Title Page

Short Communication

Efflux transport characterization of resveratrol glucuronides in UDP-glucuronosyltransferase 1A1 transfected HeLa cells: Application of a cellular pharmacokinetic model to decipher the contribution of multidrug resistance-associated protein 4

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Running Title Page

Running Title: MRP4 transport of resveratrol glucuronides

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Non-standard abbreviations

BCRP, breast cancer resistance protein; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; GUSB, β-glucuronidase; \( K_m \) or \( K'_m \), Michaelis-Menten constant; \( K_{si} \), substrate inhibition constant; MRP, multidrug resistance-associated protein; shRNA, short hairpin RNA; UDPGA, uridine diphosphoglucuronic acid; R3G, resveratrol 3-O-glucuronide; R4’G, resveratrol 4’-O-glucuronide; shRNA, short hairpin RNA; UGT, UDP-glucuronosyltransferase; UPLC, ultra performance liquid chromatography; \( J_{max} \) or \( V_{max} \), maximal velocity.
Abstract

Resveratrol undergoes extensive metabolism to form biologically active glucuronides in humans. However, the transport mechanisms for resveratrol glucuronides are not fully established. Here we aimed to characterize the efflux transport of resveratrol glucuronides using UGT1A1-overexpressing HeLa cells (HeLa1A1 cells), and to determine the contribution of multidrug resistance-associated protein 4 (MRP4) to cellular excretion of the glucuronides. Two glucuronide isomers [i.e., resveratrol 3-O-glucuronide (R3G) and resveratrol 4’-O-glucuronide (R4’G)] were excreted into the extracellular compartment after incubation of resveratrol (1-100 μM) with HeLa1A1 cells. The excretion rate was linearly related to the level of intracellular glucuronide, indicating that glucuronide efflux was a non-saturable process. MK-571 (a dual inhibitor of UGT1A1 and MRPs) significantly decreased the excretion rates of R3G and R4’G while increasing their intracellular levels. Likewise, short-hairpin RNA (shRNA)-mediated silencing of MRP4 caused a significant reduction in glucuronide excretion but an elevation in glucuronide accumulation. Furthermore, β-glucuronidase expressed in the cells catalyzed the hydrolysis of the glucuronides back to the parent compound. A cellular pharmacokinetic model integrating resveratrol transport/metabolism with glucuronide hydrolysis/excretion was well fitted to the experimental data, allowing derivation of the efflux rate constant values in the absence or presence of shRNA targeting MRP4. It was found that a large percentage of glucuronide excretion (43-46%) was attributed to MRP4. In conclusion, MRP4 participated in cellular excretion of R3G and R4’G. Integration of mechanistic pharmacokinetic modelling with transporter knock-down was a useful method to derive the contribution percentage of an exporter to overall glucuronide excretion.
Introduction

Resveratrol is a natural polyphenol widely distributed in plant kingdom (e.g., grapes, peanuts, and berries) (Shishodia and Aggarwal, 2006). It possesses numerous health-promoting effects including anti-oxidant, anti-inflammatory, chemopreventive, and cardioprotective properties (Bishayee, 2009; Brisdelli et al., 2009; Jasiński et al., 2013; Poulsen et al., 2013; Szkudelski and Szkudelska, 2015). However, bioavailability of resveratrol is exceedingly low (< 1%) after oral uptake (Walle et al., 2004; Wenzel and Somoza, 2005; Walle, 2011). Extensive glucuronidation is found to be the main cause of low oral bioavailability (Walle et al., 2004; Walle, 2011). Glucuronidation results in high levels of circulating glucuronides [3-O-glucuronide (R3G) and 4’-O-glucuronide (R4’G)] that are suggested to be one source of the beneficial effects of resveratrol (Walle et al., 2004; Walle, 2011).

Efflux transporters such as BCRP, MRP2 and MRP3 have been identified as contributors to intestinal/hepatic excretion of resveratrol glucuronides (Adachi et al., 2005; Maier-Salamon et al., 2006; van de Wetering et al., 2009). However, it remains unknown whether the MRP4 transporter (with significant expression in the liver and kidney) is involved in transport of resveratrol glucuronides (Russel et al., 2008). The objective of the present study was to determine the role of MRP4 in excretion of resveratrol glucuronides using UGT1A1 stably transfected HeLa cells. Contribution of MRP4 to glucuronide excretion was quantified by coupling shRNA-mediated silencing with mechanistic pharmacokinetic modeling.
Materials and Methods

Materials

Expressed human UGT1A1 was purchased from BD Biosciences (Woburn, MA). Anti-GUSB and anti-beta actin antibodies were purchased from Abcam (Cambridge, MA). MK-571 was obtained from Sigma-Aldrich (St Louis, MO). Resveratrol was purchased from Aladdin Reagents (Shanghai, China). R3G and R4’G were purchased from Cayman Chemical (Ann Arbor, MI).

