A Novel Prodrug of Quercetin, 3-*N*,*N*-Dimethyl Carbamoyl Quercetin (DCQ), with Improved Stability against Hydrolysis in Cell Culture Medium

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The flavonoid quercetin (3',4',4,5,7-pentahydroxyflavone) (Fig. 1) is abundant in many common foods, especially in apples, onions, grapes, red wine and green tea. Quercetin shows favorable physiological activities such as antioxidant,[1] anti-inflammatory,[2] antiviral,[3,4] immunomodulatory,[5] and anticancer effect.[6] Particularly, anticancer activity of quercetin is known to be related with inhibition of a number of enzymes involved in proliferation and in the signal transduction pathway.[7,8] However, clinical trials exploring different schedules of administration of quercetin have been held back by its extreme water insolubility, requiring formulation in dimethy sulfoxide (DMSO).[6]

Also, only very low level of quercetin is found in blood or plasma even after a quercetin-rich meal due to fast metabolism into the methylated, sulfonylated, and glucuronidated quercetins.[7-11] The quercetin metabolites may not themselves have biological activities.[12,13]

To overcome these limitations, prodrug approaches which include an inactive precursor of quercetin have been attempted.[14] The glycine carbamate moiety of QC12 (Fig. 1) is designed to help quercetin being transported into blood stream. In the blood stream, the glycine carbamate is lost by hydrolysis to give the active ingredient, quercetin. However, due to the fast hydrolysis in blood (half-life, $t_{1/2}$ = 0.39 h).[14] QC12 has low bioavailability which limits its clinical use.

Recently, we reported synthesis and pharmacokinetic properties of several novel quercetin-amino acid conjugates (Fig. 1), which showed remarkable increases in water solubilities (6.8 - 53.0 fold increase relative to quercetin).[15] Moreover, quercetin-lysine and quercetin-glutamic acid conjugates showed resistance against enzymatic hydrolysis in the cell lysate which contained various activated hydrolyzing enzymes ($t_{1/2}$ = 3 h).[15] However, quercetin-amino acid conjugates were rapidly hydrolyzed (10 - 60 min) in DMEM (Dulbecco's Modified Eagle's Medium) cell culture medium[15] which implies their low stabilities in similar conditions such as body fluid. As catechol 4'-*O*-esters (for numbering system, see quercetin in Fig. 1) are chemically labile at neutral and alkaline pH to undergo fast acyl transfer as well as hydrolysis,[16] it seems reasonable to assume that the quercetin-amino acid conjugates with amino acid carbamate functionality at 4'-*O* position of the catechol moiety are susceptible for hydrolysis. Thus, we intended to move the amino acid carbamate group from the catechol to 3-OH or 7-OH group of quercetin. Attempted synthesis started from protection of the catechol moiety with diphenylmethylene ketal[17] followed by carbamoylation to give quercetin-3,7-di-*O*-amino acid conjugates (data not shown). However, under various deprotection conditions of the diphenylmethylene ketal, amino acid carbamate functionality was lost to give complex mixtures, which initiated extensive search for more stable promoieties than amino acid carbamates. In this context, Bambuterol, the bis-dimethyl carbamate prodrug of the β_2-agonist terbutaline for use in the treatment of asthma, attracted our attention due to its remarkably increased residence time (24 h) compared with that of terbutaline (4 - 7 h).[18-22] which demonstrated the use of dimethyl carbamate group as a promoiety resistant for hydrolysis.

