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# In vitro solubility, stability and permeability of novel quercetin–amino acid conjugates

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ABSTRACT

In order to discover a quercetin prodrug with improved bioavailability, we synthesized nine quercetin–amion acid conjugates and estimated their pharmacokinetic properties including water solubility, stability against chemical or enzymatic hydrolysis, and cell permeability. Among the synthesized quercetin prodrugs, quercetin–glutamic acid conjugate Qu-E (**4g**/**5g**) showed remarkable increases in water solubility, stability, and cell permeability compared with quercetin, which warrants further development as a quercetin prodrug.



## 1. Introduction

Quercetin (3′,4′,3,5,7-pentahydroxyflavone) is a member of the class of flavonol (Fig. 1), which is abundant in many common foods including apples, onions, grapes, red wine and green tea, at an average level of 10 mg/kg.

Quercetin has many biological activities including inhibition of a number of tyrosine kinases.[1,2] Clinical trials exploring different schedules of administration of quercetin have been held back by its extreme water insolubility, requiring formulation in dimethylsulfoxide (DMSO) where 150 mg quercetin is soluble in 1 mL of DMSO.[3] To overcome these limitations, QC12 (Fig. 1), a water-soluble glycine carbamate prodrug of quercetin, was synthesized and evaluated in a clinical study to investigate its pharmacokinetics following oral administrations to cancer patients.[4] The glycine carbamate moiety of QC12 was designed to be lost in the blood stream by hydrolysis to give the active ingredient, quercetin.[4] In vitro hydrolysis of QC12 to quercetin at 37 °C in water showed the prodrug to be stable with a half-life of 16.9 h while the half-life in whole blood was only 0.39 h. Also, following oral administration neither QC12 nor quercetin was detected in the serum, demonstrating that QC12, like quercetin is not orally bioavailable.[5] Thus, the advantage presented by QC12 is its high aqueous solubility compared to that of quercetin, eliminating the need for formulation in DMSO. However, the utility of QC12 for oral dosage is limited due to the low bioavailability presumably conferred by the fast metabolism and excretion of the prodrug.

The quercetin–glucose conjugate, which is the natural form of the quercetin obtained from the plant source, is absorbed into the apical membrane of the enterocyte. Once absorbed into the apical membrane of the enterocyte, quercetin–glucose conjugate is known to be hydrolyzed to quercetin which is then exhaustively metabolized to the methylated, sulfonylated, and glucuronidated quercetins by the enterocytic transferases.[6] These quercetin metabolites are then easily transported out to the intestinal lumen by ABS/MDR translocator.[6] Analogous conjugation reactions also take place in the liver.[6,7] As a result, only very low levels of quercetin are found in blood or plasma even after a quercetin-rich meal.[8–12] Studies with everted intestine segments and analysis of blood and lymph samples have led to the identification of up to 23 different quercetin conjugates[13–19] all arising via single or multiple glucuronation, sulfation, and methylation of the hydroxyls, but the metabolites may not themselves have biological activities.[20,21]

Taken together, results from the Q12 study indicates that a good quercetin prodrug should have increased water solubility as well as decreased rate of hydrolysis to prevent the deactivating metabolism. In this study, as a part of our ongoing efforts to discover a quercetin prodrug with improved bioavailability, we designed nine quercetin–amino acid conjugates. Herein we report syntheses and evaluation of the pharmacokinetic properties of the quercetin prodrugs including water solubility, chemical stability in the buffer solution, enzymatic stability, and cell permeability across the MDCK (Madin–Darby canine kidney) cell.

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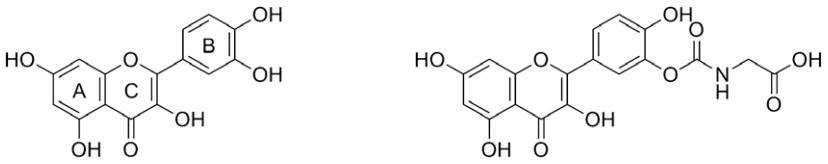



**Figure 1.** Structures of quercetin and Q12.

## 2. Results and discussion

### 2.1. Syntheses of the Q12 analogues

Among 20 amino acids, 4 nonpolar (Ala, Val, Phe, and Met), 1 positively charged (Lys), and 2 negatively charged (Asp, Glu) amino acids were attached to the B-ring of quercetin via a carbamate linkage (Scheme 1).

Syntheses of amino acid carbamate derivatives of quercetin from partly protected quercetin and amino acid ester isocyanates was previously reported by Wu et al.[22] In this study, instead of using amino acid ester isocyantes, we prepared a series of quercetin–amino acid conjugates through conversion of amino acids (**1**, Scheme 1) to the corresponding activated urethanes[23] followed by alcoholysis with quercetin. The reaction smoothly underwent to provide the quercetin–amino acid conjugates (**2** and **3**, Scheme 1) as inseparable mixtures of two regioisomers in an approximately 3:1 ratio. Deprotection of the *tert*-butyl group by treatment with TFA provided the desired quercetin prodrugs (**4** and **5**, Scheme 1). Biasuto et al. also reported that esterification on the quercetin B-ring catechol moiety gave inseparable regioisomeric mixtures of quercetin-3′-ester and 4′-ester due to the rapid intramolecular transesterification reaction.[24] Thus, the two isomers would be expected to have indistinguishable physicochemical properties. Investigation of the $^1$H NMR spectra of the final products revealed that the major product was 3′-*O*-substituted quercetin, which showed significant down-field shifts of $\delta_{H6'}$ (0.3 ppm) and $\delta_{H2'}$ (0.23 ppm) due to the introduction of the carbamate group at the 3′-OH (*para*- and *ortho*- position to H2′ and H6′, respectively, Fig. 2).[24] By the same token, the minor product was assigned to be 4′-O-substituted quercetin of which $\delta_{H5'}$ was down-field shifted (0.3 ppm) with other protons' chemical shifts unchanged (Fig. 2).[24]

