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Quercetin-Glutamic Acid Conjugate with a Non-Hydrolysable Linker; A Novel Scaffold for Multidrug Resistance Reversal Agents through Inhibition of P-Glycoprotein

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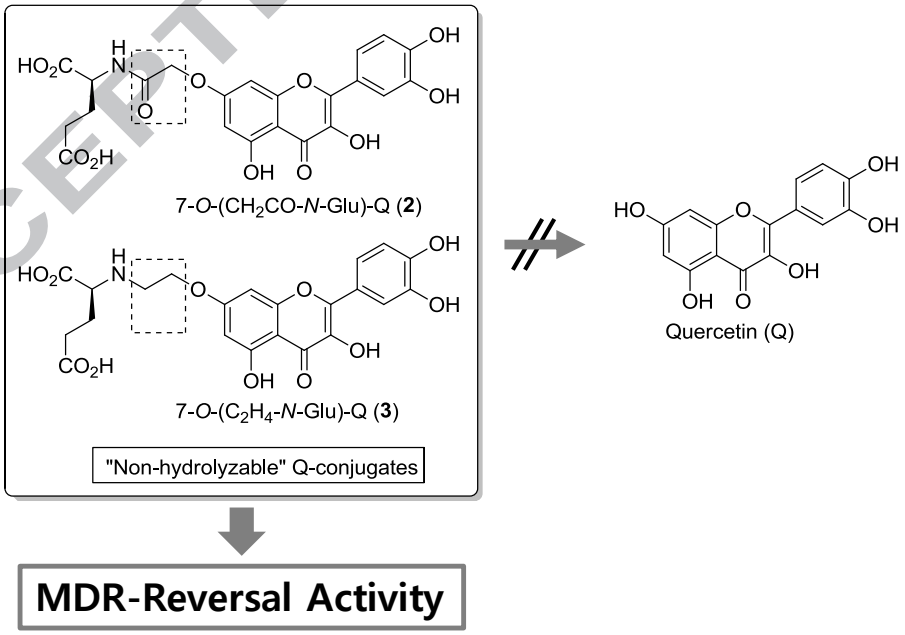
Graphical Abstract



Keywords: Multidrug resistance (MDR) · Cancer · Quercetin · Conjugate · Reversal activity

ABSTRACT

Previously, we have reported remarkable effect of a quercetin-glutamic acid conjugate to reverse multidrug resistance (MDR) of cancer cells to a broad spectrum of anticancer agents through inhibition of P-glycoprotein (Pgp)-mediated drug efflux. Due to the hydrolysable nature, MDR-reversal activity of the quercetin conjugate was attributed to its hydrolysis product, quercetin. However, several lines of evidence demonstrated that the intact quercetin-glutamic acid conjugate has stronger MDR-reversal activity than quercetin. In order to evaluate this hypothesis and to identify a novel scaffold for MDR-reversal agents, we prepared quercetin conjugates with a glutamic acid attached at the 7-*O* position via a non-hydrolysable linker. Pgp inhibition assay, Pgp ATPase assay, and MDR-reversal activity assay were performed, and the non-hydrolysable quercetin conjugates showed significantly higher activities compared with those of quercetin. Unfortunately, the quercetin conjugates were not as effective as verapamil in Pgp-inhibition and thereby reversing MDR, but it is worth to note that the structurally modified quercetin conjugates with a non-cleavable linker showed significantly improved MDR-reversal activity compared with quercetin. Taken together, the quercetin conjugates with appropriate structural modifications were shown to have a potential to serve as a scaffold for the design of novel MDR-reversal agents.

1. Introduction

Multidrug resistance (MDR) renders cancer cells insensitive to anticancer agents, and it is one of the major obstacles to the cancer chemotherapy[1]. Various mechanisms have been proposed to be involved in cancer MDR and, among those, overexpression of P-glycoprotein (Pgp) and thereby anticancer drug efflux is the most well-characterized cellular mechanism[2-3]. There have been numerous attempts to block the Pgp-mediated anticancer drug efflux by using chemical entities, but none is currently available for clinical use. First-generation agents represented by cyclosporine and verapamil showed unacceptable toxicity[4-5]. The ensuing second- (dexverapamil[6], valspodar[7-8], elacridar[9], and biricodar[10-12]) and third- (tariquidar[13], zosuquidar[14], laniquidar[15], and ONT-093[16]) generation MDR-reversal agents were obtained by structural modification of the first-generation molecules, but they suffered from unpredictable pharmacokinetic behavior and toxicity. Therefore, new chemical entities with favorable physicochemical, pharmacokinetic and safety profiles are urgently required for development of novel MDR-reversal agents.

In this context, natural flavonoids with well-known safety profiles in addition to the MDR-reversal activity have drawn special attention[17-28]. Previously, we have shown that the MDR-reversal activity of quercetin (Q, Fig. 1), a natural flavonoid abundant in fruits and vegetables, can be significantly enhanced through conjugation with glutamic acid (7-*O*-Glu-Q (**1**), Fig. 1)[27]. The quercetin conjugate **1** was designed as a prodrug and, as anticipated, it was hydrolyzed to quercetin in the intracellular milieu ($t_{1/2}$ = 9.3 h) to keep intracellular concentration of quercetin high enough to show MDR-reversal activity[27]. Thus, the MDR-reversal activity of **1** was attributed to its hydrolysis product, quercetin. On the other hand, however, a functional assay for Pgp showed that the quercetin conjugate **1** binds to the drug-binding cavity of Pgp to stimulate Pgp ATPase activity by 3.5-fold, which is significantly

higher than the quercetin-induced stimulation (1.8-folds)[27]. Also noteworthy is that, in Pgp inhibition assay, 15-minute incubation of the Pgp-overexpressing MDR cells with **1** resulted in effective inhibition of rhodamine 123 efflux[27]. Considering that **1** did not undergo hydrolysis up to 6 h under the same incubation conditions, it is highly unlikely that the hydrolysis product of **1**, quercetin, was responsible for Pgp inhibition. Taken together, there was ample reason to suspect that the intact quercetin-glutamic acid conjugate **1** has stronger MDR-reversal activity than quercetin. In order to evaluate this hypothesis and to identify a novel scaffold for MDR-reversal agents, we designed quercetin conjugates with a glutamic acid attached at the 7-*O* position via a non-hydrolysable linker. Herein, we report synthesis and biological evaluation of two non-hydrolysable quercetin-glutamic acid conjugates (**2** and **3**, Fig. 1).