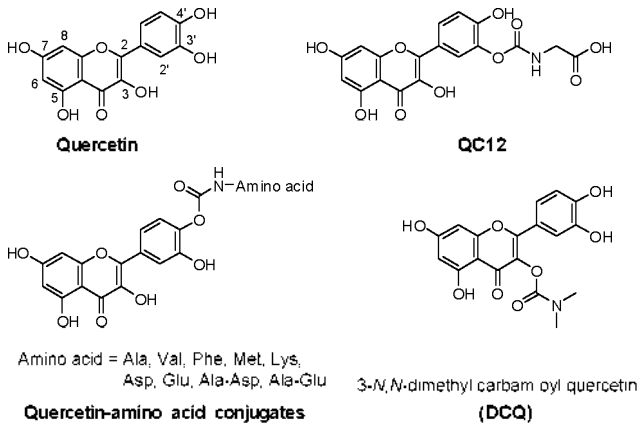


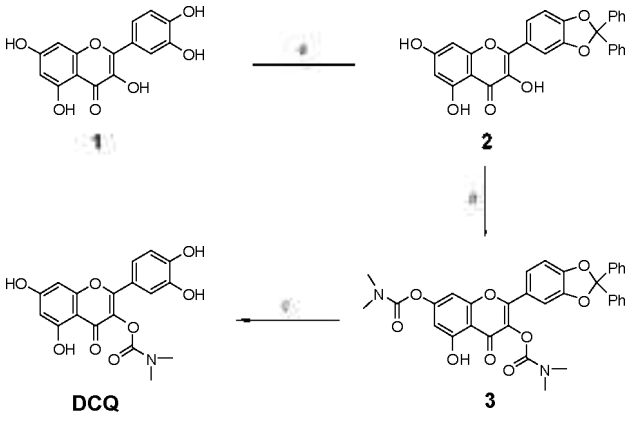
Figure 1. Structures of quercetin, QC12, quercetin-amino acid conjugates, and DCQ.

In this study, we introduced a dimethyl carbamate promoiety at the 3-*O* position of quercetin [3-*N*,*N*-dimethyl carbamoyl quercetin (DCQ), Fig. 1] and evaluated its physicochemical and *in vitro* pharmacokinetic properties including water solubility, chemical stability in buffer solution as well as cell culture medium, enzymatic stability, and cell permeability across the MDCK (Madin-Darby canine kidney) cell.

For the synthesis of the 3-*N*,*N*-dimethyl carbamoyl quercetin (DCQ), the catechol ring of quercetin was selectively protected with diphenylmethylene ketal.[17] The 3',4'-bis-protected quercetin **2** thus obtained was treated with dimethyl carbamoyl chloride to give 3,7-disubstituted quercetin **3**. Deprotection of the catechol ring proceeded with concurrent deprotection of the 7-*O* substituent to give the desired 3-*N*,*N*-dimethyl carbamoyl quercetin (DCQ) in 85% yield (Scheme 1).[25]

The synthesized DCQ was evaluated for its water solubility,

[a]These two authors contributed equally to this work



Reagents and conditions: (a) Ph_2CCl_2, 180 °C, 10 min; (b) dimethyl carbamoyl chloride, NaH, THF, 0 °C to rt; (c) Pd/C, H_2, THF/MeOH, rt.

Scheme 1. Synthesis of the 3-*N*,*N*-dimethyl carbamoyl quercetin (DCQ)

cell permeation, and stability against hydrolysis by using the same protocol as previously described.[15]

For solubility test, a stock solution of DCQ in DMSO was serially diluted with PBS (phosphate buffered saline, pH 7.4) buffer to give a series of samples with increasing concentrations of which forward light scattering were measured by the NEPHELOstar laser based microplate nephelometer.[15] DCQ showed 14 times increased water solubility (698 μM) compared with quercetin (50 μM) presumably due to the presence of a polar dimethylamido group.

Stability (half-life, $t_{1/2}$) of DCQ under non-enzymatic as well as enzymatic hydrolysis conditions was estimated by HPLC.[15] Under non-enzymatic hydrolysis conditions such as DMEM cell culture medium and PBS buffer, DCQ underwent neither decomposition nor hydrolysis up to 18 h (Fig. 2a). Compared with the half-lives of QC12 in PBS buffer ($t_{1/2}$ = 16.9 h)[14] and quercetin-amino acid conjugates in DMEM cell culture medium ($t_{1/2}$ = 10 min ~ 60 min),[15] DCQ showed remarkably increased stability against non-enzymatic hydrolysis. On the other hand, the dimethyl carbamate promoiety of DCQ smoothly underwent hydrolytic cleavage by cellular hydrolyzing enzymes obtained by lysis of MDCK cell with half-life of 0.5 h (Fig. 2b). Taken together, the 3-*N*,*N*-dimethylcarbamoyl promoiety of DCQ turned out to be strongly resistant against non-enzymatic hydrolysis in PBS buffer and DMEM cell culture medium but effectively cleaved to provide the active ingredient quercetin inside the cell by cellular enzymes.