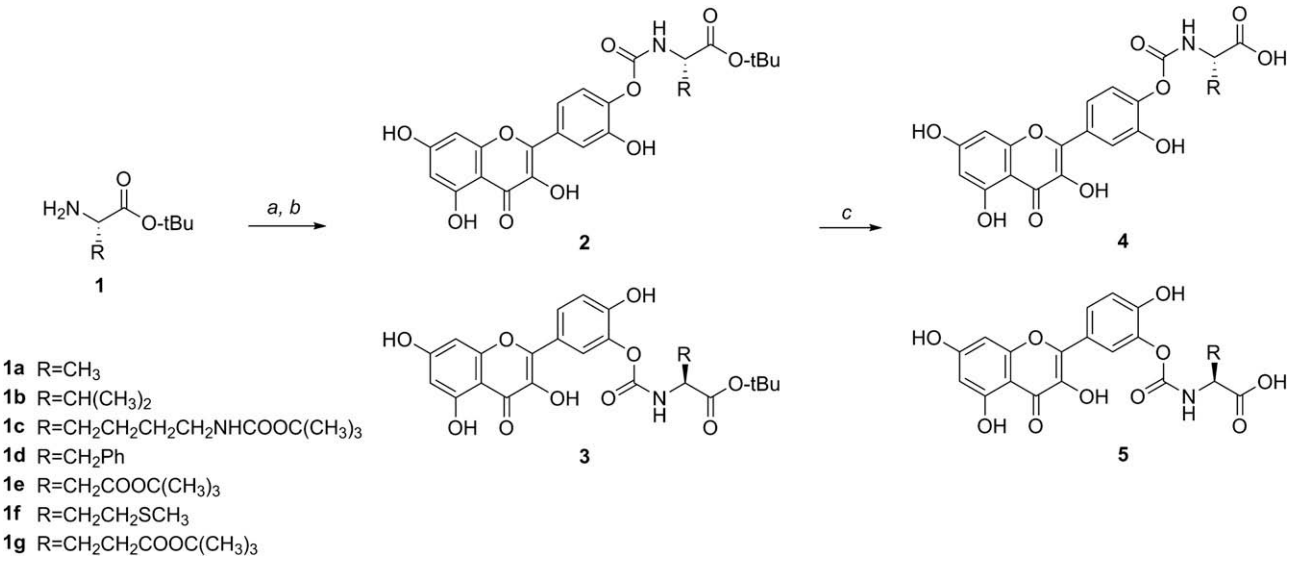
In addition to quercetin–amino acid conjugates **4** and **5** (Scheme 1), two quercetin–dipeptide conjugates such as quercetin-alanine-aspartic acid (Qu-AD) and quercetin-alanine-glutamic acid (Qu-AE) were also prepared. Usually, amino acids are transported into the intestine by help of human peptide transporter, hPepT1, which is involved in absorption of various peptides across the intestinal epithelium. As hPepT1 is known to recognize amino acids attached to a drug, it can be hijacked to transport the drug-amino acid conjugates.[25–27] Thus, the peptide transporter may be exploited to increase oral absorption of drug with poor essence permeability by attaching them to an enzymatically stable peptide, which is recognized by hPepT1. Also, recent studies on the hPepT1 revealed that it is more selective for dipeptides such as Asp-Ala and Glu-Ala.[28] Therefore we attached these two dipeptides to quercetin to improve absorption by help of the peptide transpoter (Scheme 2).

A regioisomeric mixture of the quercetin-alanine conjugates **4a** and **5a** was coupled with H-Glu(O*t*Bu)-OtBu·HCl [or H-Asp(OtBu)-O*t*Bu·HCl], and then treated with TFA to provide the corresponding quercetin–dipeptide conjugates **6h** and **7h** (**6i** and **7i**) as an inseparable mixture in 45% yield.

### 2.2. Pharmacokinetic properties of the quercetin conjugates

#### 2.2.1. Solubility

The quercetin prodrugs synthesized in this study showed dramatic increases in water solubility compared with the parent drug, quercetin. The solubilities of the quercetin-amino acid conjugates in PBS buffer are summarized in Table 1.




**Scheme 1.** Syntheses of the Q12 analogues. Reagents and conditions: (a) (4-$NO_2$-PhO)$_2$CO, DIPEA, THF/DMF, 0 °C to rt; (b) quercetin, DIPEA, DMF, 0 °C to rt; (c) TFA, $CH_2Cl_2$, 0 °C to rt.

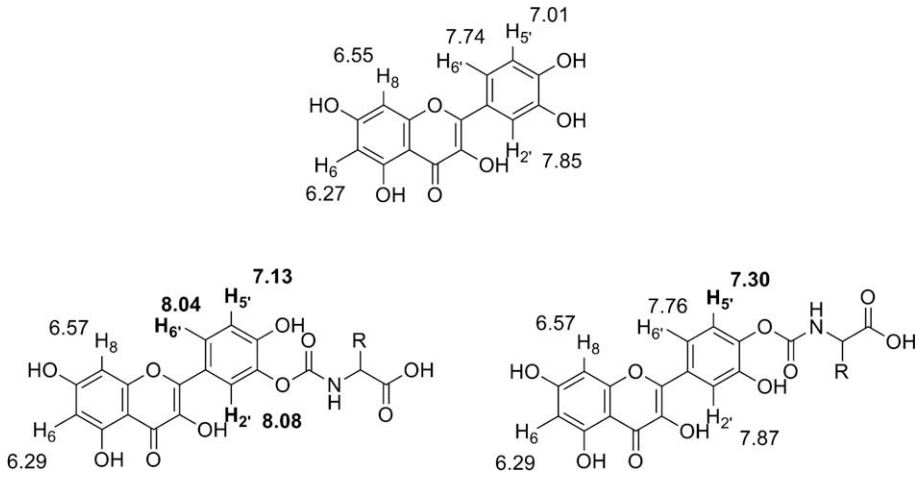
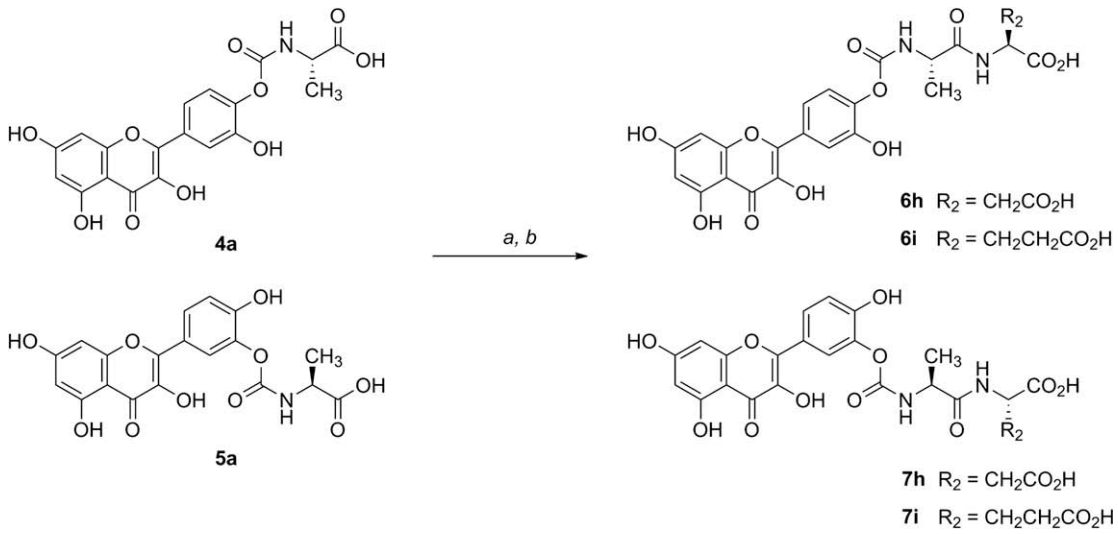



