Bioorganic & Medicinal Chemistry 21 (2013) 1671–1679

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Quercetin–POC conjugates: Differential stability and bioactivity profiles between breast cancer (MCF-7) and colorectal carcinoma (HCT116) cell lines

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ARTICLE INFO

Article history:
Received 13 December 2012
Revised 18 January 2013
Accepted 27 January 2013
Available online 4 February 2013

Keywords:
Quercetin
Isopropyloxycarbonylmethoxy (POC)
Conjugate
Stability
Metabolism

ABSTRACT

In the course of our ongoing efforts to develop novel quercetin conjugates with enhanced stability profiles, we introduced an isopropyloxycarbonylmethoxy (POC) group to 7-OH and/or 3-OH of quercetin and prepared three novel quercetin conjugates. The quercetin–POC conjugates were stable up to 96 h in PBS but slowly hydrolyzed with half-lives of 1–54 h in cell-free culture medium, which is reminiscent of the stability profiles of the previously reported quercetin–POM (pivaloxymethyl) conjugates. However, the quercetin–POC conjugates were more susceptible to passive transport, intracellular hydrolysis, and metabolism in breast cancer (MCF-7) cell line compared with their POM congeners to result in low concentration of quercetin in this cell line and thereby low antiproliferative effect. In contrast, upon incubation with colorectal carcinoma HCT116 cells, the quercetin–POC conjugates were shown to undergo slow hydrolysis and metabolism to maintain concentrations of the active quercetin species high enough to exert enhanced cytotoxicity. Taken together, the quercetin–POC conjugates synthesized in this study exhibited cell type-specific stability as well as bioactivity profiles, which warrants further investigation into the underlying mechanisms and therapeutic potential.

1. Introduction

Quercetin (**1**, Fig. 1) has various biological properties including antioxidant,[1] antiviral,[2,3] antibacterial,[4] and anticancer[5] activities. However, quercetin suffers from low stability particularly in aqueous medium with high pH. The two phenolic hydroxyl groups of quercetin, 7-OH and 3-OH, are known to be responsible for metabolic[6–8] and chemical[9] instability of quercetin, respectively. In our previous study,[10,11] to enhance the physicochemical properties of quercetin, we have prepared quercetin conjugates by introducing a POM (pivaloxymethyl) moiety at 3-O and/or 7-O position of quercetin (Fig. 1).

Conjugation of quercetin with a POM promoiety at C7 hydroxyl group resulted in increased stability as well as enhanced cell permeability, and the resulting quercetin–POM conjugates 7-*O*-POM-Q (**2**)[10] and 3,7-bis-*O*-POM-Q (**4**)[11] underwent efficient intracellular conversion to quercetin and 3-*O*-POM-Q (**3**), respectively. On the other hand, 3-*O*-POM-Q (**3**), obtained by introduction of POM promoiety at 3-O position of quercetin, exhibited excellent stability profile but, due to the lack of cell permeability, it failed to accumulate inside the cell.

In the course of our ongoing efforts to develop novel quercetin conjugates with enhanced stability profiles, we attempted to install an isopropyloxycarbonylmethoxy (POC) group, which has been successfully exploited in antiviral tenofovir disoproxil fumarate.[12] In this study, 7-OH and/or 3-OH of quercetin were blocked with POC promoiety to provide three novel quercetin conjugates [7-*O*-POC-Q (**5**), 3-*O*-POC-Q (**6**) and 3,7-bis-*O*-POC-Q (**7**)] (Fig. 2).

Herein, we report regioselective syntheses of novel quercetin–POC conjugates (**5**, **6** and **7**) and evaluation of their physicochemical as well as biological properties.

2. Results and discussion

2.1. Syntheses of the quercetin–POC conjugates

Syntheses of the quercetin–POC conjugates were accomplished by nucleophilic substitution of appropriately protected quercetin derivatives (**9**, **10** and **14**: Schemes 1 and 2) with iodomethyl isopropyl carbonate (POC-I). Thus, regioselective preparation of the protected quercetin derivatives (**9**,[13,18] **10**[14,15,19] and **14**) was the key to syntheses of the quercetin–POC conjugates.

Preparation of 7-*O*-POC-Q (**5**) was started with peracetylation of quercetin by treatment with excess amount of acetic anhydride and pyridine (Scheme 1). Selective deacetylation of 7-OAc group

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http://dx.doi.org/10.1016/j.bmc.2013.01.057

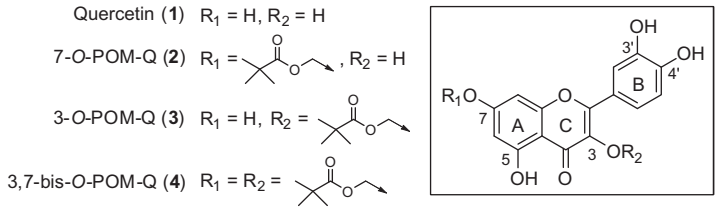


Figure 1. Structures of quercetin (**1**) and quercetin–POM conjugates (**2**, **3** and **4**).

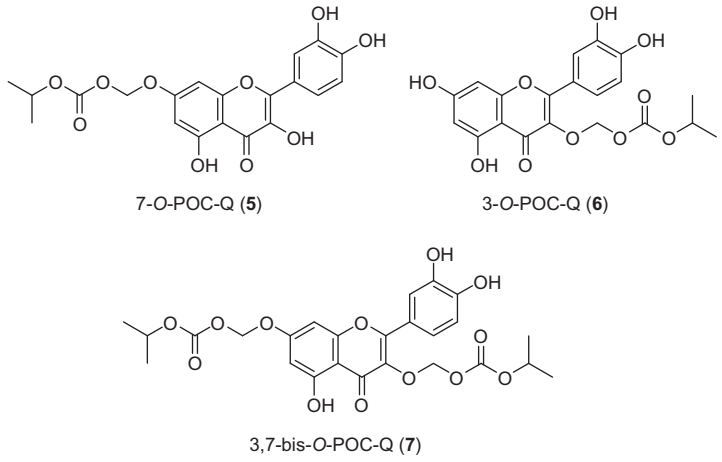
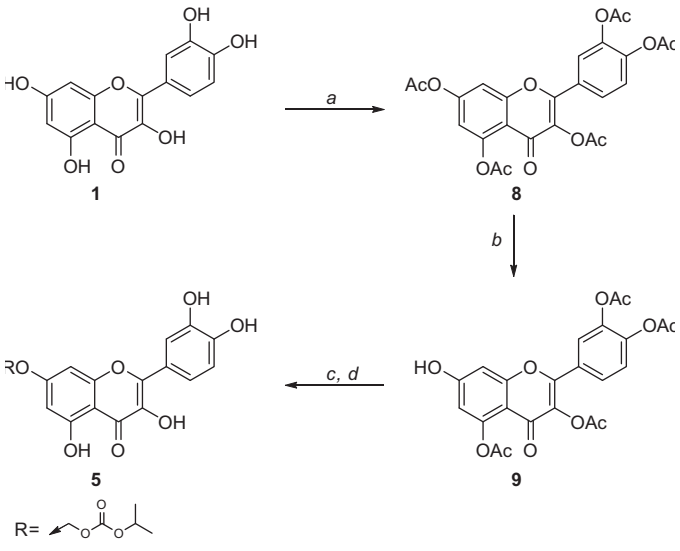


Figure 2. Quercetin–POC conjugates (**5**, **6** and **7**) investigated in this study.

of the resulting quercetin peracetate (**8**)[16,17] was accomplished by transesterification with PhSH to give the key intermediate **9** (73% yield),[18] which, upon substitution reaction with POC-I followed by deacetylation, provided the desired 7-*O*-POC-Q (**5**) in 91% yield.

