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UGT1A9-overexpressing HeLa Cells Is an Appropriate Tool to Delineate the Kinetic Interplay between BCRP and UGT and to Rapidly Identify the Glucuronide Substrates of BCRP

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Running Title: Novel Use of UGT1A9 Overexpressing HeLa

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Abbreviations: AIC, Akaike's information criterion; BCRP, Breast cancer resistance protein; CL, Clearance of efflux transporter; CTR, Control; DME, Drug metabolizing enzyme; DMEM, Dulbecco's modified Eagles medium; DMSO, Dimethyl sulfoxide; FBS, Fetal bovine serum; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; G418, Geneticin®; HBSS, Hank's balanced salt solution; HRP-conjugated, Horseradish peroxidase-conjugated; LTC_4, Leukotriene C_4; MRP, Multidrug resistance protein; OATP, Organic anion transporting polypeptide; P-gp, P-glycoprotein; UGT, UDP-glucuronosyltransferase; SULT, Sulfotransferase; W.T., Wild type.

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ABSTRACT

The interplay between phase II enzymes and efflux transporters leads to extensive metabolism and low bioavailability for flavonoids. To investigate the simplest interplay between one UGT isoform and one efflux transporter in flavonoid disposition, engineered HeLa cells stably overexpressing UGT1A9 were developed, characterized and further applied to investigate the metabolism of two model flavonoids (genistein and apigenin) and excretion of their glucuronides. The results indicated that the engineered HeLa cells overexpressing UGT1A9 rapidly excreted the glucuronides of genistein and apigenin. The kinetic characteristics of genistein or apigenin glucuronidation were similar when using UGT1A9 overexpressed in HeLa cells or the commercially available UGT1A9. siRNA-mediated UGT1A9 silencing resulted in the substantial decrease in glucuronide excretion (>75%, $p<0.01$). Furthermore, a potent inhibitor of BCRP Ko143 caused, in a dose-dependent manner, a substantial and marked reduction of the clearance (74%-94%, $p<0.01$), and a substantial increase in the intracellular glucuronide levels (4 to 8 folds, $p<0.01$), resulting in a moderate decrease in glucuronide excretion (19%-59%, $p<0.01$). In addition, a significant albeit moderate reduction in the fraction of genistein metabolized (or f_{met}) in the presence of Ko143 was observed. In contrast, LTC_4 and siRNA against MRP2 and MRP3 did not affect excretion of flavonoid glucuronides. In conclusion, the engineered HeLa cells overexpressing UGT1A9 is an appropriate model to study the kinetic interplay between UGT1A9 and BCRP in the phase II disposition of flavonoids. This simple cell model should also be very useful to rapidly identify if a phase II metabolite is the substrate of BCRP.

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INTRODUCTION

Interplay between transporters and drug metabolizing enzymes (DME), firstly proposed about 15 years ago (Wacher *et al.*, 1995), has been postulated to have a major role in determining a drug's absorption and disposition (Custodio *et al.*, 2008; Pang *et al*., 2009). Originally, this interplay was primarily related to the p-glycoprotein (P-gp) and cytochrome P 450 3A (CYP 3A). More recently, the interplay was expanded to include those between phase II enzymes such as UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) and other efflux transporters such as breast cancer resistance protein (BCRP) and multidrug resistance protein 2 (MRP2) as well as organic anion transporting polypeptide (OATP) (Liu Z *et al.*, 2007; Urquhart *et al.*, 2007).

Two of the most common and popular cell lines used to delineate the interplay in the epithelial cells are Caco-2 and MDCK II cell lines. Both cell lines are polarized cells and suitable for studying phase II metabolism of xenobiotics including flavonoids. However, the above two cell models are still too complex for us to study the interplay between UGTs and efflux transporters since multiple efflux transporters and other phase II enzymes (e.g. SULTs) are also expressed in these cells. Therefore, we modified HeLa cells for the purpose of developing a cell culture model to study the interplay between one UGT isoform and efflux transporters. There were two main reasons for selecting the HeLa cells: first, they had no detectable glucuronidation and negligible sulfation activity in metabolizing genistein and apigenin; and second, they expressed significant amount of BCRP but very little MRP2. The very low expression of MRP2 was consistent with those reported by a different group of investigators (Ahlin *et al.*, 2009). We were interested in these two efflux transporters, since both BCRP and MRP2 were capable of

mediating the efflux of glucuronides and sulfates (Enokizono *et al.*, 2007; Lee *et al.*, 2009; Lengyel *et al.*, 2008; Sakamoto *et al.*, 2008).

Human UGT1A9 was the first UGT isoform selected to be overexpressed in HeLa cells for several reasons: first, UGT1A9 was responsible for metabolism of many clinical drugs such as SN-38 and acetaminophen (Court *et al.*, 2001; Paoluzzi *et al.*, 2004); second, and more importantly, our previous data showed that UGT1A9 was able to metabolize many flavonoids at relatively high rates, consistent with results from Pritchett et al (Pritchett *et al.*, 2008); and third, UGT1A9 was more stable than other isoforms (Kurkela *et al.*, 2003).

Two typical flavonoids genistein and apigenin were used as model flavonoids. The flavonoids were selected because previous investigation presumed that it was the interplay between phase II enzymes and efflux transporters that contributed to their low bioavailabilities (Liu *Z et al.*, 2007; Zhu *et al*., 2010). Low bioavailability impedes the testing of many of their "claimed" beneficial effects in humans including anti-cancer, anti-inflammatory and anti-viral as well as prevention of cardiovascular diseases (Benavente-Garcia *et al.*, 2007; Comalada *et al.*, 2005; Lee *et al.*, 2007; Grassi *et al.*, 2009). We believe that a better understanding of the factors that govern the metabolism of flavonoids holds the key to overcome their oral bioavailability barriers. Moreover, it is likely that the simplest interplay between an efflux transporter and a phase II enzyme will form the basis to understand and delineate the complex interplays between multiple phase II enzymes and efflux transporters that determine the flavonoid bioavailability in vivo. Therefore, the purpose of this research is to study the simplest interplay between UGT1A9 and an efflux transporter in control of glucuronidation of genistein and apigenin and of excretion of their glucuronides.

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MATERIALS AND METHODS

Materials. HeLa cells and pcDNA3.1(+/-) were a kind gift from Dr. Yu Rong (University of Texas at Houston). Apigenin, genistein and testosterone were purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ). Recombinant human UGT1A9 expressed in baculovirus-infected insect cells (Supersome™) was purchased from BD Biosciences (Woburn, MA). UGT1A9 antibody was purchased from Abnova (Walnut, CA). pCMV6_XL4 vector carrying *UGT1A9* gene was from Origene (Rockville, MD) . siRNA of UGT1A9 and scrambled siRNA were purchased from Ambion (Austin, TX). siRNA of MRP2 or MRP3 and Ko143 were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). LTC_4 was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and solvents were of analytical grade or better.

***Transient transfection*.** HeLa cells were seeded at a density of $1.0X10^5$ cells per well in a 6-well plate, and maintained at 37°C under 5% CO_2 in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum (FBS). After 6-7 hours, vector carrying UGT1A9 gene (*NM_021027.2)* was introduced to the cells using the modified calcium precipitation method (Chen *et al*., 1988). The medium containing 10% FBS (DMEM with high glucose) was changed to a medium containing 2% FBS on day 2. The transiently transfected HeLa cells were ready for excretion study or UGT activity assay on day 3.

Development of stably transfected HeLa cells. *UGT1A9* gene (NM_021027.2) from vector pCMV6_XL4 (Origene, Rochville, MD) was subcloned into pcDNA3.1 (+/-) vector. Then the vector carrying *UGT1A9* gene was transiently transfected into HeLa cells by using the modified calcium precipitation method (Chen *et al*., 1988). After transfection, HeLa cells were maintained at 37°C under 5% CO_2 in DMEM containing 10% fetal bovine serum (FBS) and Geneticin® (G418, 1.2mg/ml). Media were changed every two or three days until the colonies came out.

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The colonies were picked up and cultured in a 12-well plate (1 colony/well). Once cells reached the 100% confluence, the cells from each well of the 12-well plate were split into two wells of the 6-well plates and allowed to grow until confluence. Those cells that were able to excrete significant amounts of glucuronides were considered as the positive clones. Positive cloned cells were further cultured for five generations to test the stability of glucuronide production, and stable and highly active cells were then cryopreserved for future use. Each vial of cryopreserved cells was used for ten passages before a new one was initiated for continued use. The HeLa cells stably transfected with *UGT1A9* were called engineered HeLa cells.

