

The Bioflavonoid Kaempferol is an Abcg2 Substrate and Inhibits Abcg2-mediated Quercetin Efflux.

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Abbreviations: Abcg2, Bcrp, Breast Cancer Resistance Protein; P-gp, P-glycoprotein;
MRP2, Multidrug Resistance Protein 2; MRM, multiple reactions monitoring; LOQ,
lower limit of quantification; FTC, fumitremogin C; MDCK, Madin-Darby canine kidney;
BCRP, breast cancer resistance protein; HBSS, Hank’s balanced salt solution.

ABSTRACT

The flavonoids quercetin and kaempferol are major constituents of Ginkgo biloba extract. The ABC efflux transporter, Breast Cancer Resistance Protein (Bcrp, Abcg2), is involved in the transport of quercetin and represents a possible mechanism for the low bioavailability of quercetin. Our objective was to investigate whether kaempferol inhibits Bcrp-mediated quercetin efflux and determine whether it is a substrate for BCRP. The intracellular uptake of kaempferol, with and without specific inhibitors, was determined in Bcrp-expressing cells. The transport of quercetin or kaempferol (10 μ M) across MDCK cell monolayers was investigated in both the apical (A)-to-basolateral (B) and B-to-A directions. Samples were analyzed using LC/MS/MS. Compared to quercetin alone group, the transport ratio decreased 11.6 fold (from 97.5 to 8.37) in the present of kaempferol in MDCK/Bcrp1 cells, indicating that kaempferol is a Bcrp inhibitor. The intracellular concentration of kaempferol was significantly increased in the presence of GF120918, a potent Bcrp inhibitor, suggesting that kaempferol may also be a Bcrp substrate. Moreover, in MDCK/Bcrp1 cells, the $P_{app,B-A}$ of kaempferol was much higher ($17.7 \pm 3.8 \times 10^{-6}$ cm/s) than the $P_{app,A-B}$ ($0.279 \pm 0.037 \times 10^{-6}$ cm/s), with a transport ratio of 63.4. In contrast, the transport ratio of kaempferol was only 0.68 in Bcrp1-negative MDCK/Mock cells. We report for the first time that kaempferol is a Bcrp substrate and our results indicate that kaempferol inhibits Bcrp-mediated quercetin efflux. Intestinal efflux by Bcrp may represent one possible mechanism for the low bioavailability of kaempferol. The use of flavonoids in combination may increase their bioavailability through transport interactions.

INTRODUCTION

Flavonoids are biologically active polyphenolic compounds that are widely distributed in the plant kingdom. They are composed of a common phenylchromanone structure (C6-C3-C6). Variations in the patterns of hydroxylation and substitutions in their heterocyclic ring give rise to seven flavonoid subclasses: flavonols, flavones, flavanones, flavanols, isoflavones, chalcones, and anthocyanidins. Among 8000 different flavonoids identified so far, the flavonols quercetin and kaempferol are the most abundant flavonoids. It has been reported that quercetin and kaempferol account for 70~76 % and 17~24 % of mean total flavonol intake, respectively (Hertog et al., 1993; Sampson et al., 2002). The dominant sources of the flavonols quercetin and kaempferol include black and green teas, apple, strawberry, onion, broccoli, kale and endive (Somerset and Johannot, 2008). It is estimated that the average daily consumption of quercetin and kaempferol is about 30 mg/day (de Vries et al., 1997). In addition to their presence in the diet, quercetin and kaempferol are also major constituents of several herbal supplements. For example, both quercetin and kaempferol represent the major active ingredients of ginkgo biloba, a top selling herbal product (Kang et al., 2010). Many *in vitro* and *in vivo* laboratory studies have confirmed the findings of epidemiological studies indicating that quercetin and kaempferol may reduce the occurrence of various cancers (Mukhtar et al., 1988; Nguyen et al., 2003). The pharmacological effects of quercetin and kaempferol have been well documented and both compounds demonstrate anti-oxidant, anti-inflammatory, neuroprotective, anti-anxiety and cognitive enhancing effects (Morand et al., 1998; Havsteen, 2002; Niering et al., 2005; Cho et al., 2006; Zhang et al., 2008). Although various beneficial effects have been observed *in vitro*,

whether and to what extent these *in vitro* activities can be extrapolated to *in vivo* is always a concern because both quercetin and kaempferol have poor bioavailability (de Boer et al., 2005; Barve et al., 2009). Understanding the mechanism(s) behind their low bioavailability would be valuable in order to improve the bioavailability of these flavonoids.

The extensive first pass metabolism, mainly phase II conjugation in the liver and in the intestinal wall, has been considered as the main reason leading to the low bioavailability of quercetin and kaempferol (Gugler et al., 1975; Manach and Donovan, 2004; Barve et al., 2009). Many ATP-binding cassette transporters, including BCRP, P-glycoprotein (P-gp) and Multidrug Resistance Protein 2 (MRP2), are known to be expressed in a number of normal tissues and have a major impact on drug disposition (Schinkel and Jonker 2003). Recently, Bcrp was found to be involved in the transport of quercetin: the transport of quercetin across MDCK/Bcrp1 cell monolayers was much more efficient in the B-to-A direction than in the A-to-B direction, with a transport ratio of approximately 160; no directional transport was detected in corresponding parental MDCK cells (Sesink et al., 2005). Therefore, in addition to first pass extraction by metabolism, intestinal efflux, mediated by transporters such as Bcrp, may also represent a possible mechanism for the low bioavailability of quercetin. Whether Bcrp also contributes to the poor bioavailability of kaempferol is currently unknown.