The syntheses of 3-*O*-POC-Q (**6**) and 3,7-bis-*O*-POC-Q (**7**) were summarized in Scheme 2. The catechol group of quercetin (**1**) was protected by reaction with 1,1-dichlorodiphenylmethane at 180 °C to provide **10**.[19] Alkylation of the key intermediate **10** with excess amount of POC-I followed by hydrogenolysis in the presence of Pd/C in a mixture of THF and MeOH provided the desired product 3,7-bis-*O*-POC-Q (**7**) (66% yield). On the other hand, preparation of 3-*O*-POC-Q (**6**) required orthogonal protection of the 7-, 3′-, and 4′-hydroxyl groups through tedious protection-deprotection chemistry. First, quercetin diphenylmethylketal (**10**) was peracetylated with excess amount of Ac_2O in pyridine (93% yield) to give **11** of which 7-OAc group was selectively deacetylated.[18]

The free 7-OH group of the resulting intermediate **12** was then protected with a benzyl group to provide **13** in 69% yield. The remaining acetyl functionalities in **13** (3-OAc and 5-OAc) were removed by treatment with methanolic ammonia to give the key intermediate **14** in 90% yield. Alkylation of **14** with POC-I followed by hydrogenolysis of the benzyl as well as diphenylmethyl protecting groups furnished the desired product 3-*O*-POC-Q (**6**) in 61% combined yield.



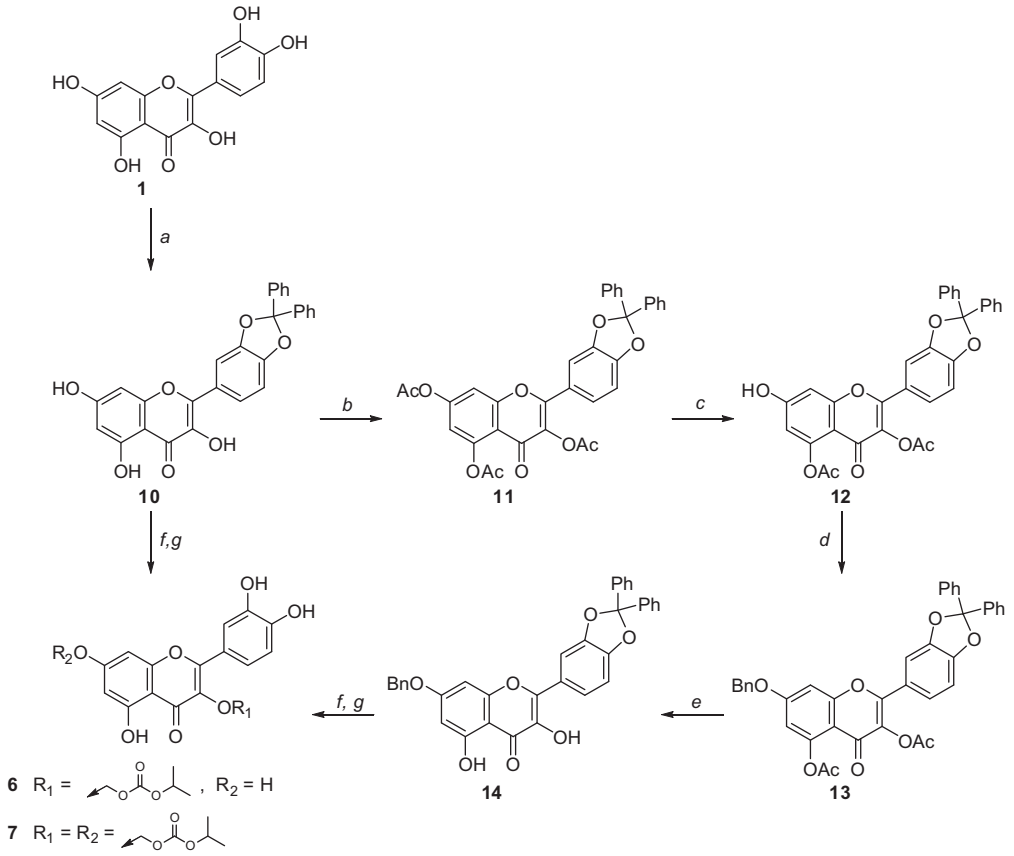
Scheme 1. Synthesis of 7-*O*-POC-Q (**5**). Reagents and conditions: (a) Ac_2o, Pyr, 70 °C; (b) PhSH, NMP, imidazole, 0 °C; (c) POC-I, K_2CO_3, acetone, rt; (d) NH_3/MeOH, 0 °C.

2.2. Properties of the quercetin–POC conjugates

2.2.1. Stability

Quercetin (**1**) is known to undergo oxidative degradation in PBS buffer (pH 7.4) with a half-life of 10 h.[10] Moreover, in oxidizing environment such as cell culture medium, quercetin is highly unstable to decompose in less than an hour ($t_{1/2}$ <0.5 h).[10] Quercetin's free hydroxyl groups were known to be responsible for its oxidative decomposition[10] and we have been anticipating that transiently blocking the 3-OH and/or 7-OH of quercetin would increase its stability against the oxidative stress.[10,11] Thus, stabilities of the POC-protected quercetin conjugates (**5**, **6**, and **7**) were measured by HPLC (Fig. 3) in PBS and cell-free cell culture medium (cDMEM), and their half-lives ($t_{1/2}$) were summarized in Table 1.

In general, stability profiles of the quercetin–POC conjugates were almost the same as the previously reported quercetin–POM conjugates;[10,11] the quercetin–POC conjugates (**5**, **6** and **7**), which showed remarkable stability in PBS with half-lives longer than



Scheme 2. Syntheses of 3-*O*-POC-Q (**6**) and 3,7-bis-*O*-POC-Q (**7**). 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, rt; (e) NH_3/MeOH, 0 °C; (f) POC-I, K_2CO_3, acetone/DMF, rt; (g) H_2, pd/C, THF/MeOH, rt.

