Short Communication

BIOFLAVONOID STIMULATION OF GLUTATHIONE TRANSPORT BY THE 190-kDa MULTIDRUG RESISTANCE PROTEIN 1 (MRP1)

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ABSTRACT:
In tumor cells, the human multidrug resistance protein 1 (MRP1), confers resistance to a broad spectrum of anticancer agents. MRP1 is also expressed in many normal tissues where it acts as an ATP-dependent transporter of organic anions. Reduced glutathione (GSH) is transported by MRP1 with very low affinity, and certain MRP1 substrates are transported in association with this tripeptide. Previous studies have shown that various dietary flavonoids stimulate the ATPase activity of MRP1 and inhibit transport of its conjugated organic anion substrates but are poor reversers of MRP1-mediated drug resistance. In contrast, many of the same flavonoids markedly stimulate GSH transport by MRP1. In the present study, we found that stimulation of GSH transport in inside-out MRP1-enriched membrane vesicles by apigenin, naringenin, genistein, and quercetin was maximum at a concentration of 30 μM. Apigenin was the most efficacious of the four bioflavonoids, showing a maximal 6-fold increase over basal levels of GSH transport. The apparent $K_m$ and $V_{max}$ for GSH uptake in the presence of 30 μM apigenin were 116 μM and 666 pmol mg$^{-1}$ min$^{-1}$, respectively. Chemosensitivity assays with control-transfected and MRP1-transfected HeLa cell lines showed that the IC$_{50}$ values for apigenin, naringenin, genistein, and quercetin were similar, demonstrating that overexpression of MRP1 does not confer resistance to these bioflavonoids. Our results suggest that flavonoids stimulate MRP1-mediated GSH transport by increasing the apparent affinity of the transporter for GSH but provide no evidence that a cotransport mechanism is involved.

The 190 kDa human multidrug resistance protein 1 (MRP1$^1$) is a member of the ATP-binding cassette transporter superfamily that in tumor cells confers resistance to a broad spectrum of xenobiotics including natural product type drugs, the antifolate methotrexate, and certain arsenical and antimonal oxyanions (Leslie et al., 2001c). In addition, many conjugated organic anions have been identified as substrates of MRP1 in vitro, including leukotriene $C_4$ (LTC$_4$), a glutathione conjugated arachidonic acid metabolite involved in the mediation of inflammatory responses (Jedlitschky et al., 1994; Loe et al., 1996; Leslie et al., 2001c). Studies of Mrp1 deficient mice have confirmed that LTC$_4$ is an endogenous substrate of the murine ortholog of MRP1 (Wijnholds et al., 1997; Robbiani et al., 2000).

In addition to amphipathic drugs and conjugated organic anions, MRP1 is a low affinity (estimated $K_m > 1$ mM) transporter of reduced glutathione (GSH) (Paulusma et al., 1999; Leslie et al., 2001b; Qian et al., 2001). Several substrates of MRP1, including most of the drugs to which it confers resistance, are not conjugated to any significant extent in vivo but may be transported by MRP1 in association with GSH. Current evidence suggests that at least some of these drugs are cotransported with GSH across the plasma membrane (Versantvoort et al., 1995; Zaman et al., 1995; Loe et al., 1996, 1998; Rappa et al., 1997). More recently, it has been shown that the transport of certain conjugated organic anions is also enhanced or completely dependent on the presence of this tripeptide (Sakamoto et al., 1999; Leslie et al., 2001b; Qian et al., 2001). However, cotransport of GSH with these conjugated substrates could not be demonstrated. Conversely, several xenobiotics, including the Ca$^{2+}$ channel antagonist verapamil, appear not to be transported by MRP1 but are potent stimulators of MRP1-mediated $[^3H]$GSH transport (Loe et al., 2000a,b). Thus, verapamil causes a substantial increase in the affinity of MRP1 for GSH, and reciprocally, GSH increases the affinity of MRP1 for verapamil although transport of verapamil is not detectable.