**Figure 2.** $^{1}$H NMR chemical shifts of quercetin, quercetin-3′-*O*-amino acid, and quercetin-4′-*O*-amino acid.




**Scheme 2.** Syntheses of quercetin–dipeptide conjugates. Reagents and conditions: (a) EDC, H-Asp(O*t*Bu)-O*t*Bu·HCl or H-Glu(O*t*Bu)-O*t*Bu·HCl, HOBt, DMF; (b) TFA, $CH_2Cl_2$.

**Table 1**
Solubility of quercetin and quercetin–amino acid conjugates in PBS buffer

| Compound | Water solubility (μM) | Fold increase[a] |
|---|---|---|
| Qu | 50 | 1.0 |
| Qu-A (**4a**/**5a**) | 1290 | 25.8 |
| Qu-V (**4b**/**5b**) | 767 | 15.3 |
| Qu-K (**4c**/**5c**) | 338 | 6.8 |
| Qu-F (**4d**/**5d**) | 1766 | 35.3 |
| Qu-D (**4e**/**5e**) | 2262 | 45.2 |
| Qu-M (**4f**/**5f**) | 675 | 13.5 |
| Qu-E (**4g**/**5g**) | 2649 | 53.0 |
| Qu-AD (**6h**/**7h**) | 2093 | 41.9 |
| Qu-AE (**6i**/**7i**) | 2628 | 52.6 |

[a] Fold increase in water solubility relative to quercetin (Qu).

The fold increase in solubility was as low as 6.8 fold in lysine conjugate (Qu-K, **4c**/**5c**) and as high as 53.0 fold in glutamic acid conjugate (Qu-E, **4g**/**5g**). Among the mono-amino acid conjugates, phenylalanine (Qu-F, **4d**/**5d**), aspartic acid (Qu-D, **4e**/**5e**), and glutamic acid (Qu-E, **4g**/**5g**) showed remarkable increases in solubility and the dipeptide conjugates such as alanine–aspartic acid (Qu-AD, **6h**/**7h**) and alanine–glutamic acid conjugates (Qu-AE, **6i**/**7i**) showed 41.9-fold and 52.6-fold increases, respectively. Quercetin-glutamic acid (Qu-E, **4g**/**5g**), quercetin-alanine-glutamic acid (Qu-AE, **6i**/**7i**), quercetin-aspartic acid (Qu-D, **4e**/**5e**) and quercetin-alanine-aspartic acid (Qu-AD, **6h**/**7h**) had two carboxylic acid groups, of which interaction with water must have increased their solubilities in water. In contrast, lysine conjugate (Qu-K, **4c**/**5c**) with the positively charged side chain showed lowest increase in solubility compared with quercetin.

### 2.2.2. Stability

Unlike QC12 ($t_{1/2}$ = 16.9 h)[4], no quercetin prodrug synthesized in this study was hydrolyzed in PBS buffer up to 17 h at 37 °C (data not shown). Presumably, the amino acid side chains provided additional stabilities to the synthesized QC12 analogues (**4**, **5**, **6**, and **7**) against hydrolysis by blocking the approach of water molecules to the carbamate linkage. On the other hand, in the presence of hydrolyzing enzymes drug-amino acid conjugates become easily hydrolyzed and the short half-life (0.39 h) of QC12 in whole blood[4] can also be attributed to the fast enzymatic hydrolysis by various endogenous hydrolyzing enzymes. In order to estimate the rate of enzymatic hydrolysis, the QC12 analogues were treated with whole cell lysates. Thus, MDCK cell was decomposed with lysis buffer (pH 7.6) to give cell lysate, in which many enzymes were activated at once to hydrolyze the quercetin prodrugs. In cell ly-

**Table 2**
Half-lifes ($t_{1/2}$) of quercetin prodrugs in cell lysate

| Compound | Half-life ($t_{1/2}$) (min) |
|---|---|
| Qu-A (**4a**/**5a**) | 30 |
| Qu-V (**4b**/**5b**) | <30 |
| Qu-K (**4c**/**5c**) | 180 |
| Qu-F (**4d**/**5d**) | <30 |
| Qu-D (**4e**/**5e**) | 85 |
| Qu-M (**4f**/**5f**) | 90 |
| Qu-E (**4g**/**5g**) | 180 |
| Qu-AD (**6h**/**7h**) | 90 |
| Qu-AE (**6i**/**7i**) | 100 |

sate, the quercetin prodrugs were hydrolyzed to quercetin but the rates of hydrolysis were dependent upon the amino acid attached to quercetin (Table 2). Amino acids with hydrophobic side chains such as alanine, valine, and phenylalanine were easily hydrolyzed from the corresponding conjugates [Qu-A (**4a**/**5a**), Qu-V (**4b**/**5b**), and Qu-F (**4d**/**5d**)] whereas quercetin conjugates with attached amino acids such as lysine [Qu-K (**4c**/**5c**)], aspartic acid [Qu-D (**4e**/**5e**)], methionine [Qu-M (**4f**/**5f**)], and glutamic acid [Qu-E (**4g**/**5g**)] showed extended half-lifes of more than 85 min. It is worth to note that polar amino acid side chains with more than two methylene units (lysine and glutamic acid) show strong resistance against enzymatic hydrolysis and dipeptide conjugates Qu-AD (**6h**/**7h**) and Qu-AE (**6i**/**7i**) are not as stable as the corresponding monoamino acids Qu-D (**4e**/**5e**) and Qu-E (**4g**/**5g**).

### 2.2.3. Cell permeability

The passive transports of quercetin and its prodrugs were estimated by non-cell-based permeability assay, first. An artificial cell membrane made of hexadecane, however, did not allow transport of neither quercetin nor its amino acid conjugates (data not shown).