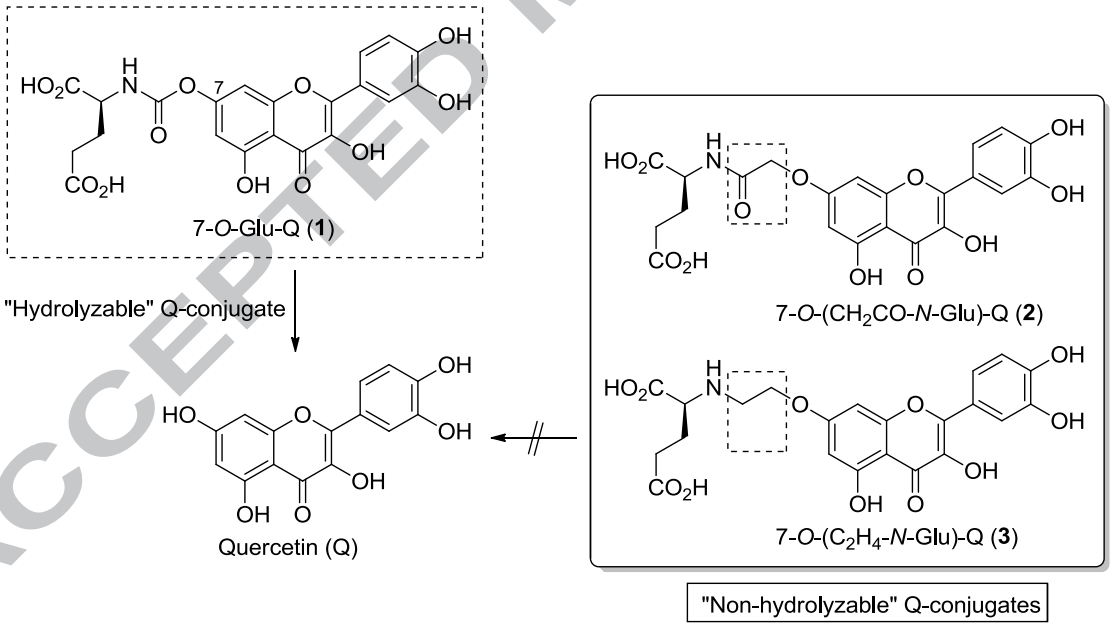
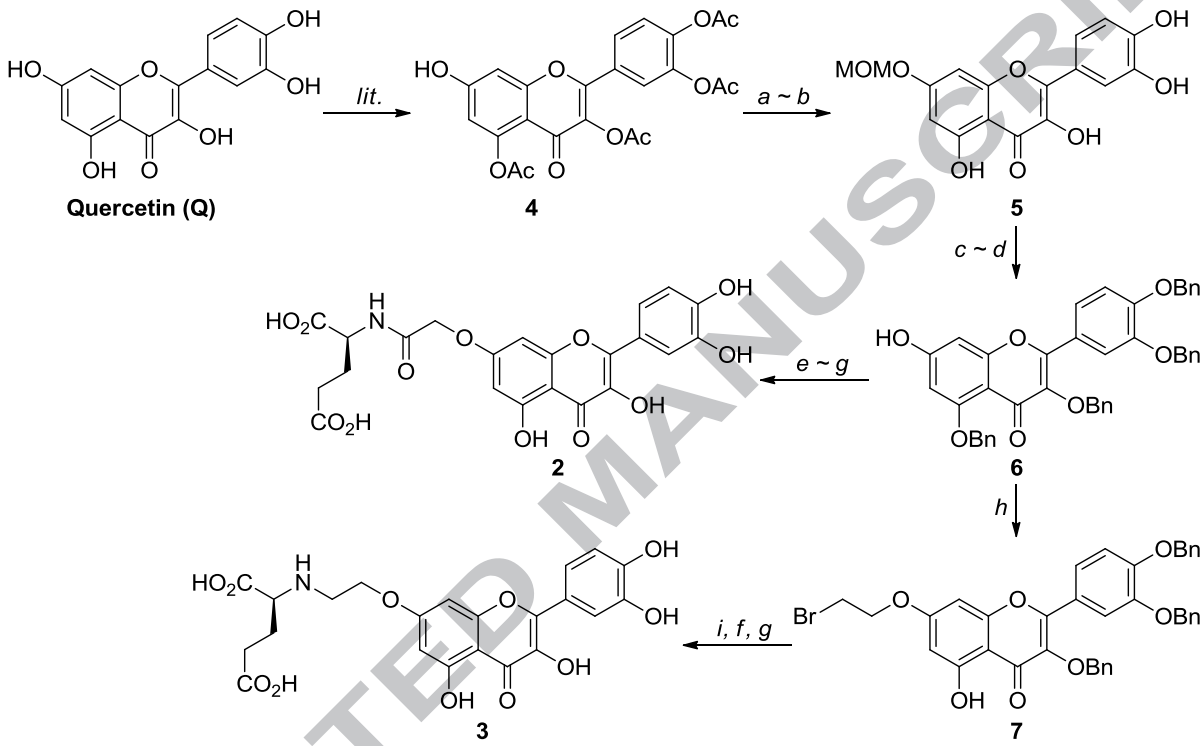


Figure 1. Structures of quercetin (Q), 7-*O*-Glu-Q (**1**) and the title compounds (**2** and **3**)

2. Results and Discussion

2.1. Chemistry

In order to synthesize the title compounds (**2** and **3**), the 7-OH position of quercetin was differentiated from other hydroxyl groups by using the well-known synthetic procedures[29-30], and the 3,5,3′,4′-*O*-tetraacetylquercetin (**4**) was prepared in 73% yield from quercetin (Scheme 1).



Reagents and conditions: a) MOMCl, K_2CO_3, acetone, rt; b) NH_3/MeOH, 0 °C to rt; c) BnBr, K_2CO_3, acetone, rt; d) 3N HCl, THF, 40 °C; e) ($ClCH_2CO$)-Glu(OtBu)-(OtBu), K_2CO_3, DMF, rt; f) Pd/C, H_2, THF, MeOH, rt; g) TFA, CH_2Cl_2, 0 °C; h) 1,2-dibromoethane, K_2CO_3, Acetone, KI, rt; i) H-Glu(OtBu)-OtBu·HCl, DIPEA, THF, DMF, rt

Scheme 1. Synthesis of title compounds **2** and **3**

The intermediate **4**, thus obtained, was converted into 7-*O*-methoxymethyl quercetin (**5**) after protection of the 7-OH functionality with a methoxymethyl (MOM) group followed by methanolysis of four acetyl groups at 3, 5, 3′, and 4′ positions. Treatment of **5** with excess amount of BnBr in the presence of K_2CO_3 in acetone provided the corresponding 3,5,3′,4′-*O*-tetrabenzyl ether which, after deprotection of the 7-*O*-MOM group under the acidic conditions, was converted into the key intermediate 3,3′,4′-*O*-tribenzylquercetin (**6**) in 78%

yield. Nucleophilic substitution of **6** with *N*-(chloromethylcarbonyl)-Glu(OtBu)-(OtBu) followed by successive deprotection of the benzyl and *tert*-butyl functionalities afforded the desired product **2** in 34% combined yield. On the other hand, treatment of **6** with 1,2-dibromoethane provided **7** in 68% yield, which, after substitution with H-Glu(OtBu)-(OtBu)·HCl followed by sequential removal of the benzyl and *tert*-butyl protecting groups, provided another desired product **3** in 27% yield.

