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Disposition of Flavonoids via Enteric Recycling: Determination of the UDP-Glucuronosyltransferase Isoforms Responsible for the Metabolism of Flavonoids in Intact Caco-2 TC7 Cells Using siRNA

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Received November 16, 2006; Revised Manuscript Received August 2, 2007; Accepted August 7, 2007

Abstract: Our recent study indicates that microsomal glucuronidation rates are not predictive of the cellular glucuronide excretion rates and whole cell systems are needed to accurately determine the metabolic rates. This study aims to determine the contribution of UGT isoforms responsible for the metabolism of flavonoids in intact Caco-2 cells and cell lysates using siRNA. The results showed that UGT1A6 activities (as measured by *p*-nitrophenol glucuronidation) and expression were typically decreased 60–80% by siRNA treatment. Using siRNA-mediated silencing, we also showed that in intact cells, siRNA treatment substantially decreased the rate of excretion of apigenin glucuronide at low and high concentrations (>35%, $p < 0.05$), although it only moderately decreased the rate of excretion of genistein glucuronide at a high concentration (29%). The results also indicated that well-expressed UGT isoforms in the Caco-2 cells, UGT1A1, UGT1A3, UGT1A6, and UGT2B7, were capable of metabolizing apigenin faster than genistein and that UGT1A6 silencing did not substantially increase the level of expression of genistein-metabolizing UGT isoforms. We also determined the contribution of UGT1A6 to the apigenin and genistein metabolisms as a function of concentration, and the results indicated that metabolism of apigenin and genistein was saturable and siRNA treatment greatly reduced the rate of metabolism of apigenin but not that of genistein. In conclusion, we show for the first time that siRNA can be used effectively to determine which UGT isoform contributes to the metabolism of its substrate in intact cells. The results also indicate that UGT1A6 is a major contributor to glucuronidation of apigenin but not genistein in intact Caco-2 cells and in cell lysates.

Keywords: UGT1A6; siRNA; UGT; flavonoid; genistein; Caco-2; apigenin

Introduction

Apigenin (Figure 1) is a nonmutagenic bioflavonoid present in leafy plants and vegetables with significant chemopreventive activity against UV radiation. Current literature indicates that it may reduce the extent of DNA oxidative damage, inhibit the growth of human leukemia cells, induce cancer cells to differentiate, block growth signal transduction, and cause apoptosis in cancer cells.[1–5] However, the bioavailability of apigenin is poor,[6] which may be a serious impediment to its clinical development since observed

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10.1021/mp0601190 CCC: $37.00
Published on Web 10/10/2007

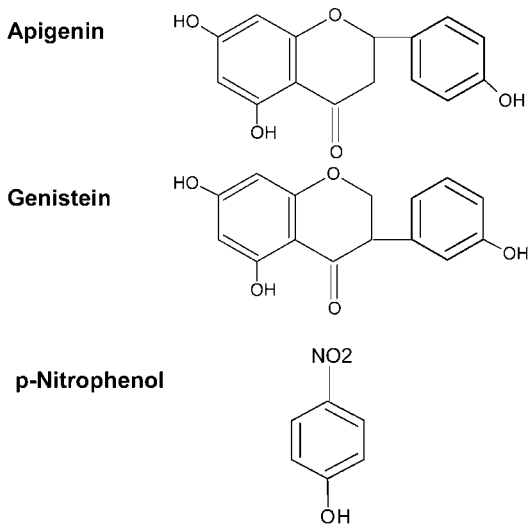

Figure 1. Chemical structures of apigenin, genistein, and *p*-nitrophenol.

IC_{50} or EC_{50} values (in the micromolar range) are significantly higher than its in vivo concentration (nanomolar) that can be achieved from dietary intake.

Genistein (Figure 1) is a constitutional isomer of apigenin, and it plays a major role as a weak estrogenic photochemical and is a known estrogen modulator in animal models. It has anticarcinogenic and anticancer properties against a variety of cancers, including breast, colon, and prostate cancers.[7] Genistein exerts its pleiotropic effects on cancer cells by affecting cell survival and growth. However, genistein is also poorly bioavailable, and most isoflavones in plasma are present as conjugated forms.[8,9] This is a serious concern because in vivo plasma concentrations of aglycones (unconjugated isoflavone) are in the range of 0.01–0.4 μM,[8–10] significantly lower than the IC_{50} or EC_{50} values of 5–50 μM commonly reported for its in vitro anticancer effects.[6,11]

To address these concerns, our previous works had aimed at finding the causes of this bioavailability problem. Our studies have shown that the low bioavailability of apigenin and genistein was not the result of poor absorption but of extensive metabolism.[6,11–15] However, we have limited understanding of the enzyme isoforms responsible for the glucuronidation of apigenin and genistein and the contribution of each UGT isoform to their metabolism. Few published reports had systemically determined the isoforms responsible for flavonoid glucuronidation,[16,17] but none has been conducted using intact cells. In expressed isoform studies, it was found that 1A1, 1A4, 1A6, 1A7, 1A9, and 1A10 were capable of metabolizing genistein.[16] Limited studies of the UDP-glucuronosyltransferase (UGTs) or UGT isoforms responsible for apigenin metabolism suggested that it was metabolized rapidly in HepG2 cells via UDP-glucuronosyltransferase 1A1 (UGT1A1).[18] However, UGT1A1 was only moderately expressed in the Caco-2 TC7 cells; in Caco-2 TC7 cells, UGT1A9 was poorly expressed and UGT1A7 and UGT1A10 were not expressed.[19] Furthermore, investigators have not actually determined the contribution of a specific UGT isoform in an intact cell system. Study using an intact cell system is highly valuable since our recent study indicates

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that microsomally derived kinetic parameters were not predictive of the intestinal or biliary excretions of glucuronides in rats.[20] Therefore, the purpose of this study is to determine the contribution of relevant UGT isoforms in the metabolism of apigenin and genistein Caco-2 TC7 cells and to develop an alternative approach to elucidating the contribution of a specific UGT isoform in intact cells.