For cell-based permeability assay, the MDCK cell is used which is a common model for studying cell growth regulation, metabolism, and transport mechanisms in distal renal epithelia.[29–34] Like Caco-2 cells, MDCK cells have been shown to differentiate into columnar epithelium and to form tight junctions when cultured on semipermeable membranes.[35,36] Before the transepithelial transport experiments, the MDCK monolayers were checked by measuring TEER (transepithelial electrical resistance). For the transport experiments, only monolayers displaying TEER values 991–1055 Ω were chosen.[37,38] Compared with quercetin, quercetin prodrugs did not show significant increases in cell permeabilities except aspartic acid [Qu-D (**4e**/**5e**)], glutamic acid [Qu-E (**4g**/**5g**)] and alanine–glutamic acid [Qu-AE (**6i**/**7i**)] conjugates (Fig. 3). In particular, the dipeptide Qu-AE (**6i**/**7i**) showed remarkable increase in cell permeability, which indicates that the human peptide transporter hPepT1 might be at work in recognition as well as transport of the quercetin–dipeptide conjugates.

## 3. Conclusion

Quercetin has many physiological activities. However, quercetin has pharmacokinetic problems such as low solubility in water and fast metabolism as well as excretion, which results in its low bioavailability. In this study, we synthesized new quercetin prodrugs such as quercetin-amino acid conjugates and quercetin–dipeptide conjugates to improve pharmacokinetic properties of quercetin. The quercetin prodrugs showed remarkable increases in water solubilities (6.8–53.0 fold increase relative to quercetin). In particular, amino acids such as aspartic acid and glutamic acid increased the water solubilities of their corresponding quercetin conjugates Qu-D (**4e**/**5e**) and Qu-E (**4g**/**5g**) by 45.2 and 53.0 folds, respectively. Like QC12, quercetin prodrugs were stable in PBS buffer ($t_{1/2}$ > 17 h) but susceptible in enzymatic hydrolysis in cell lysate which contained various activated hydrolyzing enzymes. However, quercetin–amino acid conjugates such as Qu-K (**4c**/**5c**) and Qu-E (**4g**/**5g**) showed strong resistance against hydrolases to show significantly extended half-lives (180 min) compared with that (0.39 h) of QC12.

The quercetin prodrugs Qu-D (**4e**/**5e**), Qu-E (**4g**/**5g**) and Qu-AE (**6i**/**7i**) also showed significantly increased intestinal permeability at MDCK cell in comparison with quercetin.

Taken together, through synthesis and In vitro pharmacokinetic analyses of quercetin–amino acid conjugates, we have identified a novel quercetin-amino acid conjugate, Qu-E (**4g**/**5g**), of which remarkably increased water solubility, stability, and cell permeability compared with quercetin and QC12 (Table 3) warrants further development as a quercetin prodrug.

## 4. Experimental

### 4.1. Materials and general methods

Quercetin (3′,4′,3,5,7-pentahydroxyflavone)-dihydrate, 1-hydroxybenzotriazole (HOBt), trifluoroacetic acid (TFA), dimethyl sulfox-

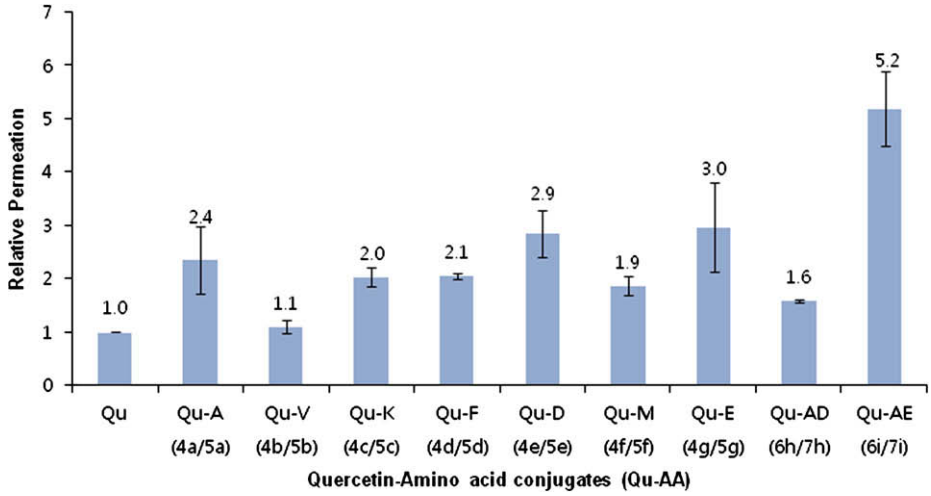



**Figure 3.** MDCK cell permeabilities of quercetin prodrugs relative to quercetin.

**Table 3**
Water solubility, stability, and permeability of quercetin (Qu), QC12, and Qu-E (**4g**/**5g**)

| Compound | Qu | QC12 | Qu-E (**4g**/**5g**) |
|---|---|---|---|
| Water solubility (μM) | 50 | ND[a] | 2649 |
| *Stability ($t_{1/2}$, h)* | | | |
| PBS buffer | — | 16.9[b] | >17 |
| Cell lysate | — | 0.39[b] | 3.0 |
| Relative permeability (MDCK cell) | 1.0 | ND[a] | 5.2 |

[a] Not determined.
[b] Taken from Ref. 3.

ide (DMSO), and RIPA buffer were purchased from Sigma–Aldrich. Various amino acids were obtained from Bachem. Bis(4-nitrophenyl) carbonate and 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide (EDC) were purchased from TCI Int. Phenylmethanesulfonyl fluoride (PMSF) was purchased from Fluka. Dulbecco's Modified Eagle Medium (DMEM), penicillin, streptomycin and fetal bovine serum (FBS) were purchased from Invitrogen. The 96-well MultiScreen Caco-2 plate (PSHT004S5) and Millicell®-ERS system ohm meter (MERS 000 01) were purchased from Millipore Corp. The STX-100M electrode was purchased from World Precision Instruments, Sarasota, FL. 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 ($\delta$) 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 ($\delta$) relative to the solvent peak. TLC was performed on silica gel-60 $F_{254}$ purchased from Merck. Column chromatography was performed using either silica gel-60 (220–440 mesh) for flash chromatography. Mass spectrometric data (MS) were obtained by electronspray ionization (ESI).