Transfection of siRNA. The engineered HeLa cells were seeded at 0.5 $X10^5$ cells/well in a 12-well plate and maintained at 37°C under 5% CO_2 in DMEM containing 10% FBS. On the next day, siRNA of UGT1A9 (sense: 5'-CGAAGUAUAUAUUCUCUAUtt; antisense: 5'-AUAGAGAAUAUAUACUUCGta), scrambled siRNA (30pmole/well) or equal volume of water was introduced to the cells by using Lipofactamine™ 2000 (Invitrogen) following the manufacturer's protocol (Ee *et al.*, 2004). Cells were ready for experiment two days after transfection. Following a similar procedure, siRNA of MRP2 (SC-35963) or MRP3 (SC-40748) was transfected into the engineered HeLa cells.

RT-PCR. Cells were collected and the RNA was extracted by using RNeasy Mini Kit (QIAGE, Valencia, CA). RT-PCR was run according to the manufacturer's protocol (OneStep RT-PCR Kit from QIAGEN). Briefly, a 50µl mixture containing 2µg total RNA, primers (final 0.6µM, sequences shown later), QIAGEN OneStep RT-PCR Enzyme Mix (2µl), dNTP mix (final 400µM of each dNTP) and QIAGEN OneStep RT-PCR Buffer as well as RNase-free water, was reversely transcribed at 50°C for 30min. Then the mixture was continuously incubated at 95°C for 15min followed by 35 cycles of expansion (94°C/0.5min, 55°C/0.5min, 72°C/1min), then by the final extension at 72°C for 10min. The forward primer of UGT1A9 is 5'GTTGCCTATGGAATTTGA, and the reverse primer is 5' GGGTGACCAAGCAGAT. The

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forward primer of BCRP is 5'TTCTCCATTCATCAGCCTCG, and the reverse primer is 5' TGGTTGGTCGTCAGGAAGA. The forward primer of β-actin is 5' GAGAAGATGACCCAGATCATGT, and the reverse primer is 5' TCGTCATACTCCTGCTTGCAG (Ee *et al.*, 2004). All these primers were shown to work previously and supplied by Sigma. The MRP2 (SC-35963-PR) and MRP3 (SC-40748-PR) primers were purchased from Santa Cruz biotechnology, Inc. together with siRNA of MRP2 (SC-35963) and siRNA of MRP3 (SC-40748), respectively. After RT-PCR, agarose gel electrophoresis and UV visualization were used to determine the relative amounts of PCR products.

Preparation of cell lysates. HeLa cells transiently transfected with *UGT1A9* or engineered HeLa cells were grown for 3-4 days, then washed and harvested in 50mM potassium phosphate buffer (KPI, pH7.4). The collected cells *were* sonicated in Aquasonic 150D sonicator (VWR Scientific, Bristol, CT) for 30min at the maximum power (135 average watts) in an ice-cold water bath (4°C) (Liu X *et al*., 2007). The glucuronidation activity of UGT1A9 was not expected to be affected during sonication process due to its thermal stability (Fujiwara *et al*., 2007). Then the cell lysates were centrifuged at 4°C (5min at 6000rpm). The supernatant to be used in the UGT activity assay or western blotting was harvested, and its protein concentration was determined by BCA assay (Pierce Biotechnology, Rockford, IL).

Western blotting. Cell lysates from wild type and engineered HeLa cells and UGT1A9 Supersomes™ were boiled at 95°C, and denatured protein was then run on a 10% SDS-PAGE gel to test the expression levels of UGTs. After electrophoresis, the separated proteins on the gel were transferred to a PVDF membrane (Millipore, Billerica, MA) following a standard protocol. After blocking in 5% nonfat milk for one hour, the membrane was incubated with UGT1A9 antibody at 1:500 dilution in 3% milk at 4°C overnight. After the membrane was washed three times, it was incubated with HRP-conjugated 2nd antibody (goat host anti-rabbit

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antibody) (1:2000 dilution) in 3% milk for one hour. Afterwards, the membrane was developed by enhanced chemiluminescence kit (Pierce Biotechnology, Rockford, IL). The same membrane was stripped for detecting glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as loading control. For commercial UGT1A9 Supersome^{TM}, GAPDH was not present and could not be detected.

UGT activity assay. The method was modified based on a previously published paper (Tang, *et al.,* 2010). Briefly, we mixed cell lysates (final protein concentration from 0.053mg/ml to 0.21mg/ml) or human UGT1A9 Supersome™ (final protein concentration from 0.013mg/ml to 0.053mg/ml), magnesium chloride (0.88mM), saccharolactone (4.4mM), and alamethicin (0.022 mg/ml), and different concentrations of genistein (0.5-50μM) or apigenin (0.5-40μM) in a 50mM potassium phosphate (or KPI) solution (pH 7.4) diluted from 100X concentrated stock solutions in organic solvent (DMSO/Methanol=1:4). Uridine diphosphoglucuronic acid (3.5mM) was added last to the previous mixture to the final volume of 170 μl, and the mixture was incubated at 37°C for 30min, 45min or 60min. At the end of the reaction, it was stopped by the addition of 50μl of 100μM testosterone solution (in 94% acetonitrile/6% glacial acetic acid) as the internal standard. Samples were ready for HPLC or UPLC analysis after centrifugation (15min at 15,000 rpm).

Excretion experiments. The engineered HeLa cells (stably transfected with *UGT1A9*) were grown on 12-well plate (1 X10^5 cells/ well) or on 6-well plate (2X10^5 cells/ well) about 3-4 days. Before experiments were started, the engineered HeLa cells were washed twice with pre-warmed (37°C) HBSS buffer (Hank's balanced salt solution, pH=7.4). Then the cells were incubated with a HBSS buffer containing genistein or apigenin with or without an inhibitor (defined as "loading solution",1ml/well for 12-well plates and 2ml/well for 6-well plates) for a predetermined time interval (40, 80 and 120min) at 37°C. For the experiments on LTC_4 and siRNA of MRP2 or MRP3, the time intervals were 60, 120, 180 and 240min (shown in

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supplements). In the loading solution, the proper concentration of genistein (2, 5, 10, 20 and 50μM) or apigenin (2, 5, 10 and 20μM) was diluted from 100X concentrated stock solutions in organic solvent (DMSO/Methanol=1:4), while the concentration of Ko143 (5 or 10μM) was diluted from 20mM stock solutions in DMSO and LTC_4 (0.1μM) was diluted from 80μM stock solutions in methanol. The sampling times were selected to ensure that the amounts excreted vs. time plots stay in the linear range. At each time point, 200μl of incubating media from each well were collected and the same volume of loading solutions was used to replenish each well. The collected incubating media were mixed with a "Stop Solution" consisted of 94%acetonitrile and 6% acetic acid containing 100uM testosterone as the internal standard. Supernatants were ready for UPLC analysis after centrifugation (15min at 15,000 rpm). For transiently transfected HeLa cells, which were used to demonstrate whether intact UGT1A9-overexpressing HeLa cells could excrete amounts of glucuronides (Fig.1), they were grown on 6-well plates and incubated overnight with the loading solution (genistein or apigenin at 10μM, 2ml/well). The collected incubating media was mixed with the Stop Solution and analyzed by HPLC after centrifugation (15min at 15,000 rpm).

Determination of intracellular glucuronides in engineered HeLa cells. The engineered HeLa cells were washed with ice-cold HBSS buffer twice after the excretion experiments at 120min. Then the cells were collected in 100 or 200 μl HBSS buffer and sonicated in Aquasonic 150D sonicator (VWR Scientific, Bristol, CT) for 30min at the maximum power (135 average watts) in an ice-cold water bath (4°C) (Liu X *et al*., 2007). UGT1A9 is not expected to function without the co-factors once cells are broken. After centrifugation at 15,500 rpm for 20min, supernatants were collected and mixed with the Stop Solution. The samples were ready for UPLC analysis after centrifugation (15min at 15,000 rpm).

***Sample analysis by HPLC*.** The conditions for HPLC analysis of genistein, apigenin and their glucuronides were modified based on a previously published method (Hu *et al*., 2003). The

conditions were: system, Hewlett Packard 1090 with dioarray detector and Hewlett Packard Chemstation; column, Aqua (Phenomenex, Gilroy, CA), 5µm, 150X0.45cm; mobile phase A, 0.1% formic acid plus 0.06% triethylamine (pH 2.6); mobile phase B, 100% acetonitrile; gradient, 0-3min 20%B, 3-22min 20-49%B, 22-26min 49%B; injection volume, 200ul; wavelength, 254nm (for genistein and its glucuronides as well as the internal standard) and 340nm (for apigenin and its glucuronides). There was a 4-min interval between the end of the run and the next injection to allow the column to be reequilibrated with 20% mobile phase B. The precision was typically better than 5% and accuracy better than 10%. The detection limits were at least 0.2 µM for both alycones and their glucuronides.