We and others have previously reported that a wide range of flavonoids can inhibit the conjugated organic anion transport properties and modulate the ATPase activity of MRP1 (Hooijberg et al., 1997, 1999, 2000; Leslie et al., 2001a). Thus a number of bioflavonoids were found to be potent inhibitors of LTC$_4$ and 17β-estradiol 17-β-glucuronide) transport by MRP1-enriched membrane vesicles (Leslie et al., 2001a). In some cases, transport inhibition was enhanced by the addition of GSH. We also made the unexpected observation that, like verapamil, several flavonoids caused a significant stimulation of $[^3H]$GSH transport by MRP1. In the present study, we have further characterized the stimulation of $[^3H]$GSH transport by four different flavonoids that are found in common foodstuffs (Ross and Kasum, 2002) and show that apigenin is the most efficacious of these with respect to enhancing transport of this tripeptide in MRP1-enriched inside-out membrane vesicles. We have also shown that apigenin markedly reduces the $K_m$ value of MRP1 for $[^3H]$GSH. Finally, using intact cells we have established that MRP1 does not confer resistance to apigenin, naringenin, genistein, or quercetin.

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Abbreviations used are: MRP1, multidrug resistance protein 1; LTC$_4$, leukotriene $C_4$; GSH, glutathione.

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et al., 1995; Zaman et al., 1995; Loe et al., 1996, 1998; Rappa et al., 1997). More recently, it has been shown that the transport of certain conjugated organic anions is also enhanced or completely dependent on the presence of this tripeptide (Sakamoto et al., 1999; Leslie et al., 2001b; Qian et al., 2001). However, cotransport of GSH with these conjugated substrates could not be demonstrated. Conversely, several xenobiotics, including the Ca$^{2+}$ channel antagonist verapamil, appear not to be transported by MRP1 but are potent stimulators of MRP1-mediated $[^3H]$GSH transport (Loe et al., 2000a,b). Thus, verapamil causes a substantial increase in the affinity of MRP1 for GSH, and reciprocally, GSH increases the affinity of MRP1 for verapamil although transport of verapamil is not detectable.

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Taken together, these results suggest that bioflavonoids stimulate \[^{3}\text{H}]\text{GSH}\) transport by a mechanism that does not involve their co-transport by MRP1.

**Materials and Methods**

**Materials and Cell Lines.** \[^{3}\text{H}]\text{GSH}\) (50 Ci/mmol) was purchased from PerkinElmer Life Science (Guelph, ON, Canada). GSH, nucleotides, MgCl\(_2\), dithiothreitol, acivicin, verapamil, apigenin, naringenin, genistein, and quercetin were obtained from Sigma-Aldrich (St. Louis, MO). The transfected HeLa cell lines and the small cell lung cancer cell line H69 and its MRP1-overexpressing variant H69AR used for the preparation of inside-out membrane vesicles have been described previously (Cole et al., 1992, 1994). The molecular volumes of drugs and flavonoids were determined using Viewer Pro 4.2 software (Accelrys Inc., Burlington, MA).

**Membrane Vesicle Transport Studies.** Inside-out plasma membrane vesicles were prepared from the cell lines using a nitrogen cavitation procedure and tested for MRP1 expression levels by immunoblot analysis with monoclonal antibody QCRL-1 as described (Loe et al., 1996). \[^{3}\text{H}]\text{GSH}\) transport assays were carried out using the rapid filtration method (Leslie et al., 2001a), and ATP-dependent transport activity was calculated by subtracting \[^{3}\text{H}]\text{GSH}\) uptake in the presence of AMP from \[^{3}\text{H}]\text{GSH}\) uptake in the presence of ATP. Uptake was measured for 20 min at 37°C in a 60-μl volume containing MRP1-enriched vesicles (20 μg of protein) prepared from the MRP1 transfected HeLa cell line, ATP or AMP (4 mM), MgCl\(_2\) (10 mM), creatine phosphate (10 mM), creatine kinase (100 μg/ml), dithiothreitol (10 mM), and \[^{3}\text{H}]\text{GSH}\) (100 μM; 120 nCi). Flavonoids were dissolved in dimethyl sulfoxide and added at the indicated concentrations such that the final concentration of dimethyl sulfoxide never exceeded 1%. To minimize GSH catabolism during transport, vesicles were preincubated with 500 μM acivicin for 10 min. Uptake was terminated by adding the entire reaction mix to 800 μl of ice-cold Tris sucrose buffer. Uptake of \[^{3}\text{H}]\text{GSH}\) in the presence of verapamil was measured as a positive control.

Kinetic parameters of GSH transport were determined by measuring \[^{3}\text{H}]\text{GSH}\) uptake at eight different substrate concentrations (10–1500 μM, 120–240 nCi) of substrate in the presence of apigenin (30 μM). For comparison, the kinetic parameters of GSH uptake in the presence of verapamil (30 μM) were also determined in the same experiment.