### 4.2. Syntheses of quercetin conjugates

#### 4.2.1. *tert*-Butyl-amino acid carbamate derivatives of quercetin (2 and 3)

Amino acids [H-Ala-O*t*Bu·HCl (**1a**), H-Val-O*t*Bu·HCl (**1b**), H-Lys(Boc)-O*t*Bu·HCl (**1c**), H-Phe-O*t*Bu·HCl (**1d**), H-Asp(O*t*Bu)-O*t*Bu·HCl (**1e**), H-Met-O*t*Bu·HCl (**1f**) and H-Glu(O*t*Bu)-O*t*Bu·HCl (**1g**)] (3 mmol) were dissolved in anhydrous THF (20 mL). Bis(4-nitrophenyl) carbonate (3 mmol) and *N*,*N*-diisopropylethylamine (6 mmol) were added to this solution, and the reaction mixture was stirred at room temperature. After 12 h, quercetin (3 mmol) was added, and the mixture was stirred for 12 h at room temperature. The resulting solution was concentrated under reduced pressure, and the residue was purified by column chromatography on silica gel (dichloromethane/acetone = 6:1) to afford inseparable mixtures of the regioisomers (**2** and **3**) as yellow solids.

**4.2.1.1. 4′-*O*-CO-(Ala-O*t*Bu)-quercetin (2a) and 3′-*O*-CO-(Ala-O*t*Bu)-quercetin (3a) (60% yield).** LC/MS (ESI) *m/z* Found: 473.3 $[M+H]^+$; Calcd for $C_{23}H_{23}NO_{10}$: 473.13; For **2a**, $^1$H NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.14 (s, 1H), 8.02–8.16 (m, 2H), 7.12 (d, $J$ = 8.7 Hz, 1H), 6.54 (s, 1H), 6.27 (s, 1H), 4.16–4.23 (m, 1H), 1.46 (s, 12H); For **3a**, $^1$H NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.09 (s, 1H), 7.89 (d, $J$ = 1.9 Hz, 1H), 7.76 (dd, $J$ = 6.8, 1.8 Hz, 1H), 7.29 (d, $J$ = 8.5 Hz, 1H), 6.54 (s, 1H), 6.27 (s, 1H), 4.16–4.23 (m, 1H), 1.46 (s, 12H).

**4.2.1.2. 4′-*O*-CO-(Val-O*t*Bu)-quercetin (2b) and 3′-*O*-CO-(Val-O*t*Bu)-quercetin (3b) (55% yield).** LC/MS (ESI) *m/z* Found: 501.3 $[M+H]^+$; Calcd for $C_{25}H_{27}NO_{10}$: 501.16; For **2b**, $^1$H NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.48 (s, 1H), 8.00–8.24 (m, 2H), 7.12 (d, $J$ = 8.6 Hz, 1H), 6.52 (s, 1H), 6.28 (s, 1H), 4.03–4.12 (m, 1H), 2.20–2.28 (m, 1H), 1.50 (s, 9H), 1.04 (s, 6H); For **3b**, $^1$H NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.12 (s, 1H), 7.89 (d, $J$ = 1.6 Hz, 1H), 7.74 (d, $J$ = 8.7 Hz, 1H), 7.31 (d, $J$ = 8.7 Hz, 1H), 6.52(s, 1H), 6.28(s, 1H), 4.03–4.12 (m, 1H), 2.20–2.28 (m, 1H), 1.50 (s, 9H), 1.04 (s, 6H).

**4.2.1.3. 4′-*O*-CO-[Lys(Boc)-O*t*Bu]-quercetin (2c) and 3′-*O*-CO-[Lys(Boc)-O*t*Bu]-quercetin (3c) (50% yield).** LC/MS (ESI) *m/z* Found: 630.4 $[M+H]^+$; Calcd for $C_{31}H_{38}N_2O_{12}$: 630.24; For **2c**, $^1$H NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.14 (s, 1H), 8.03–8.10 (m, 2H), 7.12 (d, $J$ = 8.6 Hz, 1H), 6.56 (s, 1H), 6.27 (s, 1H), 4.16 (d, $J$ = 4.9 Hz, 1H), 3.12 (d, $J$ = 4.8 Hz, 2H), 2.88 (s, 4H), 1.79–1.93 (m, 2H), 1.49 (s, 9H), 1.41 (s, 9H); For **3c**, $^1$H NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.09 (s, 1H), 7.89 (s, 1H), 7.76 (d, $J$ = 8.5 Hz, 1H), 7.30 (d, $J$ = 8.6 Hz, 1H), 6.56 (s, 1H), 6.27 (s, 1H), 4.16 (d, $J$ = 4.9 Hz, 1H), 3.12 (d, $J$ = 4.8 Hz, 2H), 2.88 (s, 4H), 1.79–1.93 (m, 2H), 1.49 (s, 9H), 1.41 (s, 9H).

**4.2.1.4. 4′-*O*-CO-(Phe–O*t*Bu)-quercetin (2d) and 3′-*O*-CO-(Phe–O*t*Bu)-quercetin (3d) (55% yield).** LC/MS (ESI) *m/z* Found: 549.3 $[M+H]^+$; Calcd for $C_{29}H_{27}NO_{10}$: 549.16; For **2d**, $^1$H NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.14 (s, 1H), 8.02 (d, $J$ = 8.6 Hz, 2H), 7.34–7.39 (m, 5H), 7.11 (d, $J$ = 8.1 Hz. 1H), 6.56 (s, 1H), 6.27 (s, 1H), 4.41–4.45 (m, 1H), 3.12–3.22 (m, 2H), 1.46 (s, 9H); For **3d**, $^1$H NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.09 (s, 1H), 7.88 (d, $J$ = 1.6 Hz, 1H), 7.72 (d, $J$ = 8.6 Hz, 1H), 7.10–7.29 (m, 6H), 6.56 (s, 1H), 6.27 (s, 1H), 4.41–4.45 (m, 1H), 3.12–3.22 (m, 2H), 1.46 (s, 9H).

**4.2.1.5. 4′-*O*-CO-[Asp(O*t*Bu)-OtBu]-quercetin (2e) and 3′-O-CO-[Ala(O*t*Bu)-O*t*Bu]-quercetin (3e) (60% yield).** LC/MS (ESI) *m/z* Found: 573.3 $[M+H]^+$; Calcd for $C_{28}H_{31}NO_{12}$: 573.18; For **2e**, $^1$H NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.14 (s, 1H), 8.08 (d, $J$ = 1.7 Hz, 1H), 8.04 (d, $J$ = 8.7 Hz, 1H), 7.12 (d, $J$ = 8.7 Hz, 1H), 6.54 (s, 1H), 6.28 (s, 1H), 4.49–4.54 (t, $J$ = 2.2 Hz, 1H), 2.04–2.06 (m, 2H), 1.48 (s, 6H); For **3e**, $^1$H NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.09 (s, 1H), 7.90 (d, $J$ = 1.9 Hz, 1H), 7.76 (d, $J$ = 8.6 Hz, 1H), 7.29 (d, $J$ = 8.6 Hz, 1H), 6.54 (s, 1H), 6.28 (s, 1H), 4.49–4.54 (t, $J$ = 2.2 Hz, 1H), 2.04–2.06 (m, 2H), 1.48 (s, 6H).