Sample analysis by UPLC The conditions for UPLC analysis of genistein, apigenin and their glucuronides were modified based on a previously published method (Tang *et al.*, 2010; Zhu *et al*., 2010). The conditions were: system, Waters Acquity with a binary pump and a 2996 DPA diode array detector (DAD, Waters, Milford, MA); column, Acquity UPLC BEH C18 column (50X2.1mm I.D.1.7 µm (Waters, Milford, MA); mobile phase A, 2.5mM ammonium acetate (pH 7.4); mobile phase B, 100% acetonitrile; gradient, 0-2.0min, 10-35%B, 2.0-3.0min, 35-70%B, 3.0-3.5min 70%B; 3.5-3.6min, 70-90%B; 3.6-4.1min, 90%B; 4.1-4.6min, 90-10%B; injection volume, 10ul; 254nm (for genistein and its glucuronides as well as the internal standard) and 340nm (for apigenin and its glucuronides). The precision and accuracy were typically within acceptable range (<15%). The detection limits were at least 0.2µM for both alycones and their glucuronides. The structures of glucuronides were further confirmed by UPLC-MS/MS as previously showed (Zhu *et al.*, 2010).

***Quantification of glucuronides*.** The conversion factors, representing the molar extinction coefficient ratio of glucuronides to aglycones, were used to quantify the amounts of genistein and apigenin glucuronides as described previously (Liu *et al.*, 2002; Liu X *et al*., 2007; Tang *et al.*, 2010).

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Kinetic study of UGT1A9. Rates of metabolism in UGT1A9 Supersome™ or HeLa cell lysates were expressed as amounts of glucuronides formed per min per mg protein (or nmole/min/mg). If the Eadie-Hofstee plot was linear, formation rates (V) of a flavonoid glucuronide at various substrate concentrations (C) were fit to the standard Michaelis-Menten equation:

$$V=\frac{V_{max}\,C}{K_m+C} \qquad (1)$$

where K_m represents the Michaelis constant, and V_{max} is the maximum formation rate. If Eadie-Hofstee plots showed characteristic profiles of atypical kinetics such as autoactivation or biphasic kinetics (Houston *et al.*, 2000; Hutzler *et al.*, 2002), the data were fit to other corresponding equations (Wang *et al.*, 2006), using an Excel program. Aside from R^2 value, the best-fit model was determined based on Akaike's information criterion (AIC) (Yamaoka *et al.*, 1978) and the rule of parsimony was applied.

Calculation of f_{met} value* and *CL. Fraction metabolized or f_{met} value was defined as the fraction of dose metabolized (equation (2)). The f_{met} value was considered as the more appropriate parameter to reflect the extent of metabolism in the presence of a transporter-enzyme interplay (Pang *et al.*, 2009).

$$f_{met}=\frac{\sum Metabolites}{\sum Metabolites+\sum Parent\ compound} \qquad (2)$$

Clearance of efflux transporter (CL) was used here since intracellular concentrations could be very different from the extracellular concentrations of glucuronides.

$$CL=\frac{J}{Ci}=\frac{J_{max}}{K_{m'}+Ci} \qquad (3)$$

where J_{max} is the excretion rates of glucuronides, K_m' is the Michaelis constant reflecting affinity of glucuronides to the efflux transporter BCRP, and C_i is the intracellular concentration of glucuronides. The J_{max} and K_m' were previously used as two kinetics parameters for transporters

(Sun *et al*., 2008). Assuming that the average volume of engineered HeLa cells was 4µl/mg protein (Yamaguchi *et al*., 2000), the intracellular concentrations of glucuronides were calculated after the total amounts of intracellular glucuronides were determined experimentally. Other investigators have estimated volume at a value that was a bit larger than 4µl/mg protein (Saito *et al*., 1986), so the intracellular concentrations might be slightly overestimated.

Statistical analysis. All the experiments were done in duplicates or triplicates and data were analyzed by one-way ANOVA or student's t-test as appropriate and the level of significance was set at $p<0.05$ or $p<0.01$.

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RESULTS

Excretion of glucuronides in control and transiently transfected HeLa cells

The results indicated (Fig.1) that there was a new peak eluting before each parent compound in the engineered HeLa cells when comparing to the control HeLa cells. For genistein, the new peak retention time was around 7min; and for the apigenin, the new peak retention time was around 9min. Based on the published data (Liu *et al.*, 2002), these new forming peaks represented the glucuronides of each parent compound. On the contrary, only peak representing genistein or apigenin was detected in control HeLa cells. Briefly, a predominant glucuronide of genistein was detected in transiently transfected HeLa cells with pseudomoleucular ion $[M-H]^-$ at m/z 445, which was 176Da higher (characteristic of the addition of glucuronic acid) than that of genistein (m/z 269). Similarly, a predominant glucuronide of apigenin was detected in transiently transfected HeLa cells by using the same UPLC-MS/MS method. The authenticity of the glucuronides was also demonstrated in an earlier paper from this lab (Zhu *et al.*, 2010).

Characterization of engineered HeLa cells overexpressing UGT1A9

The results (Fig.2A and Fig.2B) showed that UGT1A9 was well expressed at both mRNA and protein levels in engineered HeLa cells with stable transfection. Moreover, mRNA levels of three efflux transporters: BCRP, MRP2 and MRP3 were determined in untransfected HeLa cells (Fig.2C), since MRP3 was also responsible for the excretion of phytoestrogen glucuronides in addition to MRP2 and BCRP (van de Wetering *et al*., 2009). It indicated that BCRP had a relatively higher mRNA expression level than MRP2 or MRP3 in the HeLa cells, consistent with an early observation that MRP2 was poorly expressed in HeLa cells (Ahlin et al., 2009). To determine the stability of UGT1A9 expression in engineered HeLa cells with stable transfection,

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we measured the UGT1A9 activities for five consecutive generations and found the activities to be fairly stable (Supplemental Materials, Fig.1S).

Enzyme kinetics study using UGT1A9 overexpressed in HeLa cells

The kinetics profiles of UGT1A9- (transiently or stably overexpressed in HeLa cells) mediated metabolism of genistein and apigenin were determined and compared with commercially available human UGT1A9 Supersome™. The K_m and V_{max} values of these kinetic studies were summarized in Table 1 and detailed rates vs. concentration plots were shown in Fig.3. In general, K_m values were similar in transiently and stably overexpressed UGT1A9. However, K_m values were somewhat higher in UGT1A9 overexpressed in HeLa cells than those derived from UGT1A9 expressed in insect cells (Supersome™). V_{max} values from UGT1A9 overexpressed in HeLa cells were not directly comparable to those of human UGT1A9 Supersome™, since the latter was expressed in insect cells and UGT1A9 was membrane anchored enzyme. UGT1A9 from all the sources followed the classical Michaelis-Menten equation in metabolizing genistein and apigenin (Fig.3), suggesting that UGT1A9 derived from these various sources were likely the same, although the levels of expression were clearly different.

Effects of concentrations on the excretion of glucuronides

To study whether the concentrations of genistein (2, 5, 10, 20 and 50μM) or apigenin (2, 5, 10, and 20μM) had an effect on the excretion of glucuronides, different concentrations of genistein or apigenin were incubated with engineered HeLa cells (Fig.4). As expected, the rates of excretion of both genistein and apigenin glucuronides increased whereas their f_{met} decreased with a rise in concentration. The increase in excretion reached a plateau at a relatively low

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concentration (5 μM), but the decrease in f_{met} persisted throughout. In contrast, the intracellular glucuronide concentrations increased faster than the changes in the loading concentrations, which resulted in a significant and substantial decrease in the cellular clearance of these flavonoid glucuronides as the loading concentration increased.

Effects of siRNA-mediated UGT1A9 silencing on the excretion of glucuronides

siRNA of UGT1A9 was introduced to the engineered HeLa cells to determine how changes in glucuronidation activity affected cellular glucuronide excretion, due to the unavailability of specific chemical inhibitor of UGT1A9. Two types of control studies were performed: water transfection and scrambled siRNA transfection. There was no significant difference between these two control groups. Therefore, we used scrambled siRNA group as the negative control to compare the effects of siRNA against UGT1A9. The results indicated that siRNA-mediated UGT1A9 silencing inhibited the excretion of genistein and apigenin glucuronides by 85% and 89%, respectively (Fig.5A and 5B). The intracellular levels of glucuronides in the presence of UGT1A9 siRNA were reduced by 77% and by 94% for genistein and apigenin glucuronides, respectively (Fig.5C and 5D). Similarly, f_{met} of genistein with siRNA treatment was 81% less than the control, and f_{met} of apigenin was 84% less than the control (Fig.5E and 5F). For CL of genistein glucuronide, there was no significant difference between control and siRNA of UGT1A9 treated group (Fig.5G). However, CL of apigenin glucuronide in the presence of siRNA was about 2.2 folds higher than the control (Fig.5H). These changes resulting from decreased glucuronidation activities (as demonstrated by a decrease in f_{met} of both flavonoids) were consistent with the decreases in expression levels of transfected UGT1A9 shown in the western blotting (Fig.5I). According to the band intensity ratio (UGT1A9 band intensity relative to GAPDH), the siRNA treatment could inhibit the expression of UGT1A9 by 56% in the engineered HeLa cells.