Interestingly, Zhang et al. (Zhang et al., 2005) found that kaempferol is a potent BCRP inhibitor that inhibits the efflux of mitoxantrone in BCRP-overexpressing MCF-7/MX100 cells with an IC_{50} value of 6.04 μ M. Since quercetin and kaempferol are commonly both present in various fruits, vegetables and herbal supplements, we

hypothesize that an herb-herb interaction between quercetin and kaempferol may occur through the efflux transporter Bcrp. In the current study, we carried out a quercetin bi-directional transport study to investigate the effect of kaempferol on the transport of quercetin in MDCK cells transfected with murine Bcrp1. Kaempferol has a very similar structure to quercetin, with only one hydroxyl group absent in the B-ring at position 3' (Figure 1). Based on the structure similarity of these flavonoids, we hypothesized that kaempferol might also be a Bcrp substrate and may inhibit Bcrp-mediated quercetin efflux. Since the role of Bcrp in the transport of kaempferol has not been elucidated before, here we investigated the directional transport of kaempferol in both MDCK-II/Mock and MDCK-II/Bcrp1 cells. The intracellular uptake of kaempferol in Bcrp expressing MDCK/Bcrp1 cells, in the presence and absence of a Bcrp inhibitor, was also determined in a cellular uptake study. Moreover, to understand whether other efflux transporters also contribute to the low bioavailability of kaempferol, we investigated the role of two efflux transporters P-gp and MRP2 on the transport of kaempferol in P-gp-overexpressing MCF-7/ADR cells and MDCK/MRP2 cells, respectively.

MATERIALS AND METHODS

Materials

Quercetin and kaempferol were obtained from Indofine (Hillsborough, NJ). Ascorbic acid and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). RPMI 1640, phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM) and Hank's buffered salt solution (HBSS) were purchased from Invitrogen (Carlsbad, CA). [³H]Mannitol (15 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). GF120918 was purchased from Api Services Inc (Westford, MA). All the chemicals or solvents used for LC/MS/MS were commercially available and of HPLC grade.

Cell culture

The polarized Madin-Darby canine kidney cell line (MDCK-II) was used in the quercetin bi-directional transport study, kaempferol uptake study and kaempferol bi-directional transport study. The MDCK-II and its subclone that was transfected with either murine Bcrp1 or empty vector were kindly provided by Dr. Alfred Schinkel (Netherlands Cancer Institute, Amsterdam, The Netherlands). The MRP2-transfected MDCK/MRP2 cells were obtained from Dr. P. Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). P-gp over-expressing MCF-7/ADR cells (National Cancer Institute) were used in kaempferol uptake study. MCF-7/Sensitive cells and MCF7/ADR cells were obtained from Dr. Susan E. Bates (National Cancer Institute). Both MDCK cells and MCF-7 cells were grown in a 5 % CO₂ atmosphere at 37°C and cultured in culture media (DMEM and RPMI 1640, respectively) supplemented with 10 % fetal bovine serum, 100 units/ml

penicillin, and 100 µg/ml streptomycin. A solution of 0.25 % trypsin-EDTA was used to detach the cells from the 75 cm² flasks.

Kaempferol cellular uptake studies

The intracellular uptake of kaempferol was examined with or without transporter inhibitor (GF120918, 5 µM) in Bcrp1-overexpressing MDCK/Bcrp1 cells, P-gp-overexpressing MCF-7/ADR cells and P-gp negative MCF-7/Sensitive cells.

Briefly, cells were seeded into 6-well plates at a density of 5 x 10⁵ cells per well and uptake studies were started when the cells reached 80~90 % confluence. Stock solution of kaempferol was prepared in DMSO at a concentration of 10 mM. On the day of the experiment, the cells were first washed by PBS twice. The medium containing specific concentrations kaempferol, with or without transporter inhibitors, was then added to each well. The final DMSO concentration in the transport buffer was 0.1 %. After incubation for 1 hour at 37°C, the medium was aspirated and the cells were washed three times with ice-cold PBS. To lyse the attached cells, 1 ml of 0.5 % Triton-X 100 was added into each well. The concentration of kaempferol in the cell lysates were analyzed by LC/MS/MS and normalized with cellular protein content. Protein concentration in the cell lysates was determined by the bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL).

To determine the intracellular concentrations of kaempferol in MDCK/Bcrp1 cells under different initial incubating concentrations, the following equation is used:

$$A_{\text{final}} = A_{\text{passive}} - A_{\text{efflux}} \quad (1)$$

Where A_{final} , A_{passive} and A_{efflux} are the intracellular amounts of kaempferol normalized by cellular protein content. A_{final} refers to the net amount present at 1 hour while A_{passive} is the amount of kaempferol present due to passive diffusion and A_{efflux}

is the amount of kaempferol effluxed by Bcrp1. A_{efflux} was 0 (i.e. $A_{\text{final}} = A_{\text{passive}}$) when kaempferol was incubated with a BCRP inhibitor. The affinity of kaempferol binding to Bcrp1 was reflected by its K_m , which was calculated as follows:

$$A_{\text{efflux}} = E_{\text{max}} * C / (K_m + C) \quad (2)$$

where C is the concentration of kaempferol that was added into each well and E_{max} is the maximal capacity of kaempferol to bind to Bcrp1.

$$A_{\text{efflux}} = A_{\text{final}} \text{ with inhibitor} - A_{\text{final}} \text{ without inhibitor.} \quad (3)$$

Flavonoid Bidirectional Transport Studies

The transport studies of quercetin or kaempferol across MDCK/Mock, MDCK/Bcrp1 and MDCK/MRP2 cell monolayers were conducted as previously described with minor modifications (van Herwaarden et al., 2006). Briefly, MDCK cells were seeded in 24mm i.d. transwell polycarbonate inserts in 6-well plates (0.4 μm pore size; Transwell 3412; Costar, Corning, NY) at the density of $\sim 10^6$ cells/ well. The cells were grown for 7 days and the medium was replaced everyday. On the day of the transport study, the experiment was performed in triplicate in HBSS (pH 7.4). The cell monolayers were first washed twice with HBSS for 30 min and the flavonoid (10 μM) was then added to either the apical (1.5 ml) or basolateral chamber (2.5 ml) (donor chamber). The samples (120 μl) were then taken from the opposite chamber (the receiver chamber) at 15, 30 and 60 minutes after the addition of the flavonoid. To keep the volume constant the receiver side was replenished with the same volume of fresh HBSS after each sampling.

The samples were stored at -20°C until LC/MS/MS analysis. The apparent permeability coefficients (P_{app}) of [^3H]mannitol, a paracellular marker, across MDCK cell monolayers was measured to test the integrity of the monolayer.

