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Enhanced Stability and Intracellular Accumulation of Quercetin by Protection of the Chemically or Metabolically Susceptible Hydroxyl Groups with a Pivaloxymethyl (POM) Promoiety

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In order to increase stability of quercetin, its metabolically and chemically susceptible hydroxyl groups 7-OH and 3-OH respectively were transiently blocked with a pivaloxymethyl (POM) promoiety to provide two novel quercetin conjugates [7-*O*-POM-Q (**2**), 3-*O*-POM-Q (**3**)]. In the absence of stabilizer (ascorbic acid), the synthesized conjugates showed significantly increased stability in cell culture media [$t_{1/2}$ = 4 h (**2**), 52 h (**3**)] compared with quercetin ($t_{1/2}$ < 30 min) and quercetin prodrug **1** ($t_{1/2}$ = 0.8 h). In addition, the quercetin conjugate **2** underwent efficient cellular uptake and intracellular levels of its hydrolysis product, quercetin, were maintained up to 12 h. Stability and intracellular accumulation of **2** were demonstrated by its stabilizer-independent cytostatic effect and induction of apoptotic cell death. Even though **3** was more stable than **2**, it failed to penetrate cell membranes. However, the remarkable stability of **3** warrants further investigation of quercetin conjugates with various promoieties at the 3-OH position.

Introduction

Quercetin (Figure 1), a polyphenolic flavonoid, is abundant in human diet including apples, onions, grapes, red wine, and green tea, and its various health promoting effects such as antioxidant,[1] antiviral,[2,3] and anticancer[4] activities have been studied in detail. However, studies that support clinical uses of quercetin are few presumably because of its unfavorable physicochemical as well as pharmacokinetic properties including oxidative degradation[5−7] and fast metabolism.[7−9]

Pro-oxidative compounds in cell systems such as enzymes and transition metal cations are known to facilitate oxidative degradation of quercetin.[10−15] Oxidative degradation has also been reported as the main cause of rapid elimination of quercetin from the cultured human hepatocarcinoma cell line (Hep G2).[5] In spite of the lack of correct identification of the intermediates associated with the oxidative degradation, there seems to be a broad consensus that benzofuran-3(2*H*)-one **E** (Figure 2) is one of the major degradation products[16−21] that is formed by nucleophilic attack of water to the initially formed intermediate **B** (Figure 2)[21] followed by equilibration. In this respect, it is of particular interest that a free C3 hydroxyl is reported to be essential for formation of the key intermediate **B**,[22] which suggests that transient protection of the C3 hydroxyl group with an adequate promoiety would stabilize quercetin against the devastating oxidative degradation.

On the other hand, a promoiety attached at 3′-OH or 7-OH is anticipated to work as a removable metabolic blocker. After in-depth investigation of the overall percentage of phase II conjugation[23−27] at the various hydroxyl groups in the different model systems, van der Woude et al. designated the 3′- and 7-hydroxyl groups as the preferential sites of conjugation.[28−30] Among the two hydroxyl groups, 3′-OH has been actively transformed into the corresponding esters[31−39] or carbamates[40−42] by conjugation with various promoieties, which resulted in clinical investigation of quercetin-glycine carbamate, 3′-(*N*-carboxymethyl)carbomyl-3,4′,5,7-tetrahydroxyflavone (QC12) (**1**, Figure 1) as a potential anticancer agent.[40] However, the quercetin conjugates with promoieties at the catecholic hydroxyl groups (3′-OH or 4′-OH) suffer from accelerated hydrolysis due to the "ortho effect" exerted by the neighboring free −OH group[31] to result in low stability in aqueous media, and the half-life of **1** (QC12) in whole blood was reported to be only 0.39 h.[40] In contrast, only a limited number of studies have been reported to introduce promoieties at 7-OH with a cleavable linkage.[32]

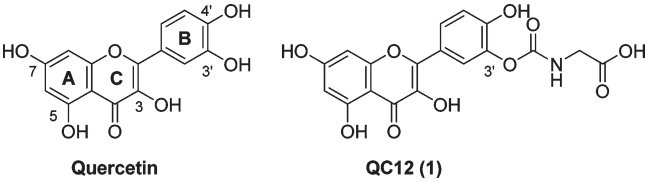

Figure 1. Structures of quercetin and quercetin prodrug, 3′-(*N*-carboxymethyl)carbomyl-3,4′,5,7-tetrahydroxyflavone (QC12) (**1**), with numbering system.

Taken together, as hydroxyl groups of quercetin such as 3-OH and 7-OH are involved in oxidative degradation and metabolism, respectively, transient protection of those hydroxyl groups with a metabolically susceptible promoiety would provide safe cell delivery followed by slow release of quercetin in intracellular compartment to result in a sustained therapeutic effect.

In this study, pivaloxymethyl (POM), which has been successfully exploited in antiviral drug of adefovir dipivoxyl,[43,44]

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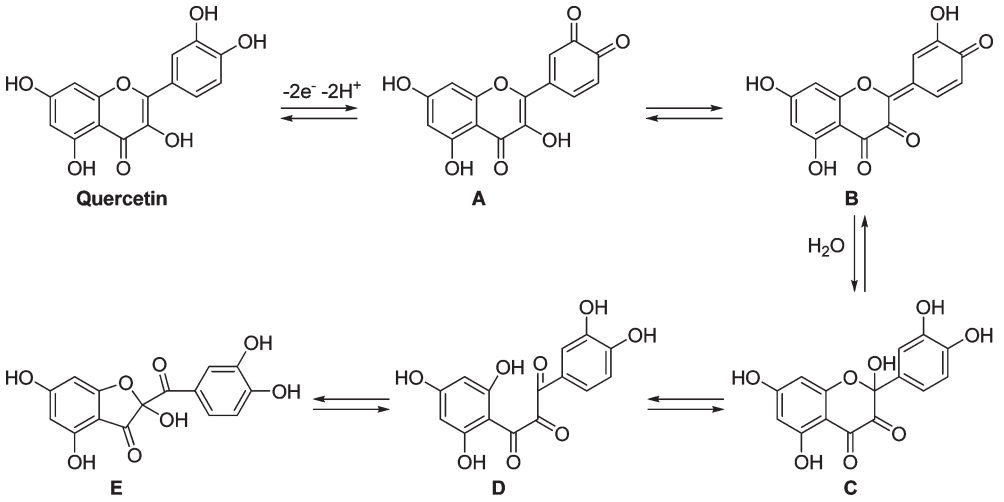


Figure 2. Proposed mechanism for oxidative degradation of quercetin.[21,22]

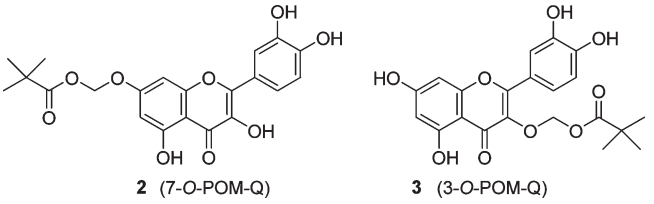


Figure 3. Quercetin-POM conjugates (**2** and **3**) investigated in this study.

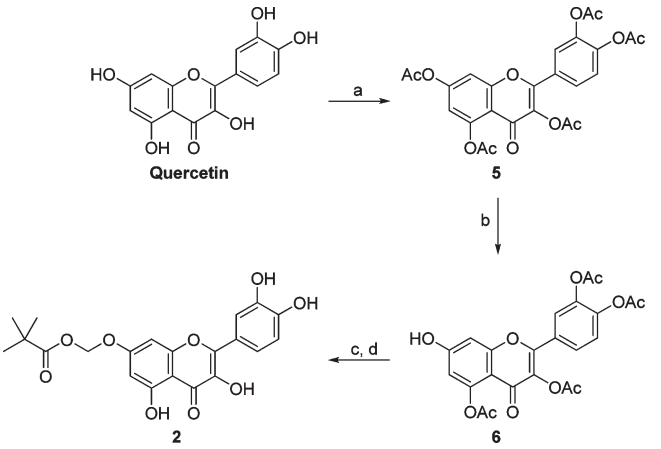
was used as our promoiety of choice. With the proven resistance to chemical hydrolysis, the POM group was expected to serve as a good protecting group of the chemically or metabolically susceptible hydroxyl groups of quercetin during its intracellular transit. Once localized inside the cell, the POM ester is known to undergo esterase-catalyzed acyl cleavage to release the parent drug molecule.[43−46] Along with the role as a transient protecting group, the neutral lipophilic character of the POM group is also expected to facilitate passive diffusion of quercetin across the cell membrane.