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Effects of siRNA-mediated MRP2 and MRP3 silencing on the excretion of glucuronides

siRNA of MRP2 or MRP3 was introduced to the engineered HeLa cells to determine how these treatments might affect the excretion of genistein and apigenin glucuronides and the results indicated that these two siRNAs had no impact on the excretion of genistein or apigenin glucuronides (Supplemental Materials, Fig.S2).

Effects of Ko143 on the excretion of glucuronides

Next, the role of BCRP in excretion of glucuronides in engineered HeLa cells was determined, using a specific and potent chemical inhibitor Ko143 (Pick *et al.*, 2010) (Fig.6). The concentrations of Ko143 (5μM and 10μM) were selected based on references that showed this compound had a K_i value of less than 1μM (Brand *et al*., 2008; Cooray *et al*., 2004). Our data showed that for genistein, the excretion amounts of its glucuronides were reduced by 42% and 59% in the presence of 5μM and 10μM Ko143, respectively. For apigenin, on the other hand, the excretion amounts of its glucuronides were reduced by 19% and 44% in the presence of 5 μM and 10μM Ko143, respectively. However, amounts of genistein glucuronides inside the cells were increased by 6 (5μM) and 8 (10μM) folds when comparing to the control, whereas amounts of apigenin glucuronides inside the cells were augmented by 4 (5μM) and 6 (10μM) folds when comparing to the control. Additional analysis of the results indicated that Ko143 had limited (or sometimes no) impact on the overall glucuronidation as represented by the f_{met} value, which was only moderately decreased (Fig.6E and 6F). For f_{met} of genistein, the decrease was about 21% (5μM, $p<0.01$) and 30% (10μM, $p<0.01$), but for apigenin, the decrease was

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statistically insignificant. Compared to limited reduction of f_{met}, cellular clearances (*or CL*) were inhibited drastically for both genistein and apigenin glucuronides by Ko143 (Fig.6G and 6H). In the presence of Ko143, CL of genistein glucuronide was reduced by 89% (5μM) and 94% (10μM), respectively, whereas CL of apigenin glucuronide was reduced by 74% (5μM) and 91% (10μM), respectively.

Effects of LTC_4 on the excretion of glucuronides

We used LTC_4 , a general inhibitor of MRPs, to determine if it would impact cellular glucuronide excretion as was demonstrated in Caco-2 cells (Hu *et al.*, 2003). The results indicated that this general MRP inhibitor did not affect the cellular excretion of either glucuronide (Supplemental Materials, Fig.3S).

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DISCUSSION

Our results clearly indicated that UGT1A9 overexpressed in the HeLa cells functioned as a conjugating enzyme, and was similar to the commercially available human UGT1A9 (Table 1, Fig.3). Expressed UGT1A9 played a critical role in controlling the concentration-dependent excretion of genistein and apigenin glucuronides in HeLa cells overexpressing UGT1A9 (Fig.4). On the other hand, the large decreases in clearances for both genistein and apigenin glucuronides in the presence of Ko143 suggested that BCRP was the predominant gate-keeper in the kinetic interplay between UGT1A9 and BCRP in the engineered HeLa cells (Fig.6G and 6H). Therefore, the engineered HeLa cells overexpressing UGT1A9 is an appropriate tool to study the kinetic interplay between UGT1A9 and BCRP, and a novel tool for rapidly identifying if a glucuronide is a BCRP substrate.

The glucuronidation of genistein or apigenin by UGT1A9 overexpressed in the engineered HeLa cells was the rate-determining step in the interplay. The glucuronides were only detectable in the transfected but not in the wild type HeLa cells (Fig.1). Furthermore, the excretions of glucuronides and f_{met} were significantly decreased when siRNA of UGT1A9 was introduced. On the other hand, BCRP was found to be the dominant efflux transporter (i.e., the gate-keeper) in the engineered HeLa cells since the intracellular levels of glucuronides were increased by several folds and the clearances of glucuronides by efflux transporter were inhibited by greater than 90% in the presence of 10 μM Ko143 (Fig.6C, 6D, 6G and 6H). However, the excretions of glucuronides with Ko143 did not decrease as much as the clearance, as one would have expected (Fig.6A and 6B). This was due to the kinetic compensation; i.e., the increasing levels of intracellular glucuronides compensated for the fact that function of efflux transporter was substantially inhibited by Ko143 (as demonstrated by large decreases in cellular CL).

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Furthermore, we excluded any major role played by MRP2 and MRP3 in the excretion of flavonoid glucuronides because neither the broad specific MRP inhibitor LTC_4 (Fig.3S) nor the potent siRNAs against MRP2 and MRP3 was effective in inhibiting the glucuronide efflux (Fig.2S). Thus, we are able to demonstrate that the observed interplay in the engineered HeLa cells is mainly the result of the kinetic interplay between UGT1A9 and BCRP.

In addition to this application in understanding kinetic interplay between UGT1A9 and BCRP, the newly engineered HeLa cells are useful for determining if a glucuronide (e.g., apigenin glucuronide) is a substrate of BCRP. This could not be routinely done previously since one would have to use purified glucuronides together with membrane vesicles that overexpress BCRP to positively identify if a glucuronide is the substrate of BCRP, a tedious and time- and resource- consuming process. As a consequence, very few glucuronides have been positively identified as a substrate of BCRP (Chen *et al.*, 2003).

Ideally, it would be desirable to directly extrapolate what we observed in the engineered HeLa cells (i.e., kinetic interplay between UGT1A9 and BCRP) to human hepatocytes or enterocytes. If there was only one pair of interaction between UGT1A9 and BCRP for the metabolism of a particular substrate, such an extrapolation could be done by measuring the relative expression levels of participating proteins (i.e., UGT1A9 and BCRP) in two model systems. Unfortunately, neither of these two compounds undergoes simple metabolism in a complex cellular system (hepatocytes or enterocytes). In these cells, genistein and apigenin are metabolized by both sulfotransferases and UGTs, each by multiple isoforms. Therefore, proper extrapolation of the simple kinetic interplay shown here requires additional development of the model systems such that each of the other possible pairs involved in the complex interplays could be well

understood. Then, a cellular model can be built to incorporate all the possible interplays so we can extrapolate what we see in simple model systems to a more complex model system. In other words, the developed HeLa cells are the first simple model and many more simple models are needed to understand the complex interplays existing in the human enterocytes or hepatocytes.

Not surprisingly, our simple model also has some additional limitations. For example, the capability of BCRP may be exaggerated. The substrates may prefer other efflux transporters, if other options are available. But here they have to depend on BCRP to get out of the cells because BCRP is the most available efflux transporter in the model. Another limitation is that the compound must be the substrate of UGT1A9, which can be alleviated by using the same approach to build additional HeLa cell models.

In conclusion, engineered HeLa cells that are stably transfected with *UGT1A9* is an appropriate tool to study the kinetic interplay between UGT1A9 and BCRP and a novel tool to **rapidly** identify the glucuronide substrates of BCRP. Our data obtained from the engineered HeLa cells clearly indicated the interplay was at least occurring at the kinetic level. Currently, more thorough investigations are ongoing to uncover the interplay by using more flavonoids and certain clinically important drugs such as SN-38 since SN-38 is a substrate of UGT1A9 (Paoluzzi *et al.*, 2004) and its glucuronide is the substrate of BCRP (Nakatomi et. al 2001). The present and future studies on the interplay between phase II conjugating enzymes and efflux transporters in the engineered HeLa cells may provide us with the insight necessary to manipulate the oral bioavailability of flavonoids and other therapeutically relevant phenolics.

Authorship Contributions

Participated in research design: Jiang, Yu and Hu.

Conducted experiments: Jiang and Xu.

Contributed new reagents or analytic tools: Yu and Hu.

Performed data analysis: Jiang, Wu and Hu.

Wrote or contributed to the writing of the manuscript: Jiang, Wu and Hu.

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FOOTNOTES

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Figure Legends

Fig.1 The HPLC chromatograms of genistein and apigenin as well as their glucuronides. The HeLa cells not transfected or transiently transfected with UGT1A9 were treated with 10μM genistein or apigenin for overnight at 37°C. The profile without the glucuronide was derived from the control cells whereas the profile with the glucuronide was from the transiently transfected cells. The chromatographic profiles were derived from HPLC analysis of the samples. Glucuronides were also previously authenticated with LC-MS/MS and authentic standards (Zhu *et al*., 2010). I.S. was an abbreviation for internal standard.