The apparent permeability coefficients (P_{app}) of kaempferol across MDCK monolayers in both the A-to-B and B-to-A directions were calculated by the following equation:

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{A \times C_0}$$

where $\Delta Q / \Delta t$ is the rate of kaempferol appearing in the receiver chamber, which is obtained from the slope of the regression line for the transport-time profile of kaempferol across the cell monolayers, C_0 is the initial concentration of kaempferol loaded in the donor chamber, and A is the cell monolayer surface area (4.71 cm^2).

Sample preparation and LC/MS/MS analysis

The concentrations of flavonoids in cells or in transport media were determined by LC/MS/MS. A 100 μl aliquot of quercetin or kaempferol samples in cell lysates or transport buffer were deproteinized using an equal volume of methanol. After vortexing, the mixture was centrifuged at 14000 rpm for 10 min. One hundred μl of the supernatant was transferred into a 200 μl vial insert for LC/MS/MS analysis.

LC/MS/MS was performed using a PE SCIEX API 3000 trip-quadruple tandem mass spectrometer (Applied Biosystem, Foster City, CA) linked to a TurboIonSpray (TIS) interface and a Shimadzu LC10 liquid chromatography. Analyst 1.4.2 software was used for data acquisition and processing. A XTerra MS C18 column (2.1 x 150mm i.d., 3.5 μm ; Waters Corporation, Milford, MA) was used and flavonols quercetin and kaempferol were eluted with a mobile phase of acetonitrile /water containing 0.1 % formic acid (50/50, v/v) at a flow rate of 200 μl /min. The retention times of quercetin and kaempferol were 3.1 min and 4.8 min, respectively. To minimize the ion suppression caused by the high concentration of salts present in the transport buffer (HBSS), the flow

from the LC column was diverted to waste for the first two minutes using an API 2000 diverter valve to prevent the early eluting salts from entering the LC/MS interface. Conditions for MS analysis of quercetin and kaempferol included an ion spray voltage of -4500 V, a nebulizing pressure of 33 psi, and a temperature of 350°C. Nebulizer and curtain gas flow were 10 ml/min and 8 ml/min, respectively. The fragment was induced with collision energy of -30 eV. The optimized declustering potential, focusing potential and collision cell exit potential were -60, -175 and -30V, respectively. The MS was performed in a negative ion mode under multiple reactions monitoring (MRM). The m/z ratio of parent ion and product ion of quercetin were 301.1 and 151.1, respectively. The m/z ratio of molecular ion and product ion of kaempferol were 285.2 and 285.2, respectively. The lower limit of quantification (LOQ) of quercetin or kaempferol was 1 ng/ml. The calibration curve was linear over the concentration range of 1~500 ng/ml for both compounds.

Statistical analysis

All statistics were performed using SPSS (SPSS 11.0; SPSS Inc; Chicago, IL). The differences between the mean values were analyzed for significance using a Student's t-test or one-way analysis of variance, followed by Dunnett's test. Differences were considered statistically significant when the p-values were less than 0.05.

RESULTS

Quercetin transport by Bcrp and inhibition by kaempferol

To investigate whether there is a Bcrp-mediated kaempferol-quercetin interaction, we examine the effect of kaempferol in Bcrp-mediated quercetin efflux in a quercetin bi-directional transport study. The mass spectra (Q1 and Q3 scan) of quercetin are shown in figure 2A and 2B. As shown in Table 1, the $P_{app,B-A}$ ($21.9 \pm 4.4 \times 10^{-6}$ cm/s) of quercetin was much higher than the $P_{app,A-B}$ ($0.232 \pm 0.078 \times 10^{-6}$ cm/s) in MDCK/Bcrp1 cells, whereas this phenomenon was not observed in corresponding control cells. This confirmed a previous report that quercetin is a substrate of Bcrp. In the presence of kaempferol, the $P_{app,A-B}$ value of quercetin was greatly increased in MDCK/Bcrp1 cells ($2.83 \pm 0.65 \times 10^{-6}$ cm/s), compared to quercetin alone group ($0.232 \pm 0.078 \times 10^{-6}$ cm/s). The transport ratio of quercetin in MDCK/Mock cells is 0.85, demonstrating little difference in the A-to-B and B-to-A flux. Compared to quercetin alone group, the transport ratio decreased 11.6 fold (from 97.5 to 8.37) in the presence of 10 μ M of kaempferol in MDCK/Bcrp1 cells. Our results indicate that kaempferol may inhibit Bcrp-mediated quercetin efflux.

Kaempferol transport by Bcrp and inhibition by a potent Bcrp inhibitor

To investigate the role of Bcrp1 in the transport of kaempferol, we conducted kaempferol bi-directional transport studies in MDCK cells transfected with empty vector (MDCK/Mock) and Bcrp1 (MDCK/Bcrp1). The transport of kaempferol (10 μ M) across MDCK/Mock and MDCK/Bcrp1 cell monolayers, in the A-to-B and B-to-A directions, is presented in Figures 3A and 3B. Table 2 shows the apparent permeability of kaempferol

(P_{app}) and transport ratios of kaempferol in MDCK cell monolayers. The B-to-A directional transport of kaempferol was much higher than its transport in the A-to-B direction in MDCK/Bcrp1 cells; this phenomenon was not observed in Bcrp1 negative MDCK/Mock cells, suggesting that Bcrp is involved in the transport of kaempferol. In MDCK/Bcrp1 cells, the P_{app,B-A} value of kaempferol was much higher ($17.7 \pm 3.8 \times 10^{-6}$ cm/s) than the P_{app,A-B} value ($0.279 \pm 0.037 \times 10^{-6}$ cm/s), with a transport ratio of 63.4. In contrast, the transport ratio of kaempferol was 0.68 in Bcrp1-negative MDCK/Mock cells; however, no statistically significant difference between the P_{app,A-B} value ($13.8 \pm 6.5 \times 10^{-6}$ cm/s) and P_{app,B-A} value ($9.29 \pm 0.63 \times 10^{-6}$ cm/s) of kaempferol was observed in MDCK/Mock cells, suggesting no directional flux. Overall, these results indicate that kaempferol is a substrate of Bcrp1.