**4.2.1.6. 4′-*O*-CO-(Met-O*t*Bu)-quercetin (2f) and 3′-*O*-CO-(Met-O*t*Bu)-quercetin (3f) (55% yield).** LC/MS (ESI) *m/z* Found: 533.2 $[M+H]^+$; Calcd for $C_{25}H_{27}NO_{10}S$: 533.14; For **2f**, $^1$H NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.08 (s, 1H), 7.99–8.10 (m, 2H), 7.12 (d, $J$ = 8.6 Hz, 1H), 6.53 (s, 1H), 6.27 (s, 1H), 4.33–4.37 (m, 1H), 3.03–3.12 (m, 2H), 2.63–2.72 (m, 2H), 2.12 (s, 3H), 1.48 (s, 9H); For **3f**, $^1$H NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.05(s, 1H), 7.90 (d, $J$ = 1.9 Hz, 1H), 7.75 (dd, $J$ = 6.6, 5.0 Hz, 1H), 7.29 (d, $J$ = 8.5 Hz, 1H), 6.53 (s, 1H), 6.27 (s, 1H), 4.33–4.37 (m, 1H), 3.03–3.12 (m, 2H), 2.63–2.72 (m, 2H), 2.12 (s, 3H), 1.48 (s, 9H).

**4.2.1.7. 4′-*O*-CO-[Glu(O*t*Bu)-O*t*Bu]-quercetin (2g) and 3′-*O*-CO-[Glu(O*t*Bu)-O*t*Bu]-quercetin (3g) (60% yield).** LC/MS (ESI) *m/z* Found: 587.3 $[M+H]^+$; Calcd for $C_{29}H_{33}NO_{12}$: 587.20; For **2g**, $^1$H NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.14 (s, 1H), 8.00–8.10 (m, 2H), 7.12 (d, $J$ = 8.6 Hz, 1H), 6.56 (s, 1H), 6.28 (s, 1H), 4.38–4.44 (m, 2H), 2.35–2.59 (m, 4H), 1.48 (s, 6H); For **3g**, $^1$H NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.08 (s, 1H), 7.94 (d, $J$ = 1.6 Hz, 1H), 7.80 (d, $J$ = 8.6 Hz, 1H), 7.37 (d, $J$ = 8.6 Hz, 1H), 6.56 (s, 1H), 6.28 (s, 1H), 4.38–4.44 (m, 2H), 2.35–2.59 (m, 4H), 1.48 (s, 6H).

#### 4.2.2. Amino acid carbamate derivatives of quercetin (4 and 5)

The *tert*-butyl-amino acid quercetin carbamates (**2** and **3**, 2.2 mmol) obtained above were dissolved in anhydrous $CH_2Cl_2$ (5 mL), and the resulting solution was treated with trifluoroacetic

acid (2 mL) at 0 °C. The reaction mixture was stirred for 4 h at room temperature, and concentrated under reduced pressure. The residue was recrystallized from a mixture of acetone (1 mL) and $CH_2Cl_2$ (10 mL). The mixture was filtered through a Bűchner or Hirsch funnel, and the filter cake was washed with $CH_2Cl_2$ to give inseparable mixtures of the regioisomers (**4** and **5**) as yellow solids.

**4.2.2.1. 4′-*O*-CO-Ala-quercetin (4a) and 3′-*O*-CO-Ala-quercetin (5a) (95% yield).** LC/MS (ESI) *m/z* Found: 418.2 $[M+H]^+$; Calcd for $C_{19}H_{15}NO_{10}$: 417.07; For **4a**, $^1H$ NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.14 (s, 1H), 8.00–8.06 (m, 2H), 7.11 (d, $J$ = 8.4 Hz, 1H), 6.54 (s, 1H), 6.27 (s,1H), 4.33–4.38 (m, 1H), 1.51 (s, 3H); For **5a**, $^1H$ NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.09 (s, 1H), 7.89 (s, 1H), 7.74 (d, $J$ = 8.4 Hz, 1H), 7.30 (d, $J$ = 8.4 Hz, 1H), 6.54 (s, 1H), 6.27 (s, 1H), 4.16–4.23 (m, 1H), 1.46 (s, 12H).

**4.2.2.2. 4′-*O*-CO-Val-quercetin (4b) and 3′-*O*-CO-Val-quercetin (5b) (89% yield).** LC/MS (ESI) *m/z* Found: 446.2 $[M+H]^+$; Calcd for $C_{21}H_{19}NO_{10}$: 445.10; For **4b**, $^1H$ NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.48 (s, 1H), 8.00–8.24 (m, 2H), 7.12 (d, $J$ = 8.6 Hz, 1H), 6.53 (s, 1H), 6.28 (s, 1H), 4.03–4.12 (m, 1H), 2.20–2.31 (m, 1H), 1.04 (s, 6H); For **5b**, $^1H$ NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.12 (s, 1H), 7.83 (d, $J$ = 1.6 Hz, 1H), 7.70 (d, $J$ = 8.6 Hz, 1H), 7.31 (d, $J$ = 8.6 Hz, 1H), 6.53(s, 1H), 6.28(s, 1H), 4.03–4.12 (m, 1H), 2.20–2.31 (m, 1H), 1.04 (s, 6H).

**4.2.2.3. 4′-*O*-CO-Lys-quercetin (4c) and 3′-*O*-CO-Lys-quercetin (5c) (90% yield).** LC/MS (ESI) *m/z* Found: 475.3 $[M+H]^+$; Calcd for $C_{22}H_{22}N_2O_{10}$: 474.13; For **4c**, $^1H$ NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.14 (s, 1H), 8.00–8.03 (m, 2H), 7.15 (d, $J$ = 9.1 Hz, 1H), 6.58 (d, $J$ = 1.6 Hz, 1H), 6.26 (d, $J$ = 1.8 Hz, 1H), 4.34 (d, $J$ = 4.1 Hz, 1H), 3.77–3.90 (m, 2H), 1.84–2.00 (m, 4H), 1.67 (q, $J$ = 7.06 Hz, 2H); For **5c**, $^1H$ NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.09 (s, 1H), 7.88 (s, 1H), 7.73 (dd, $J$ = 6.8, 1.8 Hz, 1H), 7.27 (d, $J$ = 8.5 Hz, 1H), 6.56 (d, $J$ = 1.8 Hz, 1H), 6.29 (d, $J$ = 1.8 Hz, 1H), 4.34 (d, $J$ = 4.1 Hz, 1H), 3.77–3.90 (m, 2H), 1.84–2.00 (m, 4H), 1.67 (q, $J$ = 7.06 Hz, 2H).