Fig.2 Western blotting (A) and RT-PCR (B, C and D) of wild-type and engineered HeLa cells. For western blotting, lane 1 was HeLa cells (wild type), lane 2 was engineered HeLa cells stably overexpressing UGT1A9, and lane 3 to lane7 were different amounts of commercially available human UGT1A9 Supersomes™ (amount 0.3125μg to 5μg) used as positive and quality controls. The total amount of protein loaded on to lane 1 and 2 was 80μg. For RT-PCR (B), lane 1 was marker, lane 2 was engineered HeLa cells stably overexpressing UGT1A9 and lane 3 was the control HeLa cells (wild-type). For RT-PCR (C), lane 1 was marker, and lanes 2 to 4 were all HeLa cell (wild type). For RT-PCR (D), lane 1 was HeLa cells (wild type) and lane 2 was engineered HeLa cells stably overexpressing UGT1A9.

Fig.3 In vitro metabolism study using human UGT1A9 Supersome™ and UGT1A9 overexpressed in HeLa cells (lysates of HeLa cells transiently or stably transfected with *UGT1A9*). The genistein kinetics were shown in the Panel A. Rates of metabolism were determined from 0.5μM to 50μM, and reaction time was 60min. The related Eadie-Hofstee plots

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were shown in Panel C (Supersome™), Panel E (transient overexpression) and Panel G (stable overexpression), respectively. The apigenin kinetics were shown in Panel B. Rates of metabolism were determined from 0.5μM to 40μM, and reaction time was 15, 30 or 60min. The related Eadie-Hofstee plots were shown in Panel D (Supersome™), Panel F (transient overeexpression) and Panel H (stable overexpression). Each data point was the average of two (stable overexpresion, n=2) or three (Supersome™ and transient overexpression, n=3) determinations with error bars representing the standard deviation of mean.

Fig.4 Effects of concentrations on the excretion rates (A, B), intracellular amounts (C, D), fraction metabolized (f_{met}) (E, F), and clearance (CL) (G, H) of genistein or apigenin glucuronide. Different concentrations of genistein (A, C, E and G) or apigenin (B, D, F and H) were incubated with engineered HeLa cells grown on 12-well plates, and three samples (200 μL) were taken at 40, 80 and 120min and replaced with fresh loading solution (200μL) containing the flavonoid of interest. The excretion rates of glucuronides (A and B) were calculated as slope of amounts vs. time (40, 80 and 120min) curves. The intracellular amounts of glucuronides (C and D) were determined at the end of excretion experiments (i.e., 120min) after the cells were washed three time with ice-cold buffer. The f_{met} values (determined at 120min) were shown in the 4E and 4F, and the CL values were given in the 4G and 4H. The calculated intracellular concentrations of glucuronides were shown in the X-axis of G and H followed by the actual loading flavonoid concentrations shown in brackets. Each data point was the average of three determinations with error bar representing the standard deviation (n=3). $P<0.05$,* ; $P<0.01$, **.

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Fig.5 Effects of siRNA-mediated UGT1A9 silencing on the excretion rates, intracellular amounts, fraction metabolized (f_{met}), and clearance (CL) of flavonoid glucuronides. Engineered HeLa cells stably overexpressing UGT1A9 grow on 12-well plates were treated with 10μM genistein or apigenin in the absence of siRNA (3μl water per well) or presence of 30pmole/well scrambled siRNA or siRNA targeting UGT1A9. The experiment protocol was otherwise the same as those shown in Fig.4. The amounts of excreting glucuronides as a function of time was shown in the 5A and 5B and the intracellular amounts (determined at 120min) were shown in the 5C and 5D. The f_{met} values (determined at 120min) were shown in the 5E and 5F, and the CL values were given in the 5G and 5H. For the control group treated with equal volume of water, each data point was the average of six determinations (n=6); for the control group transfected with scrambled siRNA or the group treated with siRNA targeting UGT1A9, each data point was the average of three determinations (n=3). The error bar represents the standard deviation. $p<0.05$, *; $p<0.01$,**. There was no statistically significant difference observed between the scrambled control and water transfection. In Fig.5I, effects of siRNA-mediated silencing on expression of UGT1A9 were shown. siRNA of UGT1A9 (75pmole/well of a 6-well plate, corresponding 30pmole/well of a 12-well plate) was transfected to the engineered HeLa cells. Two days after transfection, cells were incubated with apigenin (api), and genistein (gen) at 10μM for 40min to maximally mimic the condition of excretion assay. At the end of the experiments, the cells were collected and lysated for western blotting, where 25μg of protein were loaded into each lane (n=2).

Fig.6 Effects of BCRP-specific inhibitor Ko143 on the excretion rates, intracellular amounts, fraction metabolized (f_{met}), and clearance (CL) of flavonoid glucuronides. Engineered HeLa cells stably overexpressing UGT1A9 grown on 12-well plates were treated with 10μM genistein or apigenin in the absence or presence of Ko143 at 5μM or 10μM. The

experiment protocol was otherwise the same as those shown in Fig.4. The amounts of excreted glucuronides as function of time was shown in the 6A and 6B and the intracellular amounts (determined at 120min) were shown in the 6C and 6D. The f_{met} values (determined at 120min) were shown in the 6E and 6F, and the CL values were given in the 6G and 6H. Each data point was the average of three determinations with error bar representing the standard deviation (n=3). $p<0.05$, *; $p<0.01$,**

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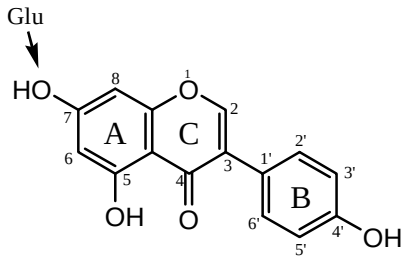
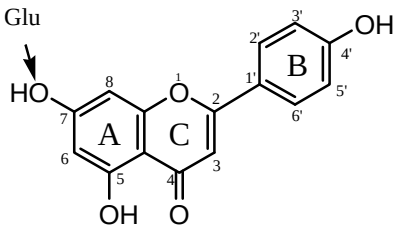
Table 1. Comparison of kinetics parameters of UGT1A9 from different sources in genistein and apigenin glucuronidation.

Structure	**Genistein** 			**Apigenin** 		
Source	Supersome™	Transient	Stable	Supersome™	Transient	Stable
K_m (μM)	2.72±0.073	3.71±0.65	*3.83±0.18	0.51±0.048	*1.76±0.11	1.68±0.037
V_{max} (nmole/ min/mg)	1.41±0.027	0.35±0.025	0.10±0.0015	2.61±0.023	1.58±0.0054	0.21±0
AIC	-15.82	-36.63	-76.62	-10.44	-18.96	-47.84
R^2	0.97	0.99	0.99	0.91	0.86	0.92

Note: A Student's t-test was used to compare the data. (*, $p<0.05$). For Supersome™ and transient overexpression of UGT1A9, three independent studies (or n=3) were run, whereas for stable overexpression of UGT1A9, only two independent studies (n=2) were run. The arrows indicated the position for glucuronidation occurring in engineered HeLa cells.