**Chemosensitivity Testing.** The MRP1 transfected and vector transfected control HeLa cell lines were used in a tetrazolium based microtiter plate assay to measure their relative sensitivity to apigenin, naringenin, genistein, and quercetin (Cole et al., 1994).

**Results and Discussion**

The purpose of the present study was to extend our investigations of bioflavonoid stimulated transport of GSH by MRP1 (Leslie et al., 2001a). As a first step we determined the dependence of \[^{3}\text{H}]\text{GSH}\) uptake on dietary flavonoids.

**Fig. 1.** Effect of dietary flavonoids on \[^{3}\text{H}]\text{GSH}\) uptake by membrane vesicles from MRP1-transfected HeLa cells.

MRP1-enriched membrane vesicles were incubated for 20 min at 37°C with 100 μM \[^{3}\text{H}]\text{GSH}\) in transport buffer. Apigenin (panel A), naringenin (panel B), genistein (panel C), quercetin (panel D), and verapamil (panel E) were added at concentrations of 0, 3, 10, 30, 70, and 100 μM. Data are expressed as fold-control uptake in the absence of flavonoid or verapamil. Data points are means (± S.D.) of triplicate determinations in a representative experiment; similar results were obtained in 1 to 2 additional independent experiments.
uptake on the concentration of apigenin, naringenin, genistein, and quercetin using MRP1-enriched membrane vesicles (Fig. 1). Apigenin stimulated ATP-dependent GSH transport most effectively, showing a maximal 4.3-fold stimulation at 30 μM (Fig. 1A). The stimulating effect of naringenin, genistein and quercetin on GSH uptake was also maximum at approximately 30 μM (2.6-, 2.4-, and 2.2-fold over basal levels, respectively) (Fig. 1, B-D). At higher concentrations (70 and 100 μM) of some of the bioflavonoids, there was a trend toward a reduction in stimulating activity, but this was not statistically significant. Verapamil, which was included in these experiments as a positive control (Loe et al., 2000a), also maximally stimulated GSH uptake 3.7-fold at a concentration of 30 μM (Fig. 1E).

Because apigenin was the most potent stimulator of MRP1-mediated GSH transport, it was investigated further. A time course of [3H]GSH uptake in the presence of apigenin (30 μM) showed that uptake by MRP1 was linear for approximately 20 min (Fig. 2A). At 20 min, the low basal level of MRP1-mediated [3H]GSH uptake in the absence of apigenin was approximately 0.50 nmol mg⁻¹H11002 which was stimulated approximately 6-fold to 2.8 nmol mg⁻¹H11002 in the presence of apigenin. The apparent decline in ATP-dependent GSH uptake observed after 20 min was caused by an increase in ATP-independent uptake in the AMP control.

To determine the effect of apigenin on the kinetic parameters of MRP1-mediated GSH transport, initial rates of ATP-dependent [3H]GSH uptake were measured in the presence of 30 μM apigenin and GSH concentrations ranging from 10 μM to 1.5 mM. The kinetics of GSH transport in the absence of apigenin could not be measured reliably because of the very low uptake levels of this tripeptide, but the K_{m} of MRP1 for GSH has been estimated previously to be >1 mM (Paulusma et al., 1999). In contrast, an Eadie-Hofstee plot of the GSH uptake data acquired in the presence of apigenin yielded an apparent K_{m} of [3H]GSH uptake of 116 μM and V_{max} of 666 pmol mg⁻¹min⁻¹ (Fig. 2B). When initial rates of [3H]GSH uptake were measured in the presence of the same concentration of verapamil, the apparent K_{m} was 236 μM whereas the V_{max} was similar (652 pmol mg⁻¹min⁻¹) (Fig. 2C). Thus stimulation of GSH transport by apigenin, as for verapamil, is associated with an increased affinity of MRP1 for this tripeptide substrate.

We next investigated whether or not bioflavonoid-stimulated GSH transport by MRP1 was accompanied by transport of the bioflavonoid. Since radiolabeled bioflavonoids are not readily available for direct transport experiments, MRP1-transfected and control-transfected HeLa cell lines were tested for their relative sensitivity to apigenin, naringenin, genistein, and quercetin. This was done to determine whether MRP1 conferred any protection from the toxicity of the flavonoids and, by inference, was capable of effluxing them. However, as shown in Fig. 3, all four flavonoids were equitoxic to MRP1 and control transfected HeLa cell lines, indicating that MRP1 does not confer resistance to these chemicals. As a positive control, cells were also tested for resistance to vincristine and as expected, the MRP1 expressing HeLa cells were 8-fold resistant to this drug (data not shown). These results, consistent with those of Versantvoort et al. (1993), who observed that MRP1-overexpressing lung cancer cells were also not resistant to genistein, indicate that although these flavonoids can stimulate GSH transport by MRP1, this transporter does not confer resistance to these agents. Thus in contrast to vincristine, which is cotransported with GSH, but similar to verapamil, which stimulates MRP1-mediated GSH transport without being transported itself, these flavonoids appear to be modulators rather than substrates of MRP1 (Loe et al., 1998, 2000a).