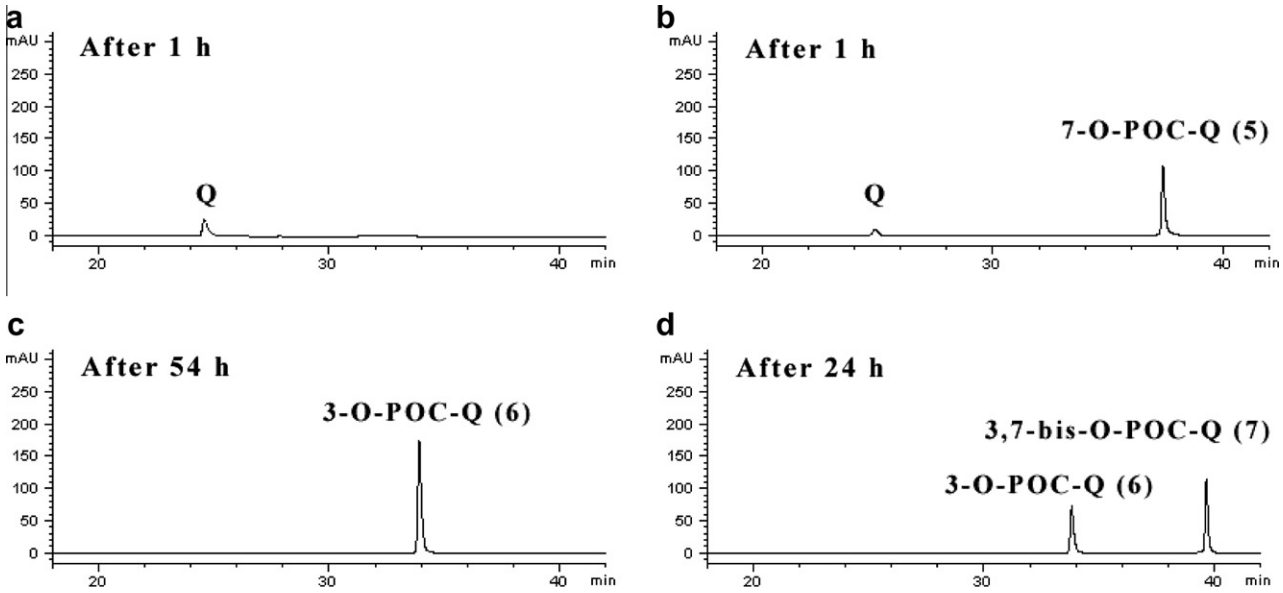


Figure 3. HPLC chromatograms of (a) quercetin (**1**, Q), (b) 7-*O*-POC-Q (**5**), (c) 3-*O*-POC-Q (**6**), and (d) 3,7-bis-*O*-POC-Q (**7**) in cell-free culture medium (cDMEM) after incubation for 1 h (a and b), 54 h (c), and 24 h (d).

96 h (Table 1), started to decomposed in cell-free culture medium. Among the series, 7-*O*-POC-Q (**5**) showed fastest decrease in concentration ($t_{1/2}$ = 1 h) followed by 3,7-bis-*O*-POC-Q (**7**) ($t_{1/2}$ = 24 h). However, it should be noted that, in comparison with 7-*O*-POC-Q (**5**) which showed hydrolysis to quercetin and/or decomposition (Fig. 3a), 3,7-bis-*O*-POC-Q (**7**) was not subject to decomposition but hydrolyzed into 3-*O*-POC-Q (**6**) (Fig. 3b). It should also be pointed out that 3-*O*-POC-Q (**6**) was the most stable quercetin–POC conjugate with high resistance to both hydrolysis and decomposition (Fig. 3c and d).

Table 1
Stability of quercetin (**1**) and quercetin–POC conjugates (**5**, **6** and **7**)

	Half-life, $t_{1/2}$ (h)			
	Quercetin (**1**)	7-*O*-POC-Q (**5**)	3-*O*-POC-Q (**6**)	3,7-Bis-*O*-POC-Q (**7**)
pH 7.4 (PBS)	10	>96	>96	>96
cDMEM[a]	<0.5	1	54	24[b]

[a] cDMEM: complete Dulbecco's modified Eagle's medium.
[b] Half-life for hydrolysis to 3-*O*-POC-Q (**6**).

Table 2
Permeability of quercetin (**1**) and quercetin conjugates (**5**–**7**) across artificial membranes[a]

Compd	LogP_e (cm/s)	
	1 h	5 h
Quercetin (**1**)	−4.14 ± 0.03	−4.44 ± 0.05
7-*O*-POC-Q (**5**)	−4.13 ± 0.02	−4.38 ± 0.03
3-*O*-POC-Q (**6**)	−4.56 ± 0.02	−5.01 ± 0.24
3,7-Bis-*O*-POC-Q (**7**)[b]	—	—

[a] Assay was performed in triplicate.
[b] Assay was not performed due to low solubility of **7** in PBS at the concentration for PAMPA (25 μM).

2.2.2. Solubility

To evaluate solubility of the quercetin–POC conjugates (**5**, **6** and **7**), forward light scattering was measured when a laser beam is directed through the solutions of the quercetin conjugates in PBS and cDMEM (Fig. 4).

Presumably due to the similar lipophilic nature of POM and POC, solubility profiles of the quercetin–POC conjugates were also reminiscent of those of the POM conjugates;[10,11] the quercetin–POC conjugates such as **5** and **7** showed relatively low aqueous solubility in PBS whereas 3-*O*-POC-Q (**6**) was highly soluble even at high concentrations. However, all the quercetin conjugates were completely soluble up to 100 μM concentration in cDMEM (Fig. 4b).