Herein, we report regioselective synthesis of two novel quercetin conjugates with POM promoieties at 7-OH [7-*O*-POM-Q (**2**), Figure 3] and 3-OH [3-*O*-POM-Q (**3**), Figure 3] and the investigation of their physicochemical as well as biological properties in comparison with quercetin.

Results

Synthesis. The quercetin prodrug **1** (Figure 1) was prepared by the method previously reported.[47] Synthesis of quercetin-POM conjugates were accomplished by nucleophilic substitution of appropriately protected quercetin derivatives (**6** and **11**; Schemes 1 and 2) with pivaloxymethyl iodide (POM-I[a]). Thus, regioselective monodeacetylation of the peracetylated quercetin[48] (Scheme 1) or regioselective protection of quercetin catechol moiety[49] (Scheme 2) was the key to the syntheses of novel quercetin conjugates, which was attempted by slight modification of the literature conditions. Treatment of quercetin with excess amount of Ac_2O in pyridine provided quercetin peracetate **5**, of which the 7-OAc group was selectively removed upon treatment with a mixture of PhSH, NMP, and imidazole[49] to give **6** in 73% yield (Scheme 1). Alkylation of **6** with POM-I followed by global deprotection with methanolic ammonia provided the desired 7-*O*-POM-Q (**2**) in 84% yield.

Scheme 1. Synthesis of 7-*O*-POM-Q (**2**)[a]



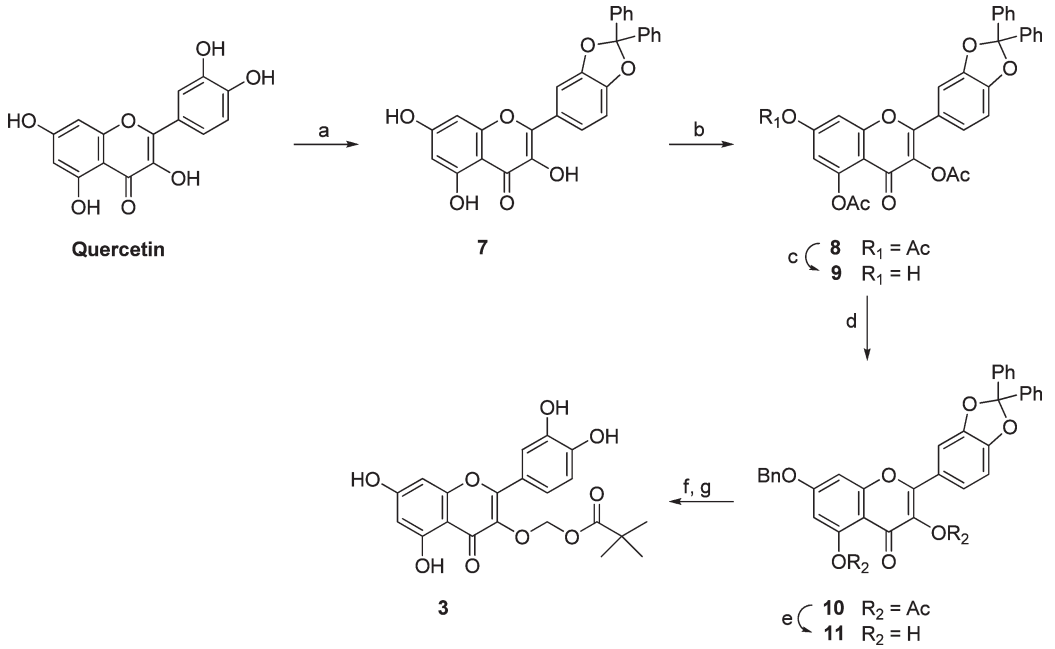
[a] Reagents and conditions: (a) Ac_2O, Pyr, 70 °C; (b) PhSH, NMP, imidazole, 0 °C; (c) POM-I, K_2CO_3, acetone, room temp; (d) NH_3, MeOH, 0 °C.

Regioselective introduction of the POM promoiety at the 3-*O* position required more tedious protection−deprotection steps to provide a quercetin derivative with orthogonal protecting groups at the catechol functionality, 7-*O* position, and 3,5-*O*-position (**10**, Scheme 2).

Thus, quercetin diphenylmethylketal (**7**, Scheme 2), obtained by reaction of quercetin with 1,1-dichlorodiphenylmethane, was peracetylated with an excess amount of Ac_2O in pyridine to give **8** in 93% yield. The 7-OAc group of **8** was selectively removed by treatment with a mixture of PhSH, NMP, and imidazole to give **9** (90% yield), which was reprotected with a benzyl group to give the key intermediate **10** in 69% yield. For selective introduction of the POM promoiety at the 3-*O* position, the acetate functionality was removed by treatment with methanolic ammonia to give **11** with free phenolic hydroxyl groups at 3 and 5 positions of quercetin (90% yield). Intramolecular hydrogen bond between the carbonyl oxygen

[a] Abbreviations: POM-I, pivaloxymethyl iodide; Ac_2O, acetic anhydride; PhSH, benzenethiol; NMP, *N*-methylpyrrolidone; PBS, phosphate buffered saline; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; PAMPA, parallel artificial membrane permeability assay; Pgp, P-glycoprotein; MRP, multidrug resistance protein.

Scheme 2. Synthesis of 3-*O*-POM-Q (**3**)[a]



[a] Reagents and conditions: (a) Ph_2CCl_2, 180 °C; (b) Ac_2O, Pyr, 70 °C; (c) PhSH, NMP, imidazole, 0 °C; (d) BnBr, K_2CO_3, acetone, room temp; (e) NH_3/MeOH, 0 °C; (f) POM-I, K_2CO_3, acetone/DMF, room temp; (g) H_2, Pd/C, THF/MeOH, room temp.

atom and 5-OH prohibits chemical reactions at the 5-OH, which resulted in regioselective alkylation of **11** at 3-*O* position upon treatment with POM-I. Simultaneous deprotection of the diphenylmethylketal and benzyl groups under hydrogenolysis conditions afforded the desired 3-*O*-POM-Q (**3**) in 67% yield.

Stability. Quercetin is reported to have low stability in PBS (pH 7.4) with a half-life of less than 10 h,[50] which can be ascribed to its fast auto-oxidation.[51] The oxidation of quercetin in PBS can be prevented by addition of antioxidants such as ascorbic acid (1 mM),[50,52,53] but because of the reinforced oxidative stress in cell culture media such as McCoy's 5A, degradation of quercetin in cell system is known to become 5 times faster even in the presence of antioxidants.[10−15]

In this study, no stabilizer was added to the incubation media for the purpose of estimation of stabilities of the quercetin conjugates in comparison with quercetin. Stabilities of quercetin and the quercetin-POM conjugates were assessed in various buffer solutions [pH 2.0 and pH 7.4 (PBS)] and DMEM culture media supplemented with FBS under cell-free conditions (cell-free cDMEM), and the result of HPLC analysis is summarized in Table 1.

