	+
Bisdemethoxycurcumin	+++	+++	+++	+
Caffeic acid phenethyl ester	O	O	+++	+
Cinnamaldehyde	+	O	O	O
Crotonaldehyde	O	O[a]	O	O
trans-2-hexenal	+	O	+[a]	O
Citral	+	O	O	O
Acrolein	O	O[a]	+	O
Compound	*Sf*9-hMRP2 vesicles		Calcein efflux from MDCKII-MRP2 cells	
	[^{14}C]EASG transport	ATPase activity	2 h pre-incubation	No pre-incubation
Curcumin	+++	O	O	O
Demethoxycurcumin	+	+	++	O
Bisdemethoxycurcumin	+	+	++	O
Caffeic acid phenethyl ester	O	+	O	O
Cinnamaldehyde	O	O	O	O
Crotonaldehyde	+	O	O	O
trans-2-hexenal	O	O	O	O
Citral	+	+	O	O
Acrolein	O	O	O	O

For experimental details see Section 2. The effects are summarized according to the percentage of inhibition in comparison to its corresponding control O: 0–25%; +: 25–50%; ++: 50–75%; +++: >75%.

[a] An enhanced effect.

was observed both in the *Sf*9 vesicle-assay and in intact MDCKII cells, suggesting that for both compounds at least the parent compounds and possibly their metabolites are responsible for potent MRP1 inhibition. Kinetic studies in cells indicated that the inhibition by these compounds is of a non-competitive character. In intact cells, a longer exposure to these demethoxy derivatives enhances their inhibitory effect on MRP1 mediated efflux, indicating that either the longer exposure time enhances inhibition by the parent compound and/or that metabolism of the compounds to more potent metabolites occurs. However, formation of metabolites that interact with MRP1 cannot be ruled out. The potent inhibition is associated with a direct inhibition of ATPase activity in MRP1.

In contrast to MRP1, inhibition of MRP2 by the demethoxy-derivatives of curcumin showed another inhibitory profile. Whereas curcumin appeared to be a very potent inhibitor of MRP2 in the vesicle assay, but only a modest inhibitor of MRP2 in intact cells, the opposite was observed for both demethoxycurcumin as bisdemethoxycurcumin. For curcumin, inhibition of MRP2 was clearly dependent on intracellular GSH levels, as higher GSH levels increased its metabolism and diminished the level of the parent compound thereby reducing the inhibition of MRP2 in cells [16]. Whether the effect of demethoxycurcumin and bisdemethoxycurcumin in comparison with curcumin in intact MRP2 cells is due to differences in metabolism and/or to a better membrane passage or passive diffusion across the membrane due to higher lipophilicity, remains to be established. In this respect it is worth noting that commercially available curcumin-supplements contain high amounts of these demethoxyderivatives of curcumin [36].

A different inhibition mechanism was observed for caffeic acid phenethyl ester (CAPE). CAPE did not inhibit either MRP1 or MRP2 in the *Sf*9 vesicle system, indicating no direct interaction of the parent compound with the MRP1 or MRP2 protein. However, in MRP1 cells, a potent inhibition of MRP-mediated transport after exposure to CAPE was observed. To a lesser extent this was observed for MRP2. The kinetic studies revealed that this inhibition of MRP1 was non-competitive. A longer pre-incubation of MRP1 cells with CAPE showed an even higher inhibition of MRP1-mediated efflux of calcein. These data indicate that not CAPE itself, but possibly a metabolite is responsible for the potent non-competitive inhibition of MRP1 in intact cells. CAPE is an ester which can easily be hydrolyzed by intracellular esterases, and the identification of its possible metabolite(s) in intact cells needs further research. In MRP2 cells, inhibition of MRP2-mediated transport by CAPE was much less compared to MRP1 cells. Cellular exposure to CAPE has been associated with high GSH depletion [23] which can indirectly affect MRP transport. Therefore, the difference in effect of CAPE between the MRP1 and MRP2 cells, can possibly be attributed to the known difference in intracellular GSH levels between these cell lines as GSH depletion is more effective when GSH levels are already low as is the case in MRP1 cells [16]. Another possibility is that MRP2 is more selective than MRP1 for inhibition by the metabolites of CAPE. This more selective inhibition of MRP2 than of MRP1 was also observed for inhibition of the MRPs by flavonoids [17].

For the other α,β-unsaturated compounds tested in this study, their inhibition potential was much lower as compared to the three curcuminoids and CAPE. MRP1 mediated activity in *Sf*9-MRP1 vesicles was inhibited by cinnamaldehyde, *trans*-2-hexenal and citral. For cinnamaldehyde and citral this effect was associated with an inhibition of MRP1 mediated calcein transport in intact cells, but *trans*-2-hexenal even enhanced MRP1-mediated transport of calcein in cells, a phenomenon which cannot be explained yet.

Overall, the results of this study show the importance to study the complex interplay between MRP-inhibitors and their metabolism, the latter affecting the ultimate potential of a compound for cellular MRP-inhibition. In respect to multidrug resistance, of the α,β-unsaturated carbonyl compounds tested especially the curcuminoids and CAPE are potent inhibitors of MRP1, and to a lesser extent MRP2. Concentration levels of curcumin and its demethoxyderivatives are expected to reach the μM range in the gastrointestinal tract upon oral intake of curcumin supplements of 2–8 g curcumin per day. In a phase I clinical trial in which humans were given orally a therapeutic recommended dose of 8 g curcumin per day, a serum concentration of about 2 μM was detected, while concentrations observed in the liver and colon mucosa were even 75- to 150-times higher than those observed in plasma [37]. Both curcumin and CAPE are known to have many chemopreventive effects [37–41]. Curcumin has also been shown to inhibit Pgp-mediated transport [42,43]. The additional fact that the curcuminoids and CAPE modulate MRP1 mediated transport in vitro suggest that these phytochemicals are promising as potentiators of the toxicity of chemotherapeutic drugs thereby possibly enhancing existing treatments for cancer patients.

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