2.2. Intrinsic cytotoxicity and non-hydrolysable nature of 2 and 3

The synthesized quercetin-glutamic acid conjugates (**2** and **3**) were first evaluated for their antioxidant activity, the characteristic bioactivity of quercetin. As it is well known that the antioxidant activity of quercetin strongly depends on its B-ring catechol moiety[31], the newly synthesized quercetin conjugates with alkyl substituents at the 7-*O* positions did not show any difference in DPPH radical scavenging activity compared with quercetin (data not shown).

The toxicity of **2** and **3** in drug-sensitive human uterine sarcoma cell line MES-SA and Pgp-overexpressed MDR MES-SA/Dx5 cells[27-28, 32] were then determined but no significant cytotoxicity was observed up to 100 μM concentration (data not shown). Next, the non-hydrolysable nature of the title compounds (**2** and **3**) was confirmed. Thus, MES-SA and MES-SA/Dx5 cells were incubated with **2** or **3**, and the cell lysates were analyzed by HPLC. No trace of quercetin was observed from the cell lysates but the title compounds **2** and **3** underwent slow decomposition with half-lives of 30 h and 56 h, respectively (Fig. 2).

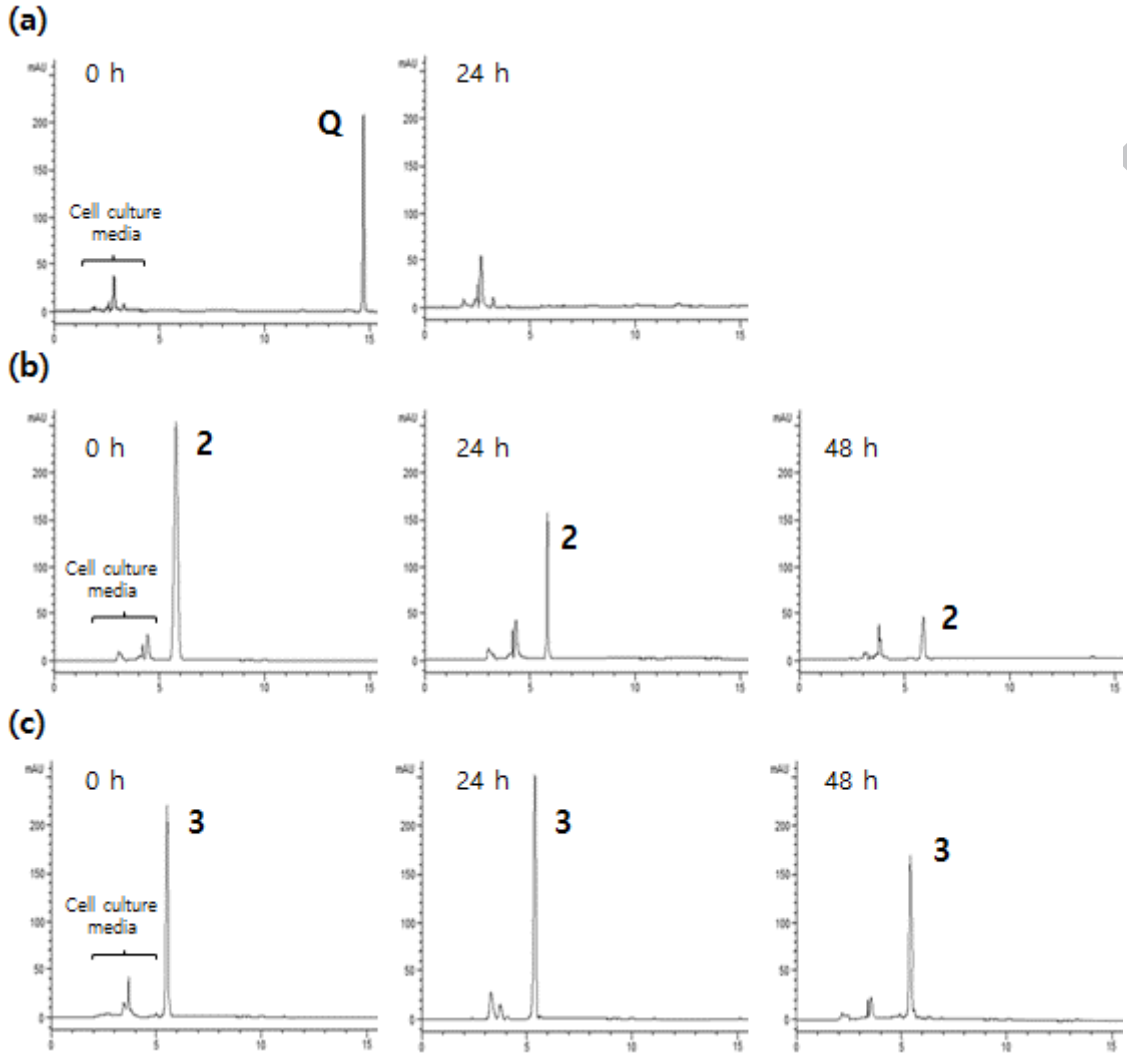


Figure 2. HPLC chromatograms of MES-SA/Dx5 cell lysates after incubation with (a) quercetin (Q), (b) **2**, and (c) **3** for 0 h, 24 h and 48 h

2.3. Pgp-inhibitory activity

Pgp-inhibitory activity of the quercetin conjugates (**2** and **3**) was then evaluated. Doxorubicin (DOX) was used as a fluorescent substrate of Pgp, and the assay was performed by measuring fluorescence of DOX inside the MES-SA or MES-SA/Dx5 cell after combined treatment of DOX with the quercetin conjugate (**2** or **3**)[33]. Flow cytometric analysis of fluorescence from intracellularly localized DOX showed that significantly decreased

fluorescence in the Pgp-overexpressed Mes-SA/Dx5 cells were recovered by addition of the Pgp inhibitor verapamil (5 μM) (Fig. 3). By the same token, the quercetin conjugates **2** and **3** (5 μM) showed similar effects albeit with a lower potency; **2** and **3** increased DOX fluorescence by 2.3-fold and 2.1-fold, respectively, while verapamil showed 5.5-fold increase (Fig. 3).