Fig. 1

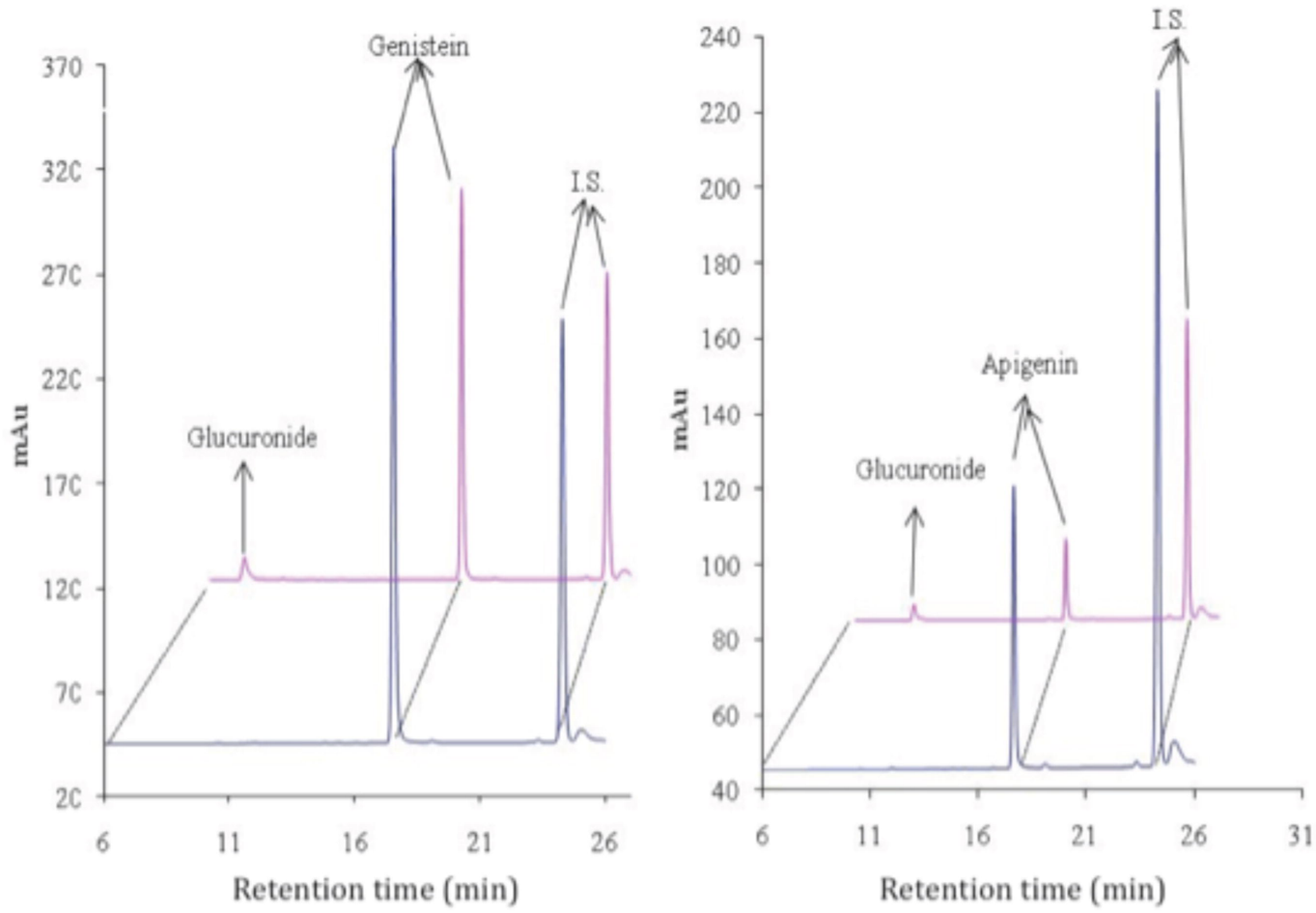


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Fig.2

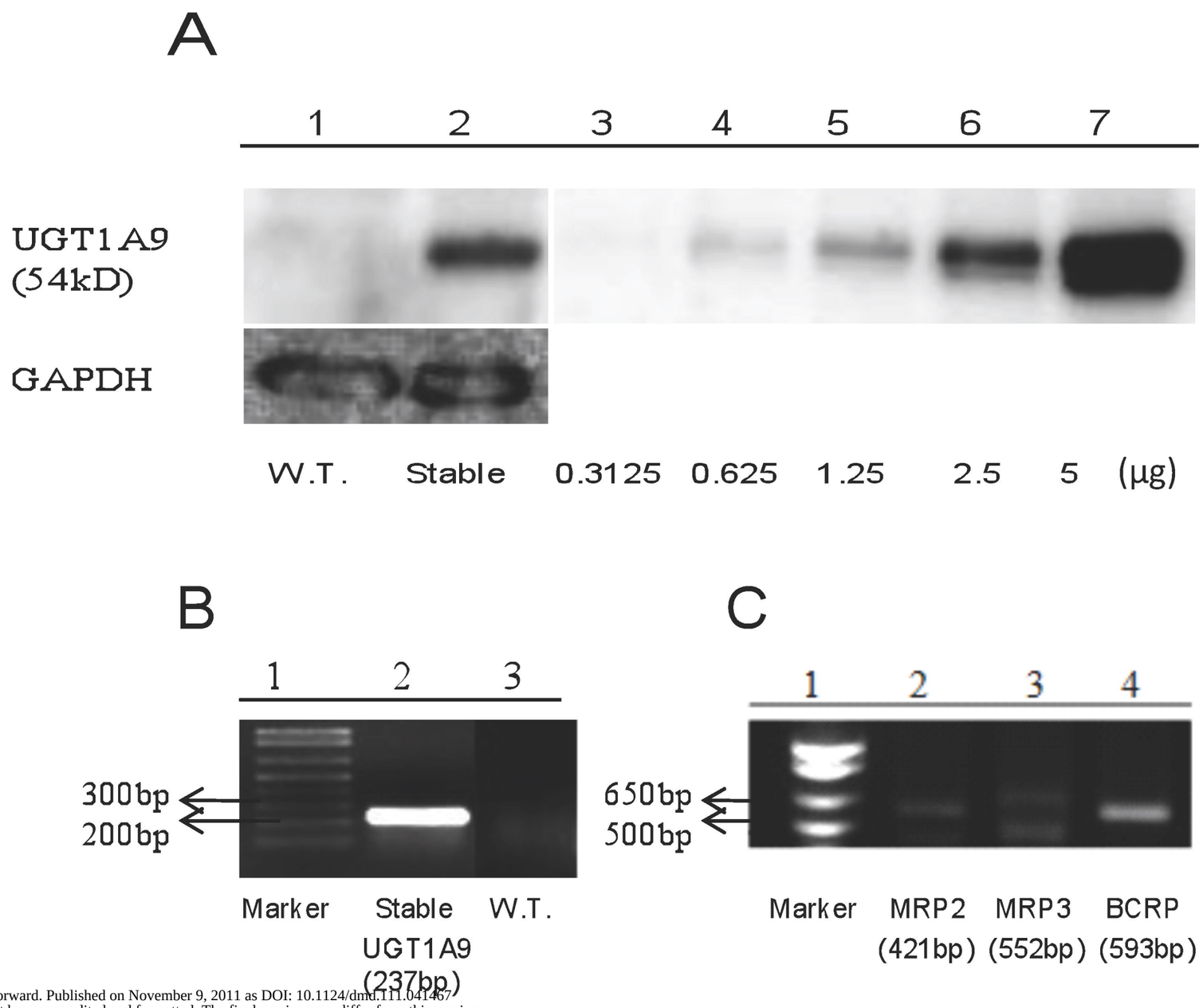

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Fig. 3

◆◇ UGT1A9 Supersome™ ▲△ Transient overexpression ●○ Stable overexpression