The structural features that determine whether or not a compound can stimulate [3H]GSH transport by MRP1 are presently unknown. Given the vast differences in the molecular volumes of the three most

![Fig. 2. Time course and kinetic analyses of MRP1-mediated [3H]GSH uptake.](image-url)

Panel A, time course of ATP-dependent [3H]GSH (100 μM, 120 nCi) uptake by membrane vesicles (10 μg of protein per time point) prepared from H69 (open symbols) and H69AR (closed symbols) cell lines measured in the presence (○, ○) and absence (●, □) of apigenin (30 μM). Panels B and C, kinetic parameters of [3H]GSH uptake by H69AR membrane vesicles were measured at GSH concentrations ranging from 10 to 1500 μM (120–240 nCi) for 20 min at 37°C in the presence of (panel B) 30 μM apigenin or (panel C) 30 μM verapamil. Kinetic parameters were determined from regression analysis of the Eadie-Hofstee transformation of the data (insets). All data points are means (± S.D.) of triplicate determinations in a representative experiment; similar results were obtained in a second independent experiment.
potent stimulators of [3H]GSH transport identified to date [vincristine (615 A²), verapamil (367 A²), and apigenin (186 A²)], overall steric bulk does not appear to be a critical determinant. We have previously reported that some degree of hydrophobicity is required for flavonoid interaction with MRPI in its membrane environment, although there is no significant correlation between the relative ability of flavonoids to stimulate GSH transport and their relative hydrophobicity (Leslie et al., 2001a). On the other hand, the flavone apigenin and the flavanone naringenin are structurally very similar to one another, differing by only one carbon-carbon double bond in the C-ring (Fig. 1, A and B), and yet these two compounds show a 2-fold difference in their maximal stimulation of GSH transport by MRPI.

Upon first consideration, flavonoid-stimulated GSH transport by MRPI might be expected to have toxic effects through depletion of cellular GSH stores. Indeed, there have been reports of cellular oxidative stress in cultured cells exposed to flavonoids including apigenin and naringenin, thought to be induced by redox cycling (Ratty and Das, 1988; Galati et al., 1999). On the other hand, MRPI is located on basolateral membranes of epithelial cells in many tissues including lung, kidney, and testes (Leslie et al., 2001c), and therefore would transport GSH into the interstitial space of these tissues where it could have a protective function by acting in a local fashion as a scavenger of electrophiles. Thus it is of interest that high levels of GSH are found in normal alveolar epithelial lining fluid, whereas even higher levels of GSH are found in fluid from the lungs of smokers (Cantin et al., 1987). GSH efflux across basolateral membranes of epithelial cells in lung, kidney, or possibly even liver into the circulation via MRPI could also lead to increased pools of cysteine for uptake into other tissues, where the availability of free cysteine may be rate-limiting for GSH biosynthesis (Ookhtens and Kaplowitz, 1998). In addition, several flavonoids including apigenin and quercetin have been shown to induce the expression of γ-glutamylcysteinyl synthetase, the rate limiting enzyme for GSH biosynthesis (Myhrstad et al., 2002). Thus, although certain flavonoids could cause a decrease in cellular GSH pools in cells expressing MRPI through increased efflux of GSH, this may be balanced, at least in part, by a flavonoid-induced increase in GSH biosynthesis. Whether or not the present findings are of physiological relevance is not known, but it should be noted that serum levels corresponding to the tablet forms of these bioflavonoids. Mrp1 knock-out mice offer a potentially relevant model in which to investigate the effects of diets high in flavonoids on GSH homeostasis.

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Vector control transfected HeLa cells (□) and MRPI transfected HeLa cells (●) were incubated in the presence of apigenin (panel A), naringenin (panel B), genistein (panel C), and quercetin (panel D) for 96 h and then cell viability was obtained in two additional independent experiments.

**Fig. 3.** Effect of apigenin, naringenin, genistein, and quercetin on the viability of transfected HeLa cells.