Materials and Methods

Materials. Cloned Caco-2 TC7 cells were a kind gift from M. Rousset of INSERMU178 (Villejuit, France). Genistein and apigenin were purchased from Indofine Chemicals (Somerville, NJ). *p*-Nitrophenol and *p*-nitrophenol glucuronide (PNP-g) were purchased from Sigma-Aldrich (St. Louis, MO). A siRNA mixture or *SMART*pool against UGT1A6 (catalog no. M-020196-00-0050), four individual duplex siRNAs, and the negative control pool (catalog no. D-001206-13-20) were products of Dharmacon Inc. (Dallas, TX). Lipofectamine 2000 and NOVEX precast gels were from Invitrogen. Expressed human UGT1A1, -1A3, -1A4, -1A6, -2B7, -2B15, and -2B17 were purchased from BD Biosciences (Woburn, MA). All other materials (typically analytical grade or better) were used as received.

Cell Culture. The culture conditions for growing Caco-2 cells have been described previously.[15,21,22] In the experiments described here, we used cells that were grown for a short period of time using the same growth medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum). Caco-2 TC7 cells were fed every other day.

siRNA Design. On the basis of the rules suggested by Elbashir et al.,[23] the antisense strand of siRNA was targeted against an $AA(N)_{19}$ sequence at least 100 nucleotides downstream of the start codon. The GC content of the duplexes was kept within the 40–70% range. In addition to these rules, a BLAST search was conducted against GenBank to minimize the possibility of silencing another gene. A *SMART*pool that contained an equal molar mixture of four short siRNAs was generated by Dharmacon. The four pairs of siRNA have the following sequences: GUACAGGAAUAACAUGAUUU (sense, pair 1) and 5′-P-AAUCAUGUUAUUCCUG UACUU (antisense, pair 1), GAUAUGACUUUGUGCUUGAUU (sense, pair 2) and 5′-P-UCAAGCACAAAGUCAUAUCUU (antisense, pair 2), GAUCCUGGCUGAGUAUUUGUU (sense, pair 3) and 5′-P′-CAAAUACUCAGCCAGGAUCUU (antisense, pair 3), and GAACCGUUACCAAUCAUUUUU (sense, pair 4) and 5′-P-AAAUGAUUGGUAACGGUUCUU (antisense, pair 4).

Transient Transfection. Caco-2 TC7 cells were seeded at a density fo 5×10^4 cells/cm^2 and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS, Hyclone), but without antibiotics. Twelve hours post-plating, the cells were doubly transfected (6 h each) with serum-free medium containing Lipofectamine along with either 50 ng of total siRNA mixture or *SMART*pool, an equimolar amount of an individual siRNA duplex, or the negative control pool, whereas the control cells were treated with normal growth medium with Lipofectamine. At 48 h post-transfection, cells were harvested and cellular lysates were made and used for analyzing UGT1A6 enzyme activities in vitro and for blotting UGT1A6 protein levels as described later.

Caco-2 Cell Lysate. Caco-2 cell monolayers (3.3 cm^2 each) were harvested, placed in 0.5 mL of 50 mM potassium phosphate (pH 7.4 buffer), and sonicated in Aquasonic 150D sonicator (VWR Scientific, Bristol, CT) for 30 min at the maximum power (135 average watts) in an ice-cold water bath. The resulting cell lysate was then used in the UGT activity assay.

Lysate Protein Concentration. Protein concentrations of cell lysates were determined using the Bio-Rad (Hercules, CA) protein assay kit, using bovine serum albumin as standards.

Measurement of UGT Activities Using Cell Lysate. The incubation procedures for measuring UDP-glucuronosyltransferase (UGT) activities using cell lysate are as follows. (1) Mix 114.3 μL of cell lysate (final concentration, ~0.5 mg of protein/mL), magnesium chloride (0.88 mM), saccharolactone (4.4 mM), and alamethicin (0.022 mg/mL); different concentrations of substrates in a 50 mM potassium phosphate buffer (pH 7.4); and uridine diphosphoglucuronic acid (UDPGA) (3.5 mM, add last) to a final volume of 170 μL. (2) Incubate the mixture at 37 °C for 45, 90, and 480 min for *p*-nitrophenol, apigenin, and genistein, respectively. (3) Stop the reaction via the addition of 50 μL of a 94% acetonitrile/6% glacial acetic acid mixture containing 100 μM testosterone as an internal standard. In the determination of the kinetic parameters, apigenin concentrations were varied from 0.5 to 100 μM and genistein concentrations from 0.5 to 200 μM.

Following this reaction scheme, the reaction progressed linearly for as long as the substrate is less than 50% exhausted or up to 12 h, whichever is shorter. When assessing glucuronidation at different concentrations, we followed the time and percent substrate exhaustion limit so we could accurately determine the rate of glucuronidation.

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Measurement of UGT Activities Using Expressed Human UGTs. The incubation procedures for measuring UDP-glucuronosyltransferase (UGT) activities using expressed Supersomes are similar to what was used for measuring UGT activities in cell lysate except the concentration of Supersomes in the final reaction mixture was 0.026 mg/mL (for apigenin) or 0.052 mg/mL (for genistein) since the microsomes have a much higher concentration than the cell lysate. The reaction time was 30 min for apigenin and 60 min for genistein. With these reaction times and microsomal protein concentrations, the maximal percent substrate loss was 21% for apigenin and 26% for genistein, well within the linear range of no more than 50% substrate loss.

Screening UGT Supersomes for Apigenin, Genistein, and *p*-Nitrophenol Glucuronidation Activity. Glucuronidation activity of expressed UGT1A1, -1A3, -1A4, -1A6, -2B7, -2B15, and -2B17 was measured using the same assay conditions described in Measurement of UGT Activities Using Cell Lysate except that the final protein concentration was 0.05 mg/mL.

Western Blot Analyses of UGT1A6 Protein Levels. Caco-2 cell lysate (12 μg of protein) was separated via SDS–PAGE (12% Tris-Glycine gel), and then the separated proteins were transferred onto a nitrocellulose membrane by a standard protocol. After nonspecific binding had been blocked using a Tris buffer solution containing 5% nonfat milk and 1% Tween 80, the nitrocellulose membrane was incubated with WB-UGT1A6 (BD Biosciences) antibody at 1:2000 dilution for 1 h (at room temperature). After being washed three times to remove nonspecifically bound antibody, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody followed by detection with enhanced chemiluminescence using SuperSignal West Femto reagents from Pierce (Rockford, IL). Western blotting of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control. Densities of the Western blot bands were quantified using the density measurement software supplied by FluoroChem.