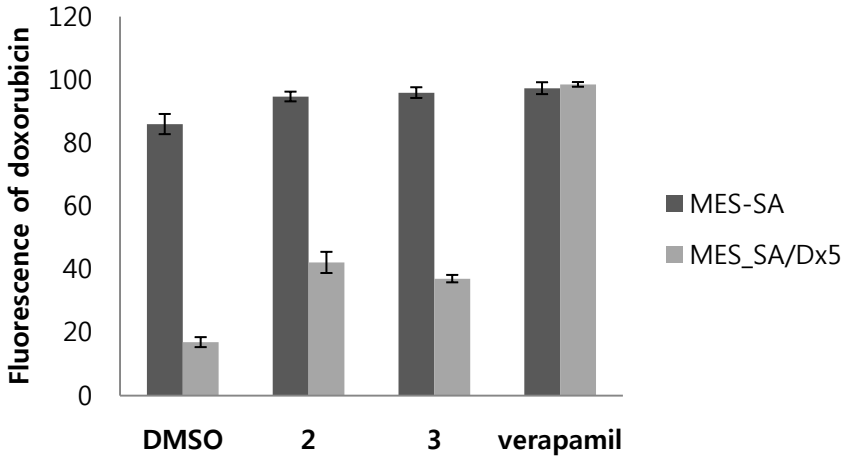


Figure 3. Fluorescence from intracellularly localized doxorubicin (DOX) in MES-SA and MES-SA/Dx5 cells in the absence (DMSO) and presence of the Pgp inhibitors (**2**, **3** and verapamil) (5 μM). Results are presented as mean ± SD from three experiments

2.4. Pgp ATPase stimulation

Pgp inhibitors bind to one of the two binding sites (drug binding cavity and NTP binding domain) inside Pgp and, depending on the binding site, they show either inhibition[24] or stimulation[34] of Pgp-mediated ATP hydrolysis: compounds that bind to the NBD inhibit ATP hydrolysis by Pgp[24], while others that bind to the drug-binding cavity typically stimulate ATPase activity of Pgp[34]. Therefore, evaluation of Pgp ATPase activity upon addition of the Pgp inhibitor is known to be the method of choice for determining its binding site inside Pgp[24,26,27-28]. In this study, effects of the quercetin conjugates (**2** and **3**) (100 μM) on the Pgp ATPase activity were evaluated by Pgp-Glo™ assay (Promega, Madison, WI), and the results

are summarized in Fig. 4.

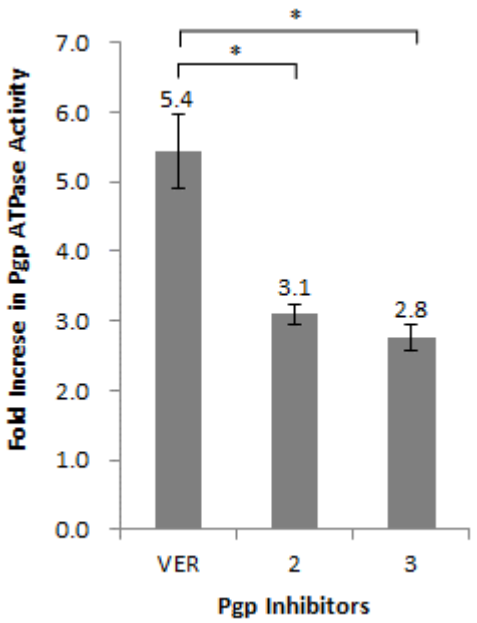


Figure 4. Fold increases in Pgp ATPase activity conferred by Pgp inhibitors (100 μM). VER; verapamil. The means ± SD (error bars) of three independent experiments are shown. Asterisks represent significant differences compared with the verapamil-stimulated Pgp ATPase activity; $p < 0.001$.

Verapamil (100 μM), a known binder to the drug-binding site in TBD[35], showed to stimulate Pgp ATPase activity over the basal level by 5.4-fold (Fig. 4, "VER"). Compound **2** (or **3**) (100 μM) was also found to stimulate ATPase activity by 3.1-fold (or 2.8-fold). Even though **2** (or **3**) is a weaker stimulator than verapamil, this result clearly indicates that, like verapamil, **2** (or **3**) also binds to the drug-binding site in TBD rather than nucleotide binding domain (NBD). This result is reminiscent of the previous reports that the substituted flavonoids preferentially bind to the drug-binding site of Pgp[19,24,26,27-28].

2.5. MDR-reversal activity

The quercetin conjugates (**2** and **3**) with inhibitory activity against Pgp-mediated

doxorubicin efflux were then evaluated for their MDR-reversal activity (Fig. 5 and Table 1).

(a) (b)

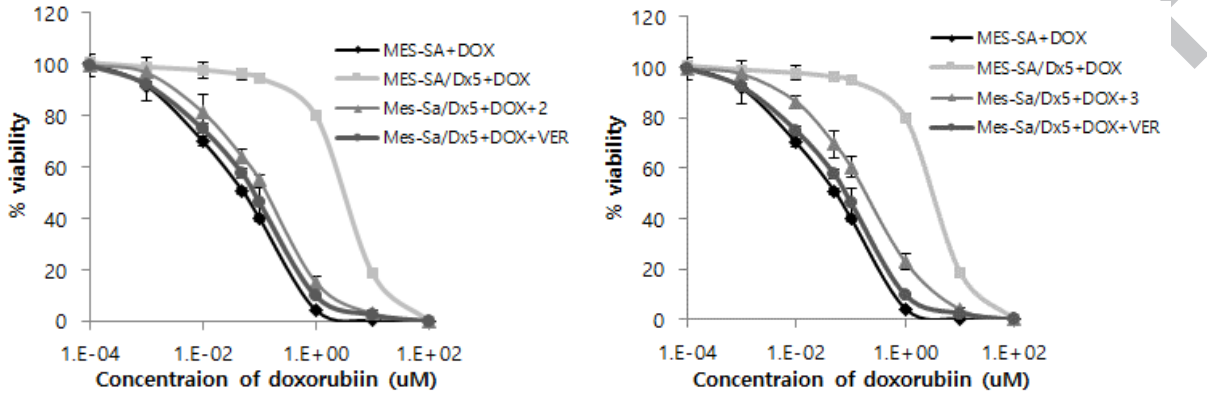


Figure 5. Cell viabilities of MES-SA and MES-SA/Dx5 cells before and after combination of doxorubicin (DOX) with (a) **2** and (b) **3**. Verapamil (VER) was used as a positive control and concentrations of VER, **2** and **3** were fixed at 5 μM. Assay was performed in triplicate

Table. 1 The MDR-reversal activity of quercetin (Que), quercetin conjugates (**2** and **3**), and verapamil (VER) on the sensitivities of MES-SA/Dx5 to anticancer agents[a]