In line with literature,[10−15,50] without added stabilizer, quercetin showed a moderate to low stability profile with half-lives ($t_{1/2}$) of 7 h, 10 h, and less than 30 min in acidic buffer solution (pH 2.0), PBS (pH 7.4), and cell-free cDMEM, respectively (Table 1). Previously, the water-soluble quercetin prodrug **1** was reported to be stable against in vitro hydrolysis in water ($t_{1/2}$ = 16.9 h) but unstable in blood with a half-life of only 0.39 h.[40] In our study, a similar trend was observed in the hydrolysis of **1** to quercetin (Table 1). In contrast, the quercetin conjugates **2** and **3** did not provide the hydrolysis product, quercetin, in any conditions tested. As anticipated by introduction of the carbamate linkage to the noncatecholic hydroxyl groups of quercetin (7-OH and 3-OH), the resulting quercetin conjugates **2** and **3** must have acquired resistance against hydrolytic cleavage. In PBS, HPLC analysis showed

Table 1. Stability of Quercetin and Quercetin-POM Conjugates in PBS and Complete DMEM

		half-life $t_{1/2}$ (h)			
		quercetin	**1**[a]	7-*O*-POM-Q (**2**)	3-*O*-POM-Q (**3**)
buffer	pH 2.0	7	3	7	20
	pH 7.4 (PBS)	10	10	>72	>72
cDMEM		< 0.5	0.8	4	52

[a] Half-life for hydrolysis of **1** to quercetin.

that the levels of **2** and **3** remained constant up to 72 h. In acidic buffer solution (pH 2.0) and cell-free cDMEM, the amount of **2** decreased to half at 7 and 4 h after incubation, respectively, but the lack of quercetin in the incubation media attributes this to direct oxidative decomposition of the quercetin conjugate itself. In this context, it is noteworthy that introduction of the POM carbamate at the noncatecholic and chemically labile 3-OH group of quercetin provided the resulting conjugate **3** with near-optimal in vitro stability profile. Neither chemical hydrolysis nor oxidative decomposition of **3** seems feasible (Table 1).

Solubility. Solubilities of quercetin, quercetin prodrug (**1**), and quercetin-POM conjugates (**2** and **3**) in PBS, DMEM, and cDMEM were estimated by measuring forward scattered light when a laser beam is directed through the solution,[54] and the results are summarized in Table 2 and Figure 4. In order to prevent decomposition of quercetin, analysis was performed immediately after dissolving the solutes in PBS, but solubility of quercetin in cell culture media was not amenable to estimation because of its instability.

In PBS, quercetin was soluble up to 100 μM, after which the solubility decreased abruptly (Table 2, Figure 4a, ◆). As reported previously,[40] the quercetin prodrug **1** was completely soluble in PBS as well as cell culture media, and no light scattering caused by insoluble solute molecules was observed up to 400 μM (Table 2, Figure 4, ●). Because of the presence of the lipophilic POM promoiety, limited solubility of the quercetin conjugate **2** (Figure 4a, ▲) in PBS was observed

(5 μM, Table 2). On the contrary, the conjugate **3** showed almost the same solubility profile as quercetin in PBS (Figure 4a, ■). The high solubility of 3-*O*-POM-Q (**3**) compared with 7-*O*-POM-Q (**2**) might be attributed to the bulky POM group at 3-*O* position which shifts the planarity of the molecule to an out-of-plane conformation to disrupt the crystal packing and increase solubility. In DMEM without added FBS (Figure 4b), no significant change in solubility was observed in comparison with PBS (Figure 4a, Table 2). However, upon addition of FBS to DMEM (Figure 4c), forward light scattering intensity from the samples of both **2** and **3** showed significant decrease, which may be attributed to interactions of the lipophilic POM promoiety of the conjugates with the serum proteins in FBS.

Intracellular Localization. With proven solubility as well as stability in cell-free culture media, intracellular localization remains as the next biological barrier for the quercetin conjugates **2** and **3**, which was visualized by fluorescence microscopy. It is known that quercetin exhibits specific fluorescence (488 nm_{ex}/500−540 nm_{ex}) upon internalization in cells due to binding to intracellular target proteins.[55−58] Therefore, different levels of fluorescence were anticipated depending on efficiencies of cellular uptake of quercetin and its conjugates.

Table 2. Solubilities of Quercetin, Quercetin Prodrug (**1**), and Quercetin-POM Conjugates (**2** and **3**) in PBS and Cell Culture Media

	solubility (μM)			
media	quercetin	**1**	7-*O*-POM-Q (**2**)	3-*O*-POM-Q (**3**)
PBS	100	>400	5	100
DMEM	ND[a]	>400	50	100
cDMEM	ND[a]	>400	200	>400

[a] ND: not determined because of instability of quercetin in cell culture media.

Following incubation of human colon cancer cells (HCT116) with quercetin and the conjugate **2**, fluorescent staining was observed in a confocal microscope at two different time points (1 and 12 h after incubation), and the results are visualized in Figure 5.

After short incubation (1 h), quercetin (Figure 5a, left panel) showed strong fluorescence which, however, did not persist until 12 h (Figure 5a, right panel). Taking short half-life of quercetin (< 30 min, Table 1) in the extracellular media into consideration, efflux of the internalized quercetin followed by extracellular decomposition might provide a reasonable explanation for this observation. 7-*O*-POM-Q (**2**) also showed bright fluorescence signal upon incubation with the cells for 1 h (Figure 5b, left panel) and the fluorescence was maintained up to 12 h (Figure 5b, right panel). However, unlike quercetin and **2**, no significant fluorescence was observed from the cells treated with the conjugate **3** regardless of the incubation time (data not shown). The lack of intracellular fluorescence was in good agreement with the low PAMPA[59] permeability of **3** (Table 3). Whereas quercetin (5.6−15.9%) and **1** (40.3−55.1%) were observed in moderate to high amounts in the acceptor plates after penetration of the artificial membrane, the quercetin conjugate **3** could be found only in the donor plate.

Membrane permeability is much higher for more lipophilic molecules than for polar molecules because molecules must pass through a highly nonpolar lipid bilayer membrane. In this context, it is worth remembering the high water-solubility of **3** in spite of the presence of the lipophilic POM group (Table 2), and the polar nature of **3** might be related to its low membrane permeability and thereby low cellular uptake.

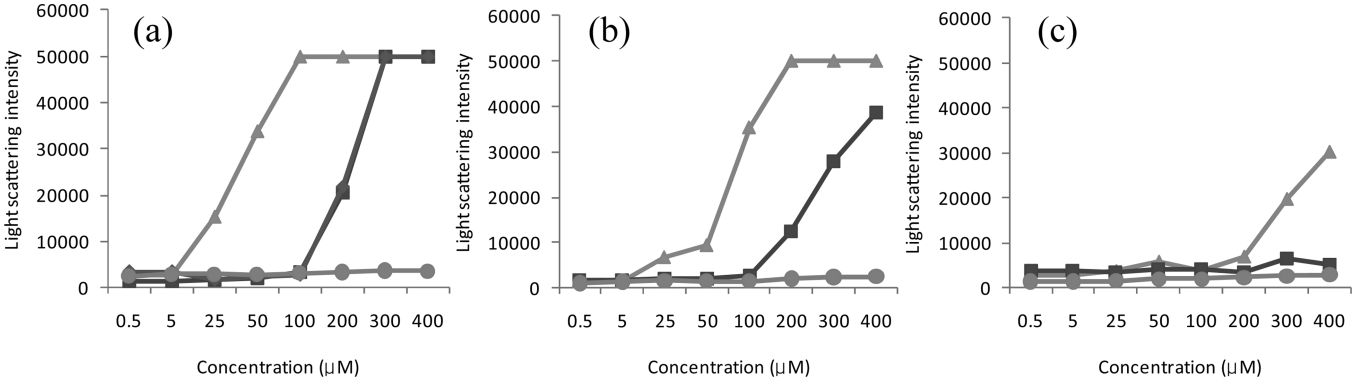


Figure 4. Forward light scattering intensity of solutions of quercetin (◆), **1** (●), 7-*O*-POM-Q (**2**, ▲), and 3-*O*-POM-Q (**3**, ■) dissolved in (a) PBS, (b) DMEM, and (c) cDMEM. In PBS (a), light scattering intensities from the solutions of quercetin (◆) and 3-*O*-POM-Q (**3**, ■) are almost the same and the data labels for the two compounds are overlapped.