Excretion of Apigenin and Genistein Glucuronides by Intact Caco-2 Cells. The Caco-2 cell monolayers were washed three times with 37 °C, pH 7.4 Hank's balanced salt solution (HBSS). The monolayers were incubated with the buffer for 1 h, and the incubation medium was then aspirated. Afterward, the solution containing the compound of interest was loaded, and samples (400 μL) were taken at the different incubation times, followed by the addition of 400 μL of fresh buffer to keep the constant volume. To each sample was added 50 μL of 6% glacial acetic acid in an acetonitrile solution containing 100 μM testosterone as the internal standard. Afterward, the mixture was centrifuged at 13 000 rpm for 15 min, and the supernatant was analyzed by HPLC (Table 1).

Sample Analysis. The conditions for HPLC analysis of isoflavones were modified on the basis of a previously published method.[13] The HPLC conditions for analyzing *p*-nitrophenol, apigenin, genistein (see Figure 1 for structures), and their conjugates are as follows: system, Hewlett Packard (Wilmington, DE) 1090 with a diode array detector and HP Chemstation; column, Synergi Polar-RP (Phenomenex, Torrence, CA), 4 μm, 150 mm × 4.60 mm; mobile phase A (MBA), 100% acetonitrile; mobile phase B (MPB), 0.04% (w/v) phosphoric acid and 0.06% (v/v) triethylamine (pH 2.8). For *p*-nitrophenol and its glucuronide, the gradient is as follows: from 0 to 2 min, 2% MPA; from 2 to 15 min, 2 to 20% MPA; from 15 to 20 min, 20 to 33% MPA; from 20 to 21 min, 45.5% MPA. For apigenin and its glucuronide, the gradient is as follows: from 1 to 3 min, 20% MPA; from 3 to 23 min, 20 to 48.5% MPA. For genistein and its glucuronide, the gradient is as follows: from 0 to 3 min, 2% MPA; from 3 to 35 min, 2 to 45.5% MPA. Each injection is followed by a 5 min equilibrium time before the next injection. The retention times of *p*-nitrophenol, apigenin, genistein, and their conjugates are listed in Table 1. The limit of detection of this method is 0.25 μM or 50 pmol in the 200 μL sample, and the average accuracy was 92.7% for genistein and 91.1% for apigenin standards (eight total), using standard curves forced through the origin. Typical CV% is less than 5% between injections. Representative HPLC profiles of *p*-nitrophenol, genistein, and apigenin as well as their glucuronides are shown in Figure 2.

Table 1. Retention Times of PNP, Apigenin, Genistein, and Their Glucuronides[a]

compound	wavelength (nm)	retention time (min)
PNP (P)	254	21.9
PNP-glucuronides (Pg)	264	14.3
apigenin (A)	340	17.5
apigenin glucuronides (Ag)	340	9.7
genistein (G)	254	30.0
genistein glucuronides (Gg)	242	21.2
testosterone (internal standard, IS)	254	variable, but always the last peak

[a] The HPLC conditions are described in *Materials and Methods*.

Quantitation of Glucuronides. We have previously described how to calculate the concentration of metabolites.[15] Briefly, the metabolites are obtained from rat intestinal perfusate, which contains both glucuronides and parent flavonoid aglycones. After the perfusate is extracted with methylene chloride, the resulting aqueous phase contains only glucuronide. We then make serial dilution of phase II conjugate metabolites. Each solution of metabolites is divided into two haves. In one half, we add glucuronidase in water and the other water only. We allow the mixture to incubate in water batch for 4–8 h (time varied) to allow the sample with glucuronidase to hydrolyze completely. We then measure the peak areas of original glucuronides and that of the released aglycones. We found a linear correlation between the released aglycone and the glucuronides, and the correlation coefficient is then used to normalize the metabolite peak we measured in the experiments. In this way, we can quantify the metabolites using a standard curve for the parent compound which was done previously.[15]

Data Analysis. The apparent kinetic parameters of K_m and V_{max} were estimated. For apigenin, as suggested by the

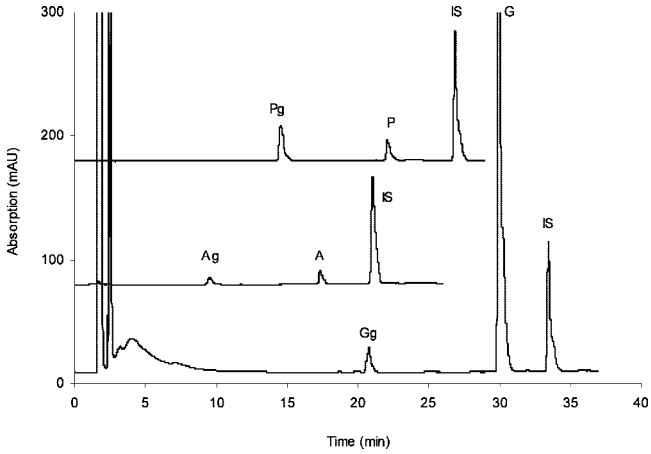

Figure 2. Representative HPLC profiles of *p*-nitrophenol (P), *p*-nitrophenol glucuronide (Pg), apigenin (A), apigenin glucuronide (Ag), genistein (G), genistein glucuronide (Gg), and testosterone (IS). The HPLC conditions are described in Materials and Methods. The retention time of each aglycone and its glucuronide is listed in Table 1.

Eadie–Hofstee analysis, three saturable (Michaelis–Menten-based) models with and without autoactivation were fit to the observations between the rate of reaction and substrate concentrations, using ADAPT II (University of Southern California, Los Angeles, CA). The general model structure is shown in eq 1. To determine the best-fit model, the model candidates were discriminated using Akaike's information criterion (AIC),[24] and the rule of parsimony was applied.

$$\text{reaction rate} = \frac{[V_{\max-0} + V_{\max-d}(1 - e^{-CR})]C}{K_m + C} \qquad (1)$$

where $V_{max\text{-}0}$ is the intrinsic maximum enzyme reaction rate, $V_{max\text{-}d}$ is the maximum induction of enzyme activity, R is the rate of enzyme activity induction, C is the concentration of substrate, and K_m is the concentration of substrate to achieve 50% of $V_{max\text{-}0} + V_{max\text{-}d}$.