	Anticancer Agents							
	Doxorubicin		Actinomycin D		Vinblastine		Paclitaxel	
	EC_{50} (μM)[b]	FR[c]	EC_{50} (μM)	FR	EC_{50} (μM)	FR	EC_{50} (μM)	FR
None[d]	8.71 ± 0.35	—	14.52 ± 0.22	—	13.26 ± 0.18	—	11.08 ± 0.12	—
Que	4.48 ± 0.14*	1.94	4.75 ± 0.32*	3.06	4.82 ± 0.26*	2.75	4.46 ± 0.24*	2.48
2	0.34 ± 0.03*	25.6	0.30 ± 0.05*	48.4	0.51± 0.02*	26.0	0.35 ± 0.03*	34.6
3	0.39 ± 0.05*	22.3	0.71 ± 0.03*	20.5	0.82± 0.03*	16.2	0.64 ± 0.02*	17.3
VER	0.16 ± 0.02	54.4	0.18 ± 0.01	80.7	0.24 ± 0.01	55.3	0.20 ± 0.03	55.4

[a] Concentrations of the MDR-reversal agents were fixed at 5 μM.

[b] Amounts of the anticancer agents required to reduce 50% viability of the drug-resistant (MES-SA/Dx5) cells in the presence of the MDR-reversal agents. The means ± SD (error bars) of three independent experiments are shown.

[c] FR = Fold Reversal = IC_{50} (untreated) / IC_{50} (treated with MDR-reversal agents)

[d] The row "None" denotes IC_{50} values of the anticancer agents obtained in the absence of the MDR-reversal agents. Asterisks represent significant differences compared with these control values; $p < 0.01$. The means ± SD (error bars) of three independent experiments are shown.

In drug-sensitive MES-SA cells, anticancer drug doxorubicin showed potent cytotoxic activity with IC_{50} value of 0.067 μM (♦, Fig. 5a and 5b). In contrast, doxorubicin showed significantly decreased activity in the Pgp-overexpressed MDR MES-SA/Dx5 cells with 130-fold increase in IC_{50} value (IC_{50} = 8.71 μM) (■, Fig. 5a and 5b; Table 1). Upon combination with verapamil (5 μM), however, doxorubicin showed dramatically increased cytotoxicity in MES-SA/Dx5 cells, and its IC_{50} value (IC_{50} = 0.16 μM) was comparable to that obtained in drug-sensitive MES-SA cells (●, Fig. 5a and 5b; Table 1). Quercetin also showed MDR-reversal activity but the cytotoxicity of doxorubicin increased by only 1.94-fold upon combination with quercetin (Table 1). The quercetin conjugates **2** and **3**, however, exhibited more potent MDR-reversal activity than quercetin, and they potentiated doxorubicin by 25.6-fold (**2**) (▲, Fig. 5a and Table 1) and 22.3-fold (**3**) (▲, Fig. 5b and Table 1), respectively, in MES-SA/Dx5 cells.

The quercetin conjugates **2** and **3** were also effective in reversing MDR upon combination with other anticancer drugs such as actinomycin D, vinblastine and paclitaxel (Table 1). Thus, in MES-SA/Dx5 cells, 5 μM of **2** potentiated cytotoxicity of these drugs by 48.4, 26.0 and 34.6-fold, respectively (Table 1). Compound **3** was less effective than **2**, and it provided 20.5, 16.2 and 17.3-fold increases in the cytotoxic activity of actinomycin D, vinblastine and paclitaxel, respectively (Table 1).

The MDR-reversal activity of **2** and **3** were further evaluated at various concentrations (0, 0.5, 1, 2.5, 5 and 10 μM), and their potencies were determined by the amounts required to reduce IC_{50}s of the anticancer agents by half in the drug-resistant MES-SA/Dx5 cells (EC_{50} values)[27]

(Fig. 6 and Table 2).

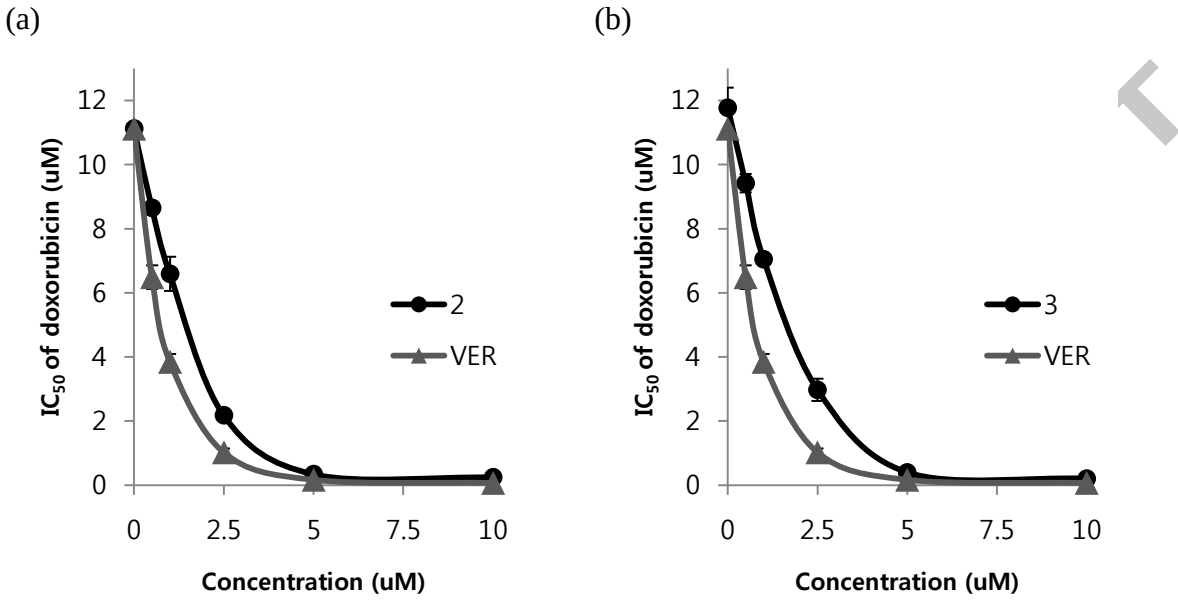


Figure 6. Concentration-dependent effects of (a) **2** and (b) **3** for lowering IC_{50}s of doxorubicin in MDR MES-SA/Dx5 cells. Verapamil (VER) was used as a positive control. Assay was performed in triplicate

Table. 2 MDR-reversal potency (EC_{50}) of the quercetin conjugates (**2** and **3**) in the drug-resistant (MES-SA/Dx5) cells

	EC_{50} (μM)[a]			
	Doxorubicin	Actinomycin D	Vinblastine	Paclitaxel
2	2.2 ± 0.3*	2.3 ± 0.2*	2.5 ± 0.4*	2.1 ± 0.3*
3	2.8 ± 0.2*	2.7 ± 0.4*	2.4 ± 0.7*	2.5 ± 0.2*
Verapamil	0.7 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.8 ± 0.1

[a]Amount of the MDR-reversal agents required to reduce the IC_{50}′s of the anticancer agents by half in the drug-resistant MES-SA/Dx5 cell line. The means ± SD of three independent experiments are shown. Asterisks represent significant differences compared with EC_{50} of verapamil; $p < 0.01$.