Solid symbol: genistein Open symbol: apigenin

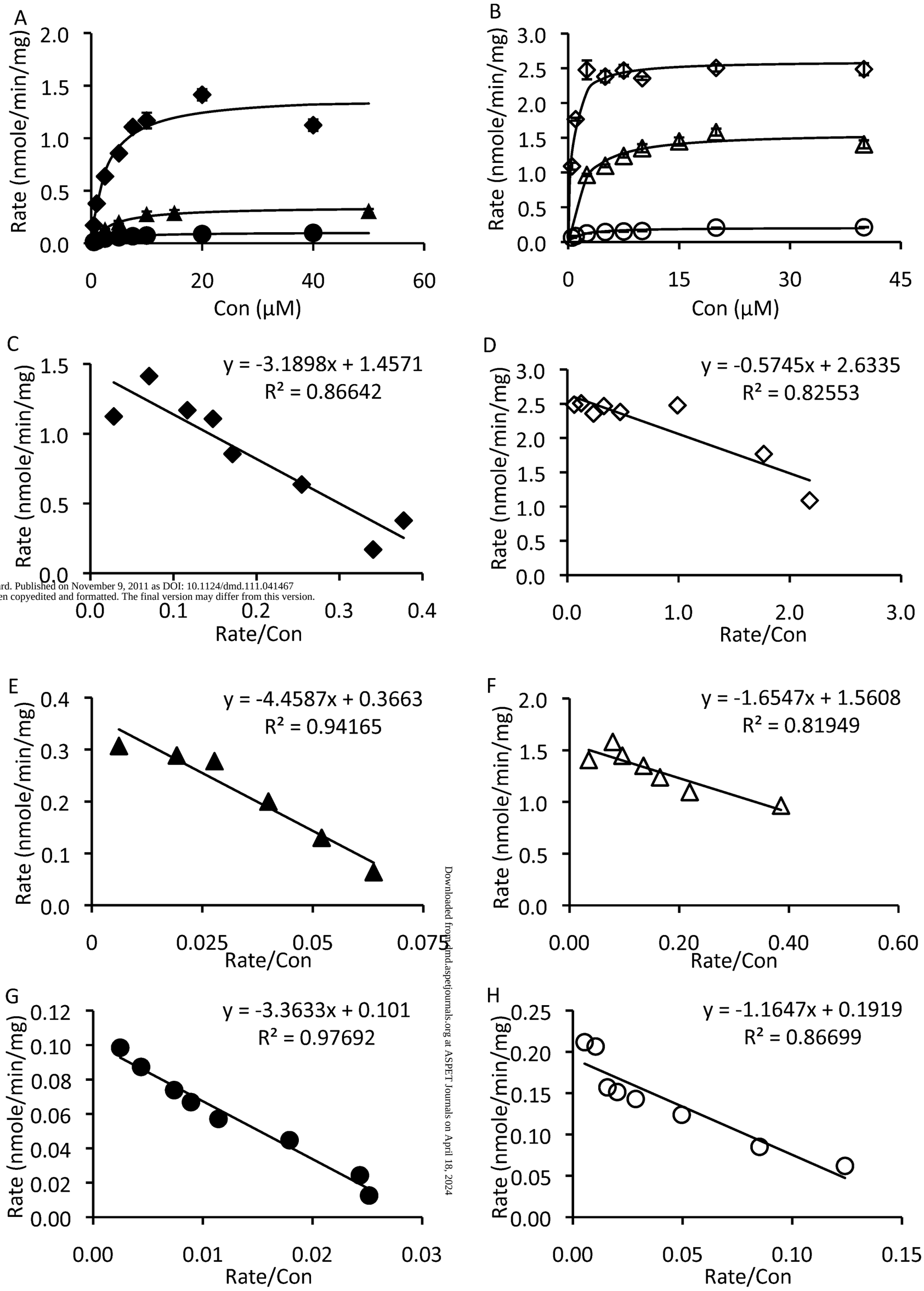

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Fig. 4

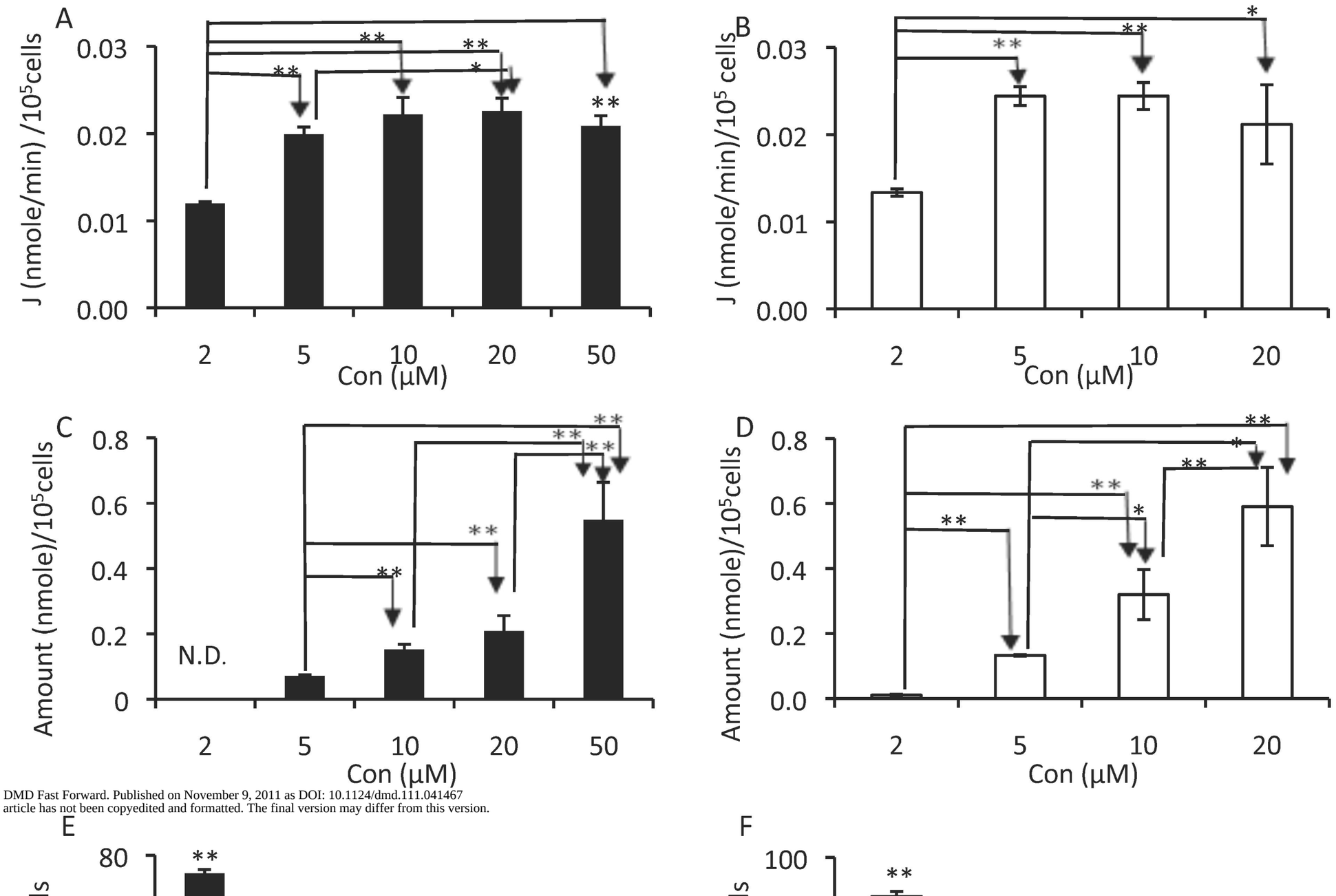

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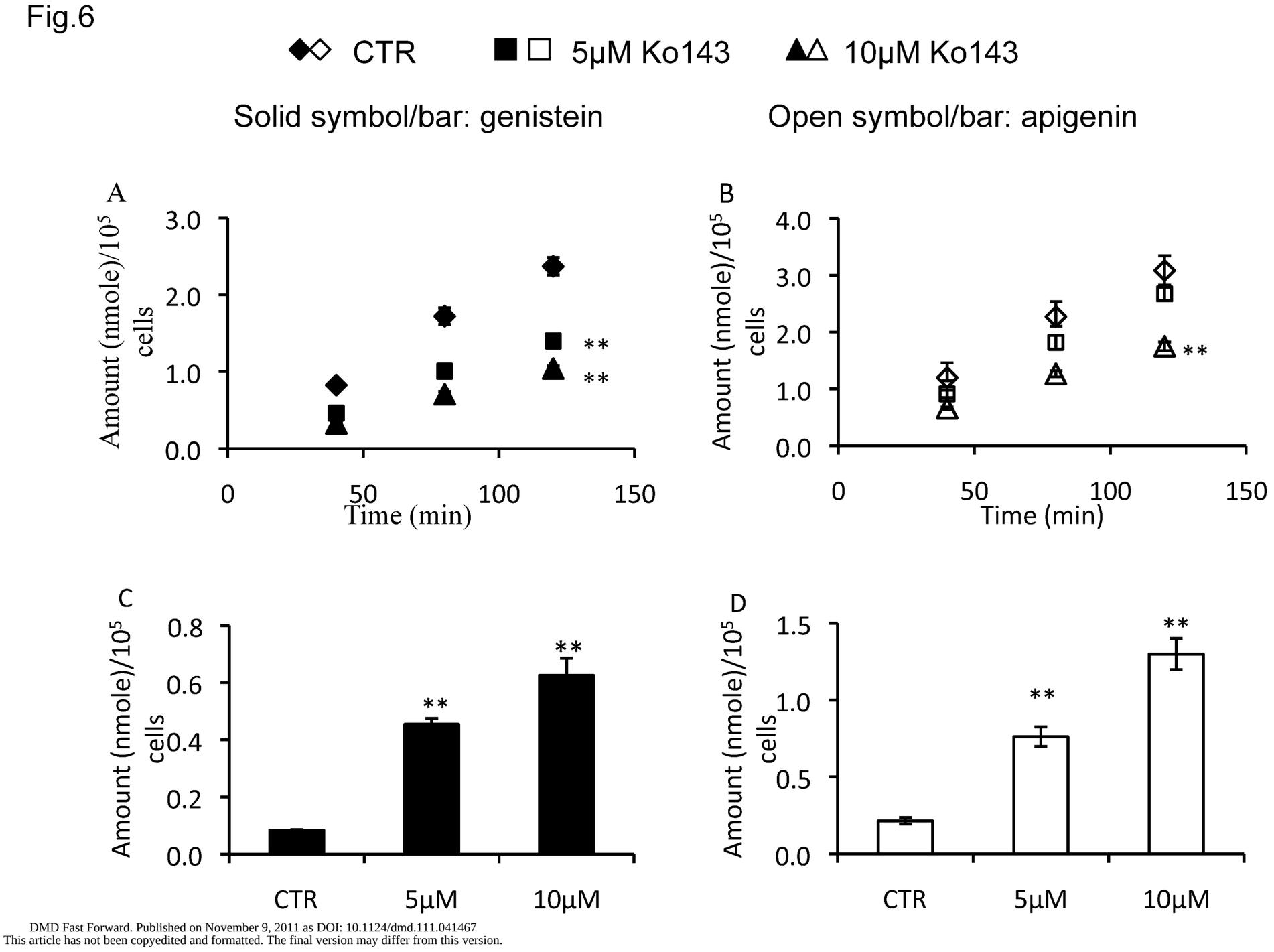
Fig. 5