Establishment of UGT1A1-overexpressing and GUSB-overexpressing HeLa Cells

UGT1A1- or GUSB-overexpressing HeLa cells were generated using the lentiviral transfection method as described (Quan et al., 2015; Sun et al., 2015). Western blotting against GUSB was performed as described (Quan et al., 2015).

Glucuronide excretion experiments

Glucuronide excretion experiments were performed following the published procedures (Quan et al., 2015). Resveratrol and its glucuronides (R3G and R4’G) were quantified using Waters ACQUITY UPLC system.

Statistical analysis

Data are expressed as mean ± SD. Statistically significant differences were analyzed by unpaired Student’s t test. The level of significance was set at p < 0.05 (*) or p < 0.01 (**) or p < 0.001 (***)
Results and Discussion

Effects of MK-571 on disposition of resveratrol glucuronides

MK-571 (a pan-MRP inhibitor) caused a significant reduction (>36%, p < 0.01) in excretion of resveratrol glucuronides (Figure 1A). A maximal reduction was associated with the highest MK-571 concentration of 20 μM (Figure 1A). By contrast, MK-571 led to a significant elevation (>140%, p < 0.05) in glucuronide accumulation (Figure 1). The extent of elevation was positively correlated with the MK-571 concentration (Figure 1). The results suggested that MRP family proteins may be responsible for excretion of resveratrol glucuronides.

Effects of MK-571 on resveratrol glucuronidation by UGT1A1

The effects of MK-571 on glucuronidation of resveratrol were determined using expressed UGT1A1 enzyme. Formation of both R3G and R4’G was significantly inhibited by MK-571 (Figure 1C/D). The inhibition kinetics for R3G formation was best described by the competitive inhibition model (Supplemental eq.4) (Figure 1C & Supplemental Tab.1). The inhibition kinetics for R4’G formation was well fitted by a two-site inhibition model (Supplemental eq.10) (Figure 1D & Supplemental Tab.1). The models consistently suggested that MK-571 inhibited glucuronidation of resveratrol through its binding to the reaction site of UGT1A1. Since the enzyme activity was inhibited by MK-571, reduced glucuronide excretion may not be ascribed to suppression of MRP transporters by MK-571 (Figure 1A). However, the significant increase in glucuronide accumulation caused by MK-571 was strong evidence that one or more MRP family
proteins contributed to cellular excretion of resveratrol glucuronides (Figure 1B). This was because elevated glucuronide accumulation must result from blocked glucuronide efflux though the action of MK-571 on MRPs.

**Effects of MRP4 knock-down on glucuronide disposition**

MRP4 was knocked-down by shRNA-mediated gene silencing. The selected shRNA was able to decrease the expression of MRP4 by 65% in HeLa cells (Quan et al., 2015). Knock-down of MRP4 caused a significant reduction (p < 0.05) in excretion of resveratrol glucuronides (Supplemental Fig.1A). On the contrary, MRP4 knock-down led to a significant elevation (p < 0.05) in intracellular glucuronide (Supplemental Fig.1B). The alterations in glucuronide disposition indicated that the MRP4 transporter was involved in excretion of resveratrol glucuronides. Transport of the resveratrol glucuronides by MRP4 was further confirmed by the vesicular transport assays (Supplemental Fig.2). It was a novel finding that MRP4 was involved in the transport of resveratrol glucuronides. Previous studies indicated that BCRP, MRP2, and MRP3 were responsible for excretion of resveratrol glucuronides (Adachi et al., 2005; Maier-Salamon et al., 2006; van de Wetering et al., 2009). BCRP and MRP2 (two apical exporters) facilitated elimination of the glucuronides via biliary and intestinal routes. By contrast, MRP3 and MRP4 (two basolateral exporters) contributed to systemic exposure of the glucuronides via basolateral excretion.
GUSB-mediated hydrolysis of resveratrol glucuronides

The hydrolysis potential of resveratrol glucuronides (R3G and R4’G) in HeLa cells was determined using the lysate preparations from HeLa (control lysate) and GUSB-overexpressing HeLa cells (GUSB lysate). Overexpression of GUSB was confirmed by Western blotting (Figure 2A). Both control lysate and GUSB lysate were able to catalyze hydrolysis of resveratrol glucuronides (R3G and R4’G) (Figure 2B/C). As expected, GUSB lysate showed a much higher hydrolysis activity toward the glucuronides compared to control lysate (Figure 2B/C). The intrinsic clearance (CLint) for the hydrolysis reaction was estimated by performing linear regression (CLint equaled the slope). The GUSB enzyme possessed a similar activity toward the two glucuronide isomers (4.3 vs 4.7 μl/h/mg, p > 0.05) (Figure 2B/C).