To further confirm the results of the kaempferol bi-directional transport study, kaempferol uptake, in the absence and presence of the potent Bcrp inhibitor GF120918 (5 μ M) was examined in MDCK/Bcrp1 cells. The representative chromatograms of kaempferol (5 μ M) in the presence or absence of GF120918 in Bcrp1-transfected MDCK cells are shown in figure 4B. The peak area of kaempferol increased more than 10 fold when 5 μ M of kaempferol was co-incubated with 5 μ M of GF120918. Figure 4C presents the intracellular concentrations of kaempferol when 5, 10, or 15 μ M of kaempferol, with or without GF120918, was incubated with Bcrp1-expressing MDCK/Bcrp1 cells for 1 hour. In the presence of GF120918, kaempferol cellular uptake was significantly increased compared with the control group, at all concentrations tested in current study, indicating that kaempferol is a substrate of Bcrp.

When MDCK/Bcrp1 cells were incubated with different concentrations of kaempferol, we observed that the cellular accumulation of kaempferol was not proportional to its incubation concentration. In the presence of 5 μ M of GF120918, the intracellular amount of kaempferol increased 14.8 fold at 5 μ M, 2.11 fold at 10 μ M, and 1.49 fold at 15 μ M (Table 3). Since the final cellular concentration of kaempferol is dependent on both passive diffusion and active efflux by Bcrp1, the efflux of kaempferol was calculated as described under Methods and the results are given in Table 2. The estimated K_m and E_{max} of kaempferol to Bcrp1 were 1.26 μ M and 1.89 μ mol/mg protein, respectively (figure 5).

Kaempferol transport by MRP2 and P-gp

In addition to Bcrp1, we also examined the role of MRP2 in the transport of kaempferol in MDCK/MRP2 cells. As shown in figure 3C, the transport of kaempferol in MDCK/MRP2 cells was similar to that in MDCK/Mock cells, suggesting that kaempferol is not a MRP2 substrate. The $P_{app,A-B}$ value of kaempferol was $18.9 \pm 4.2 \times 10^{-6}$ cm/s and $P_{app,B-A}$ value was $11.6 \pm 4.0 \times 10^{-6}$ cm/s in MDCK/MRP2 cells, with a transport ratio of 0.61.

Since P-gp is similar to BCRP in that it has a broad substrate spectrum and plays an important role in the disposition of various compounds, we examined the role of P-gp in the transport of kaempferol (10 μ M) in P-gp-overexpressing MCF-7/ADR cells and P-gp negative MCF-7/Sensitive cells. It has been reported that only P-gp, but not BCRP or MRP2, was detected in MCF-7/ADR cells (Zhang et al., 2004). As is shown in figure 6, the intracellular concentration of kaempferol was greatly increased (~3.2 fold) in the presence of P-gp inhibitor in MCF-7/ADR cells; whereas this phenomenon was not

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observed in corresponding P-gp negative control cells. This result suggested that kaempferol is also a substrate of P-gp.

DISCUSSION

Although herb-herb interactions between flavonoids have not been extensively investigated, such interactions may explain why individual flavonoids in many cases exhibit greater bioavailability or pharmacological effects when administered as a mixed extract or product rather than in a pure form. Increased bioavailability of individual flavonoids was observed in several studies when a flavonoid was administered with other flavonoids. For example, when Chen et al. (Chen et al., 1997) investigated the pharmacokinetics of tea polyphenols in rats, they coincidentally found that epigallocatechin-3-gallate (EGCG) displayed a different pharmacokinetic behavior when it was given in decaffeinated green tea (DGT), compared with pure EGCG. Their results revealed that the absorption rate constant (k_a) increased 3.6-fold for EGCG in DGT than for EGCG administered alone. A lower clearance and higher AUC were also observed for EGCG when administered as a combination. Moon and Morris (Moon and Morris, 2007) investigated the effect of quercetin and EGCG on the disposition of biochanin A in rats. They found that the AUC of biochanin A, after both i.v and oral administration, increased significantly when it was co-administered with quercetin and EGCG. For these previously reported herb-herb interactions, competitive inhibition of UDP-glucuronosyltransferases and sulfotransferases, the main metabolic pathways for these flavonoids, was proposed as the possible mechanism. In the current study, we investigated the effect of kaempferol on the transport of quercetin in BCRP-overexpressing cells and our results clearly showed that there is an interaction between kaempferol and quercetin involving the efflux transporter BCRP.

In the quercetin bi-directional transport study, we found that in MDCK/Bcrp1 cells the $P_{app,A-B}$ value of quercetin was greatly increased in the presence of kaempferol compared to quercetin alone group ($2.83 \pm 0.65 \times 10^{-6}$ cm/s and $0.232 \pm 0.078 \times 10^{-6}$ cm/s, respectively). Since A-to-B directional transport is physiologically more relevant to drug absorption, based on our results we would expect an increase in quercetin bioavailability when it is co-administered with kaempferol. It should be noted that although the $P_{app,A-B}$ value of quercetin in MDCK/Bcrp1 increased more than 10 fold in the presence of kaempferol, it is still much lower than the $P_{app,A-B}$ value obtained for quercetin transport in MDCK/Mock cells. To what extent the in vitro observation can be extrapolated to in vivo situation depend on several factors, such as the Bcrp expression level in the intestine and the intestine concentration of kaempferol after oral administration.

Since kaempferol and quercetin are similar in structure, we conducted a kaempferol bi-directional transport study in MDCK-II cells to examine whether kaempferol was also a Bcrp1 substrate. Usually a compound is considered to be a substrate of certain efflux transporter(s) if the transport ratio ($P_{app,B-A} / P_{app,A-B}$) > 2 (Balimane et al., 2006). In our study, the transport ratio of kaempferol in MDCK/Bcrp1 cells is 63.4 and this transport pattern was not observed in parental MDCK/Mock cells, which has a transport ratio of 0.68. Our results strongly indicated that kaempferol is a substrate of Bcrp. The fact that kaempferol is not only a Bcrp inhibitor but also a Bcrp substrate suggests that kaempferol may inhibit Bcrp-mediated quercetin efflux through competitive inhibition. Moreover, kaempferol is known to be poorly absorbed with a bioavailability as low as 2 % (Barve et al., 2009). Our results also suggested that, in addition to first pass extraction due to

metabolism, efflux transporters such as Bcrp may also represent a possible mechanism for the low bioavailability of kaempferol.