For genistein, the reaction kinetic data showed biphasic kinetics (in which two isoforms with different kinetic behaviors were responsible for the glucuronidation). Therefore, formation rates (V) of isoflavone glucuronides at various substrate concentrations (C) were fitted to the following equation (eq 2):

$$\text{reaction rate} = \frac{V_{max1}C}{K_{m1} + C} + \frac{V_{max2}C}{K_{m2}C} \qquad (2)$$

where V_{max1} is themaximum enzyme reaction rate of one UGT isoform, V_{max2} is the maximum enzyme reaction rate of another UGT isoform, K_{m1} is theconcentration of substrate to achieve 50% of V_{max1}, and K_{m2} is the concentration of substrate to achieve 50% of V_{max2}.

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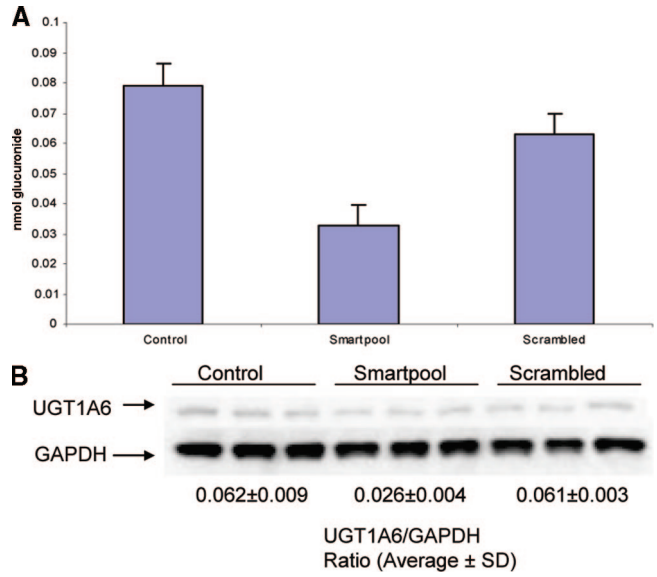

Figure 3. Effects of siRNA treatment on cellular UGT1A6 activities and expression. In panel A, *p*-nitrophenol (50 μM) glucuronidation rates were measured in Caco-2 cells treated with Lipofectamine alone (control), negative control (nonsense), siRNA pool (Noncontrol), or siRNA targeted to human UGT1A6. Panel B shows protein levels of UGT1A6 (top) and GAPDH (bottom) in Caco-2 cells transfected with control (buffer alone) (lanes 1–3), 50 nM siRNA-UGT1A6 (lanes 4–6), and nontarget control (lanes 7–9) using a Western blot. The numerical values below the bands were averages of three densitometer readings and the associated standard deviation of the means. The cells were doubly transfected within 24 h and grown for 72 h before experiments. Each data point represents the average of three determinations, and the error bar represents the standard deviation of the mean.

Statistical Analysis. One-way ANOVA or an unpaired Student's *t* test (Microsoft Excel) was used to analyze the data. The prior level of significance was set at 5%, or $p < 0.05$.

Results

Inhibition of UGT1A6 Expression in Caco-2 Cells using siRNA. On the basis of the expression pattern of UGT1As in the Caco-2 cells and activities of various UGT1As reported in the literature,[19] we expect UGT1A6 to be an important isoform for flavonoid glucuronidation. The results showed that UGT1A6 activities, as measured by *p*-nitrophenol (a prototypic UGT1A6 substrate) glucuronidation rates, decreased significantly in siRNA-treated Caco-2 cells ($p < 0.05$) (Figure 3A). This decrease in activities was corroborated by a decrease in UGT1A6 protein level in Western blots, as signified by a 59% decrease in the band density ratio (UGT1A6/GAPDH) from 0.062 to 0.026 (Figure 3B). The same experiment was replicated several times, and UGT1A6 protein levels typically decreased by 60–80%. Furthermore, when the four siRNA duplexes were tested

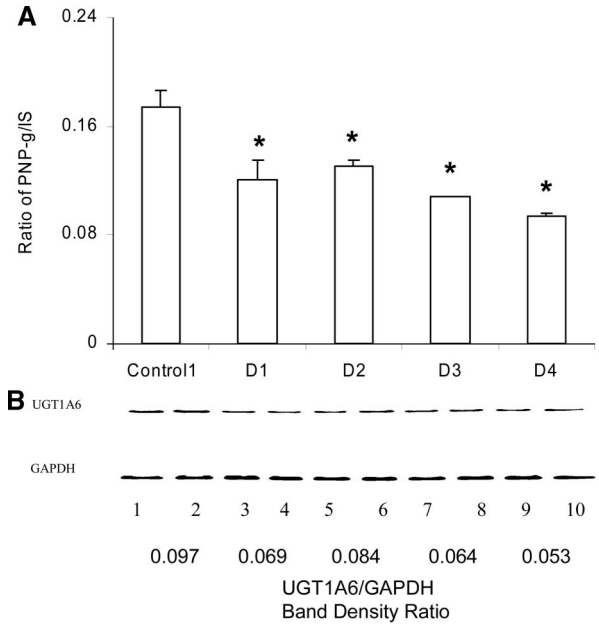

Figure 4. Effect of different siRNA sequences on cellular glucuronidation rates of *p*-nitrophenol (50 μM) in Caco-2 cell lysates. All siRNAs were targeted to human UGT1A6 but had different sequences, and each was used at an equal molar amount for 72 h. Each bar represents the average of three determinations (normalized arbitrary values), and the error bar represents the standard deviation of the mean (A). Panel B shows the Western blot for protein levels of UGT1A6 (top) and GAPDH (bottom) in Caco-2 cells transfected with control (buffer alone) (lanes 1 and 2), 50 nM siRNA-D1 (lanes 3 and 4), siRNA-D2 (lanes 5 and 6), siRNA-D3 (lanes 7 and 8), and siRNA-D4 (lanes 9 and 10). The band density ratio below the bands is the average of the two lanes.