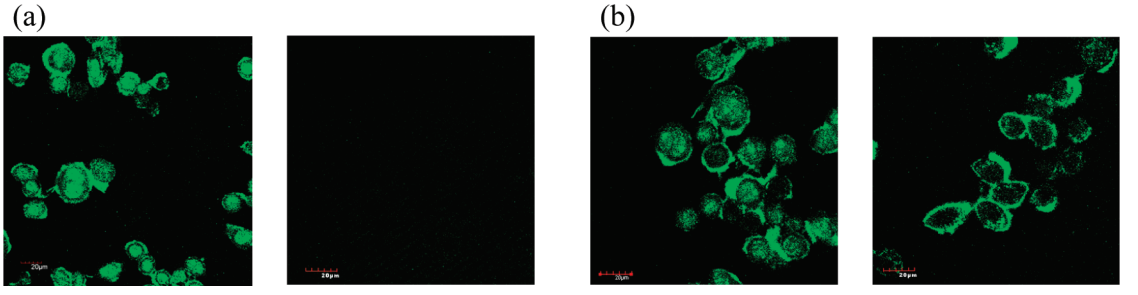


Figure 5. Cellular uptake of quercetin and its conjugates visualized by fluorescence microscopy. HCT116 cells were treated with 5 μM (a) quercetin and (b) 7-*O*-POM-Q (**2**) for 1 h (left panel) and 12 h (right panel). After a wash with PBS, fluorescent staining was observed in a confocal microscope at 488 nm_{ex}/500−540 nm_{em}.

Table 3. PAMPA Permeability: Percentage of Quercetin or Quercetin Conjugates Contained in the Donor and Acceptor Plate after Incubation for 1 and 5 h

	percentage (%)[a]							
	quercetin		**1**		s7-*O*-POM-Q (**2**)		3-*O*-POM-Q (**3**)	
incubation time (h)	D[b]	A[c]	D[b]	A[c]	D[b]	A[c]	D[b]	A[c]
1	45	16	6	55	ND[d]	ND[d]	87	0
5	8	6	18	40	ND[d]	ND[d]	74	0

[a] Distribution of contents in each plate was calculated from the integrated HPLC peak area. [b] D: donor plate. [c] A: acceptor plate. [d] ND: not determined because of low solubility of 7-*O*-POM-Q (**2**) in PBS at the concentration for PAMPA assay (50 μM).

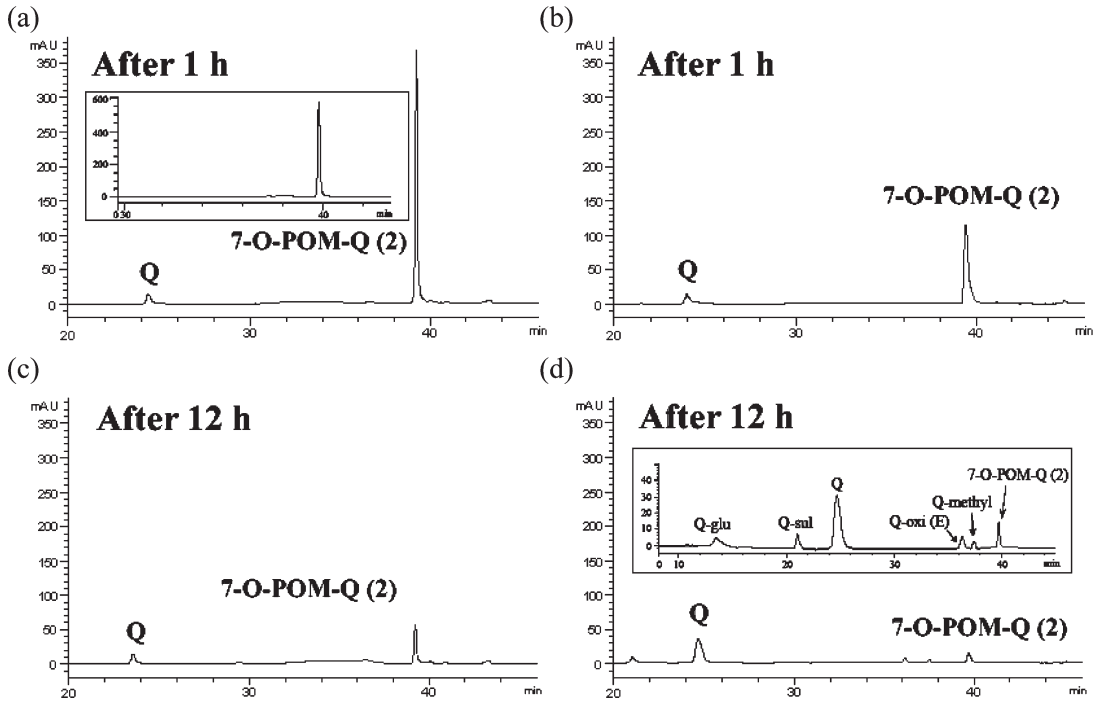


Figure 6. HPLC chromatograms of (a) cell culture media after 1 h, (b) cell lysate after 1 h, (c) cell culture media after 12 h, and (d) cell lysate after 12 h of incubation of MCF-7 cells with the quercetin conjugate **2**. Chromatogram of **2** before incubation is presented as an inset in (a). Expanded chromatogram of the cell lysate sampled 12 h after incubation was inserted in (d). Q: quercetin. Q-glu: quercetin glucuronide. Q-sul: quercetin sulfate. Q-oxi (E): oxidized product of quercetin (compound **E**, Figure 2). Q-methyl: methyl quercetin.

Addition of Pgp inhibitor (verapamil) or MRP inhibitor (gemfibrozil) to the cell culture media did not have any effect on accumulation of **3**, and no intracellular fluorescence was observed by confocal live-cell imaging. This result suggests that, along with the lack of passive permeation, active transport followed by efflux mechanism of **3** is not in action, either.

Next, as another measure of the cellular uptake and efflux, chemical species remaining in extracellular as well as intracellular compartment were analyzed by HPLC after incubation of human breast cancer cells (MCF-7) with the conjugate **2**, and the result is summarized in Figure 6 and Table 4.

After short incubation (1 h) with 7-*O*-POM-Q (**2**), HPLC analysis of the cell culture media (Figure 6a) showed the native conjugate **2** with only 62% (Table 4) of its initial amount (Figure 6a, insert) along with a small amount of quercetin (5%, Table 4). On the basis of the lack of nonenzymatic hydrolysis of **2** described above, identification of quercetin in the cell culture media suggests a series of events that includes cellular uptake of **2**, intracellular hydrolysis of **2** to quercetin, and efflux of quercetin out of the cell. The HPLC chromatogram of the cell lysate at the same detection point (1 h after incubation, Figure 6b) supports this hypothesis with significant intracellular accumulation of the conjugate **2** (21%, Table 4) along with quercetin (7%, Table 4). Significant reduction of **2** in the extracellular fluid after 12 h (Figure 6c, Table 4) was well correlated with its stability as well as cellular uptake profile. Once again, extracellular quercetin level (5%, Table 4), kept steady up to 12 h, (Figure 6c), provides support for the aforementioned intercellular trafficking and intracellular hydrolysis mechanism of **2** and quercetin. Intracellularly, along with quercetin (17%, Table 4), several minor peaks were observed in the HPLC chromatogram (Figure 6d, insert), and they were

Table 4. Quantitative Analysis of 7-*O*-POM-Q (**2**), Quercetin, and Quercetin Metabolites Remaining in the Cell Culture Media and Cell Lysate after Incubation of **2** in MCF-7 Cell Line[a]

	percentage (%)[b]			
	after 1 h		after 12 h	
compd	culture media	cell lysate	culture media	cell lysate
7-O-POM-Q (**2**)	62	21	12	4
Q	5	7	5	17
Q metabolites				Q-Glu (6), Q-Sul (3), Q-Oxi (2), Q-Me (1)

[a] Q: quercetin. Q-glu: quercetin glucuronide. Q-sul: quercetin sulfate. Q-oxi (E): oxidized product of quercetin (compound **E**, Figure 2). Q-methyl: methyl quercetin. [b] Percentage (%) of each content in culture media and cell lysate was calculated from the integrated HPLC peak area.

identified as either oxidized quercetin (Q-oxi, compound **E** in Figure 2) or quercetin metabolites [quercetin-glucuronide (Q-glu), quercetin-sulfate (Q-sul), and methylated quercetin (Q-methyl)] by mass analysis (Figure 6d, insert). High intracellular level (17%, Table 4) and low metabolism (1−4%, Table 4) of quercetin, however, might be attributed to low oxidative and metabolic capacity of MCF-7 cells, and the cell type-specific intracellular accumulation of quercetin was proven by repeating the same experiment in metabolically competent hepatocytes (Huh-7 cell line). Incubation of the conjugate **2** in the Huh-7 cell line for 1 h and HPLC analysis of the extracellular and intracellular contents provided almost the same chromatograms as in Figure 6a and Figure 6b, respectively (data not shown). However, presumably because of the increased oxidative stress in the Huh-7 hepatoma cell line,[60] the quercetin conjugate **2** was not stable enough in this cell culture system and started to decompose after 1 h. Therefore, 12 h after incubation, quercetin was observed only at a trace level and intracellular accumulation of the quercetin metabolites such as Q-sul and Q-methyl was not significant (< 5%).