2.2.3. Membrane permeability

Besides the aqueous solubility of a drug substance, its permeability is a second fundamental parameter for controlling oral drug absorption. One of the in vitro methods that can be used to estimate the drug permeability is the parallel artificial membrane permeability assay (PAMPA) which utilizes an artificial membrane immobilized between a donor and an acceptor compartment.[20] With passively transported drugs, an excellent correlation between the flux across the artificial membrane and the extent of absorption has been demonstrated.[21–23] Thus, passive transport of quercetin (**1**) and its POC conjugates (**5**–**7**) were evaluated by PAMPA. Unfortunately, due to low solubility in PBS (Table 1), 3,7-bis-*O*-POC-Q (**7**) was not amenable to PAMPA. Briefly, quercetin (**1**) and the POC conjugates (**5** and **6**) were dissolved in donor plates and then the donor and acceptor plates were incubated together for 1–5 h after which, the plates were separated, and the concentration of compound in the accepter compartment was determined by UV_{340} measurement. The PAMPA data summarized in Table 2 shows that, in terms of passive permeability, 7-*O*-POC-Q (**5**) is as efficient as quercetin whereas 3-*O*-POC-Q (**6**) is less permeable than others. Nevertheless, the passive permeability of 3-*O*-POC-Q (**6**) is worth mentioning because the previously reported 3-*O*-POM-Q (**3**) was not permeable at all through the artificial membrane.[10]

2.2.4. Metabolite analysis

Combining profiles of stability, solubility, and membrane permeability, the quercetin–POC conjugates were anticipated to safely cross the cell membrane. Once localized inside the cell, the quercetin conjugates were supposed to undergo hydrolytic cleavage to release quercetin. Also, in the cellular milieu, the released quercetin would be subject to metabolic changes to the corresponding glucuronide (Q-Glu), methyl ether (Q-Me), and sulfate (Q-Sul).[24] For this reason, analysis of the cellular contents was performed, which was expected to provide information about intracellular metabolism of the quercetin conjugates. Thus, two different human cancer cell lines, MCF-7 (breast cancer) and HCT116 (colorectal carcinoma), were treated with the quercetin conjugates (**5**–**7**) and, after incubation for 1, 3, 6, and 12 h, cells were harvested and lysed. The cell lysates were then analyzed by HPLC to identify the remaining quercetin conjugates, its hydrolysis product (quercetin), and/or quercetin metabolites, and the results were summarized in Table 3.

In breast cancer (MCF-7) cell line, all the quercetin conjugates were shown to undergo facile conversion to quercetin as well as the quercetin metabolites and, after incubation for 12 h, neither quercetin nor quercetin metabolite was observed from the cell lysate. As already reported,[24] the quercetin glucuronide (Q-Glu) was identified as the major metabolite in all quercetin conjugates, which was shown to increase until 6 h of incubation. Interestingly, 3-*O*-POC-Q (**6**), which was highly resistant to hydrolysis to quercetin in cell-free culture medium ($t_{1/2}$ = 54 h, Table 1), was also transformed into quercetin with a half-life of 3 h, and this result clearly indicates that cellular uptake followed by intracellular hydrolysis of **6** was underway in MCF-7 cell line. By the same token,

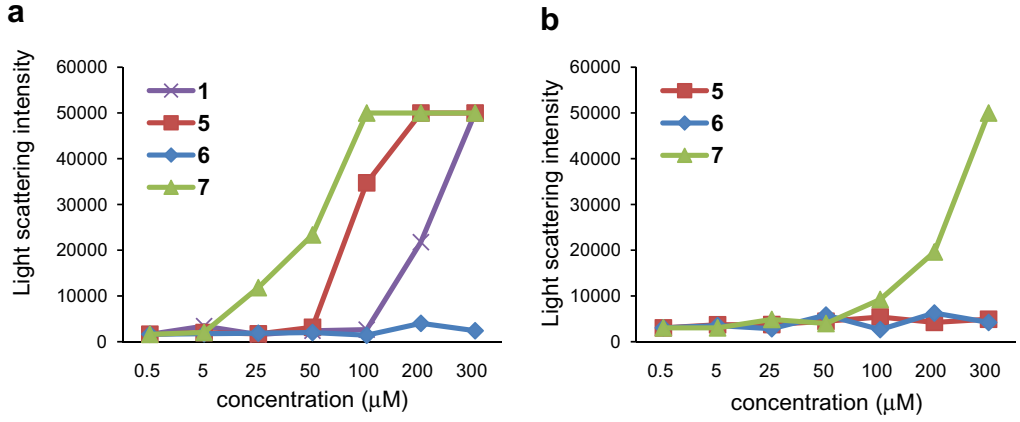


Figure 4. Aqueous solubility of quercetin (**1**) and quercetin–POC conjugates (**5**, **6** and **7**) in (a) PBS and (b) cDMEM measured by forward light scattering intensity. Quercetin's solubility was evaluated only in PBS due to its low stability in cell culture medium.

Table 3
Quantitative analysis of quercetin (**1**), quercetin conjugates (**5**–**7**), and quercetin metabolites (Q-Met)[a] in cell lysates after incubation of the quercetin conjugates (**5**–**7**) in breast cancer (MCF-7) and colorectal carcinoma (HCT116) cell lines

Q-Conj.	Contents in cell lysates	Relative concentration[b]							
		MCF-7				HCT116			
		1 h[c]	3 h	6 h	12 h	1 h	3 h	6 h	12 h
7-*O*-POC-Q (**5**)	7-*O*-POC-Q (**5**)	51	6	2	–[d]	65	34	18	12
	Quercetin (**1**)	24	11	1	–	4	11	13	4
	Q-Glu	–	21	23	–	1	–	–	–
	Q-Me	–	6	8	–	–	–	–	–
	Q-Sul	–	–	2	–	–	1	2	1
3-*O*-POC-Q (**6**)	3-*O*-POC-Q (**6**)	92	49	4	–	80	76	71	65
	Quercetin (**1**)	3	26	21	–	–	–	–	–
	Q-Glu	–	7	27	–	–	–	–	–
	Q-Sul	–	2	2	–	–	–	–	–
3,7-Bis-*O*-POC-Q (**7**)	3,7-Bis-*O*-POC-Q (**7**)	25	6	1	–	64	47	20	3
	7-*O*-POC-Q (**5**)	14	–	–	–	–	–	–	–
	3-*O*-POC-Q (**6**)	8	3	1	–	34	40	63	69
	Quercetin (**1**)	13	24	6	–	–	–	–	
	Q-Glu	2	9	22	–	–	–	–	–
	Q-Sul	–	1	1	–	–	–	–	–

[a] Q-Glu = quercetin glucuronide, Q-Me = methyl quercetin, Q-Sul = quercetin sulfate.
[b] Percentage (%) of each content in cell lysate calculated from integrated HPLC peak area.
[c] Incubation time.
[d] Not detected.

hydrolysis of 3,7-bis-*O*-POC-Q (**7**) into 7-*O*-POC-Q (**5**) and 3-*O*-POC-Q (**6**), which was unamenable in cell-free culture medium ($t_{1/2}$ = 24 h for transformation into **6** only, Table 1), could also be explained by the intracellular event. At this stage, it should be pointed out that, in the same breast cancer (MCF-7) cell line, the quercetin–POC conjugates were more susceptible to intracellular hydrolysis followed by metabolism compared with their POM congeners.[10,11] Also noteworthy is that 3-*O*-POC-Q (**6**) underwent facile hydrolysis and metabolic changes while neither of those events has not been observed in the case of quercetin-3-*O*-POM conjugates such as 3-*O*-POM-Q (**3**)[10] and 3,7-bis-*O*-POM-Q (**4**).[11]