Mechanistic pharmacokinetic modeling of resveratrol disposition in control versus MRP4 knock-down cells

The concentration-time profiles were determined for resveratrol and its glucuronides in both extracellular and intracellular compartments after administration of 50 nmol resveratrol. A cellular pharmacokinetic model was used to describe the data (Supplemental Fig.3 and 4). Mechanistic fitting was performed by fixing binding affinity parameters (Km and Ks, independent of model system) to their corresponding values derived from in vitro glucuronidation assay (Supplemental Tabs. 2 and 3). The coefficients of variation for all fitted parameters were less than 20%, suggestive of adequate fitting of the model to the data (Supplemental Tab.2). The estimated values for all parameters except the efflux rate constants (Kef,1 and Kef,2) were similar (p > 0.05).
between control and MRP4 knock-down cells. The $K_{ef,1}$ and $K_{ef,2}$ values were reduced to $72 \pm 4.2\%$ and $70 \pm 3.6\%$ of the control, respectively. Based on the relative expression level of MRP4, it was estimated that MRP4 contributed $43 \pm 1.6\%$ of R3G excretion and $46 \pm 2.4\%$ of R4'G excretion in HeLa1A1 cells.

The established pharmacokinetic model (Supplemental Fig.3) was scientifically solid. First, the model assumed that resveratrol passively diffused across cell membranes. Passive diffusion as the main transport mechanism for resveratrol had been documented in the literature (Henry et al., 2005). Second, the model included a deglucuronidation process. This was necessary because GUSB enzyme expressed in the cells was able to hydrolyze resveratrol glucuronides back to the parent molecule. Third, the model assumed that glucuronide efflux was a non-saturable process. This assumption appeared to be valid because the excretion rate of glucuronide was linearly related to the intracellular glucuronide (Supplemental Fig.5). Four, it was assumed that intracellular binding of glucuronides was negligible. This was well supported by the fact the hydrophilic glucuronides hardly bound to lysate proteins (Supplemental Fig.6).

In summary, this study for the first time demonstrated that MRP4 participated in cellular excretion of resveratrol glucuronides and β-glucuronidase was a key determinant to total cellular glucuronidation. Further, integration of mechanistic pharmacokinetic modelling with transporter knock-down was a useful method to derive the contribution percentage of an exporter to overall glucuronide excretion in a cell system expressing multiple transporters.
Authorship Contributions

Participated in research design: Wang, Li, Dong and Wu.

Conducted experiments: Wang, Li, Quan and Dong.

Contributed new reagents or analytic tools: Dong.

Performed data analysis: Wang, Li and Wu.

Wrote or contributed to the writing of the manuscript: Wang, Li and Wu.
References


Footnotes

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SW and FL contributed equally to this work.
Legends for Figures

Figure 1  Effects of MK-571 on disposition of resveratrol glucuronides after incubation of HeLa1A1 cells with resveratrol (25 and 100 μM). (A) Effects of MK-571 on the excretion rates of resveratrol glucuronides; (B) Effects of MK-571 on the intracellular levels of resveratrol glucuronides. Each data point was the average of three determinations with error bar representing the standard deviation. *p < 0.05; **p < 0.01; ***p < 0.001 compared with control group. (C) Kinetic modeling of the effects of MK-571 on R3G formation by UGT1A1; (D) Kinetic modeling of the effects of MK-571 on R4’G formation by UGT1A1.

Figure 2  Functional characterization of β-glucuronidase (GUSB) in hydrolysis of resveratrol glucuronides. (A) Protein expression of GUSB in wild-type HeLa (WT) and GUSB-overexpressing HeLa cells (HeLa_GUSB). (B) Hydrolysis kinetics of R3G by HeLa lysate (control) and HeLa_GUSB lysate. (C) Hydrolysis kinetics of R4’G by HeLa lysate (control) and HeLa_GUSB lysate. The hydrolysis activity of GUSB enzyme was derived by subtracting the hydrolysis rate of HeLa cell lysate from that of HeLa_GUSB lysate. Each data point was the average of three determinations with error bar representing the standard deviation.
**Figure 1**

(A) Relative rate of excretion (%)

Resveratrol@25 μM  
Resveratrol@100 μM

MK-571, μM

(B) Fold change (accumulation)

MK-571, μM

(C) Rate of 3-O-glucuronidation

UGT1A1

(D) Rate of 4-O-glucuronidation

UGT1A1
**Figure 2**

(A) Western blot showing GUSB and β-actin bands. WT and HeLa_GUSB samples are compared.