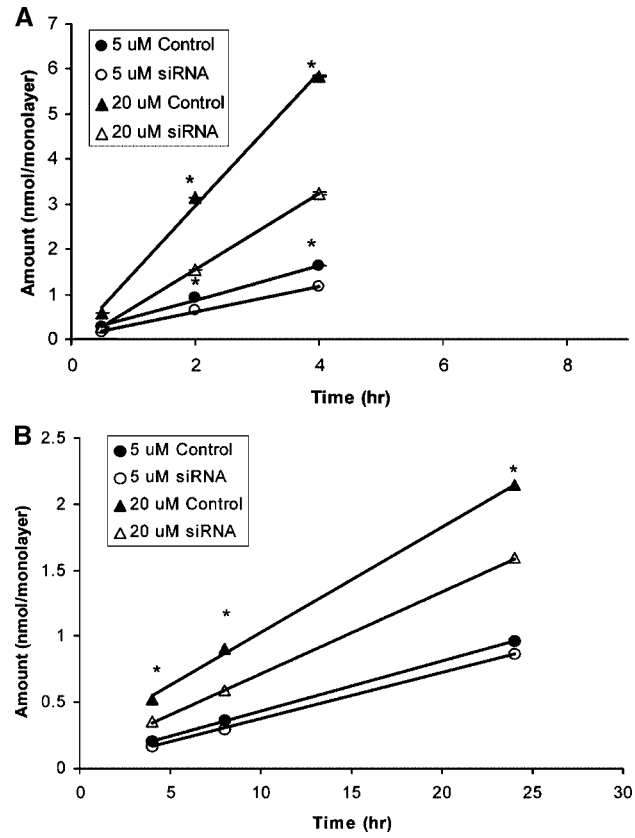

Figure 5. Effects of siRNA treatment on the cellular excretion of apigenin glucuronides (A) and genistein glucuronides (B) from the Caco-2 cells. Cell monolayers were incubated with two different concentrations of the flavonoids (5 and 20 μM), and metabolites produced were followed as a function of time, with or without siRNA treatment using the protocol described in Materials and Methods. Each data point represents the average of three determinations, and the error bar represents the standard deviation of the mean.

individually at the same molar concentration, results indicated that UGT1A6 activities and protein levels were decreased by a similar extent, which was also reflected by a maximum of 45% in the band density ratio (UGT1A6/GAPDH) from 0.097 to 0.053 (Figure 4A,B). On the basis of these results, and the fact that a mixture of siRNA was easier to use and provided more effective and consistent gene suppression, the rest of the experiments were performed with the siRNA mixture or simply siRNA.

Effect of siRNA on the Cellular Excretion of Apigenin and Genistein Glucuronides. To determine the contribution of each UGT isoform to apigenin glucuronide excretion in intact Caco-2 cells, we used siRNA since isoform specific chemical inhibitors were not available and UGT reaction rates were always the rate-controlling step in apigenin glucuronide excretion.[14] The results indicated that siRNA treatment in intact Caco-2 cells affected apical excretion of glucuronides in intact Caco-2 cells. At a low loading concentration of 5 μM, siRNA treatment significantly decreased ($p < 0.05$) the rate of excretion of apigenin glucuronide from 0.0386 to 0.0286 nmol min^{-1} (mg of protein)$^{-1}$ (or 35%). On the other hand, the same siRNA treatment did not affect excretion of genistein glucuronide [0.0038 vs 0.00348 nmol min^{-1} (mg of protein)$^{-1}$ or 8%] (Figure 5). In contrast, siRNA treatment appeared to have a more substantial effect on the excretion of both flavonoid glucuronides at a higher concentration (20 μM). The excretion rates decreased from 0.149 to 0.085 nmol min^{-1} (mg of protein)$^{-1}$ for apigenin glucuronide (79% decrease, $p < 0.05$) and from 0.00801 to 0.00621 nmol min^{-1} (mg of protein)$^{-1}$ for genistein glucuronide (29% decrease, $p < 0.05$) (Figure 5).

Glucuronidation Activities by Human UGT Isoforms toward Apigenin, Genistein, and *p*-Nitrophenol. We then determined which UGT isoforms may contribute to the metabolism of apigenin and genistein by measuring the glucuronidation activities of expressed human UGT1A1, -1A3, -1A4, -1A6, -2B4, -2B7, -2B15, and -2B17. These four UGT1As were selected because they are well expressed by Caco-2 TC7 cells, whereas other UGT1As such as UGT1A7, UGT1A8, UGT1A9, and UGT1A10 were either poorly expressed (UGT1A8 and UGT1A9) or not expressed (UGT1A7 and UGT1A10).[19] UGT2Bs were chosen since

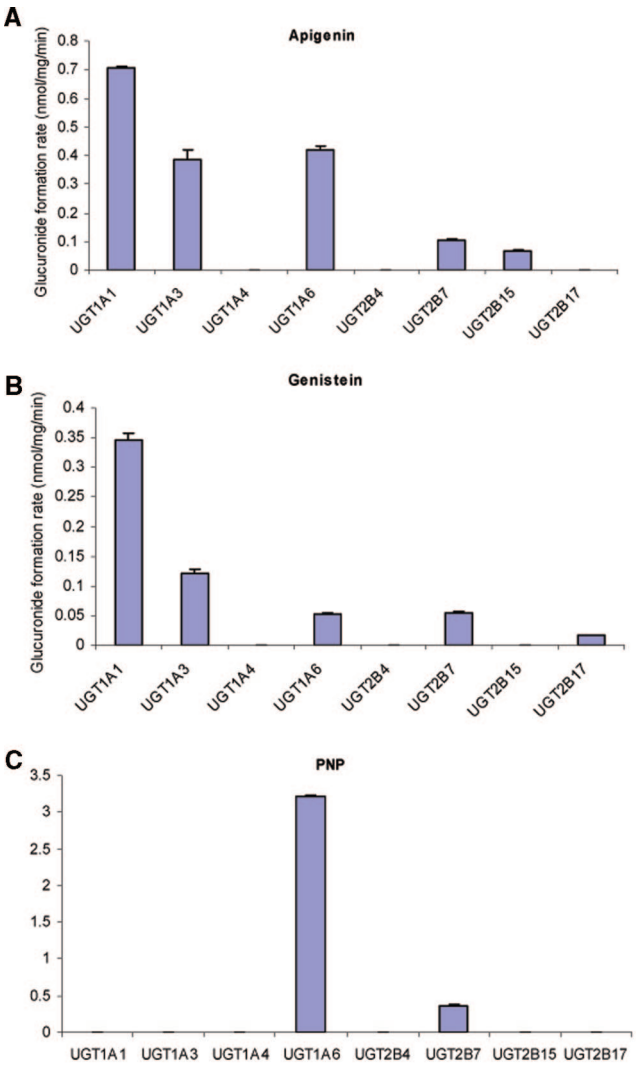

Figure 6. Metabolism of apigenin (A), genistein (B), and *p*-nitrophenol (C) by expressed human UGTs. The experiments were performed using 10 μM flavonoids and 50 μM *p*-nitrophenol for 4 h using procedures described in Materials and Methods.