Fig.6



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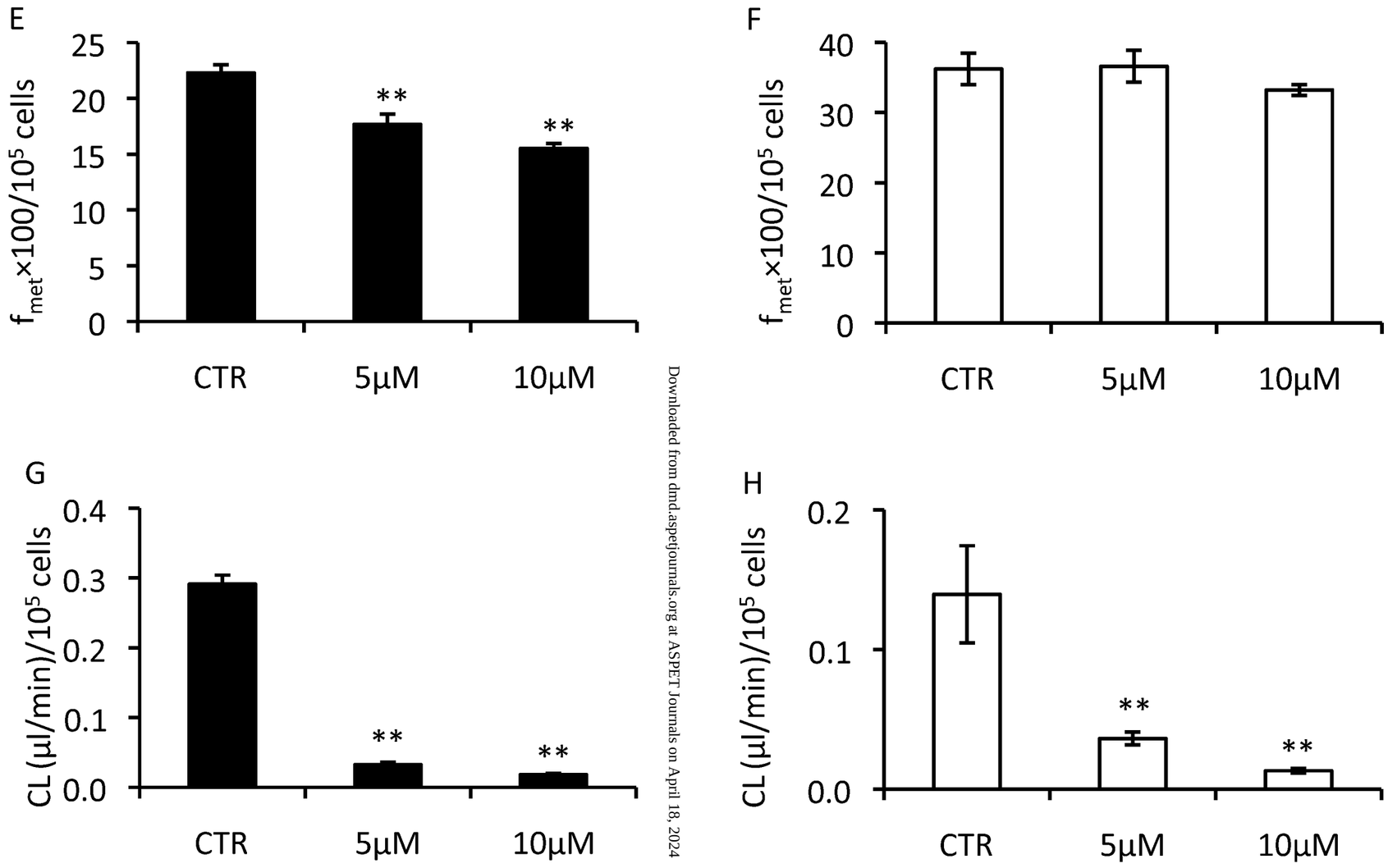

UGT1A9-overexpressing HeLa Cells Is an Appropriate Tool to Delineate the Kinetic Interplay between BCRP and UGT and to Rapidly Identify the Glucuronide Substrates of BCRP

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Drug Metabolism and Disposition

Fig.1S

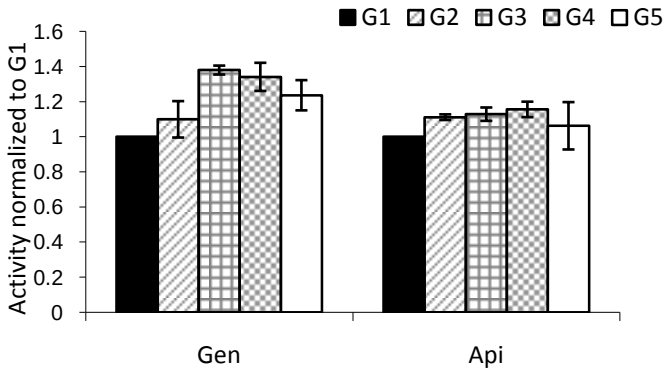

Fig.1S Stability of expressed UGT1A9 in engineered (or stably transfected) Hela cells. Genistein (Gen) or apigenin (Api) (10μM) were incubated with engineered HeLa cells grown on 6-well plates for five consecutive generations. Each cell generation was marked by a cell passage every 4 days. The activities were all normalized to the first generation. Each data point was the average of three determinations with error bar representing the standard deviation (n=3).

Fig.2S

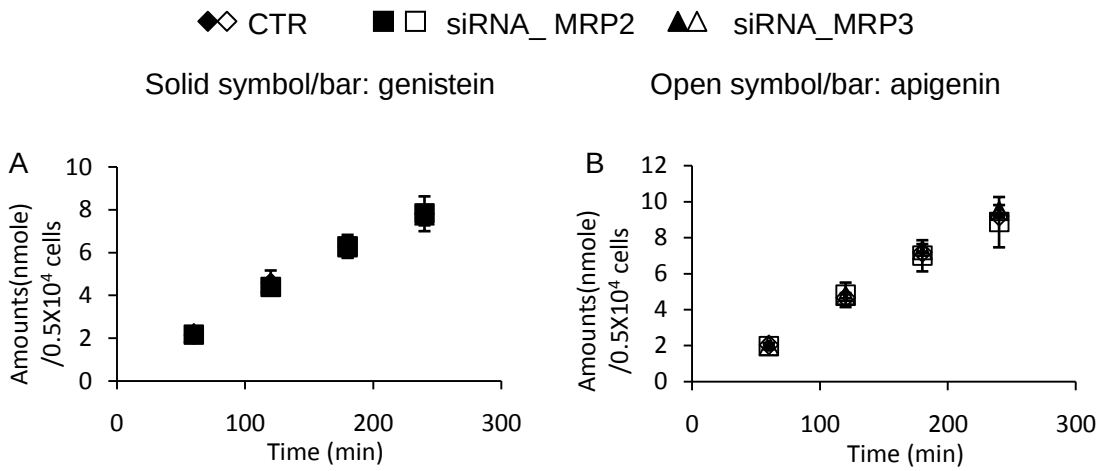

Fig.2S Effects of siRNA-mediated MRP2/MRP3 silencing on the excretion of glucuronides. Genistein (A) or apigenin (B) (10μM) were incubated with engineered HeLa cells in the presence or absence of siRNA of MRP2 or MRP3 (40pmole/well of a 12 well-plate) at 37ºC. The incubating media were taken at 60, 120, 180 and 240min. Each data point was the average of three determinations with error bar representing the standard deviation (n=3). $p<0.05$, *

Fig.3S

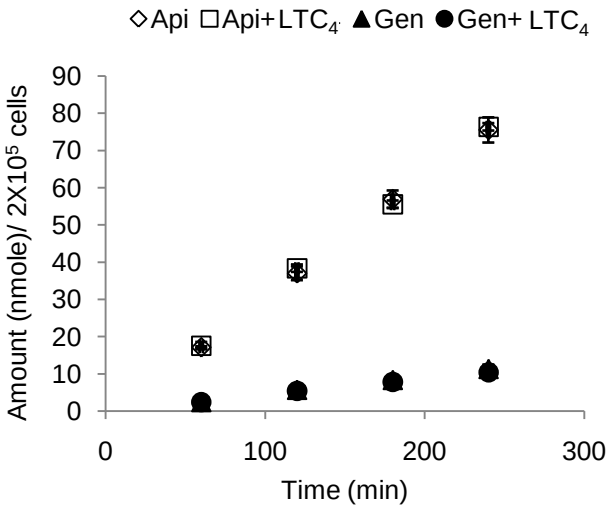

Fig.3S Effects of LTC_4 (0.1μM) on the excretion of glucuronides. Genistein (Gen) or apigenin (Api) (10μM) were incubated with engineered HeLa cells grown on 6-well plates in the presence or absence of LTC_4 at 37ºC. The incubating media were taken at 60, 120, 180 and 240min. Each data point was the average of three determinations with error bar representing the standard deviation (n=3). $P<0.05$, *