(B) Hydrolysis of GUSB and control lysates as a function of R3G concentration. CL_int values are calculated as follows:
- GUSB lysate: CL_int = 5.2 ± 0.3 µl/h/mg
- Control lysate: CL_int = 4.3 ± 0.2 µl/h/mg
- GUSB: CL_int = 0.8 ± 0.1 µl/h/mg

(C) Hydrolysis of GUSB and control lysates as a function of R4'G concentration. CL_int values are calculated as follows:
- GUSB lysate: CL_int = 5.4 ± 0.5 µl/h/mg
- Control lysate: CL_int = 4.7 ± 0.3 µl/h/mg
- GUSB: CL_int = 0.7 ± 0.1 µl/h/mg
Supplemental data

Manuscript title:
Efflux transport characterization of resveratrol glucuronides in UDP-glucuronosyltransferase 1A1 transfected HeLa cells: Application of a cellular pharmacokinetic model to decipher the contribution of multidrug resistance-associated protein 4

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Journal name:
Drug Metabolism and Disposition
Methods and Materials

Protein Binding Assay

Binding of resveratrol and its glucuronides to cell lysate (0.2 mg protein/ml) was measured using the rapid equilibrium dialysis system (RED) (Thermo Scientific, Rockford, IL, USA) according to the manufacturer’s instructions. The fraction of unbound (fu) was calculated as described (Sun et al., 2015).

Preparation of cell lysate

The cells were collected and suspended in potassium phosphate buffer (50 mM, pH 7.4). This was followed by sonication for 15 min in an ice-cold water bath. Cell lysate was obtained by centrifugation (4°C) at 1,000 g for 5 min. Total protein concentration of cell lysate was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

Glucuronidation assay

Glucuronidation activities were determined following the incubation procedures as described (Quan et al., 2015). In brief, potassium phosphate buffer (pH 7.4) was used as the incubation medium. The final incubation mixture of 300 μl maintained at 37°C contained resveratrol (at a desired concentration), saccharolactone (4.4 mM), MgCl₂ (0.88 mM), alamethicin (22 μg/ml), UDPGA (3.5 mM), and the enzyme material (cell lysate or expressed UGT1A1; 0.1 mg/ml). The modifier (MK-571) or vehicle (control) was added into the microsomal incubation to determine its effects on the glucuronidation activity. The reaction was terminated by the addition of 100 μl ice-cold acetonitrile. The incubation samples were vortexed, followed by centrifugation for 15 min at 18,000 g. The resulting supernatant was subjected to UPLC analysis. Preliminary experiments were performed to ensure that the formation rates of R3G and R4′G were determined under linear conditions with respect to incubation time and protein concentration. All experiments were performed in triplicate.

Transient transfection of shRNA plasmids

The shRNA plasmid construct targeting MRP4 was established in our previous study (Quan et al.,
The forward and reverse sequences of the shRNA fragments were 5′-GATCCGCACAGAAGCCTTTTTAACATTCAAGAGATGTTAAAGGCTTTGTGCTTTTTTACGCGTC-3′ and 5′-TCGAGACGCGTAAAAAAGCACAGAAGCCTTTTTAACATCTCTTGAATGTTAAAGAAGGCTTTGTGCG-3′, respectively. HeLa1A1 cells were transiently transfected with shRNA plasmid as described (Quan et al., 2015). In brief, the cells were seeded in a six-well plate (at a density of 2.0 × 10⁵ cells/well) and cultured in DMEM supplemented with 10% FBS. On day 2, the shRNA construct or scramble (4 μg) was introduced into the cells using the Polyfectine transfection reagent (Biowit Technologies, Shenzhen, China). Cells were used for glucuronide excretion experiments 2 days after shRNA transfection.

Vesicular transport assay

Transport of resveratrol glucuronide into human MRP4 vesicles was measured by the rapid filtration method using a 96-well MultiScreen vacuum manifold (Millipore, Billerica, MA). In brief, different concentrations of resveratrol glucuronide were added to a reaction mixture containing TS buffer (10 mM Tris/HCl and 250 mM sucrose, pH 7.4), 10 mM MgCl₂, 4 mM ATP, 3 mM GSH, 10 mM DTT, and 37.5 μg of MRP4 or control vesicles. Transport of resveratrol glucuronide into the vesicles was stopped by adding ice-cold TS buffer. The membrane vesicles were separated from the mixture by filtration through a PVDF filter plate (MultiScreenHTS-HV, 0.45 μm, Millipore). Filters were collected, cut and transferred to 20% ACN solution. After 30-min sonication and centrifugation at 18,000 g, the supernatant was analyzed by UPLC. Vesicular accumulation of resveratrol glucuronide was calculated by subtracting the value derived with the MRP4 vesicles from that obtained with the control vesicles.