their expression levels in Caco-2 TC7 cells were unknown. The results indicated that human UGT1A1, -1A3, -1A6, -2B7, and -2B15 glucuronidated apigenin at different rates (Figure 6A), with UGT1A1 having the highest activity (set as 100%), followed by UGT1A6 (60% of that of UGT1A1), UGT1A3 (55%), UGT2B7 (15%), and UGT2B15 (9%), whereas these same UGT isoforms also glucuronidated genistein, but at rates slower than that of apigenin. Once again, UGT1A1 glucuronidated the fastest followed by UGT1A3 (35% of that of UGT1A1), UGT2B7 (16%), UGT1A6 (15%), and UGT2B17 (4%) (Figure 6B). In addition, we found that *p*-nitrophenol (at a low concentration of 50 μM) is a nearly specific substrate for the UGT1A6 isoform (Figure 6C), since UGT2B7 made only a minor contribution. This result is consistent with a previous finding that showed *p*-nitrophenol as a specific UGT1A6 substrate.[25]

Table 2. Glucuronidation of Apigenin in Caco-2 Cell Lysate without (control) or with siRNA Treatment[a]

	glucuronidation rate[b] (pmol min^{-1} mg^{-1}) $\pm$ SD	
	control	siRNA-treated
0.5 μM apigenin	2.86 $\pm$ 0.33	below the level of detection[c]
5.0 μM apigenin	57.8 $\pm$ 6.5	30.2 $\pm$ 2.1[c]
20 μM apigenin	297.9 $\pm$ 44.5	128.5 $\pm$ 9.8[c]
0.5 μM genistein	1.45 $\pm$ 0.21	2.00 $\pm$ 0.25
10 μM genistein	12.5 $\pm$ 2.2	9.60 $\pm$ 1.53
50 μM genistein	45.8 $\pm$ 1.5	31.4 $\pm$ 3.4[c]

[a] The siRNA was targeted to human UGT1A6, and the cells were treated twice with siRNA and used 72 h after the first treatment (see *Materials and Methods* for details). [b] Each data point represents the average of three determinations. [c] There are statistically significant differences between rates of metabolism in Caco-2 cell lysate with UGT1A6 gene silencing and control ($p < 0.05$).

Effect of UGT1A6 Silencing on the Metabolism of Apigenin and Genistein by Caco-2 Cell Lysate. To determine the effects of UGT1A6 silence (by siRNA) on the metabolism of apigenin and genistein, the conjugation activities were measured at low, medium, and high concentrations in Caco-2 cell lysates with or without siRNA treatment. The results indicated that in siRNA-treated cells, glucuronidation rates of apigenin decreased significantly at all tested concentrations (57%, $p < 0.05$), whereas that of genistein was only modestly decreased (32%, $p < 0.05$) at a high concentration (50 μM) (Table 2).

Kinetic Analysis of Apigenin and Genistein Glucuronidation by Caco-2 Cell Lysates. We determined the glucuronidation rates as a function of concentration because changes in rates of flavonoid glucuronidation as the result of siRNA treatment are dependent on the concentration of flavonoids (Table 2). Results indicated that the rates of glucuronidation increased with concentration and reached a plateau as the concentration of apigenin increased (Figure 7A). An Eadie–Hofstee plot of the same data (a hooked curve) was consistent with autoactivation kinetics[26] (Figure 7B). Therefore, the data were fit using an atypical Michaelis–Menten model with autoactivation, and the best-fit model was found to be an autoactivation model with three parameters (Table 3). The atypical K_m values were 13.14 μM for control and 5.32 μM for siRNA-treated cells. The V_{max} values were determined to be 0.574 nmol min^{-1} mg^{-1} for control and 0.191 nmol min^{-1} mg^{-1} for siRNA-treated cells.

The extent of genistein glucuronidation as a function of concentration was also determined in Caco-2 cells without or with UGT1A6 gene silencing (Figure 8). The Eadie–Hofstee plot indicated that the reaction kinetics followed a

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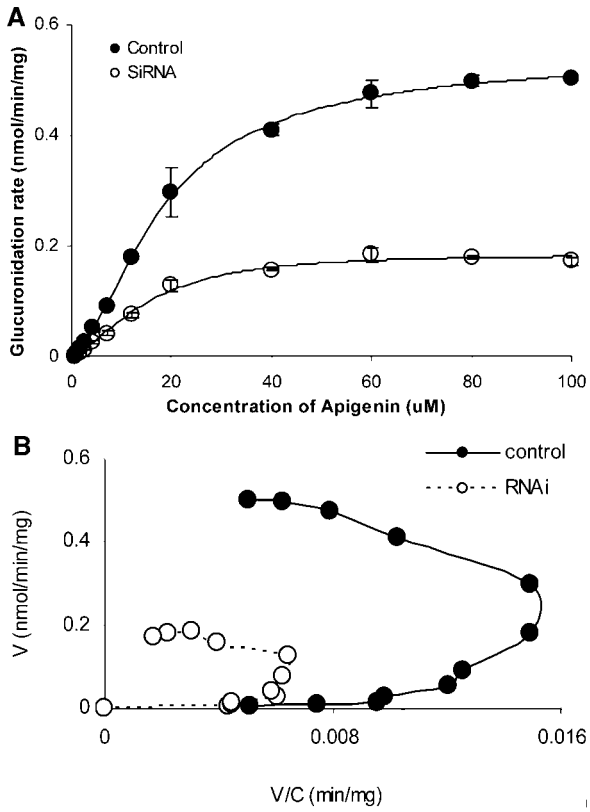

Figure 7. Metabolism of apigenin in Caco-2 cell lysates without (solid line) or with siRNA treatment (dashed line) ($n = 3$). Rates of metabolism were determined from 0.56 to 100 μM, and the reaction time was 90 min (A). Each data point represents the average of three determinations, and the error bar represents the standard deviation of the mean. Panel B shows the Eadie–Hofstee plots of the same data without (filled circles) or with siRNA treatment (empty circles).

biphasic pattern regardless of siRNA treatment (Figure 8B). Furthermore, the plot showed very sharp angles, suggesting two isoforms with significantly different K_m and/or V_{max} values. We first fitted these data using eq 2 with ADAPTII, but the fitting did not consistently generate meaningful kinetic parameters, most likely due to technical issues associated with model identifiability. Since there were minor differences in glucuronidation of genistein in siRNA-treated cells and in control cells, we did not obtain the kinetic parameters to describe genistein glucuronidation.