In sharp contrast to this, the quercetin–POC conjugates were shown to undergo slow hydrolysis and low, if any, metabolism in the presence of the colorectal carcinoma (HCT116) cells. In addition, contents of the lysed HCT116 cells incubated with the quercetin–POC conjugates were the same as those obtained from the cell-free culture medium (Table 1). Thus, 7-*O*-POC-Q (**5**) underwent hydrolysis to quercetin leaving no trace of the quercetin metabolites. As was the case with the cell-free culture medium, 3-*O*-POC-Q (**6**) was stable up to 12 h and quercetin was not observed. Likewise, after incubation with 3,7-bis-*O*-POC-Q (**7**), the lysates of the HCT116 cells were shown to contain **7** and 3-*O*-POC-Q (**6**), the only hydrolysis product in the cell-free culture medium (Table 1). Taken together, unlike MCF-7, HCT116 cells did not affect the hydrolysis pattern and metabolism of the quercetin–POC conjugates but expedited hydrolysis of 3,7-bis-*O*-POC-Q (**7**) ($t_{1/2} \approx 3$ h) compared with that in cell-free conditions ($t_{1/2}$ = 24 h, Table 1), which indicates a series of intercellular and intracellular events surrounding **7** and its hydrolysis product **6**.

Overall, the quercetin–POC conjugates turned out to have differential susceptibility to hydrolysis as well as metabolism by MCF-7 and HCT116 cell lines, which was not the case in the quercetin–POM series.

2.2.5. Cytotoxicity

Previously described results obtained from PAMPA and cell lysate analysis all together suggest possible cellular uptake followed by intracellular hydrolysis of the quercetin–POC conjugates, and evaluation of the bioactivity of the quercetin conjugates in cell level would support this hypothesis. Among various bioactivities associated with quercetin, cytotoxicity against cancer cell lines was chosen. As the quercetin conjugates showed different profiles of hydrolysis and metabolism in breast cancer (MCF-7) and colorectal carcinoma (HCT116) cell lines, comparison of the cytotoxicity of the quercetin conjugates in those two cancer cell lines was also expected to provide correlation between stability of the quercetin conjugates and their bioactivity. Thus, after treating MCF-7 and HCT116 cell lines with quercetin and its POC-conjugates, viability of these cells were measured, and the results were summarized in Figure 5.

Interestingly, the quercetin conjugates showed cell line-dependent cytotoxicity; in MCF-7 cell line, the quercetin conjugates were as cytotoxic as quercetin (Fig. 5a) while 7-*O*-POC-Q (**5**) and 3,7-bis-*O*-POC-Q (**7**) were found to be more cytotoxic than others in HCT116 cell line (Fig. 5b). In MCF-7 cell line, all of the quercetin conjugates underwent fast hydrolysis, metabolism, and decomposition to result in low concentrations of the active species such as quercetin and/or quercetin conjugates, which is in good accordance with their low cytotoxic effects in this cell line. In contrast, concentration of quercetin and/or quercetin conjugates was kept relatively high in HCT116 cell line due to slow hydrolysis as well as resistance to metabolism, and the enhanced cytotoxicity of 7-*O*-POC-Q (**5**) and 3,7-bis-*O*-POC-Q (**7**) compared with quercetin might be attributed to their stability in this cell line. However, it should be noted that 3,7-bis-*O*-POC-Q (**7**) showed higher cytotoxicity than 7-*O*-POC-Q (**5**) (Fig. 5b) even though the conjugate **7** did not produce quercetin (Table 3). Also noteworthy is that the most stable quercetin conjugate 3-*O*-POC-Q (**6**), the only hydrolysis product of **7** in HCT116 cell line (Table 3), did not show cytotoxic effect (Fig. 5b). Taken together, these results clearly show that 3,7-bis-*O*-POC-Q (**7**) has intrinsic cytotoxicity which is higher than that of 7-*O*-POC-Q (**5**) or quercetin.

3. Conclusion

In this study, suboptimal stability of quercetin was tackled by novel quercetin conjugates which were prepared by regioselective introduction of the isopropyloxycarbonylmethoxy (POC) group at 3-OH and/or 7-OH of quercetin. Compared with quercetin, the quercetin–POC conjugates showed significantly enhanced stability in PBS and cell culture medium. In comparison with the previously reported quercetin–POM conjugates, the quercetin–POC

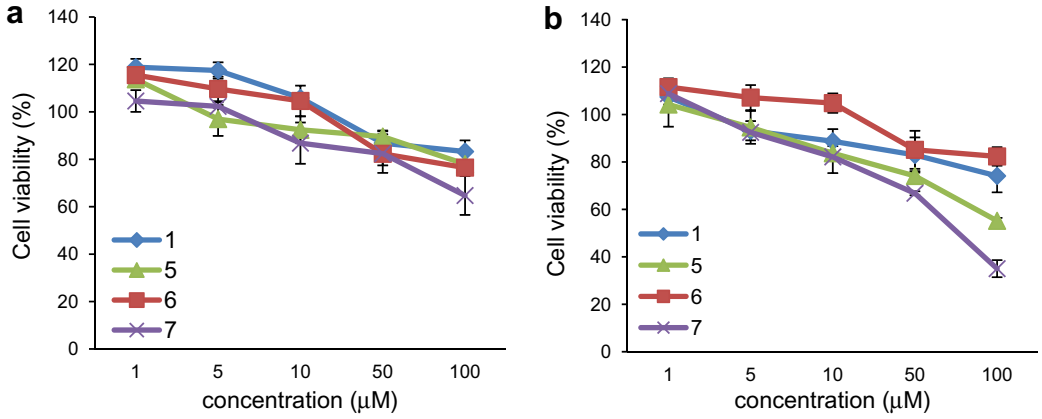


Figure 5. Viabilities of (a) MCF-7 and (b) HCT116 cell lines treated with quercetin (**1**), 7-*O*-POC-Q (**5**), 3-*O*-POC-Q (**6**), and 3,7-bis-*O*-POC-Q (**7**).

conjugates showed characteristic profiles in passive transport, intracellular hydrolysis, metabolism, and bioactivity. In particular, the quercetin–POC conjugates were more susceptible to passive transport, intracellular hydrolysis, and metabolism in breast cancer (MCF-7) cell line compared with their POM congeners to result in low antiproliferative effect against this cancer cell line. In contrast, upon incubation with colorectal carcinoma (HCT116) cells, the quercetin conjugates such as 7-*O*-POC-Q (**5**) and 3,7-bis-*O*-POC-Q (**7**) were shown to undergo slow hydrolysis and metabolism to maintain concentrations of the quercetin conjugates as well as their hydrolyzed products high enough to exert enhanced cytotoxicity.