Modeling of enzyme/transport kinetics

Enzyme kinetic parameters were derived by fitting Michaelis-Menten equation (eq.1) or substrate inhibition equation (eq.2) to the data. In the model equations, $K_m$ is the Michaelis constant. $V_{\text{max}}$ is the maximal velocity and $K_{\text{si}}$ is the substrate inhibition constant. Parameter estimation was performed using Graphpad Prism 5 (San Diego, CA).
Transport kinetic parameters were derived by fitting the Michaelis-Menten like equation (eq.3) to the data. In the equation, $K_m'$ is the Michaelis constant. $J_{\text{max}}$ is the maximal transport rate. Parameter estimation was performed using Graphpad Prism 5 (San Diego, CA).

\[
V = \frac{V_{\text{max}} \cdot [S]}{K_m' + [S]} \quad \text{eq.1}
\]

\[
V = \frac{V_{\text{max}} \cdot [S]}{K_m + [S](1 + \frac{[S]}{K_{si}})} \quad \text{eq.2}
\]

\[
J = \frac{J_{\text{max}} \cdot [S]}{K_m' + [S]} \quad \text{eq.3}
\]

**Evaluation of inhibition kinetics**

Due to distinct reaction kinetics for generation of R3G and R4′G by UGT1A1, two different sets of inhibition models (i.e., eqs.4-7 and eqs.8-11) were used to describe the inhibition kinetics for formation of the two glucuronide isomers. Formation of R3G obeyed the classical Michaelis-Menten kinetics, suggestive of a typical (one-site) enzymatic reaction. Hence, the four conventional inhibition models [i.e., competitive (eq.4), noncompetitive (eq.5), uncompetitive (eq.6) and mixed-type (eq.7)] were used to fit the inhibition data of R3G. In model equations, $[S]$ and $[I]$ were the concentrations of the substrate (resveratrol) and inhibitor (MK-571), respectively.

Formation of R4′G followed the substrate inhibition kinetics, an atypical enzymatic behavior that was well explained by the existence of two substrate-binding sites in the enzyme (Wu, 2011). Accordingly, various mechanistic two-site models (eqs.8-11) were used to describe the inhibition data of R4′G. All models assumed that the enzyme had two binding sites (i.e., one reaction site and one inhibitory site). Equation 8 assumed that the inhibitor bound to the reaction site only. In equation 9, binding of the inhibitor to the inhibitory site required the occupancy of the reaction site with the
substrate. In equations 10 and 11, the inhibitor bound to both binding sites within the enzyme. Equation 10 assumed equivalent binding of the inhibitor to both sites, whereas equation 11 assumed that binding affinity of the inhibitor to the inhibitory site was altered due to prior binding of the substrate to the reaction site. $K_s$ and $K_i$ were disassociation (or binding affinity) constants of the substrate and inhibitor, respectively. Constant $\beta$ reflected the changes in catalytic efficiency associated with the binding of a second substrate molecule. Constant $\alpha$ was the factor by which the inhibitor dissociation constant of the vacant site changed when the first substrate molecule was bound. $V_{max}$ was the maximal velocity.

$$V = \frac{V_{max} \cdot [S]}{1 + \frac{[S]}{K_s} + \frac{[I]}{K_i}}$$  
Eq.4

$$V = \frac{V_{max} \cdot [S]}{1 + \frac{[S]}{K_s} + \frac{[I]}{K_i} + \frac{[I] \cdot [S]}{K_i \cdot K_s}}$$  
Eq.5

$$V = \frac{V_{max} \cdot [S]}{1 + \frac{[S]}{K_s} + \frac{[I]}{K_i} \cdot [S]}$$  
Eq.6

$$V = \frac{V_{max} \cdot [S]}{1 + \frac{[S]}{K_s} + \frac{[I]}{K_i} + \frac{[I] \cdot [S]}{\alpha \cdot K_i \cdot K_s}}$$  
Eq.7

$$V = \frac{V_{max} \cdot \left( \frac{[S]}{K_s} + \frac{\beta \cdot [S]^2}{K_s^2} \right)}{1 + \frac{[S]}{K_s} + \frac{[I]}{K_i} + \frac{[S]^2}{K_s^2}}$$  
Eq.8

$$V = \frac{V_{max} \cdot \left( \frac{[S]}{K_s} + \frac{\beta \cdot [S]^2}{K_s^2} \right)}{1 + \frac{[S]}{K_s} + \frac{[S]^2}{K_s^2} + \frac{[I] \cdot [S]}{K_i \cdot K_s}}$$  
Eq.9
Kinetic modeling and parameter estimation were performed using the curve fitting tool in Matlab™ (Mathworks Inc, Natick, MA). Goodness of fit was determined by AIC and R².