Discussion

This study is a continuation of our effort to dissect the mechanisms involved in the intestinal disposition of flavonoids via the enteric recycling mechanism. It seeks to use siRNA to determine the contribution of a single UGT isoform in the metabolism of a flavonoid because siRNA appeared to be one of the few viable approaches to completing this task. The results of this study clearly indicate that siRNA is effective in defining the contribution of a particular UGT isoform inside intact cells and in cell lysates. The results of cell lysate studies in siRNA-treated cells are consistent with

Table 3. Kinetic Parameters of Apigenin Glucuronidation in Caco-2 Cell Lysates Prepared from Control and siRNA-Treated Cells Using Three Different Kinetic Models

best-fit parameter (±CV)	model 1[a]	model 2[b]	model 3[c]
control			
$V_{max\text{-}0}$ (nmol min^{-1} mg^{-1})	0.711 ± 0.067	0 (fixed)	0.030 ± 1.11
$V_{max\text{-}d}$ (nmol min^{-1} mg^{-1})	0 (fixed)	0.574 ± 0.03	0.542 ± 0.068
K_m (μM)	33.7 ± 0.2	13.1 ± 4.2	12.6 ± 0.4
R	0 (fixed)	0.091 ± 0.238	0.0833 ± 0.292
r^2	0.99	0.999	0.999
AIC	−55.0	−86.3	−85.1
siRNA-treated			
$V_{max\text{-}0}$ (nmol min^{-1} mg^{-1})	0.235 ± 0.074	0 (fixed)	0.8×10^{-8}
$V_{max\text{-}d}$ (nmol min^{-1} mg^{-1})	0 (fixed)		0.191 ± 0.08
K_m (μM)	23.3 ± 0.2	5.31 ± 1.03	5.32 ± 1.86
R	0 (fixed)	0.0788 ± 0.46	0.0788 ± 0.63
r^2	0.979	0.994	0.994
AIC	−73.3	−89.3	−87.3

[a] Classic Michaelis–Menten model, with two parameters. [b] Autoactivation model without residual reactivity, with three parameters. [c] Autoactivation model with residual reactivity, with four parameters.

the fact that multiple human UGTs in both UGT1A and UGT2B subfamilies are involved in the metabolism of apigenin and genistein in the Caco-2 cells, and in the human intestinal and liver cells as well. On the basis of the fact that UGT1A6 is expressed well in Caco-2 cells but not particularly active against genistein, it was not surprising to see that siRNA-mediated UGT1A6 silencing in the Caco-2 cells decreased the extent of glucuronidation of apigenin but not genistein. It was somewhat unexpected, however, to observe that a 60–80% decrease in cellular UGT1A6 activity resulted in a similar decrease in the rate of cellular excretion of apigenin conjugates, since we have expected a more moderate effect as an earlier study using mature Caco-2 cells showed that the efflux transporter was the rate-limiting step.[14]

It is difficult to separate the contribution of a single UGT isoform in the metabolism of a UGT substrate for the following reasons. First of all, UGT substrates such as flavonoids and raloxifene are often metabolized by multiple UGT isoforms (Figure 6).[16–18] Second, expression patterns of UGT isoforms are organ and tissue specific and have high intersubject variability.[27,28] Third, there is a lack of isoform specific substrate, or UGT isoform specific chemical inhibitor or inhibitory antibody. Therefore, an alternative approach is

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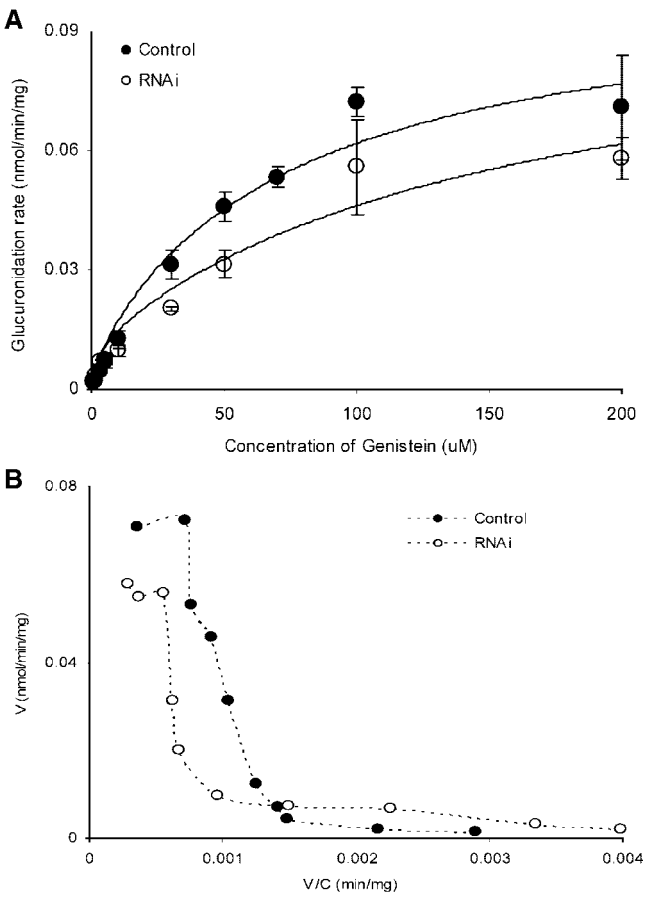

Figure 8. Metabolism of genistein in Caco-2 cell lysates without (solid line) or with siRNA treatment (dashed line) ($n = 3$). Rates of metabolism were determined from 0.5 to 200 μM, and the reaction time was 480 min (A). Each data point represents the average of three determinations, and the error bar represents the standard deviation of the mean. Panel B shows the Eadie–Hofstee plots of the same data without (filled squares) or with siRNA treatment (empty circles).

needed to define the contribution of a single UGT isoform to the metabolism of a substrate in functional cells.