As anticipated by the inhibitory activity against Pgp, both **2** (Fig. 6a) and **3** (Fig. 6b) showed remarkable effects in reversing resistance toward doxorubicin with EC_{50} values of 2.2 μM and 2.8 μM, respectively (Table 2). Also, compounds **2** and **3** potently sensitized

MDR MES-SA/Dx5 cells to a broad spectrum of anticancer drugs (actinomycin D, vinblastine and paclitaxel) with EC_{50} values of 2.1−2.8 μM (Table 2). Compared with verapamil (EC_{50} = 0.7−1.0 μM), compounds **2** and **3** were 2.6-4 folds less potent, but it should be noted that the substituted quercetins represent a novel scaffold for MDR-reversal agents.

3. Conclusions

Based on our previous observation that the intact quercetin-glutamic acid conjugate might have stronger MDR-reversal activity than quercetin, we prepared quercetin conjugates with a glutamic acid attached at the 7-*O* position via a non-hydrolysable linker (**2** and **3**). Biological activity of the quercetin conjugates (**2** and **3**) were assessed by Pgp inhibition assay, Pgp ATPase assay, and MDR-reversal activity assay, and the non-hydrolysable quercetin conjugates showed significantly higher activities compared with those of quercetin. Unfortunately, however, the quercetin conjugates were not as effective as verapamil in Pgp-inhibition and thereby reversing MDR. Nevertheless, it is worth to note that the structurally modified quercetin conjugates with a non-cleavable linker showed significantly improved MDR-reversal activity compared with quercetin. Thus, this study established that the quercetin conjugate with appropriate structural modifications has a potential to serve as a scaffold for the design of novel MDR-reversal agents. Further studies are warranted to explore the molecular determinants for Pgp-inhibition of the quercetin conjugates and the structure-activity relationships.

4. Experimental

4.1. Materials and General Methods

Chemicals were purchased from Sigma-Aldrich unless noted otherwise. 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. ESI mass spectrometric data were obtained at Korea Basic Science Institute (Daegu, Korea) and reported in the form of *m/z* (intensity relative to base peak=100). Nuclear magnetic resonance spectra were recorded on Bruker 400 AMX spectrometer (Karlsruhe, Germany) at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR with tetramethylsilane as an internal standard. Chemical shifts were reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br s (broad singlet). Coupling constants were reported in hertz (Hz). Chemical shifts were reported as parts per million (δ) relative to the solvent peak.

4.2. Synthesis of the title compounds

4.2.1. 2-(3,4-Dihydroxyphenyl)-3,5-dihydroxy-7-(methoxymethoxy)-4*H*-chromen-4-one (5)

A mixture of potassium carbonate (269 mg, 2.1 mmol) and chloromethyl methyl ether (0.2 mL, 2.7 mmol) were added to a solution of **4** (1.0 g, 2.1 mmol) in acetone (20 mL). After stirring for 4 h at room temperature, the reaction mixture was filtered and the filtrate was concentrated under reduced pressure to give a pale yellow syrup. The crude product was purified by column chromatography on silica gel (2:1 = Hexane:EtOAc) to give a yellow powder (1.1 g, 2.1 mmol, 100% yield). The resulting methoxymethyl (MOM)-protected quercetin (1.1 g, 2.1 mmol) was dissolved in NH_3/MeOH (10 mL) and stirred at 0 ^{o}C for 4 h. The reaction mixture was concentrated under reduced pressure, and the residue was purified by column chromatography on silica gel (1:1 = Hexane:EtOAc) to afford **5** (692 mg, 2.0 mmol, 95% yield) as a yellow powder: ^{1}H NMR (400 MHz, Acetone-d_6) δ 12.34 (s, OH),

7.87 (d, *J* = 2.0 Hz, 1H, $H_{2'}$), 7.74 (dd, *J* = 2.0, 8.4 Hz, 1H, $H_{6'}$), 7.00 (d, *J* = 8.5 Hz, 1H, $H_{5'}$), 6.77 (d, *J* = 2.0 Hz, 1H, H_8), 6.41 (d, *J* = 2.0 Hz, 1H, H_6), 5.34 (s, 2H, -CH_2), 3.48 (s, 3H, -CH_3).

4.2.2. 3,5-Bis(benzyloxy)-2-(3,4-bis(benzyloxy)phenyl)-7-hydroxy-4*H*-chromen-4-one (6)

A mixture of potassium carbonate (1.0 g, 8.4 mmol) and benzyl bromide (1.2 mL, 8.4 mmol) were added to a solution of **5** (750 mg, 2.1 mmol) in DMF (10 mL). After stirring at 80 ^oC for 4 h, the reaction mixture was filtered and the filtrate was concentrated under reduced pressure to give pale yellow syrup, which was used for the next step without further purification. The product obtained above was dissolved in 3N HCl, and the resulting solution was stirred at 40 ^oC for 4 h. The reaction mixture was concentrated under reduced pressure, and the residue was purified by column chromatography on silica gel (2:1 = Hexane:Acetone) to give **6** (1.1 g, 1.6 mmol, 78% yield) as a yellow powder: ^1H NMR (400 MHz, Acetone-d_6) δ 12.84 (s, OH), 7.84 (d, *J* = 2.1 Hz, 1H, $H_{2'}$), 7.70 (dd, *J* = 2.1, 8.6 Hz, 1H, $H_{6'}$), 7.54 (d, *J* = 7.4 Hz, 2H, -ArH), 7.46 (d, *J* = 7.4 Hz, 2H, -ArH), 7.37-7.42 (m, 10H, -ArH), 7.29-7.34 (m, 6H, -ArH), 6.94 (d, *J* = 8.6 Hz, 1H, $H_{5'}$), 6.50 (d, *J* = 2.0 Hz, 1H, H_8), 6.28 (d, *J* = 2.0 Hz, 1H, H_6), 5.20 (s, 2H, -CH_2), 5.11 (s, 2H, -CH_2), 5.04 (s, 2H, -CH_2), 4.89 (s, 2H, -CH_2).