**4.2.2.4. 4′-*O*-CO-Phe-quercetin (4d) and 3′-*O*-CO-Phe-quercetin (5d) (90% yield).** LC/MS (ESI) *m/z* Found: 494.3 $[M+H]^+$; Calcd for $C_{25}H_{19}NO_{10}$: 493.10; For **4d**, $^1H$ NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.14 (s, 1H), 8.02 (d, $J$ = 8.6 Hz, 2H), 7.34–7.37 (m, 5H), 7.11 (d, $J$ = 8.1 Hz, 1H), 6.57 (s, 1H), 6.28 (s, 1H), 4.51–4.59 (m, 1H), 3.12–3.33 (m, 2H); For **5d**, $^1H$ NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.09 (s, 1H), 7.88 (d, $J$ = 1.6 Hz, 1H), 7.72 (d, $J$ = 8.6 Hz, 1H), 7.10–7.29 (m, 6H), 6.57 (s, 1H), 6.28 (s, 1H), 4.51–4.59 (m, 1H), 3.12–3.33 (m, 2H).

**4.2.2.5. 4′-*O*-CO-Asp-quercetin (4e) and 3′-*O*-CO-Asp-quercetin (5e) (92% yield).** LC/MS (ESI) *m/z* Found: 462.2 $[M+H]^+$; Calcd for $C_{20}H_{15}NO_{12}$: 461.06; For **4e**, $^1H$ NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.14 (s, 1H), 8.08 (d, $J$ = 1.7 Hz, 1H), 8.04 (d, $J$ = 8.8 Hz, 1H), 7.13 (d, $J$ = 8.7 Hz, 1H), 6.57 (s, 1H), 6.28 (s, 1H), 4.68–4.69 (t, $J$ = 2.2 Hz, 1H), 3.00 (d, $J$ = 5.4 Hz, 2H); For **5e**, $^1H$ NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.09 (s, 1H), 7.90 (d, $J$ = 1.9 Hz, 1H), 7.77 (d, $J$ = 8.4 Hz, 1H), 7.29 (d, $J$ = 8.6 Hz, 1H), 6.57 (s, 1H), 6.28 (s, 1H), 4.68–4.69 (t, $J$ = 2.2 Hz, 1H), 3.00 (d, $J$ = 5.4 Hz, 2H).

**4.2.2.6. 4′-*O*-CO-Met-quercetin (4f) and 3′-*O*-CO-Met-quercetin (5f) (90% yield).** LC/MS (ESI) *m/z* Found: 478.3 $[M+H]^+$; Calcd for $C_{21}H_{19}NO_{10}S$: 477.07; For **4f**, $^1H$ NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.08 (s, 1H), 7.98–8.10 (m, 2H), 7.13 (d, $J$ = 8.5 Hz, 1H), 6.54 (s, 1H), 6.27 (s, 1H), 4.52–4.56 (m, 1H), 2.63–2.72 (m, 2H), 2.02–2.05 (m, 2H), 1.69 (s, 3H); For **5f**, $^1H$ NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.05 (s, 1H), 7.90 (d, $J$ = 2.0 Hz, 1H), 7.76 (d, $J$ = 8.7 Hz, 1H), 7.29 (d, $J$ = 8.6 Hz, 1H), 6.54 (s, 1H), 6.27 (s, 1H), 4.52–4.56 (m, 1H), 2.63–2.72 (m, 2H), 2.02–2.05 (m, 2H), 1.69 (s, 3H).

**4.2.2.7. 4′-*O*-CO-Glu-quercetin (4g) and 3′-*O*-CO-Glu-quercetin (5g) (88% yield).** LC/MS (ESI) *m/z* Found: 476.2 $[M+H]^+$; Calcd for $C_{21}H_{17}NO_{12}$: 475.08; For **4g**, $^1H$ NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.14 (s, 1H), 8.00–8.10 (m, 2H), 7.12 (d, $J$ = 8.6 Hz, 1H), 6.56 (s, 1H), 6.28 (s, 1H), 4.38–4.44 (m, 2H), 2.35–2.59 (m, 4H); For **5g**, $^1H$ NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.08 (s, 1H), 7.94 (d, $J$ = 1.6 Hz, 1H), 7.80 (d, $J$ = 8.6 Hz, 1H), 7.37 (d, $J$ = 8.6 Hz, 1H), 6.56 (s, 1H), 6.28 (s, 1H), 4.38–4.44 (m, 2H), 2.35–2.59 (m, 4H).

### 4.2.3. Dipeptide carbamate derivatives of quercetin (6 and 7)

Quercetin–alanine conjugate (**4a** and **5a**) (2 mmol) was dissolved in dimethylformamide (DMF) (4 mL), and 1-hydroxybenzotriazole (HOBT) (4 mmol) and H-Glu(O*t*Bu)-O*t*Bu·HCl (2 mmol) or H-Asp(O*t*Bu)-O*t*Bu·HCl (2 mmol) was added to the solution. And then 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide (EDC) (2 mmol) was added and the solution was stirred for 3 days at 0 °C. The resulting solution was concentrated under reduced pressure, and the residue was filtered through a short silica gel column washing with a mixture of $CH_2Cl_2$ and acetone (6:1). After concentration of the filtrate under reduced pressure, the residue was used for the next step without further purification. The *tert*-butyl-dipeptide quercetin carbamates obtained above (1.8 mmol) were dissolved in $CH_2Cl_2$ (5 mL). To this solution, trifluoroacetic acid (2 mL) was added at 0 °C, and the solution was stirred for 4 h at room temperature. The resulting solution was concentrated under reduced pressure, and the residue was recrystallized from a mixture of acetone (1 mL) and $CH_2Cl_2$ (10 mL). The mixture was filtered through a Bűchner or Hirsch funnel, and the filter cake was washed with $CH_2Cl_2$ to give mixtures of the regioisomers (**6** and **7**) as yellow solids.