Unlike **2**, the extracellular amount of **3** remained constant regardless of the cell type and incubation time. Also, HPLC analysis of the cell lysate showed neither **3** nor quercetin (metabolites). This result provides another piece of evidence that, even though introduction of a POM promoiety at the chemically susceptible 3-OH position provided remarkable stability to the resulting quercetin conjugate **3** in cell culture media, the 3-*O*-POM group failed to transport the resulting conjugate **3** across the cell membrane.

By combination of information obtained from confocal microscopy, PAMPA permeability, and HPLC analysis, it can be concluded that 7-*O*-POM-Q (**2**) is efficiently absorbed into the cell and undergoes intracellular conversion to quercetin and its metabolites over a relatively long period. Unlike **2**, 3-*O*-POM-Q (**3**) is neither localized into the cell nor hydrolyzed to quercetin.

Cell Viability. Studies have shown that quercetin has antiproliferative effects[61,62] and can induce apoptotic cell death in several cancer cell lines such as leukemia,[63] breast,[64] lung,[65] hepatoma,[66] oral,[67] and colon[62] cancer but not in normal cells.[68] Viabilities of cancer cell lines such as breast (MCF-7), colon (HCT116), and prostate cancer (DU145) as well as normal human diploid fibroblast cell line (HS27) treated with quercetin and quercetin conjugates (**2** and **3**) in the absence of ascorbic acid were estimated, and the results are summarized in Table 5.

Table 5. Effects of Quercetin and Its Conjugates (**2** and **3**) on Viability (EC_{50}) of Three Different Cancer Cell Lines (MCF-7, HCT116, and DU145) and a Normal Cell Line (HS27)

	EC_{50} (μM)			
compd	MCF-7	HCT116	DU145	HS27
2	20	36	10	>100
3	>100	>100	>100	>100
quercetin	>100	>100	>100	>100

Quercetin, because of the instability in cell culture media, was not cytotoxic at all up to 100 μM (Table 5). In contrast, because of the enhanced stability as well as efficient intracellular transport, the conjugate **2** showed significant cytostatic effects in three cancer cell lines tested (Table 5). On the other hand, as expected by lack of intracellular accumulation, 3-*O*-POM-Q (**3**) did not affect the cell viability at all even at concentration of 100 μM (Table 2). Neither quercetin nor its conjugates had effects on viability of normal diploid cell line HS-27 (Table 5). Interestingly, 7-*O*-POM-Q (**2**) showed preferential effect on DU145 with EC_{50} value of 10 μM (Table 5).

The stability-limited cytotoxicity of quercetin was confirmed by addition of an antioxidative stabilizer, ascorbic acid, to the cell culture media. As ascorbic acid shows cell type-specific cytotoxicity in MCF-7 cell line,[10] HCT116 was used as a cell line of choice in this study (Figure 7). Whereas ascorbic acid rescued the cytotoxic effect of quercetin (Figure 7a), it had no effect on the viability of the cancer cells treated with 7-*O*-POM-Q (**2**, Figure 7b). In combination with the stability profiles observed for quercetin and its conjugate **2**, this result clearly shows that resistance to oxidative decomposition is one of the key issues in determining the biological effect of quercetin.

Cell Cycle Analysis. Antiproliferative effect of the conjugate **2** promoted our interest in its effect on cell cycle distribution. Quercetin is known to arrest the cell cycle in S/early G2 phase,[40,62] and the same effect was anticipated by **2** which gives quercetin in the intracellular compartment. Considering poor stability and thereby low antiproliferative effect of quercetin, cells were treated with quercetin in higher concentration (50 μM) compared with **2** (30 μM). Untreated cells or cells treated with quercetin or **2** were submitted to cell cycle analysis after 12 h of treatment, and the results are shown in Figure 8.

In line with literature,[62] cell cycle analysis showed a decrease in the percentage of cells in the G0/G1 phase (21.0%, Figure 8d) with a concurrent increase in the S-phase (78.0%, Figure 8d) when cells were exposed to 50 μM quercetin (Figure 8b) compared to control (Figure 8a). Interestingly, after exposure to the conjugate **2** (Figure 8c), the percentage of cells in the G0/G1-phase decreased further (an approximately

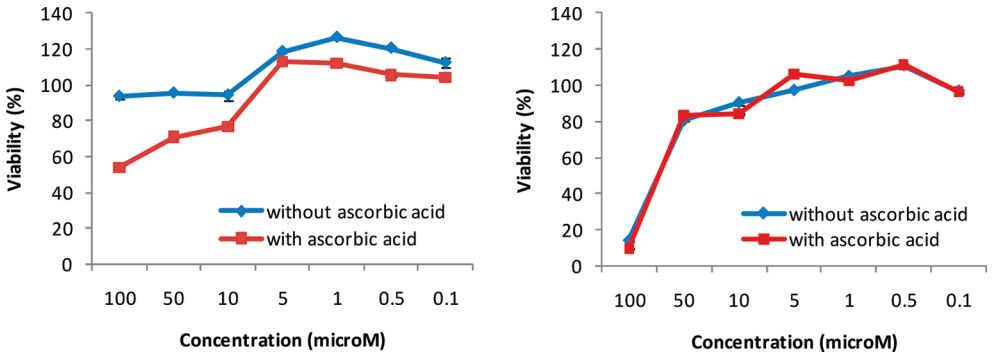


Figure 7. Effects of ascorbic acid on the viability of HCT116 cells treated with (a) quercetin and (b) 7-*O*-POM-Q (**2**).