Taken together, the quercetin–POC conjugates synthesized in this study exhibited cell type-specific stability as well as bioactivity profiles, which warrants further investigation into the underlying mechanisms and therapeutic potential.

4. Experimental

4.1. Materials and general methods

All chemicals 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 plate were manufactured by BD Bioscience Discovery Labware (Bedford, MA) using a polyvinylidene fluoride (PVDF) 96-well filter plate with 0.4 μm pore size. The 100 mm cell culture plate was purchased from Dishes Biousing™. Nuclear magnetic resonance spectra were recorded on a Bruker 400 AMX spectrometer (Karlsruhe, Germany) at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR with tetramethylsilane as the internal standard. Chemical shifts (δ) are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br s (broad singlet). Coupling constants are reported in hertz. The chemical shifts are reported as parts per million (δ) relative to the solvent peak. TLC was performed on silica gel 60 F_{254} purchased from Merck. Column chromatography was performed using silica gel-60 (220–440 mesh) for flash chromatography. Mass spectrometric data (MS) were obtained by electronspray ionization (ESI). High resolution fast atom bombardment (FAB) mass spectra were recorded using a JEOL JMS-700 mass spectrometer at the Daegu center of KBSI, Korea. All tested compounds were $\geqslant$95% purity, as determined by reverse phase HPLC. HPLC was performed on Agilent 1050 (Hewlett–Packard) equipment with variable wavelength (VW) UV detector using Polaris 5, C18-A 250 × 4.6 mm (Varian) column. Analytical conditions were as follows: gradient used was 20–25% 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), 100% acetonitrile in water containing 0.1% formic acid (45–50 min), 100–20% acetonitrile in water containing 0.1% formic acid (50–54 min) and 20% acetonitrile in water containing 0.1% formic acid (54–60 min). Flow rate was 1 mL/min. UV was detected at 340 nm.

4.2. Syntheses of the quercetin–POC conjugates

4.2.1. Acetic acid 3,5-diacetoxy-2-(3,4-diacetoxy-phenyl)-4-oxo-4*H*-chromen-7-yl ester (8)[16,17]

To a solution of quercetin (**1**) (5 g, 16.5 mmol) in pyridine (35 mL) was added acetic anhydride (12.5 mL, 132 mmol), and the reaction mixture was stirred at rt. The reaction was monitored by TLC. After starting material was consumed, the reaction mixture was concentrated under reduced pressure. The crude compound was purified by column chromatography on silica gel (4:1:1 = hexane/acetone/CH_2Cl_2) as eluent and was recrystallized from CH_2Cl_2 to afford **8** (4.5 g, 8.8 mmol, 54% yield) as off-white powder; mp 190 °C (rec. CH_2Cl_2) (lit.[16] mp 190 °C); ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 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); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 169.1, 168.6, 168.2, 168.0, 167.8, 167.6, 156.2, 154.2, 153.2, 149.3, 144.3, 142.0, 133.0, 126.9, 126.5, 124.3, 123.6, 114.5, 113.9, 109.9, 20.7, 20.6, 20.2, 20.17, 20.0; HR-FABMS (m/z): Found: 512.1030 $[M+H]^+$; Calcd for $C_{25}H_{20}O_{12}$: 511.0951.

4.2.2. Acetic acid 2-acetoxy-4-(3,5-diacetoxy-7-hydroxy-4-oxo-4*H*-chromen-2-yl)-phenyl ester (9)[13,18]

To a stirred mixture of **8** (3 g, 5.85 mmol) and imidazole (80 mg, 1.17 mmol) in NMP (30 mL) was slowly added PhSH (0.48 mL, 4.69 mmol) at 0 °C. The reaction mixture was stirred for 2 h at rt. 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 product was purified by column chromatography on silica gel (2:1:1 = hexane/acetone/EtOAc) as eluent and was recrystallized from CH_2Cl_2 to give **9** (2 g, 4.25 mmol, 73% yield) as white powder; mp 204 °C (rec. CH_2Cl_2); ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 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); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm); 169.0, 168.8, 168.3, 168.1, 168.0, 162.9, 157.6, 152.4, 150.2, 144.2, 142.2, 132.8, 127.4, 126.5, 124.5, 123.6, 109.4, 109.1, 101.0, 20.9, 20.5,

20.4, 20.3; HR-FABMS (*m*/*z*): Found: 471.0926 $[M+H]^+$; Calcd for $C_{23}H_{18}O_{11}$: 470.0847.

4.2.3. Carbonic acid 2-(3,4-dihydroxy-phenyl)-3,5-dihydroxy-4-oxo-4*H*-chromen-7-yloxymethyl ester isopropyl ester (5)

To a solution of **9** (150 mg, 0.32 mmol) in acetone (8 mL) was added K_2CO_3 (88 mg, 0.64 mmol) and POC iodide (234 mg, 0.96 mmol). The reaction mixture was stirred for 4 h at rt, filtered, and concentrated under reduced pressure to give pale yellow syrup, which was used for the next step without further purification. The degassed suspension of the quercetin conjugate obtained above and Pd/C (15 mg) in a mixture of THF (3 mL) and MeOH (3 mL), under an atmosphere of hydrogen gas (balloon), was vigorously stirred for 12 h at rt. The reaction mixture was filtered through a short celite pad and purified by column chromatography on silica gel (hexane/EtOAc = 1:1) to afford **5** (122 mg, 0.29 mmol, 91% yield) as yellow powder; ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) 12.63 (s, 1H), 7.73 (d, J = 2.0 Hz, 1H), 7.60 (dd, J = 8.5, 2.1 Hz, 1H), 6.90 (d, J = 8.5 Hz, 1H), 6.85 (d, J = 2.1 Hz, 1H), 6.47 (d, J = 2.1 Hz, 1H), 5.95 (s, 2H), 4.87–4.80 (m, 1H), 1.24 (d, J = 6.2 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 176.4, 161.5, 160.9, 156.0, 153.2, 148.3, 148.1, 145.5, 136.6, 122.1, 120.6, 116.0, 115.6, 105.5, 98.7, 94.1, 87.2, 73.1, 21.7; LC/MS (ESI) *m*/*z* Found: 417.20 $[M-H]^-$; Calcd for $C_{20}H_{18}O_{10}$: 418.09.

4.2.4. 2-(2,2-Diphenyl-benzo[1,3]dioxol-5-yl)-3,5,7-trihydroxy-chromen-4-one (10)[14,15,19]

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 (hexane/EtOAc = 4:1) on silica gel as eluent and was recrystallized from $CHCl_3$ to afford **10** (2.4 g, 5.2 mmol, 35% yield) as yellow powder; mp 222–224 °C (rec. $CHCl_3$) (lit.[14] mp 222–224 °C); ^{1}H NMR (400 MHz, acetone-d_6) δ (ppm) 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); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 176.1, 164.2, 160.8, 156.3, 147.7, 146.8, 145.6, 139.5, 136.5, 129.5, 128.7, 125.8, 125.3, 123.1, 117.1, 108.9, 107.9, 101.1, 98.4, 93.7; HR-FABMS (*m*/*z*): Found: 467.1135 $[M+H]^+$; Calcd for $C_{28}H_{18}O_7$: 466.1056.