**4.2.3.1. 4′-*O*-CO-Ala-Asp-quercetin (6h) and 3′-*O*-CO-Ala-Asp-quercetin (7h) (90% yield).** LC/MS (ESI) *m/z* Found: 547.3 $[M+H]^+$; Calcd for $C_{24}H_{22}N_2O_{13}$: 546.11; For **6h**, $^1H$ NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.16 (s, 1H), 7.96–8.00 (m, 2H), 7.11 (d, $J$ = 8.5 Hz, 1H), 6.56 (s, 1H), 6.27 (s, 1H), 4.50–4.58 (m, 1H), 4.30–4.36 (m, 1H), 2.40–2.50 (m, 4H), 1.46 (s, 3H); For **7h**, $^1H$ NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.09 (s, 1H), 7.88 (d, $J$ = 1.6 Hz, 2H), 7.73 (d, $J$ = 8.7 Hz. 1H), 7.31 (d, $J$ = 8.7 Hz, 1H), 6.56 (s, 1H), 6.27 (s, 1H), 4.50–4.58 (m, 1H), 4.30–4.36 (m, 1H), 2.40–2.50 (m, 4H), 1.46 (s, 3H).

**4.2.3.2. 4′-*O*-CO-Ala-Glu-quercetin (6i) and 3′-*O*-CO-Met-quercetin (7i) (92% yield).** LC/MS (ESI) *m/z* Found: 533.3 $[M+H]^+$; Calcd for $C_{23}H_{20}N_2O_{13}$: 532.10; For **6i**, $^1H$ NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.14 (s, 1H), 8.02–8.06 (m, 2H), 7.11 (d, $J$ = 8.6 Hz, 1H), 6.55 (s, 1H), 6.28 (s, 1H), 4.33–4.42 (m, 2H), 2.35–2.61 (m, 2H); For **7i**, $^1H$ NMR (400 MHz, acetone-$d_6$) $\delta$ (ppm) 12.08 (s, 1H), 7.92 (d, $J$ = 1.6 Hz, 1H), 7.78 (d, $J$ = 8.6 Hz, 1H), 7.35 (d, $J$ = 8.6 Hz, 1H), 6.55 (s, 1H), 6.28 (s, 1H), 4.33–4.42 (m, 2H), 2.35–2.61 (m, 2H).

## 5. Solubility test

Stock solution of the quercetin prodrugs were prepared at 10 mM, 25 mM, 50 mM, 100 mM, 150 mM, 200 mM and 250 mM in 1% DMSO, and then serially diluted in 99% phosphate buffered saline (PBS, pH 7.4) buffer. As a result, the diluted compounds have a final concentration of 100 μM, 250 μM, 500 μM, 1000 μM, 1500 μM, 2000 μM and 2500 μM. The volume of the test compound in each 96-well plate was set to be 250 μl, and the solubility was measured by the NEPHELOstar laser based microplate nephe-

lometer 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.

### 6. Stability test

Lysis buffer [RIPA buffer with PMSF (1 mM)] (60 μl, 10 mM) were added to $2.5 \times 10^4$ cells/well MDCK cells. This could break the cell membrane of the cells and release the cytoplasmic enzymes. PBS buffer (180 μl) was added which neutralized the medium to the optimum pH value (pH 7) for the enzymes. The quercetin prodrugs (60 μM) were added into the reaction mixture 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, filtered, 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).

### 7. Cell permeability assay

#### 7.1. Non-cell-based permeability

For non-cell-based permeability assay, a 5% solution (v/v) of hexadecane in hexane was prepared. This mixture was pipetted 15μ006C each in the 96-well MultiScreen permeability donor plate. The plates were allowed to dry for 1 h in a fume hood to ensure complete evaporation of the hexane resulting in a uniform layer of hexadecane, and added 300 μl of 1% DMSO in PBS buffer to each well of the MultiScreen permeability acceptor plate. The TEER (transepithelial electrical resistance) values were measured using a Millicell-ERS voltohmmeter. Inserts with TEER values >25 kΩ in layer were selected for transport experiments. The quercetin prodrugs were dissolved in a solution of 1% DMSO, 99% PBS buffer to the desired concentration range of 50–800 μM. The drug solution 150 μl was added to each well in the donor plate, the plate lid was placed, and the plate was incubated at room temperature for 5 h. After incubation, UV–vis absorption of donor and acceptor solution was measured by UV–vis spectrophotometer.

#### 7.2. MDCK cell culture

For drug transport experiments, MDCK cells were seeded onto 100 mm Cell Culture plate at a density of $2.5 \times 10^6$ cells/cm$^2$. Culture conditions were MDCK cell in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were grown at 37 °C in a humidified incubator with an atmosphere of 5% $CO_2$.

#### 7.3. MDCK cell-based permeability

For culture of MDCK cells, cell suspension was divided into sterile 15 mL centrifuge tubes and diluted with MDCK cell culture medium (DMEM) to $2.5 \times 10^4$ cells/well. Then, 75 μL of cell dilution was dispensed into the filter wells of the 96-well MultiScreen Caco-2 plates. Also, 250 μL of MDCK cell culture medium (DMEM) was added into each of the receiver plate of the 96-wells MultiScreen Caco-2 plates. The two compartments were gently reassembled and placed in the cell culture incubator, and incubated for 2–4 days at 37 °C in a humidified incubator with an atmosphere of 5% $CO_2$. When the cells have reached confluence and are differentiated, the MultiScreen Caco-2 plate was removed from the incubator and the electrical resistance for each well was determined. The TEER values across the cell monolayers were measured using a Millicell-ERS voltohmmeter. The electrical resistance of MDCK cell monolayers at the densities chosen ranged from 991 to 1055 Ω. And then, the monolayer formed by exchanging with the sterile PBS buffer, pH 7.4, in the filter plate was washed three times the volume of the culture medium. To determine the rate of drug transport in the apical to basolateral direction, 75 μL of the quercetin prodrug solution, diluted with PBS buffer, pH 7.4, to adjust drug concentration ranging from 0.05 μM to 10 μM, was added to the filter well (donor, apical plate). The transport analysis plate (acceptor, basolateral plate) was filled with 250 μL of PBS buffer. On the other hand, to determine transport rates from the basolateral to apical direction, 250 μL of the quercetin prodrugs was added to the transport analysis plate wells and filled the filter wells (apical compartment) were filled with 75 μL of the PBS buffer. The 96-well MultiScreen Caco-2 plates were incubated at 37 °C, 5% $CO_2$, 95% relative humidity and typical incubation times were 1–2 h. After incubation, UV–vis absorption of the 96-well filter plates and 96-well transport analysis plates were measured by UV–vis spectrophotometer.

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