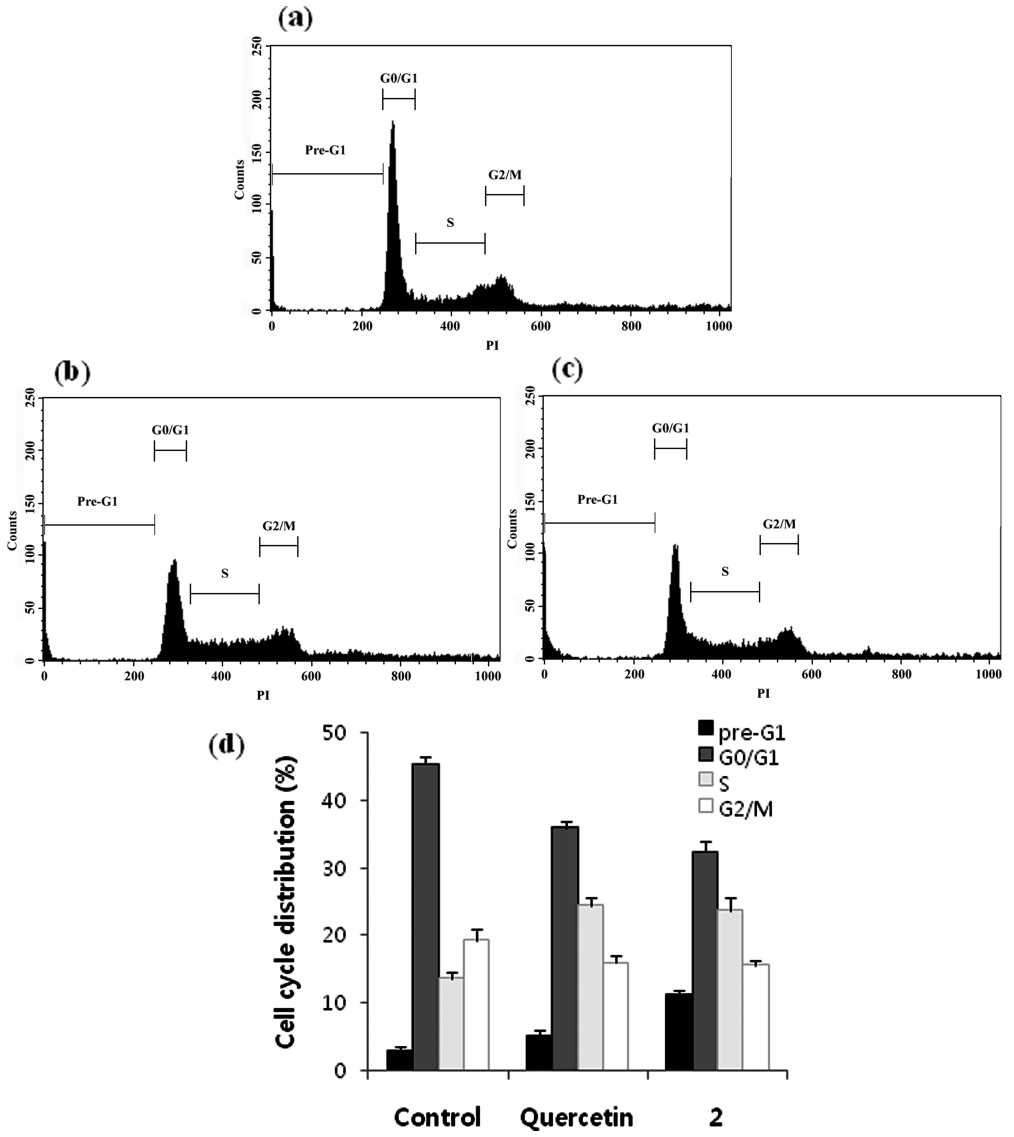


Figure 8. Cell cycle distribution of MCF-7 cells by propidium iodide staining and flow cytometry after treatment with (a) 1% DMSO, (b) 50 μM of quercetin, and (c) 30 μM of **2** for 12 h. The percentage of cells in each phase is summarized in (d).

10% decrease in comparison with quercetin, Figure 8d), while the number of cells in the sub-G1 phase increased (an approximately 120% increase when compared to quercetin, Figure 8d). This result indicates that inhibition of cell proliferation by the conjugate **2** could partly be attributed to an increase in the number of apoptotic cells in the sub-G1 phase.

Discussion

Because of the chemical instability against oxidative stress, quercetin decomposes in the cell culture media and cells experience lower concentrations of quercetin than originally added. As a result, simultaneous addition of ascorbic acid as an antioxidant[50,52,53] or refreshment of the media during incubation is in general practice to suppress the devastating oxidative degradation of quercetin. However, addition of stabilizers such as ascorbic acid may lead to overestimation of the in vitro biological activity of quercetin. In particular, the cell-type-specific antiproliferative effect of ascorbic acid[10,69,70] should be carefully taken into consideration before adding it into the cell culture. Alteration of the quercetin structure through metabolism also results in complication of its biological activity as well as reduction in bioavailability.

In this study, in order to increase stability of quercetin, the metabolically (7-OH) and chemically (3-OH) susceptible hydroxyl groups of quercetin were transiently blocked with a POM promoiety to provide the corresponding conjugates 7-*O*-POM-Q (**2**) and 3-*O*-POM-Q (**3**).

In extracellular fluid without added stabilizing factor, quercetin was highly unstable to decompose rapidly ($t_{1/2}$ < 30 min). The water-soluble quercetin prodrug **1** also underwent fast hydrolysis to quercetin ($t_{1/2}$ = 0.8 h). In contrast, under the same conditions, the half-life of 7-*O*-POM-Q (**2**) was increased to 4 h. In addition, both confocal live-cell imaging and HPLC analysis of the extracellular and intracellular contents after incubation of cells with 7-*O*-POM-Q (**2**) revealed that the quercetin conjugate **2** undergoes efficient cellular uptake, and the intracellular levels of **2** and its hydrolysis product, quercetin, are maintained up to 12 h. Extracellular stability and facile intracellular accumulation of the quercetin conjugate **2** were demonstrated by its stabilizer-independent cytostatic effect and induction of apoptotic cell death.

On the other hand, as anticipated by protection of the chemically susceptible 3-OH with a POM group, 3-*O*-POM-Q (**3**) was stable in every condition tested up to 24 h. Unfortunately,

however, the remarkably stable conjugate **3** failed to accumulate in the cell.

In conclusion, our results demonstrate that conjugation of quercetin with a POM promoiety at C7 hydroxyl group (**2**) can enhance stability and intracellular accumulation of the corresponding quercetin conjugate as well as quercetin. Even though 3-*O*-POM-Q failed to localize inside the cell, its high solubility as well as remarkable stability in extracellular fluid warrants further investigation of quercetin conjugates with various promoieties at the 3-OH position.

Experimental Section

Materials and General Method. All chemicals including MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)], verapamil, and gemfibrozil were purchased from Sigma-Aldrich. Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin, and fetal bovine serum (FBS) were purchased from Invitrogen. The precoated PAMPA plates were manufactured by BD Biosciences Discovery Labware (Bedford, MA) using a polyvinylidene fluoride (PVDF) 96-well filter plate with 0.4 μm pore size. Nuclear magnetic resonance spectra were recorded on a Bruker 400 AMX spectrometer (Karlsruhe, Germany) at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR with tetramethylsilane as the internal standard. Chemical shifts are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br s (broad singlet). Coupling constants are reported in hertz (Hz). The chemical shifts are reported as parts per million (δ) relative to the solvent peak. All tested compounds were ≥95% purity, as determined by reverse phase HPLC. HPLC was performed on Agilent 1050 (Hewlett-Packard) equipment with variable wavelength (VW) UV dectctor. The column was Polaris 5, C18-A 250 mm × 4.6 mm (Varian). Analytical conditions were as follows: Gradient used was 25% acetonitrile in water containing 0.1% formic acid (0−8 min), 25−35% acetonitrile in water containing 0.1% formic acid (8−18 min), 35% acetonitrile in water containing 0.1% formic acid (18−25 min), 35−80% acetonitrile in water containing 0.1% formic acid (25−40 min), 80−100% acetonitrile in water containing 0.1% formic acid (40−45 min), and 100% acetonitrile in water containing 0.1% formic acid (45−50 min). Flow was 1 mL/min. UV was detected at two different wavelengths (340 and 254 nm). TLC was performed on silica gel-60 F254 purchased from Merck. Column chromatography was performed using silica gel 60 (220−440 mesh) for flash chromatography. Mass spectrometric data (MS) were obtained using a MALDI-TOF-TOF mass spectrometer (Ultraflex III, Bruker Daltonik).

Chemistry. Synthesis of 2-(3,4-Diacetoxyphenyl)-4-oxo-4*H*-chromene-3,5,7-triyl Triacetate (5). Acetic anhydride (12.5 mL, 132 mmol) was added to a solution of quercetin (**1**) (5 g, 16.5 mmol) in pyridine (35 mL). The reaction was monitored by TLC. After starting material was consumed, the organic layer was concentrated under reduced pressure. The crude compound was purified by column chromatography on silica gel (4:1:1 = hexanes/acetone/CH_2Cl_2) to afford **5** (4.5 g, 8.8 mmol, 54% yield) as a white-yellow powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.72 (dd, J = 8.6, 2.0 Hz, 1H), 7.70 (d, J = 1.9 Hz, 1H), 7.35 (d, J = 8.6 Hz, 1H), 7.33 (d, J = 2.2 Hz, 1H), 6.88 (d, J = 2.2 Hz, 1H), 2.43 (s, 3H), 2.34 (s, 12H).