4.2.3. 3-(Benzyloxy)-2-(3,4-bis(benzyloxy)phenyl)-7-(2-bromoethoxy)-5-hydroxy-4*H*-chromen-4-one (7)

Potassium carbonate (83 mg, 0.6 mmol), 1,2-dibromoethane (0.1 mL, 0.3 mmol) and KI (1 mg) were added to a solution of **6** (200 mg, 0.3 mmol) in acetone (6 mL). After stirring at room temperature for 4 h, the reaction mixture was filtered and the filtrate was concentrated under reduced pressure to give **7** (153 mg, 0.2 mmol, 68% yield) as a dark yellow syrup: ^1H NMR (400 MHz, Acetone-d_6) δ 12.84 (s, OH), 7.72 (d, *J* = 2.1 Hz, 1H, $H_{2'}$), 7.62 (d, *J* = 7.5

Hz, 2H, -ArH), 7.54 (dd, *J* =2.1, 8.4Hz, 1H, $H_{6'}$), 7.46 (d, *J* = 7.5 Hz, 2H, -ArH), 7.37-7.42 (m, 6H, -ArH), 7.28-7.36 (m, 5H, -ArH), 6.95 (d, *J* = 8.5 Hz, 1H, $H_{5'}$), 6.64(d, *J* = 2.2 Hz, 1H, H_8), 6.43 (d, *J* = 2.2 Hz, 1H, H_6), 5.28 (s, 2H, -CH_2), 5.14 (s, 2H, -CH_2), 4.96 (s, 2H, -CH_2), 4.26 (t, *J* = 6.3 Hz, 2H, -C$\underline{H_2}$$CH_2$), 3.63 (t, *J* = 6.3 Hz, 2H, -CH_2C$\underline{H_2}$).

4.2.4. (*S*)-2-(2-((2-(3,4-Dihydroxyphenyl)-3,5-dihydroxy-4-oxo-4*H*-chromen-7-yl)oxy)-acetamido)pentanedioic acid (2)

Potassium carbonate (25 mg, 0.2 mmol) and (*S*)-di-*tert*-butyl 2-(2-chloroacetamido)pentanedioate (50 mg, 0.2 mmol) were added to a solution of **6** (100 mg, 0.2 mmol) in DMF (4 mL). After stirring at room temperature for 6 h, the reaction mixture was filtered and the filtrate was concentrated under reduced pressure to give a pale yellow syrup, which was used for the next step without further purification. Under an atmosphere of hydrogen gas (balloon), the degassed suspension of quercetin conjugate obtained above and Pd/C (10% w/w, 15 mg) in a mixture of THF (3 mL) and MeOH (1 mL) was vigorously stirred at room temperature for 8 h. The reaction mixture was filtered through a short celite pad and the filtrate was recrystallized from CH_2Cl_2 to afford a yellow powder. The quercetin conjugate, thus obtained, was treated with trifluoroacetic acid (1 mL) at 0 °C. After stirring at 0 °C for 4 h, the reaction mixture was concentrated under reduced pressure. The residue was recrystallized from a mixture of acetone (1 mL) and CH_2Cl_2 (10 mL) to give **2** (34 mg, 0.07 mmol, 34% yield) as a yellow solid: m.p. 262 ^{o}C; ^{1}H NMR (400 MHz, Acetone-d_6) δ 12.63 (s, OH), 7.73 (d, *J* = 2.0 Hz, 1H, $H_{2'}$), 7.58 (dd, *J* = 2.1, 8.4 Hz, 1H, $H_{5'}$), 6.91 (d, *J* = 8.4 Hz, 1H, $H_{6'}$), 6.82 (d, *J* = 2.1 Hz, 1H, H_8), 6.47 (d, *J* = 2.1 Hz, 1H, H_6), 4.34-4.38 (m, 1H, -CH), 4.06 (s, 2H, -$COCH_2$), 2.34-2.60 (m, 4H, -CH_2CH_2); ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.0 (-$CH_2CH_2\underline{C}O_2H$), 176.7 ($C_4$), 174.6 (-CH$\underline{C}O_2H$), 168.8 (-$OCH_2\underline{C}$ONH), 164.2 ($C_7$), 155.8 ($C_5$),

154.2 (C_9), 148.4(C_2), 148.0 ($C_{4'}$), 145.5 ($C_{3'}$), 136.5 (C_3), 122.1 ($C_{1'}$), 120.5 ($C_{6'}$), 116.0 ($C_{5'}$), 115.6 ($C_{2'}$), 105.4 (C_{10}), 98.6 (C_6), 94.2 (C_8), 68.2 (-O<u>C</u>H_2CONH), 55.6 (α-CH), 30.5 (CH_2<u>C</u>H_2CO_2H), 28.5 (<u>C</u>$H_2CH_2CO_2H$); MS (ESI) *m/z* Found: 490.11 $[M+H]^+$; Calcd for $C_{22}H_{20}NO_{12}$: 490.09.

4.2.5. (*S*)-2-((2-((2-(3,4-Dihydroxyphenyl)-3,5-dihydroxy-4-oxo-4*H*-chromen-7-yl)oxy)-ethyl)amino)pentanedioic acid (3)

To a solution of **7** (100 mg, 0.2 mmol) in DMF (5 ml) were added H-Glu(OtBu)-OtBu·HCl (44 mg, 0.2 mmol) and DIPEA (0.1 mL, 0.3 mmol). After stirring at room temperature for 12 h, solvent was removed under reduced pressure to give a crude product, which was used for the next step without further purification. Pd/C (10% w/w, 20 mg) was added to a solution of crude product in a mixture of THF (3 mL) and MeOH (1 mL), and the reaction flask was charged with hydrogen gas (balloon, 1 atm). After stirring at room temperature for 4 h, the reaction mixtue was filtered through a Celite pad. The filtrate was concentrated under reduced pressure and the residue was treated with trifluoroacetic acid (1 mL) at 0 °C. After stirring at 0 °C for 4 h, the reaction mixture was concentrated under reduced pressure, and the residue was recrystallized from a mixture of acetone (1 mL) and CH_2Cl_2 (10 mL) to give **3** (26 mg, 0.05 mmol, 27% yield) as a yellow solid: m.p. 230 ^oC; ^1H NMR (400 MHz, Acetone-d_6) δ 12.64 (s, OH), 7.70 (d, J = 2.2 Hz, 1H, $H_{2'}$), 7.56 (dd, J = 2.2, 8.4 Hz, 1H, $H_{5'}$), 6.86 (d, J = 8.4 Hz, 1H, $H_{6'}$), 6.82 (d, J = 2.2 Hz, 1H, H_8), 6.46 (d, J = 2.2 Hz, 1H, H_6), 4.30-4.34 (m, 1H, -CH), 4.24 (t, J = 6.3 Hz, 2H, -OCH_2), 3.61 (t, J = 6.3 Hz, 2H, -CH_2N), 2.30-2.54 (m, 4H, -$CH_2CH_2CO_2H$); ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.0 (-CH_2CH_2<u>C</u>O_2H), 176.7 (C_4), 174.6 (-CH<u>C</u>O_2H), 165.4 (C_7), 160.2 (C_5), 158.2 (C_9), 148.4(C_2), 148.0 ($C_{4'}$), 145.5 ($C_{3'}$), 136.4 (C_3), 122.1 ($C_{1'}$), 120.5 ($C_{6'}$), 116.0 ($C_{5'}$), 115.6 ($C_{2'}$), 105.4 (C_{10}), 98.2 (C_6), 92.4 (C_8), 70.4 (-O<u>C</u>H_2CH_2NH), 67.2 (α-CH), 47.4 (-OCH_2<u>C</u>H_2NH), 30.5 (CH_2<u>C</u>H_2CO_2H), 28.5