Concentration-dependent transport of kaempferol was studied by examining kaempferol uptake study in MDCK/Bcrp1 cells. Consistent with our transport data, our results from the kaempferol uptake study showed that the intracellular concentrations of kaempferol, at all three concentrations tested (i.e. 5 μ M, 10 μ M and 15 μ M), were significantly increased in the presence of the Bcrp inhibitor GF120918 (5 μ M), indicating the involvement of Bcrp in the transport of kaempferol. Interestingly, with different concentrations of kaempferol, we observed a nonlinear intracellular kaempferol accumulation when kaempferol was incubated alone. The accumulation was proportional to concentration (i.e. linear) when kaempferol was co-incubated with GF120918. Since the intracellular accumulation of kaempferol is dependent on both passive diffusion and active efflux by Bcrp1, the linear cellular uptake of kaempferol when it was co-incubated with GF120918 suggests that Bcrp1-mediated kaempferol efflux was fully reversed by 5 μ M of GF120918. Based on the efflux of kaempferol at different incubation concentrations, we estimated that kaempferol is a Bcrp substrate with high affinity (1.26 μ M) and low capacity (1.89 μ mol/mg protein).

To understand whether other efflux transporters, such as MRP2 and P-gp, also play a role in the transport of kaempferol, we conducted a kaempferol bi-directional transport study in MRP2-transfected MDCK/MRP2 cells and a kaempferol uptake study in P-gp-overexpressing MCF-7/ADR cells. Our previous western blot data showed that only P-gp, but not BCRP and MRP2, is expressed in MCF-7/ADR cells (Zhang et al., 2004). Therefore, even though GF120918 is not only a P-gp inhibitor but also a BCRP inhibitor,

the increase of kaempferol uptake in MCF7/ADR cells in the presence of GF120918 indicates that kaempferol is a P-gp substrate. Although MRP2 is well known to transport organic anions and P-gp usually mediates the efflux of uncharged or cationic hydrophobic compounds, our results showed that kaempferol, an organic anion, is a substrate of P-gp but not MRP2 (Leslie et al., 2005; Choudhuri and Klaassen, 2006). Considering the high expression level of P-gp in intestine, P-gp, in addition to Bcrp, may also contribute to the poor absorption of kaempferol after oral consumption.

Endogenous expression of drug transporters in various cell lines have been investigated by Goh et al. (Goh et al., 2002) and their results demonstrated the endogenous MDR1 mRNA expression in MDCK-II cells. The same group also found that canine MDR1 shares 78 % amino acid identity with the human MDR1. However, when kaempferol was transported across parental MDCK-II cell monolayers, no directional transport of kaempferol was observed although kaempferol was also found to be a P-gp substrate in our uptake study. This observation might be due to the very low expression of MDR1 in MDCK-II cells, the low affinity of kaempferol to P-gp, or functional differences of P-gp among different species. Therefore, it is not expected that P-gp would contribute to the flux observed in MDCK/Bcrp1 cells.

In conclusion, we report for the first time that kaempferol is a Bcrp substrate with high affinity (K_m 1.26 μ M) and low capacity (E_{max} 1.89 μ mol/mg protein). Kaempferol influenced Bcrp-mediated transport of quercetin, indicating its role as a BCRP inhibitor. Whether kaempferol inhibits Bcrp-mediated quercetin efflux through competitive inhibition requires further investigation. Other than first pass metabolism, intestinal efflux by efflux transporters Bcrp and P-gp may also represent one possible mechanism

for the poor bioavailability of kaempferol. The use of flavonoids in combination may increase their bioavailability through transport interactions.

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Authorship Contributions

Participated in research design: An and Morris

Conducted experiments: An and Gallegos

Contributed new reagents or analytical tools: Morris

Performed data analysis: An

Wrote or contributed to the writhing of the manuscript: An and Morris

Other: Morris write acquired funding for the research

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Footnote

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Figure Legends

Fig. 1. Chemical structures of flavonols quercetin and kaempferol.

Fig. 2. ESI-MS-MS spectra of quercetin A) Q1 scan; B) Q3 scan.

Fig. 3. The transport of kaempferol (10 μ M) across MDCK cell monolayers in both apical-to-basolateral (A-to-B) and basolateral-to-apical (B-to-A) directions. A) MDCK/Mock cells; B) MDCK/Bcrp1 cells; and C) MDCK/MRP2 cells. The concentrations of kaempferol were determined by LC/MS/MS. Data are presented as mean \pm SD; n =3.

Fig. 4. A) Q1 and Q3 scan of kaempferol; B) Representative chromatograms of kaempferol when MDCK/Bcrp1 cells were incubated with 5 μ M of kaempferol in the presence or absence of the BCRP inhibitor GF120918 (5 μ M); C) Intracellular concentrations of kaempferol when Bcrp1-expressing MDCK/Bcrp1 cells were incubated with 5, 10, or 15 μ M of kaempferol. GF120918, a BCRP inhibitor, was used as a positive control. Data are presented as mean \pm SD; n =3; **, p<0.01; ***, p<0.001

Fig. 5. Concentration-dependent efflux of kaempferol in MDCK/Bcrp1 cells.

Fig. 6. The uptake of kaempferol (10 μ M) in the presence or absence of 5 μ M GF120918, in both P-gp-overexpressing MCF-7/ADR cells and P-gp negative MCF-7/Sensitive cells.

Data are presented as mean \pm SD; n =3; ***, p<0.001.

Table 1. Transport of quercetin across MDCK cell monolayers transfected with empty vector or Bcrp1.

| | Quercetin (10 μ M) | Papp _{A-B} (cm/s, $\times 10^{-6}$) | Papp _{B-A} (cm/s, $\times 10^{-6}$) | Transport ratio (Papp _{B-A} /Papp _{A-B}) |
|-----------|---------------------------|--|--|--|
| Without K | MDCK/Mock | 11.8 \pm 0.35 | 10.0 \pm 0.72 | 0.85 |
| Without K | MDCK/Bcrp1 | 0.232 \pm 0.078 | 21.9 \pm 4.4 | 97.5 |
| With K | MDCK/Bcrp1 | 2.83 \pm 0.65 | 23.7 \pm 11 | 8.37 |

The apparent permeability was calculated as described in the 'Materials and Methods'.