4.2.5. Acetic acid 3,5-diacetoxy-2-(2,2-diphenyl-benzo[1,3]dioxol-5-yl)-4-oxo-4*H*-chromen-7-yl ester (11)

To a solution of **10** (1 g, 2.14 mmol) in anhydrous pyridine (10 mL) was added acetic anhydride (1 mL, 10.7 mmol) at rt. The reaction mixture was stirred for 6 h at 70 °C. After concentration, the crude product was purified by column chromatography (hexane/EtOAc = 2:1) on silica gel to give **11** (1.2 g, 2.0 mmol, 93% yield) as white powder; ^{1}H NMR (500 MHz, $CDCl_3$) δ (ppm) 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 (ppm) 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: 593.31 $[M+H]^+$; Calcd for $C_{34}H_{24}O_{10}$: 592.14.

4.2.6. Acetic acid 5-acetoxy-7-hydroxy-2-(2-methyl-2-phenyl-benzo[1,3]dioxol-5-yl)-4-oxo-4*H*-chromen-3-yl ester (12)

To a stirred mixture of **11** (1.2 g, 2.0 mmol) and imidazole (27 mg, 0.4 mmol) in NMP (24 mL) was slowly added PhSH (0.16 mL, 1.62 mmol) at 0 °C. The reaction mixture was stirred for 2 h at rt. 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 product was purified by column chromatography on silica gel (hexane/EtOAc = 1:1) to give **12** (1 g, 1.8 mmol, 90% yield) as pale yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 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 (ppm) 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: 549.32 $[M-H]^-$; Calcd for $C_{32}H_{22}O_9$: 550.13.

4.2.7. Acetic acid 5-acetoxy-7-benzyloxy-2-(2,2-diphenyl-benzo[1,3]dioxol-5-yl)-4-oxo-4*H*-chro-men-3-yl ester (13)

To a solution of **12** (1 g, 1.8 mmol) in acetone (20 mL) was added K_2CO_3 (251 mg, 1.8 mmol) and BnBr (0.32 mL, 2.7 mmol). The reaction mixture was stirred for 12 h at rt. After filtration, the filterate was concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (hexane/EtOAc = 2:1) to give **13** (800 mg, 1.25 mmol, 69% yield) as pale yellow oil; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 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 (ppm) 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: 641.34 $[M+H]^+$; Calcd for $C_{39}H_{28}O_9$: 640.17.

4.2.8. 7-Benzyloxy-2-(2,2-diphenyl-benzo[1,3]dioxol-5-yl)-3,5-dihydroxy-chromen-4-one (14)

A mixture of **13** (800 mg, 1.25 mmol) in sat'd NH_3 in MeOH (12 mL) at 0 °C was stirred for 2 h at rt. After concentration under reduced pressure, the residue was purified by column chromatography on silica gel (hexane/CH_2Cl_2/EtOAc = 6:2:1) to give **14** (624 mg, 1.12 mmol, 90% yield) as yellow powder; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 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 (ppm) 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: 557.32 $[M+H]^+$; Calcd for $C_{35}H_{24}O_7$: 556.15.

4.2.9. Carbonic acid 2-(3,4-dihydroxy-phenyl)-5,7-dihydro-xy-4-oxo-4*H*-chromen-3-yloxymethyl ester isopropyl ester (6)

To a solution of **14** (200 mg, 0.36 mmol) in a mixture of acetone (7 mL) and DMF (4 mL) was added K_2CO_3 (55 mg, 0.4 mmol) and POC iodide (132 mg, 0.54 mmol). The reaction mixture was stirred for 12 h at rt, filtered, and concentrated under reduced pressure to give pale yellow syrup, which was used for the next step without further purification. The degassed suspension of the quercetin conjugate obtained above and Pd/C (15 mg) in a mixture of THF (3 mL) and MeOH (3 mL), under an atmosphere of hydrogen gas (balloon), was vigorously stirred for 12 h at rt. The reaction mixture was filtered through a short celite pad and purified by column chromatography on silica gel (hexane/CH_2Cl_2/EtOAc = 2:1:1) to afford **6** (92 mg, 0.22 mmol, 61% yield) as yellow powder; ^{1}H NMR (400 MHz, acetone-d_6) δ (ppm) 12.62 (s, 1H), 7.63 (d, J = 2.2 Hz, 1H), 7.55 (dd, J = 8.5, 2.2 Hz, 1H), 6.96 (d, J = 8.5 Hz, 1H), 6.52 (d, J = 2.1 Hz, 1H), 6.29 (d, J = 2.1 Hz, 1H), 5.72 (s, 2H), 4.58 (septet, J = 6.3 Hz, 1H), 1.08 (s, 3H), 1.06 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 177.2, 164.8, 161.6, 157.1, 156.8, 153.5, 149.2, 145.5, 134.6, 121.6, 120.7, 116.1, 115.8, 104.3, 99.2, 94.1, 92.1, 72.4, 21.4; LC/MS (ESI) *m*/*z* Found: 417.11 $[M-H]^-$; Calcd for $C_{20}H_{18}O_{10}$: 418.09.

4.2.10. Carbonic acid 2-(3,4-dihydroxy-phenyl)-5-hydroxy-7-isopropoxycarbonyloxymethoxy-4-oxo-4*H*-chrom-en-3-yl-oxymethyl ester isopropyl ester (7)

To a solution of **10** (232 mg, 0.32 mmol) in acetone (8 mL) was added K_2CO_3 (88 mg, 0.64 mmol) and POC iodide (234 mg, 0.96 mmol). The reaction mixture was stirred for 4 h at rt, filtered, and concentrated under reduced pressure to give pale yellow syrup, which was used for the next step without further purification. The degassed suspension of quercetin conjugate obtained above and Pd/C (15 mg) in a mixture of THF (3 mL) and MeOH (3 mL), under an atmosphere of hydrogen gas (balloon), was vigorously stirred for 12 h at rt. The reaction mixture was filtered through a short celite pad and purified by column chromatography on silica gel (hexane/EtOAc = 1:1) to afford **7** (113 mg, 0.21 mmol, 66% yield) as yellow powder; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm) 12.36 (s, 1H), 7.83 (d, 1.8 Hz, 1H), 7.54 (dd, J = 8.5, 1.8 Hz, 1H), 7.00 (d, J = 8.5 Hz, 1H), 6.64 (d, J = 2.1 Hz, 1H), 6.50 (d, J = 2.0 Hz, 1H), 4.93–5.03 (m, 1H), 4.54–4.64 (m, 1H), 1.33 (d, J = 6.3 Hz, 6H), 1.09 (d, J = 6.3 Hz, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) 177.5, 162.0, 161.4, 157.8, 156.4, 153.5, 153.2, 149.5, 145.6, 135.0, 121.7, 120.5, 116.2, 115.8, 106.5, 99.5, 94.6, 90.1, 87.2, 73.1, 72.4, 21.7, 21.5; LC/MS (ESI) m/z Found: 533.33 $[M-H]^-$; Calcd for $C_{25}H_{26}O_{13}$: 534.14.