(C$H_2CH_2CO_2H$); MS (ESI) *m/z* Found: 476.24 $[M+H]^+$; Calcd for $C_{22}H_{22}NO_{11}$: 476.11.

4.3. Biological Assay

4.3.1. Materials for Biological Studies

DMSO (dimethyl sulfoxide), MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide)], and verapamil were purchased from Sigma-Aldrich. Roswell Park Memorial Institute (RPMI) 1640 media, penicillin, streptomycin and fetal bovine serum (FBS) were purchased from HyClone Laboratories.

4.3.2. Cell Lines

MES-SA, a human uterine sarcoma cell line, was grown in monolayer. Resistant MES-SA/Dx5 cell line was isolated by stepwise selection upon culture with increasing concentrations of doxorubicin (DOX). Briefly, MES-SA/Dx5 cells were treated with DOX started from a sub-IC_{50} concentration, 5 μM. The medium was changed every other day with an increment of 5 μM DOX each time. Within 2 weeks, the MES-SA/Dx5 cells acquired DOX resistance and were cultured in a medium containing 5 μM DOX thereafter. Cells were harvested and analyzed for Pgp expression by Western blot analysis.

4.3.3. Cytotoxicity

Cytotoxicity was determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) assay. Doxorubicin-sensitive MES-SA cells and doxorubicin-resistant MES-SA/Dx5 cells were seeded (5×10^3 cells per well) in tissue-cultured COSTAR clear bottom 96-well plate in complete RPMI-1640 and incubated for 24 h (37 ˚C , 5% CO_2). Quercetin (**Q**) and quercetin conjugates (**2** and **3**) dissolved in DMSO were serially diluted (100 μM, 10 μM, 1 μM, 0.1 μM, 0.01 μM, 0.001 μM,), and the resulting solutions were added to the media. After 72 h, cell viability was estimated by MTT assay.

Every assay was repeated three times.

4.3.4. Modulation of Doxorubicin Efflux

The quercetin conjugates **2** and **3** were screened for their ability to block the Pgp-dependent doxorubicin efflux by flow cytometry analysis. Briefly, 24 h before the experiment, MES-SA/Dx5 cells in the logarithmic phase of growth were seeded in 24-well plates at a density of 10^5 cells per well. Afterward, cells were incubated for 30 min at 37 °C in DMEM + 10% FBS in the presence or absence of each quercetin conjugate (10 μM) or verapamil with doxorubicin (2 μM). Finally, cells were washed twice with ice-cold PBS, trypsinized, and resuspended in 0.2 mL of ice-cold PBS for immediate analysis. Fluorescence measurements of individual cells were made with FACSCalibur (Becton Dickinson, USA).

4.3.5. Pgp ATPase Assay

Pgp ATPase activity was measured with the Pgp-Glo™ assay system with human Pgp membrane by following the manufacturer's instructions (Promega, Co. USA). The assay relies on the ATP dependence of the light-generating reaction of firefly luciferase. Briefly, 25 μg of Pgp membrane was incubated at 37 °C with either Na_3VO_4 (100 μM), solvent control (0.1% DMSO), quercetin (100 μM), **2** (or **3**) (100 μM), verapamil (100 μM), or verapamil (100 μM) plus **2** (or **3**) (100 μM). The ATPase reaction was initiated by addition of 5 mM MgATP and followed by incubation for 40 min at 37 °C. The reaction was stopped, and the remaining unmetabolized ATP was detected as a luciferase-generated luminescence signal by addition of ATP detection reagent. Following a room-temperature signal-stabilization period (20 min), luminescence was read on a Veritas microplate luminometer (Turner Designs, San Francisco, CA). Pgp ATPase activity was presented as a drop in luminescence of samples compared to that treated with Na_3VO_4.

4.3.6. Determining MDR-modulatory effect of the quercetin conjugates on the

sensitivities of MES-SA and MES-SA/Dx5 to anticancer drugs

Doxorubicin-sensitive MES-SA cells and doxorubicin-resistant MES-SA/Dx5 cells were seeded (5×10^3 cells per well) in tissue-cultured COSTAR clear bottom 96-well plate. The cultures were incubated at 37 ˚C in 5% CO_2 for 24 h. To the plates, 100 μL of the solution (final concentrations: 100 μM, 10 μM, 1 μM, 0.1 μM, 0.05 μM, 0.01 μM, 0.001 μM, 0.0001 μM) of anticancer drugs (doxorubicin, vinblastine, paclitaxel, actinomycin) with or without the MDR-reversal agents [quercetin conjugates (5 μM, 1% DMSO) or verapamil (5 μM, 1% DMSO)] in the complete RPMI-1640 medium was added and the cells were incubated for 72 h. To the control well, 1% DMSO in 100 μL of the growth medium was added and the cells were incubated for 72 h. To the blank well, which contains no cell, 100 μL of the growth medium was added. The growth medium was removed and cell viability was estimated by MTT assay. Optical absorbance at 570 nm was then recorded with a microplate reader (SpectraMax M2e, Molecular Devices). Every assay was repeated three times. The relative survival rates were calculated according to the equation

$$\text{Survival \%} = (M_{Dx} - M_{blank})/(M_{control} - M_{blank}) \times 100$$

Here, M_{Dx} presents light absorption values of cell, growth medium, anticancer drugs and MDR-reversal agents, $M_{control}$ presents light absorption values of cell and growth medium, and M_{blank} presents light absorption values of growth medium alone. The relative survival rates, thus calculated, were plotted against concentrations of anticancer drugs to define the IC_{50}'s of anticancer drugs against MES-SA and MES-SA/Dx5 cells.

4.3.7. Statistical analysis

Data represent the means of at least three separate experiments. Statistical analysis was performed using Student's *t* test. A value of $p < 0.05$ was considered significant.

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