**Pharmacokinetic modeling and data fitting**

A cellular pharmacokinetic model (Figure 3), consisting of medium and cell compartments, was constructed to describe the disposition processes of resveratrol and its glucuronides in HeLa1A1 cells. The mass balance equations were shown below (eqs.12-17). The subscripts “m” and “c” denoted the medium and cell compartments, respectively. Transport of resveratrol (R) was a passive diffusion process described by the clearance parameter CL_d. Formation of R3G (G1) by UGT1A1 followed the Michaelis-Menten kinetics (V_max,1 and K_m,1), whereas formation of R4’G (G2) obeyed the substrate inhibition kinetics (V_max,2, K_m,2 and K_si,2). Hydrolysis of glucuronide was a non-saturable process described by K_de (K_de,1 for G1; K_de,2 for G2). Likewise, glucuronide excretion was a linear process described by K_ef (K_ef,1 for G1; K_ef,2 for G2). f_u denoted the unbound fraction of resveratrol in the cell compartment. The model assumed that intracellular binding of the hydrophilic glucuronides was negligible.

\[
\frac{dR_m}{dt} = \frac{CL_d}{V_m} R_m + \frac{CL_d}{V_c} f_u R_c
\]

\[
\frac{dR_c}{dt} = \frac{CL_d}{V_m} R_m - \frac{CL_d}{V_c} f_u R_c - \frac{f_u R_c V_{max,1}}{V_c K_{m,1} + f_u R_c} - \frac{f_u R_c V_{max,2}}{V_c K_{m,2} + f_u R_c + \left(\frac{f_u R_c}{V_c K_{si,2}}\right)^2}
\]
\[
\frac{dG_{1m}}{dt} = K_{d_{1}}G_{1c} \quad \text{eq. 14}
\]

\[
\frac{dG_{2m}}{dt} = K_{d_{2}}G_{2c} \quad \text{eq. 15}
\]

\[
\frac{dG_{1c}}{dt} = \frac{f_{u}R_{v_{1}}}{V_cK_{m_{1}} + f_{u}R_{c}} - K_{d_{1}}G_{1c} \quad \text{eq. 16}
\]

\[
\frac{dG_{2c}}{dt} = \frac{f_{u}R_{v_{2}}}{V_cK_{m_{2}} + f_{u}R_{c} + \left(\frac{f_{u}R_{c}}{V_cK_{m_{2}}}ight)^2} - K_{d_{2}}G_{2c} \quad \text{eq. 17}
\]

Model building and data fitting were performed using the Simbiology module contained in MATLAB® software package (The Mathworks Inc, Natick, MA). In data fitting, the \( K_m \) and \( K_{si} \) values were fixed as the corresponding values derived from the \textit{in vitro} glucuronidation assay.
Table 1