Table 2. Transport of kaempferol across MDCK cell monolayers transfected with empty vector, Bcrp1 or MRP2.

| Kaempferol (10 μ M) | Papp, _{A-B} (cm/s, $\times 10^{-6}$) | Papp, _{B-A} (cm/s, $\times 10^{-6}$) | Transport ratio (Papp, _{B-A} /Papp, _{A-B}) |
|----------------------------|---|---|--|
| MDCK/Mock | 13.8 \pm 6.5 | 9.29 \pm 0.63 | 0.68 |
| MDCK/Bcrp1 | 0.279 \pm 0.037 | 17.7 \pm 3.8*** | 63.4 |
| MDCK/MRP2 | 18.9 \pm 4.2 | 11.6 \pm 4.0 | 0.61 |

The apparent permeability was calculated as described in the ‘Materials and Methods’.

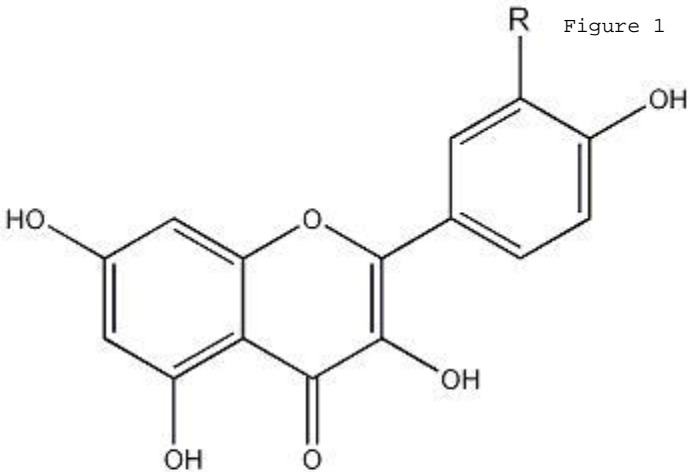
Data are presented as mean \pm SD; n =3. ***, p<0.001

Table 3. The intracellular accumulation of kaempferol (K) with different incubation concentrations.

| Conc of K (μ M) | Without inhibitor (A _{final} , μ mol/mg protein) | With inhibitor (A _{passive} , μ mol/mg protein) | Fold increase | Intracellular amount of K (A _{efflux} , μ mol/mg protein) |
|-------------------------|--|---|------------------|---|
| 5 | 0.11 \pm 0.042 | 1.63 \pm 0.56** | 14.8 | 1.52 \pm 0.74 |
| 10 | 1.50 \pm 0.33 | 3.15 \pm 0.27** | 2.11 | 1.65 \pm 0.60 |
| 15 | 3.63 \pm 0.15 | 5.40 \pm 0.23*** | 1.49 | 1.77 \pm 0.25 |