4.3. Stability test

4.3.1. Stability in PBS buffer

The synthesized quercetin–POC conjugates (**5**, **6** and **7**) (50 μM) were added to PBS buffer (pH 7.4) and incubated at 37 °C. At different time points (0, 1, 3, 6, 12, 24, 48, 96 h), an aliquot (100 μL) of the incubation mixture was taken out and the component were analyzed by HPLC equipped with a C-18 reverse phase column; flow rate, 1 mL/min; detection, UV 340 nm; mobile phase, 0–8 min (20–25% aqueous acetonitrile and 0.1% formic acid), 8–18 min (25–35% aqueous acetonitrile and 0.1% formic acid), 18–25 min (35% aqueous acetonitrile and 0.1% formic acid), 25–40 min (35–80% aqueous acetonitrile and 0.1% formic acid), 40–45 min (80–100% aqueous acetonitrile and 0.1% formic acid), 45–50 min (100% aqueous acetonitrile and 0.1% formic acid), 50–54 min (100–20% aqueous acetonitrile and 0.1% formic acid) and 54–60 min (20% aqueous acetonitrile and 0.1% formic acid).

4.3.2. Stability in DMEM cell culture medium

Quercetin–POC conjugates (**5**, **6** and **7**) were dissolved in culture medium to a final concentration of 50 μM from a 10 mM stock solution in DMSO. The solutions were incubated at 37 °C. The media were collected at different time points (0, 1, 3, 6, 12, 24, 48, 96 h), vortexed and analyzed by HPLC under the same analysis conditions described above.

4.4. Solubility test

Stock solution of the quercetin conjugates were prepared at 0.05, 0.5, 2.5, 5, 10 mM in 1–3% DMSO, and then serially diluted with either PBS (pH 7.4, 97–99%) or cDMEM [DMEM with 10% fetal bovine serum (FBS)], resulting final concentrations of 0.5, 5, 25, 50, 100, 200 (2% DMSO) and 300 μM (3% DMSO). Volume of the test compound in each 96 well plate was set to be 100 μL, and the solubility was measured by the NEPHELOstar laser based microplate nephelometer which checks the solubility of compounds by measuring forward light scatter in microplates. All raw data were processed using the BMG LABTECH NEPHELOstar Galaxy Evaluation software.

4.5. Membrane permeability assay [PAMPA (parallel artificial membrane permeability assay)]

The precoated PAMPA plate was warmed to rt for 30 min. Quercetin (**1**) and quercetin–POC conjugates (**5**, **6** and **7**) were dissolved in 95% PBS buffer solution (5% DMSO) to final concentrations of 25 μM. The solutions were added to the wells (300 μL/well) of the donor plates, and PBS buffer was added to the wells (200 μL/well) of the acceptor plates. The acceptor plate was placed on top of the donor plate, and the plate assembly was incubated at rt for 1 and 5 h. After incubation, concentration of the test compound in the acceptor, donor and reference wells was determined by transferring 100 μL of solution from each well to a new 96 well plate and quantitating using the UV plate reader. The effective permeability coefficients P_e (cm/s) was calculated using the following published equation:[25]

$$P_e = \frac{-\ln\left[1 - \frac{C_A(t)}{C_{equilbium}}\right]}{A \times \left(\frac{1}{V_D} + \frac{1}{V_A}\right) \times t} \text{ where } C_{equilibrium} = \frac{C_D(t) \times V_D + C_A(t) \times V_A}{V_D + V_A}$$

where A is the filter area (0.3 cm^2), t is the incubation time (s), V_A and V_D are, respectively, the volumes in the acceptor (0.2 mL) and the donor (0.3 mL) wells, $C_A(t)$ and $C_D(t)$ are, respectively, the concentration of the compound (mM) in the acceptor and donor wells at time t.

4.6. Metabolite analysis

Each of MCF-7 and HCT116 cell was seeded in tissue-cultured COSTAR clear bottom six-well plates (2×10^5 cells/well) and incubated for 24 h (37 °C, 5% CO_2). After addition of the quercetin–POC conjugates (30 μM) to each well, the plates were incubated (37 °C, 5% CO_2). At each time point (1, 3, 6 and 12 h), the cells were washed by PBS, trypsinized, and sonicated with Vibra-Cell VCX-130 (SONICS). After filtration, samples were analyzed by HPLC (Agilent) equipped with a C-18 reverse phase column; flow rate, 1 mL/min; detection, UV 340 nm; mobile phase, 0–8 min (20–25% aqueous acetonitrile and 0.1% formic acid), 8–18 min (25–35% aqueous acetonitrile and 0.1% formic acid), 18–25 min (35% aqueous acetonitrile and 0.1% formic acid), 25–40 min (35–80% aqueous acetonitrile and 0.1% formic acid), 40–45 min (80–100% aqueous acetonitrile and 0.1% formic acid), 45–50 min (100% aqueous acetonitrile and 0.1% formic acid), 50–54 min (100–20% aqueous acetonitrile and 0.1% formic acid) and 54–60 min (20% aqueous acetonitrile and 0.1% formic acid). Each peak was manually collected, lyophilized, and analyzed by mass spectrometry (MALDI-TOF, Bruker Daltonics).

4.7. Cytotoxicity

Cells (MCF-7 and HCT116) were seeded (5×10^3 cells per well) in tissue-cultured COSTAR clear bottom 96-well plate in cDMEM and incubated for 1 day (37 °C, 5% CO_2). Prepared solutions of quercetin (**1**) and quercetin–POC conjugates (**5**, **6** and **7**) in 1% DMSO were diluted into 6 different concentrations (1, 5, 10, 50, 100, 250 μM) and added to the media. After 12 h, cell viability was estimated by MTT assay. Every assay was repeated three times.

Acknowledgments

This research was supported by Priority Research Centers Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012-0006686), by a Grant of the Korean Health Technology R&D project, Ministry of Health & Welfare, Republic of Korea (A111698), and by a Grant from Next Generation Bio-Green 21 PJ007982 (Korea Rural Development Administration).

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