This is the first report that details the use of siRNA as a plausible and effective tool for determining the contribution of a UGT isoform to the cellular metabolism of its substrate, which is a flavonoid in this case. Whereas the use of siRNA is not without its challenge (e.g., possible compensatorily increased level of expression of alternative UGT isoforms), the results suggest that the levels of expression of several important UGT isoforms (UGT1A1, UGT1A5, and UGT2B7) did not increase substantially to compensate for UGT1A6 silencing in our study. This is because genistein metabolism rates at higher concentrations would have increased if expression levels of highly active UGT1A1, UGT1A5, and UGT2B7 (Figure 6B) were increased substantially to compensate for the of UGT1A6 silencing. Therefore, our data suggest that siRNA treatment has sufficient selectivity and did not cause substantial compensatory increases in the levels of expression of other (at least four) UGT isoforms in the Caco-2 cells.

It is fairly remarkable that siRNA-mediated UGT1A6 silencing decreased apigenin glucuronidation rates without affecting genistein glucuronidation in Caco-2 cell lysates (Table 2) since these two compounds are constitutional isomers that have very similar structures (Figure 1). On the basis of the PCR expression factor of 2.6 for UGT1A6, 1.3 for UGT1A3, and 1 for UGT1A1,[19] we had predicted that a 50% decrease in the level of apigenin glucuronidation in cell lysates was likely (Figure 7) since UGT1A1 was 40% more effective and UGT1A3 was as effective as UGT1A6 in the glucuronidation of apigenin (Figure 6). The results showed a 60% decrease in metabolism rates by siRNA at high apigenin concentrations (Figure 7A), slightly exceeding our expectation. In addition, we expect a less than 25% difference in genistein metabolism in cell lysates as the result of UGT1A6 silence, and the results (32% decrease) were mostly consistent with our expectation (Figure 8A). Taken together, these results suggest that siRNA is an effective method for determining the contribution of a dominant UGT isoform in the glucuronidation of drugs and chemicals in cell lysates.

We also expect siRNA silencing to significantly impact the kinetics of apigenin reaction but not that of genistein in the cell lysates on the basis of the fact the UGT1A6 is a major isoform for metabolism of apigenin but not of genistein (Figure 6). As expected, there was a large difference in the rates of metabolism as a consequence of siRNA silencing (Figure 7A and Table 3). V_{max} values of apigenin glucuronidation decreased 67% as the result of siRNA treatment (Table 3). This decrease was comparable to a maximum reduction of 60–80% in silencing efficiency as measured by Western blots and *p*-nitrophenol glucuronidation (Figures 3 and 4). In contrast, siRNA silencing did not significantly affect the rates of genistein glucuronidation at the tested concentrations (Figure 8A).

Whereas we have expected that siRNA treatment will be highly useful for the determination of the contribution of the UGT isoform to the metabolism of its substrate in cell lysates, we could not predict what might happen to cellular excretion of phase II conjugates in intact Caco-2 cells. This is because our studies in Caco-2 cells suggest that cellular UGT activities are often not the rate-limiting step in the cellular excretion of phase II conjugates.[12,14] Moreover, recent studies in the rat models clearly indicate that microsomal (and therefore other subcellular preparations) glucuronidation rates are not predictive of cellular glucuronide excretion rates.[20] Therefore, we were not surprised by the fact that siRNA silencing had a smaller effect on the cellular excretion of glucuronides of apigenin (35%) and genistein (8%), which were obtained using a flavonoid concentration of 5 μM (Figure 5). We were somewhat surprised that the cellular excretion of glucuronides was more affected at a higher flavonoid concentration of 20 μM since the rates of excretion of glucuronides of apigenin and genistein decreased 79 and 29%, respectively (Figure 5). These decreases in the rates of excretion were similar to or exceeded decreases in cellular lysate metabolism, which were not expected. We attributed these larger-than-expected

decreases in the rate of cellular excretion due to inhibition of BCRP by high concentrations of flavonoids[29,30] since BCRP has been shown recently to be important for the excretion of glucuronides,[31,32] but further investigation into this mechanism is necessary.

Taken together, the effect of siRNA treatment on intact cellular excretion points to the significance of a coupled action between a flavonoid-conjugating enzyme and efflux transporters in that functions of efflux transporters can significantly impact the cellular excretion of phase II conjugates. As a consequence of this coupling effect, the cells can at least partially compensate for the deficiency in enzyme (UGT1A6 in this case) activities to maintain the metabolic barrier of the intestine against flavonoids. Specifically, at 5 μM, a 50% decrease in the level of apigenin glucuronide formation (Table 2) in cell lysate resulted in an only 32% decrease in its rate of cellular excretion. This ability to compensate and therefore maintain barrier properties would help explain why flavonoids have poor bioavailabilities in humans.

In conclusion, this study demonstrates for the first time that siRNA can be used effectively to determine the contribution of a single UGT isoform in the metabolism of a substrate in intact cells and in cell lysates. The results show that UGT1A6 is mainly responsible for the cellular metabolism of apigenin, but not genistein, in Caco-2 TC7 cells. This study also provides direct evidence at the cellular level that multiple UGT isoforms are involved in the metabolism of flavonoids since silencing of UGT1A6 did not completely abolish the metabolism of apigenin or genistein. Lastly, a coupled mechanism between efflux transporters and UGT enzyme isoforms may serve as a viable mechanism for compensating for the deficiency in enzyme function.

Abbreviations Used

RNAi, RNA interference; siRNA, small interfering RNA; UGT1A6, UDP-glucuronosyltransferase 1A6; PNP-G, *p*-nitrophenol glucuronide; UDPGA, uridine diphosphoglucuronic acid; HBSS, Hanks' balanced salt solution; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MPA, mobile phase A; MPB, mobile phase B; TBS, TRIS buffer solution; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Acknowledgment. This work was supported by National Institutes of Health Grant CA87779. Our work started when X.L. and M.H. were at Washington State University, where we received outstanding technical contribution from Ms. Huimin Lin and Dr. Jun Chen. The work was completed at the University of Houston, College of Pharmacy.

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