Data are presented as mean \pm SD; n =3. **, p<0.01; ***, p<0.001

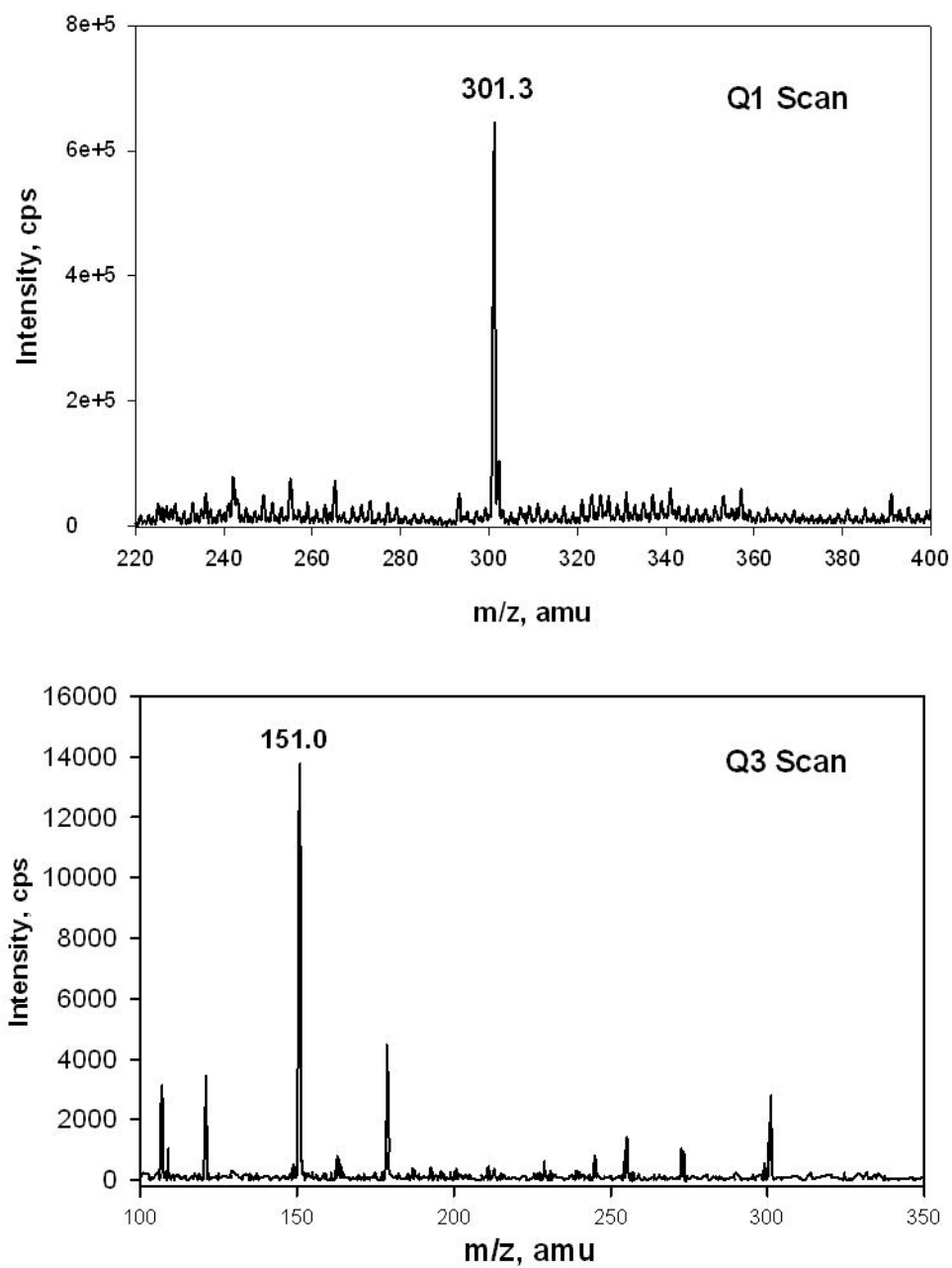
Figure 1



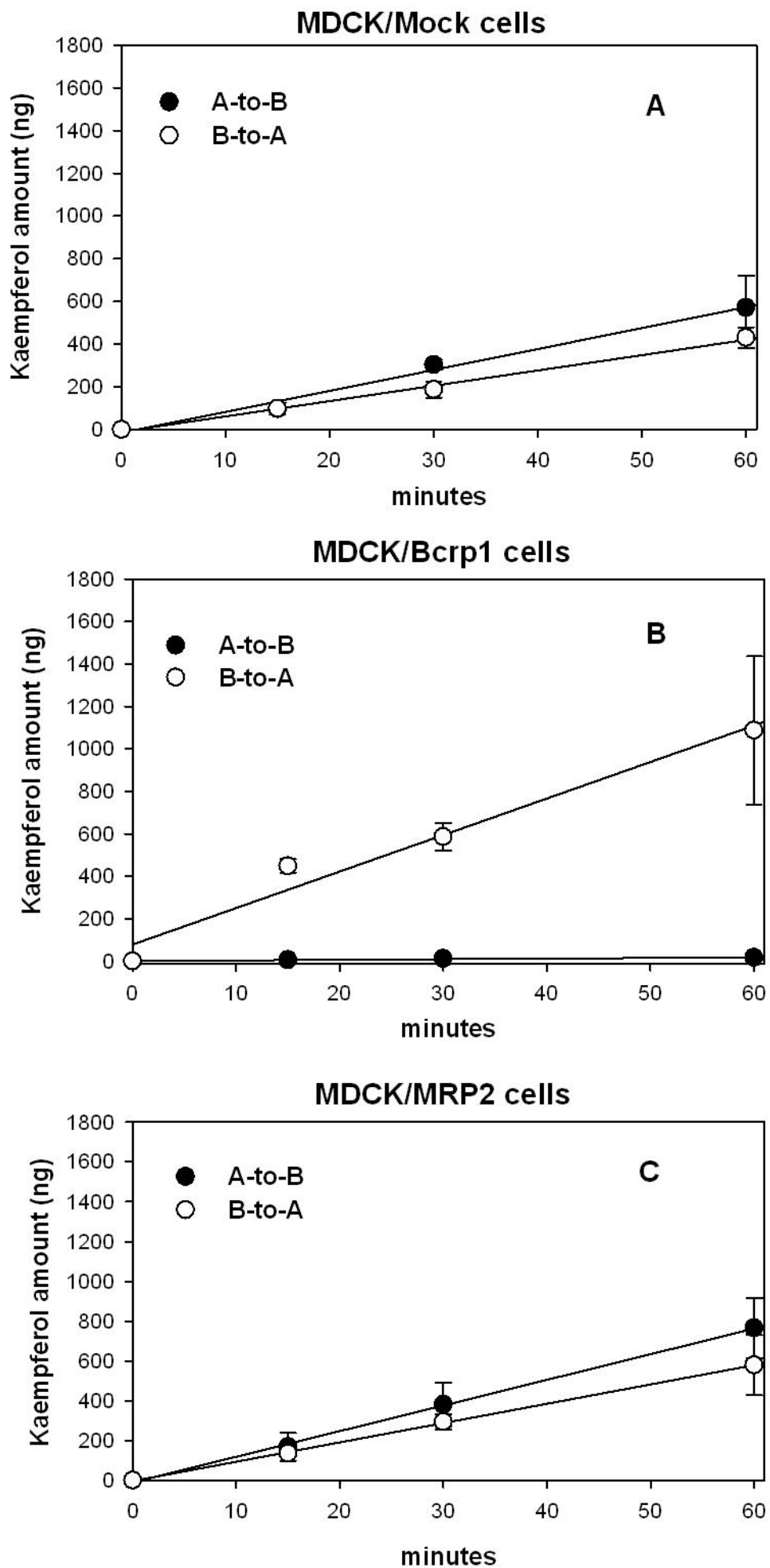
Quercetin R=OH

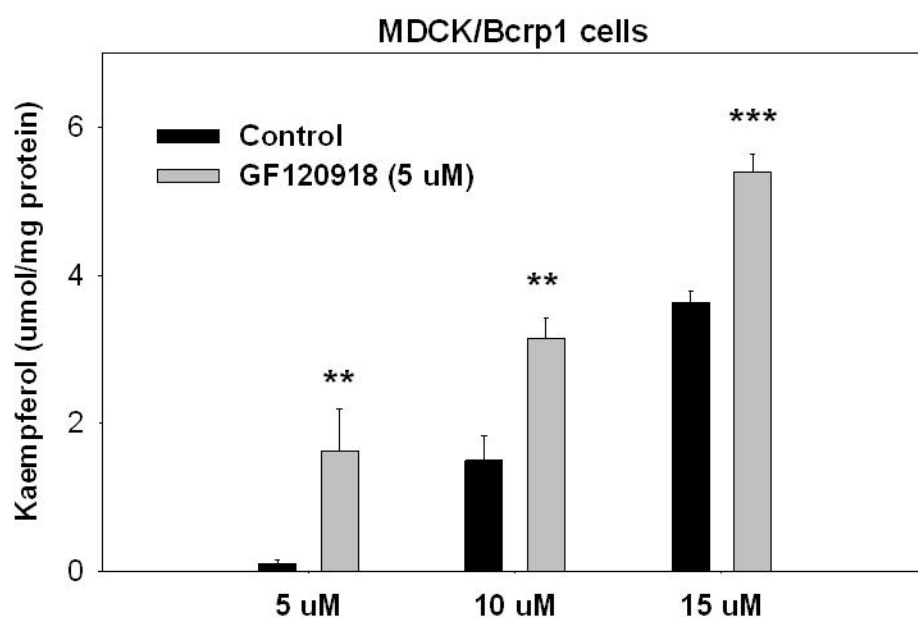