For permeability test of DCQ, monolayers of an artificial hexadecane as well as MDCK cell were prepared in the filter well of the 96-well MultiScreen permeability plate composed of three compartments; donor plate, filter well, and acceptor plate.[24] The formation of monolayer was confirmed by measuring TEER (transepithelial electrical resistance) values with Millicell-ERS voltohmmeter. DCQ or quercetin was added to the donor plate and, after incubation, concentrations of DCQ or quercetin in the donor as well as acceptor plate were measured by UV-Vis spectrophotometer.[15] Neither quercetin nor DCQ showed passive transport across an artificial membrane made of hexadecane (data not shown). On the contrary, compared with quercetin,

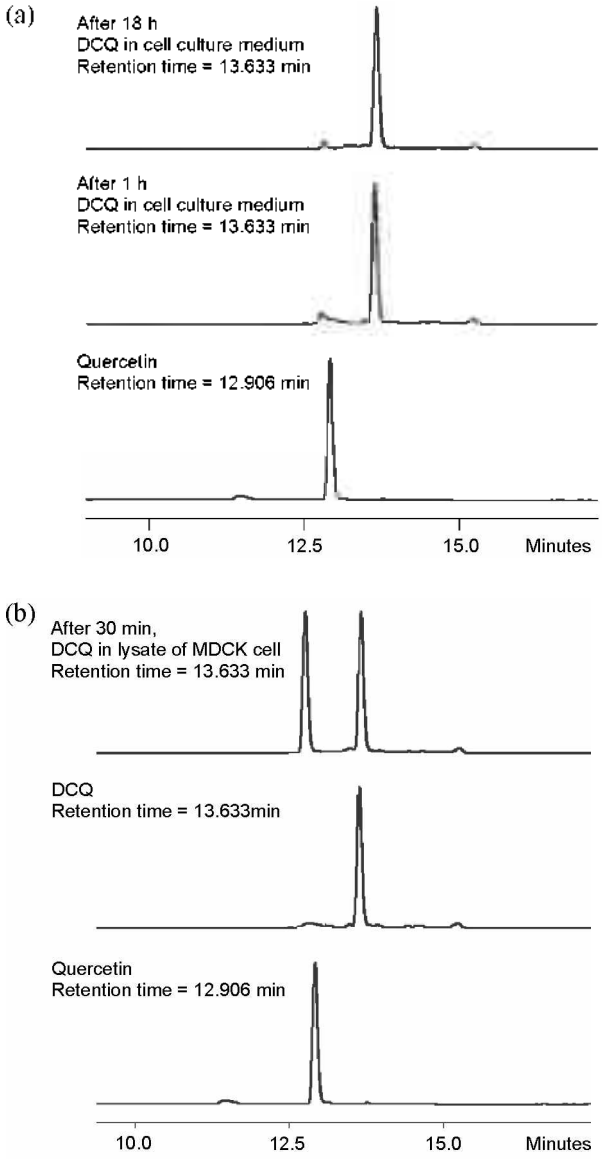


Figure 2. Half-life of DCQ in cell culture medium (a) and lysate of MDCK cell (b).

Table 1. Water solubility, stability, and permeability of quercetin (Qu), QC12, Qu-E[a] and DCQ

Compound		Qu	QC12	Qu-E[a]	DCQ
Water solubility (μM)		50	ND[b]	2,649	698
Stability ($t_{1/2}$, h)	PBS buffer	–	16.9[c]	> 17	> 18
	Cell culture medium	–	0.39[c,d]	0.5	> 18
	Cell lysate	–	ND[b]	3.0	0.5
Relative Permeability (MDCK cell)		1.0	ND[b]	5.2	1.6

[a]3'-*O* or 4'-*O* quercetin-glutamic acid conjugate (taken from reference 15). [b]Not determined. [c]Taken from reference 14. [d]In blood stream.