Synthesis of 4-(3,5-Diacetoxy-7-hydroxy-4-oxo-4*H*-chromen-2-yl)-1,2-phenylene Diacetate (6). Thiophenol (0.48 mL, 4.69 mmol) was slowly added to a stirred mixture of **5** (3 g, 5.85 mmol) and imidazole (80 mg, 1.17 mmol) in NMP (30 mL) at 0 °C. The reaction mixture was stirred for 2 h at room temperature. The mixture was diluted with EtOAc and washed with 2 N HCl. The organic layer was concentrated under reduced pressure and dried over $MgSO_4$. The crude compound was purified by column chromatography on silica gel (2:1:1 = hexanes/acetone/EtOAc) to give **6** (2 g, 4.25 mmol, 73% yield) as a white powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.33 (s, 1H), 7.83−7.80 (m, 2H), 7.51 (d, J = 8.5 Hz, 1H), 6.94 (s, 1H), 6.65 (s, 1H), 2.33 (s, 6H), 2.30 (s, 6H).

Synthesis of [2-(3,4-Dihydroxyphenyl)-3,5-dihydroxy-4-oxo-4*H*-chromen-7-yloxy]methyl Pivalate (2). Potassium carbonate (88 mg, 0.64 mmol) and POM iodide (232 mg, 0.96 mmol) were added to a solution of **6** (150 mg, 0.32 mmol) in acetone (8 mL). The reaction mixture was stirred for 4 h at room temperature, filtered, and concentrated under reduced pressure to give a pale yellow syrup, which was used for the next step without further purification. A mixture of the quercetin conjugate obtained above was dissolved in NH_3/MeOH (12 mL) and stirred for 2 h at 0 °C. After concentration under reduced pressure, the residue was purified by column chromatography on silica gel (1:1 = hexanes/EtOAc) to afford **2** (112 mg, 0.27 mmol, 84% yield) as a yellow powder: ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.64 (s, 1H), 7.73 (d, J = 2.0 Hz, 1H), 7.58 (dd, J = 8.4, 2.0 Hz, 1H), 6.91 (d, J = 8.5 Hz, 1H), 6.82 (d, J = 2.0 Hz, 1H), 6.47 (d, J = 2.0 Hz, 1H), 5.90 (s, 2H), 1.15 (s, 9H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.7, 176.4, 161.8, 160.9, 156.0, 148.4, 148.1, 145.5, 136.6, 122.1, 120.5, 116.0, 115.6, 105.4, 98.6, 94.2, 84.8, 38.8, 26.9. LC/MS (ESI) m/z found: 415.2 $[M - H]^-$ (loss of phenolic proton). Calcd for $C_{21}H_{20}O_9$: 416.11. HPLC: retention time of 12.28 min, >95% pure at 340 and 254 nm.

Synthesis of 2-(2,2-Diphenylbenzo[1,3]dioxol-5-yl)-3,5,7-trihydroxychromen-4-one (7). A mixture of quercetin (**1**) (5 g, 14.8 mmol) and dichlorodiphenylmethane (8.5 mL, 44.3 mmol) was stirred for 30 min at 180 °C. The reaction mixture was taken with $CHCl_3$ and concentrated under reduced pressure. The residue was purified by column chromatography (4:1 = hexanes/EtOAc) on silica gel to afford **7** (2.4 g, 5.2 mmol, 35% yield) as a yellow powder. ^{1}H NMR (400 MHz, acetone-d_6) δ 12.18 (s, 1H), 7.89−7.92 (m, 2H), 7.63−7.69 (m, 5H), 7.45−7.49 (m, 5H), 7.19 (d, J = 10.5 Hz, 1H), 6.58 (s, 1H), 6.28 (s,1H).

Synthesis of Acetic Acid 3,5-Diacetoxy-2-(2,2-diphenylbenzo-[1,3]dioxol-5-yl)-4-oxo-4*H*-chromen-7-yl Ester (8). Acetic anhydride (1 mL, 10.7 mmol) was added to a solution of **7** (1 g, 2.14 mmol) in anhydrous pyridine (10 mL) at room temperature. The reaction mixture was stirred for 6 h at 70 °C. After concentration, the crude compound was purified by column chromatography (2:1 = hexanes/EtOAc) on silica gel to give **8** (1.2 g, 2.0 mmol, 93% yield) as a white powder. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.57−7.59 (m, 4H), 7.38−7.44 (m, 7H), 7.36 (s, 1H), 7.30 (d, J = 1.6 Hz, 1H), 6.98 (d, J = 6.8 Hz, 1H), 6.85 (d, J = 1.6 Hz, 1H), 2.43 (s, 3H), 2.34 (s, 3H), 2.32 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.5, 169.1, 168.7, 168.1, 156.6, 154.8, 154.6, 149.8, 149.4, 147.4, 139.5, 133.0, 130.0, 129.0, 126.2, 124.3, 123.2, 117.9, 114.9, 114.3, 110.3, 109.7, 108.6, 21.2, 21.1, 20.7. LC/MS (ESI) m/z found: 593.3 $[M + H]^+$. Calcd for $C_{34}H_{24}O_{10}$: 592.14.

Synthesis of 2-(2,2-Diphenylbenzo[*d*][1,3]dioxol-5-yl)-7-hydroxy-4-oxo-4*H*-chromene-3,5-diyl Diacetate (9). Thiophenol (0.16 mL, 1.62 mmol) was slowly added to a stirred mixture of **8** (1.2 g, 2.0 mmol) and imidazole (27 mg, 0.4 mmol) in NMP (24 mL) at 0 °C. The reaction mixture was stirred for 2 h at room temperature. The mixture was diluted with EtOAc and washed with 2 N HCl. The organic layer was concentrated under reduced pressure and dried over $MgSO_4$. The crude compound was purified by column chromatography on silica gel (1:1 = hexanes/EtOAc) to give **9** (1 g, 1.8 mmol, 90% yield) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.55−7.58 (m, 4H), 7.37−7.41 (m, 8H), 6.92 (d, J = 8.1 Hz, 1H), 6.63 (d, J = 2.2 Hz, 1H), 6.50 (d, J = 2.2 Hz, 1H), 2.35 (s, 3H), 2.29 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.4, 169.2, 168.4, 163.1, 158.0, 154.0, 150.6, 149.3, 147.5, 139.6, 132.7, 130.1, 129.1, 126.3, 124.1, 123.7, 118.0, 109.7, 109.6, 109.4, 108.6, 101.5, 21.3, 20.8. LC/MS (ESI) m/z found: 549.3 $[M - H]^-$ (loss of phenolic proton). Calcd for $C_{32}H_{22}O_9$: 550.13.

Synthesis of Acetic Acid 5-Acetoxy-7-benzyloxy-2-(2,2-diphenylbenzo[1,3]dioxol-5-yl)-4-oxo-4*H*-chro-men-3-yl Ester (10). Potassium carbonate (251 mg, 1.8 mmol) and benzyl bromide (0.32 mL, 2.7 mmol) was added to a solution of **9** (1 g, 1.8 mmol)

in acetone (20 mL). The reaction mixture was stirred for 12 h at room temperature. After filtration, the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (2:1 = hexanes/EtOAc) to give **10** (800 mg, 1.25 mmol, 69% yield) as a pale yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57−7.59 (m, 4H), 7.39−7.39 (m, 13H), 6.97 (d, J = 8.3 Hz, 1H), 6.88 (d, J = 2.3 Hz, 1H), 6.70 (d, J = 2.3 Hz, 1H), 5.14 (s, 2H), 2.42 (s, 3H), 2.31 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 169.5, 169.2, 168.3, 163.1, 157.9, 154.2, 150.4, 149.4, 147.5, 139.6, 136.2, 132.9, 130.1, 129.1, 120.0, 128.8, 128.5, 126.3, 124.2, 123.6, 118.0, 110.6, 109.7, 108.6, 100.8, 70.9, 21.3, 20.8. LC/MS (ESI) m/z found: 641.3 $[M + H]^+$. Calcd for $C_{39}H_{28}O_9$: 640.17.