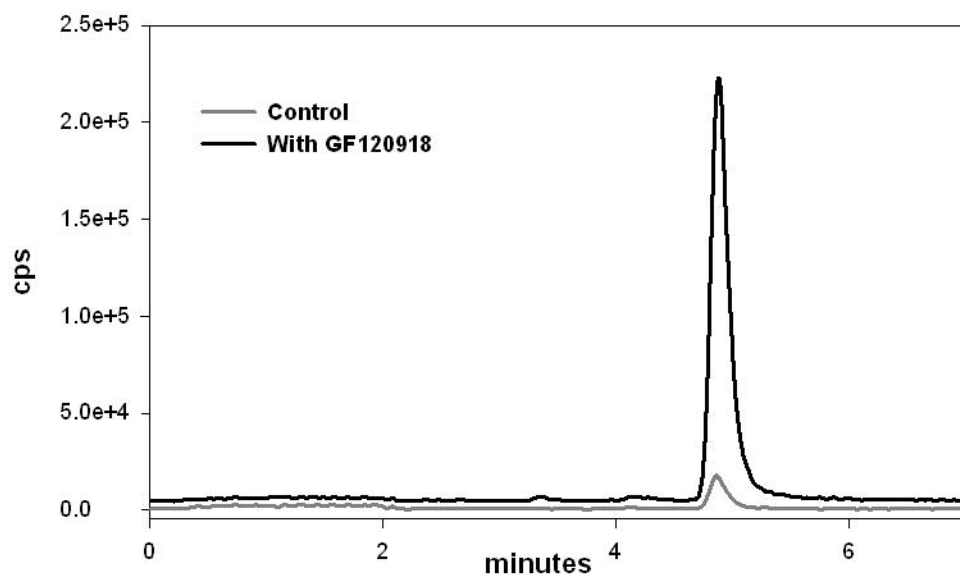
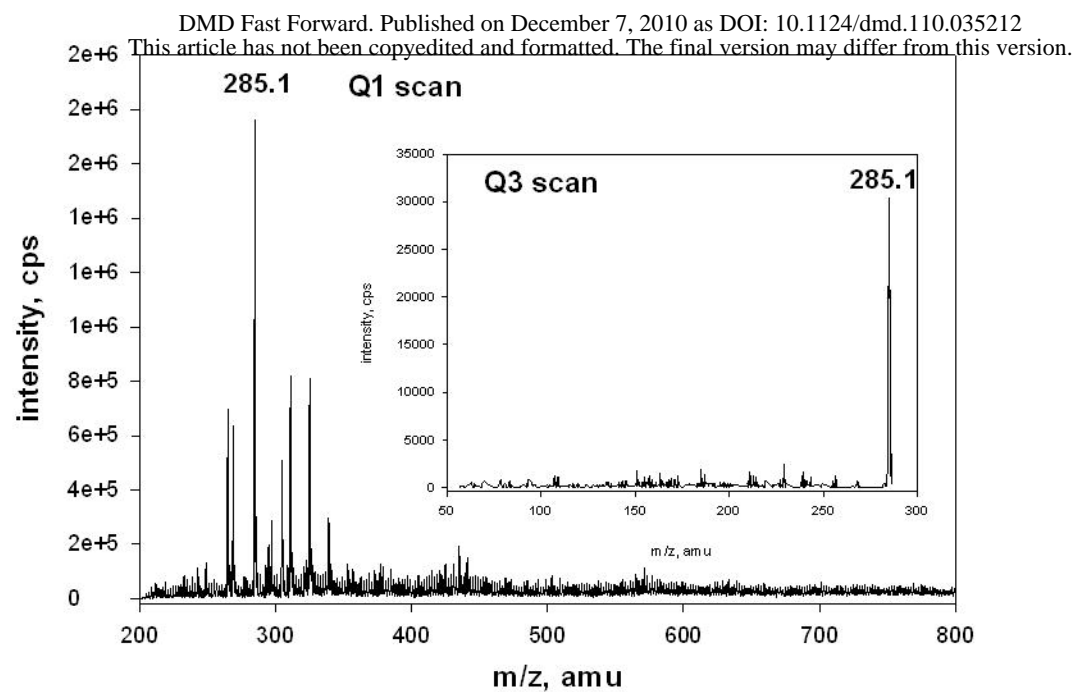
Kaempferol R=H

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Efflux of kaempferol in MDCK/Bcrp1 cells Figure 5

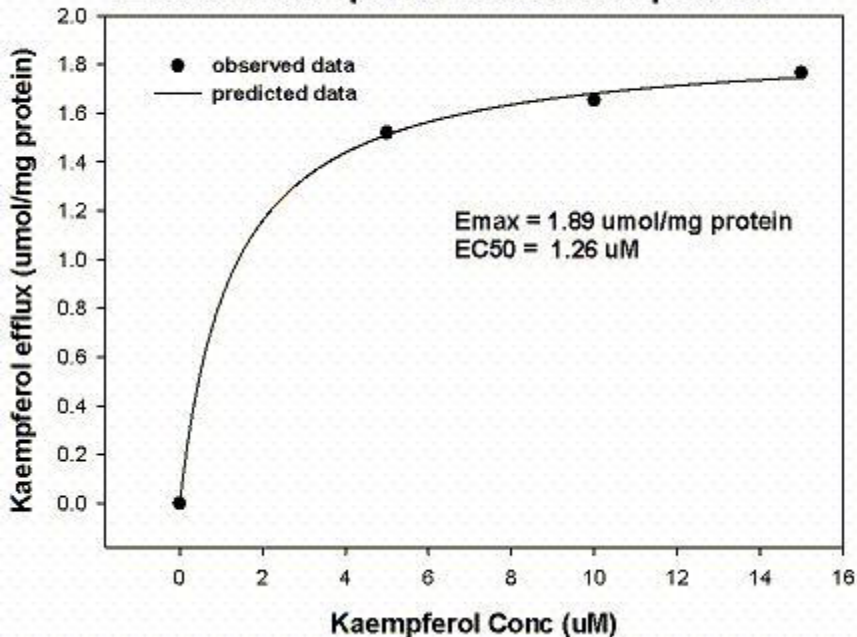


Figure 6