Kinetic parameters obtained by fitting various models to the inhibition kinetic data. Data are represented as mean ± SD.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme</th>
<th>Modifier</th>
<th>K_s (μM)</th>
<th>V_m (pmol/mg/min)</th>
<th>K_i (μM)</th>
<th>α</th>
<th>β</th>
<th>Model</th>
<th>R^2</th>
<th>AIC</th>
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</thead>
<tbody>
<tr>
<td>Resveratrol (3-OH)</td>
<td>UGT1A1</td>
<td>MK-571</td>
<td>32.1 ± 2.6</td>
<td>937 ± 25</td>
<td>3.9 ± 0.3</td>
<td>/</td>
<td>/</td>
<td>Eq.4</td>
<td>0.992</td>
<td>144</td>
</tr>
<tr>
<td>Resveratrol (3-OH)</td>
<td>UGT1A1</td>
<td>MK-571</td>
<td>45.0 ±5.7</td>
<td>1047 ± 53.1</td>
<td>14.4 ± 1.3</td>
<td>/</td>
<td>/</td>
<td>Eq.5</td>
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<td>158</td>
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<td>Resveratrol (3-OH)</td>
<td>UGT1A1</td>
<td>MK-571</td>
<td>51.8 ± 10.8</td>
<td>1090 ± 96.9</td>
<td>9.4 ± 1.6</td>
<td>/</td>
<td>/</td>
<td>Eq.6</td>
<td>0.939</td>
<td>168</td>
</tr>
<tr>
<td>Resveratrol (3-OH)</td>
<td>UGT1A1</td>
<td>MK-571</td>
<td>32.9 ± 2.9</td>
<td>495 ± 27.3</td>
<td>4.3 ± 0.6</td>
<td>28.2 ± 36.5</td>
<td>/</td>
<td>Eq.7</td>
<td>0.9992</td>
<td>146</td>
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<td>Resveratrol (4'-OH)</td>
<td>UGT1A1</td>
<td>MK-571</td>
<td>11.0 ± 1.1</td>
<td>178 ± 0.6</td>
<td>2.3 ± 0.4</td>
<td>/</td>
<td>0.13 ± 0.02</td>
<td>Eq.8</td>
<td>0.916</td>
<td>104</td>
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<td>Resveratrol (4'-OH)</td>
<td>UGT1A1</td>
<td>MK-571</td>
<td>1.0 ± 2.2</td>
<td>117 ± 90.2</td>
<td>0.8 ± 1.4</td>
<td>/</td>
<td>0.42 ± 0.3</td>
<td>Eq.9</td>
<td>0.615</td>
<td>127</td>
</tr>
<tr>
<td>Resveratrol (4'-OH)</td>
<td>UGT1A1</td>
<td>MK-571</td>
<td>11.0 ±1.0</td>
<td>170 ± 6.9</td>
<td>5.4 ± 0.6</td>
<td>/</td>
<td>0.17 ± 0.02</td>
<td>Eq.10</td>
<td>0.943</td>
<td>99</td>
</tr>
<tr>
<td>Resveratrol (4'-OH)</td>
<td>UGT1A1</td>
<td>MK-571</td>
<td>1.0 ± 1.9</td>
<td>124 ± 117</td>
<td>0.8 ± 2.1</td>
<td>1.22 ± 2.3</td>
<td>0.39 ± 0.36</td>
<td>Eq.11</td>
<td>0.617</td>
<td>130</td>
</tr>
</tbody>
</table>

AIC: Akaike information criteria; K_s, binding affinity of the substrate; K_i, binding affinity of the modifier; V_max, maximal rate of metabolism
Table 2

Fitted parameters for glucuronidation of resveratrol and excretion of formed glucuronides in control versus MRR4 knock-down HeLa1A1 cells

<table>
<thead>
<tr>
<th>PK parameters</th>
<th>Control</th>
<th>MRP4_shRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{CL}_a$ (ml/h)</td>
<td>33.5 ± 6.56</td>
<td>33.3 ± 2.69</td>
</tr>
<tr>
<td>$K_{m,1}$ (µM)</td>
<td>61.3$^a$</td>
<td>61.3$^a$</td>
</tr>
<tr>
<td>$V_{\text{max,1}}$ (pmol/h)</td>
<td>3860 ± 156</td>
<td>3599 ± 105</td>
</tr>
<tr>
<td>$K_{m,2}$ (µM)</td>
<td>82.0$^a$</td>
<td>82.0$^a$</td>
</tr>
<tr>
<td>$K_{s,2}$ (µM)</td>
<td>84.9$^a$</td>
<td>84.9$^a$</td>
</tr>
<tr>
<td>$V_{\text{max,2}}$ (pmol/h)</td>
<td>476 ± 19.0</td>
<td>442 ± 12.4</td>
</tr>
<tr>
<td>fu</td>
<td>0.053 ± 0.001</td>
<td>0.051 ± 0.001</td>
</tr>
<tr>
<td>$K_{\text{de,1}}$ (1/h)</td>
<td>2.23 ± 0.30</td>
<td>2.52 ± 0.33</td>
</tr>
<tr>
<td>$K_{\text{de,2}}$ (1/h)</td>
<td>2.12 ± 0.34</td>
<td>2.48 ± 0.26</td>
</tr>
<tr>
<td>$K_{\text{ef,1}}$ (1/h)</td>
<td>7.54 ± 0.51</td>
<td>5.40 ± 0.27**</td>
</tr>
<tr>
<td>$K_{\text{ef,2}}$ (1/h)</td>
<td>6.52 ± 0.44</td>
<td>4.55 ± 0.29**</td>
</tr>
</tbody>
</table>

$^a$Assigned values from *in vitro* glucuronidation kinetics with cell lysate (Table 3 & Figure 7)