DCQ showed 1.6 times increased permeability from the apical to the basolateral compartment of the MDCK cell, which

indicates effective transport of DCQ into the cell presumably *via* cellular transporters.

In summary, in order to prevent fast hydrolysis in blood stream or cell culture medium and thereby low bioavailability of a quercetin-amino acid conjugates with promoieties attached at the labile catechol moiety (3′-*O* or 4′-*O*), we introduced a dimethyl carbamoyl promoiety at 3-*O* position of quercetin (DCQ). Analysis of DCQ revealed several favorable physicochemical and *in vitro* pharmacokinetic properties of a prodrug including increased water solubility, stability and cell permeability. In particular, the chemical stability of DCQ against nonenzymatic hydrolysis in cell culture medium is noteworthy, which might have resulted from regioselective introduction of the carbamate at the non-labile 3-*O*-position of quercetin. This result warrants further investigation of quercetin prodrugs by regioselective introduction of various promoieties at non-labile 3-*O* or 7-*O* position of quercetin.

Experimental Section

Preparation of DCQ (**4**): Sodium hydride (1.1 mmol) was added to a solution of compound **2** (1.1 mmol) in THF (5 mL) at 0 °C. After 30 min, dimethyl carbamoyl chloride was added and the solution was stirred for 4 h at 0 °C. The mixture was neutralized by ammonium chloride. And then, the resulting solution was extracted with CH_2Cl_2 (3 × 50 mL), dried over $MgSO_4$, and filtered. The filtrate was concentrated under reduced pressure, and the residue was purified by column chromatography on silica gel (Hexane : EtOAc = 1 : 2) to afford **3** as dark yellow solid in 55% yield: ^{1}H NMR (400 MHz, Acetone-d_6) δ 12.28 (s, 1H), 7.57 - 7.62 (m, 6H), 4.47 (d, J = 1.79 Hz, 1H), 7.40 - 7.42 (m, 5H), 6.98 (d, J = 11.2 Hz, 1H), 6.87 (d, J = 2.0 Hz, 1H), 6.57 (d, J = 2.0 Hz, 1H), 3.15 (s, 3H), 3.03 (s, 6H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 175.8, 160.0, 156.9, 155.3, 152.6, 152.3, 149.0, 148.9, 146.8, 139.3, 139.2, 131.7, 131.1, 129.6, 128.4, 125.8, 124.2, 123.8, 123.6, 122.8, 117.7, 109.2, 108.4, 107.7, 105.6, 103.8, 101.6, 98.1, 37.5, 37.4, 31.2, 31.1.

Palladium on charcoal (10% 0.06 mmol) was added to a stirred solution of **3** (0.6 mmol) obtained above in MeOH/THF (1:2.5 mL) under hydrogen atmosphere. The suspension was stirred for 24 h and then filtered through a plug of Celite and eluted with acetone (50 mL). The filtrate was concentrated under reduced pressure, and the residue was purified by column chromatography on silica gel (Hexane : EtOAc = 1 : 1) to afford the 3-*N*,*N*-dimethyl carbamoyl quercetin (DCQ) (**4**) as dark yellow solid in 85% yield: ^{1}H NMR (400 MHz, Acetone-d_6) δ 2.48 (s, 1H), 7.48 (s, 1H), 7.40 (d, J = 8.9 Hz, 1H), 7.00 (d, J = 7.9 Hz, 1H), 6.63 (s, 1H), 6.29 (s, 1H), 3.15 (s, 3H), 2.97 (s, 3H); ^{13}C NMR (125 MHz, DMSO-d_6) δ 175.8, 159.0, 155.0, 153.9, 151.0, 144.5, 144.2, 142.1, 128.1, 118.5, 117.6, 114.1, 113.4, 100.4, 97.9, 92.9, 31.2, 31.1; LC/MS (ESI) m/z Found: 374.1 $[M + H]^+$; Calcd for $C_{18}H_{15}NO_8$: 373.08.

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