Synthesis of 7-Benzyloxy-2-(2,2-diphenylbenzo[1,3]dioxol-5-yl)-3,5-dihydroxy-chromen-4-one (11). A mixture of **10** (800 mg, 1.25 mmol) in NH_3/MeOH (12 mL) at 0 °C was stirred for 2 h at room temperature. After concentration under reduced pressure, the residue was purified by column chromatography on silica gel (6:2:1 = hexanes/CH_2Cl_2/EtOAc) to give **11** (624 mg, 1.12 mmol, 90% yield) as a yellow powder. ^{1}H NMR (400 MHz, $CDCl_3$) δ 11.69 (s, 1H), 7.77−7.81 (m, 2H), 7.59−7.61 (m, 4H), 7.34−7.44 (m, 10H), 7.09 (d, J = 8.3 Hz, 1H), 6.60 (s, 1H), 6.54 (d, J = 2.1 Hz, 1H), 6.45 (d, J = 2.2 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 176.6, 164.4, 160.8, 156.4, 148.1, 147.1, 146.4, 139.8, 137.2, 136.5, 129.9, 129.0, 128.9, 128.5, 128.2, 126.1, 125.5, 123.5, 117.5, 109.3, 108.2, 104.6, 98.5, 93.4, 70.3; LC/MS (ESI) m/z found: 557.3 $[M + H]^+$. Calcd for $C_{35}H_{24}O_7$: 556.15.

Synthesis of 2,2-Dimethylpropionic Acid 2-(3,4-Dihydroxy-phenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-3-yloxymethyl Ester (3). Potassium carbonate (55 mg, 0.4 mmol) and POM iodide (130 mg, 0.54 mmol) were added to a solution of **11** (200 mg, 0.36 mmol) in a mixture of acetone (7 mL) and DMF (4 mL). The reaction mixture was stirred for 12 h at room temperature, filtered, and concentrated under reduced pressure to give a pale yellow syrup, which was used for the next step without further purification. The degassed suspension of quercetin conjugate obtained above in a mixture of THF (3 mL) and MeOH (3 mL), and Pd/C (10% w/w, 15 mg), under an atmosphere of hydrogen gas (balloon), was vigorously stirred for 12 h at room temperature. The reaction mixture was filtered through a short Celite pad and purified by column chromatography on silica gel (2:1:1 = hexanes/CH_2Cl_2/EtOAc) to afford **3** (100 mg, 0.24 mmol, 67% yield) as a yellow powder: ^{1}H NMR (400 MHz, acetone-d_6) δ 12.63 (s, 1H), 7.68 (d, J = 2.0 Hz, 1H), 7.60 (dd, J = 8.5, 2.1 Hz, 1H), 6.96 (d, J = 8.5 Hz, 1H), 6.52 (s, 1H), 6.28 (s, 1H), 5.80 (s, 2H), 0.88 (s, 9H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 177.2, 177.0, 164.7, 161.6, 156.7, 156.5, 149.3, 145.5, 134.8, 121.7, 120.8, 116.2, 115.8, 104.3, 99.1, 94.1, 87.3, 38.4, 26.6. LC/MS (ESI) m/z found: 415.2 $[M - H]^-$ (loss of phenolic proton). Calcd for $C_{21}H_{20}O_9$: 416.11. HPLC: retention time of 11.94 min, >97% pure at 340 and 254 nm.

Stability in PBS Buffer. The synthesized quercetin conjugates (50 μM) were added to PBS buffer (pH 7.4) and incubated at 37 °C. At different time points (0, 30, 60, 90, 120, 150, 180, 210, 240, 300, and 360 min), an aliquot (300 μL) of the reaction mixture was taken out and injected into the HPLC equipped with a C-18 reverse phase column; flow rate, 1 mL/min; detection, UV 340 nm; mobile phase, 0−8 min (10% aqueous acetonitrile and 0.1% formic acid), 8−12 min (40% aqueous acetonitrile and 0.1% formic acid), 12−15 min (90% aqueous acetonitrile and 0.1% formic acid) and 15−20 min (10% aqueous acetonitrile and 0.1% formic acid).

Stability in DMEM Cell Culture Media. Quercetin conjugates were dissolved in culture media to a final concentration of 50 μM from a 10 mM stock solution in DMSO. The quercetin conjugate solutions were incubated at 37 °C and 5% CO_2 in 24-well plates. The media were harvested at different time points up to 24 h and stored at −80 °C until analysis. After thawing, the samples were vortexed and analyzed with HPLC under the same analysis conditions described above.

Solubility Test. Stock solutions of the quercetin conjugates were prepared at 0.5, 2.5, 5, 10, 20, 30, 40, and 50 mM in 1% DMSO and then serially diluted with either 99% phosphate buffered saline (PBS, pH 7.4) buffer or Dulbecco's modified Eagle medium (DMEM) or DMEM with fetal bovine serum (FBS to final concentrations of 5, 25, 50, 100, 200, 300, 400, and 500 μM. The volume of the test compound in each 96-well plate was set to be 200 μL and the solubility was measured by the NEPHELOstar laser based microplate nephelometer which checks the solubility of compounds by measuring forward light scattering in microplates. All raw data were processed using the BMG LABTECH NEPHELOstar Galaxy evaluation software.

Intracellular Localization Test (Confocal Microscopy). HCT-116 cell was seeded in poly-L-lysine-coated coverslip in 35 mm dishes (10^5 cells/well) and incubated for 1 day (37 °C, 5% CO_2). Quercetin (5 μM) and quercetin conjugates (5 μM) were provided for 1 and 12 h and then quickly washed with PBS and fixed with 4% formaldehyde for 5 min followed by mounting with PBS. Specimens were observed with FV-1000 spectral (Olympus, USA) at 488 nm_{ex}/500−540 nm_{em} using 40× objective lens.

PAMPA (Parallel Artificial Membrane Permeability Assay) Study. The precoated PAMPA plate was warmed to room temperature for 30 min. Quercetin conjugates were dissolved in a solution of 95% PBS buffer solution (5% DMSO) to a final concentration of 50 μM. The initial solutions were added to the wells (300 μL/well) of the donor plate, and PBS buffer was added to the wells (200 μL/well) of the acceptor plate. The acceptor plate was placed on top of the donor plate, and the plate assembly was incubated at room temperature for 1 and 5 h. After incubation, the plates were separated and aliquots (100 μL) were taken from each well plate and injected into the HPLC.

Cellular Uptake and Intracellular Stability. MCF-7 cell was seeded in tissue-cultured COSTAR clear bottom six-well plates (105 cells/well) and incubated for 24 h (37 °C, 5% CO_2). Quercetin conjugates (10 μM) were provided for 12 h. The cells were washed in PBS, trypsinized, and sonicated with Vibra-Cell VCX-130 (SONICS). After filtration, samples were analyzed by HPLC (Agilent). Each peak was manually collected, lyophilized, and analyzed by mass spectrometry (MALDI-TOF, Bruker Daltonics).

Cell Viability Study. Cells (MCF-7, HCT116, DU145, and HS27) were seeded (5×10^3 cells/well) in tissue-cultured COSTAR clear bottom 96-well plate in complete DMEM (Dulbecco's modified Eagle medium) and incubated for 1 day (37 °C, 5% CO_2). Prepared quercetin conjugates which dissolved in DMSO were diluted into seven different concentrations (0.1, 0.5, 1, 5, 10, 50 μM) and added to the medium. After 24 h, cell viability was estimated by MTT assay. Each experiment was performed in triplicate and repeated three times.

Cell Cycle Distribution Analysis by FACS. MCF-7 cell was seeded in 35 mm dishes (10^5 cells/well) and incubated for 12 h (37 °C, 5% CO_2). After treatment with quercetin (30 or 100 μM), quercetin conjugates (30 μM), and control (1% DMSO) for 12 h (37 °C, 5% CO_2), cells were trypsinized, collected, washed with cold PBS, and fixed in 70% EtOH at 4 °C for at least 30 min. The fixed cells were washed with cold PBS and stained with 50 μL/mL of propidium iodide in the presence of 25 μg/mL of RNase A. Cell cycle phase distribution was analyzed using FACSCalibur (Becton Dickinson).

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