**p<0.01 compared with the control group
# Table 3

Kinetic parameters derived for resveratrol glucuronidation by cell lysate preparation; Data are represented by Mean ± SD.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>Metabolite</th>
<th>$V_{\text{max}}$ (pmol/min/mg)</th>
<th>$K_m$ (μM)</th>
<th>$K_{\text{si}}$ (μM)</th>
<th>Fitted model</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa1A1 lysate</td>
<td>R3G</td>
<td>26.4 ± 1.41</td>
<td>61.3 ± 12.4</td>
<td>/</td>
<td>MM</td>
</tr>
<tr>
<td></td>
<td>R4’G</td>
<td>7.10 ± 1.60</td>
<td>82.0 ± 25.4</td>
<td>84.9 ± 26.1</td>
<td>SI</td>
</tr>
</tbody>
</table>

MM, Michaelis-Menten model; SI, substrate inhibition model
**Figure 1** Effects of MRP4 knock-down on glucuronide disposition. (A) Effects of MRP4 knock-down on the excretion rates of resveratrol glucuronides; (B) Effects of MRP4 knock-down on the intracellular levels of resveratrol glucuronides; Each data point was the average of three determinations with error bar representing the standard deviation. *p < 0.05; **p < 0.01.
Figure 2  Kinetic profiles for transport of R3G (A) and R4’G (B) with human MRP4 membrane vesicles. Each data point was the average of three determinations with error bar representing the standard deviation. The uptake rates of resveratrol glucuronides into MRP4 vesicles were determined at a series of substrate concentrations (2.5-60 μM), allowing adequate evaluation of transport kinetics. Transport of both R3G and R4’G followed the Michaelis-Menten like kinetics ($J_{\text{max}} = 675$ pmol/min/mg and $K'_m = 5.99$ μM for R3G; $J_{\text{max}} = 699$ pmol/min/mg and $K'_m = 6.98$ μM for R4’G). The similar kinetic profiles with similar $J_{\text{max}}$ and $K'_m$ values ($p > 0.05$) indicated that MRP4 had an equal activity in transporting the two glucuronide isomers.
Figure 3  Schematic representation of a cellular pharmacokinetic model describing the disposition processes of resveratrol and formed glucuronides in HeLa1A1 cells. Please refer to the text for definition of each parameter. R, resveratrol; R3G/G1, resveratrol 3-O-glucuronide; R4’G/G2, resveratrol 4’-O-glucuronide. The subscripts “m” and “c” denote the extracellular and cellular compartments, respectively. ET, efflux transporter.
Figure 4  Pharmacokinetic modeling of disposition of resveratrol and formed glucuronides in scramble (control) cells versus MRP4 knock-down cells at a dose of 50 nM resveratrol. (A) Extracellular resveratrol levels versus time profile; (B) Extracellular R3G levels versus time profile; (C) Extracellular R4’G levels versus time profile; (D) Intracellular resveratrol levels versus time profile; (E) Intracellular R3G levels versus time profile. (F) Intracellular R4’G levels versus time profile. Each data point was the average of three determinations with error bar representing the standard deviation. Solid lines are predicted data from the mechanistic pharmacokinetic model (Figure 3).
Figure 5  Excretion measurements of resveratrol glucuronides (R3G and R4‘G) in HeLa1A1 cells at different doses of resveratrol. (A) Excretion rate versus intracellular level profile for R3G generated with a series of resveratrol doses (i.e., 1, 2, 5, 10, 25, 50, and 100 μM). (B) Excretion rate versus intracellular level profile for R4‘G generated with a series of resveratrol doses (i.e., 1, 2, 5, 10, 25, 50, and 100 μM). Each data point was the average of three determinations with error bar representing the standard deviation. *p < 0.05; **p < 0.01; ***p < 0.001. A linear relationship was observed for glucuronide excretion versus intracellular level of the glucuronide. This indicated that efflux of the glucuronide was a non-saturable process at resveratrol doses of 1-100 μM. The slope of fitted straight line was used to estimate the rate constant for glucuronide efflux. The efflux rate constants of R3G and R4G were similar (6.59 vs 5.88 h⁻¹, p > 0.05), suggesting that the two glucuronide isomers were equally transported.
Figure 6  Protein binding measurements of resveratrol (A), R3G (B), and R4’G(C) to HeLa1A1 cell lysate (0.2 mg/ml). Each data point was the average of three determinations with error bar representing the standard deviation. Resveratrol bound significantly to lysate proteins. The fraction of unbound (fu) was independent of resveratrol concentrations. The mean fu value was 0.62. By contrast, the average fu values of resveratrol glucuronides (0.95 for R3G and 0.96 for R4’G) were close to 1, indicating that protein binding of the glucuronides was negligible.
Figure 7  Kinetic profiles for 3-O-glucuronidation (A) and 4’-O-glucuronidation (B) of resveratrol by HeLa1A1 cell lysate. Each data point was the average of three determinations with error bar representing the standard deviation.
References
