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Article

Design, Synthesis and MDR Modulatory Activity of the Apigenin Conjugates

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Abstract: Much effort has been devoted on searching for effective modulators of multidrug resistance (MDR) in cancer. In this study, as a part of our ongoing efforts to discover safe and effective flavonoid-based MDR modulators, we prepared a series of apigenin conjugates and evaluated their MDR-reversal activity. Among those, 7,4′-bis-O-pivaloxymethyl(POM)-apigenin showed the most potent MDR-reversal activity, particularly at low concentration of the anticancer drug.

Keywords: apigenin; conjugate; multidrug resistance (MDR); P-glycoprotein (Pgp); modulator

1. Introduction

Cross-resistance to a wide spectrum of anticancer agents, which is called multidrug resistance (MDR), continues to be a major problem in anticancer therapy. The best characterized resistance mechanism is ATP-driven transmembrane transport of the structurally and functionally unrelated cytostatic agents caused by overexpression of the ATP-binding cassette (ABC) superfamily of membrane transporters such as P-glycoprotein (Pgp) [1, 2], multidrug resistance protein 1 (MRP1) [3, 4], and breast cancer resistance protein (BCRP) [5]. Ever since the pivotal roles of the ABC transporters in MDR were demonstrated, there has been intensive search for potent MDR modulators via inhibition of this class of membrane transporters. The first discovery of verapamil as a Pgp inhibitor [6] was followed by numerous compounds identified to sensitize MDR cells to cytostatic agents by inhibition of drug efflux. However, these first-generation Pgp inhibitors were not specifically developed for inhibiting MDR. Rather, this class consists of drugs already approved for other medical treatment, e.g. calcium channel blockers (verapamil), immunosuppressants (cyclosporine A), antibiotics (erythromycin), antimalarials (quinine), psychotropic phenothiazines and indole alkaloids (fluphenazine and reserpine), steroid hormones and anti-steroids (progesterone and tamoxifen), and detergents (cremophor EL) [7]. Thus, due to lack of MDR specificity, the first-generation MDR modulators were ineffective or toxic at the doses required to attenuate Pgp function. Investigations of less toxic and more potent Pgp inhibitors were then prompted by structural modification of the first-generation drugs to remove their non-MDR pharmacological effects and resulted in discovery of the second-generation MDR modulators such as dexverapamil [8], dexniguldipine [9], valspodar (PSC833) [10, 11], and biricodar (VX-710) [12-14]. Even though the side effects disappeared, clinical trials of the second-generation drugs were performed with little success due to their unpredictable pharmacokinetic interactions with chemotherapy, which elevated plasma concentration of the anticancer drugs beyond acceptable toxicity by limiting drug clearance and metabolism [15]. Third-generation Pgp inhibitors such as laniquidar (R101933) [16], oc144-093 (ONT-093) [17], zosuquidar (LY335979) [18], elacridar (GF-120918) [19], and tariquidar (XR9576) [20] were then designed specifically for high transporter affinity and low pharmacokinetic interaction. However, even though these compounds showed potent and selective inhibition of Pgp-mediated drug efflux in vitro, phase III clinical trials of tariquidar and zosuquidar were failed due to toxicity and lack of pharmacological effect [18]. Taken together, the development of MDR-modulating agents has been hampered by unacceptable toxicities as well as unpredictable pharmacokinetic interactions, which warrants investigation of new chemical entities with proven safety profiles.

Flavonoids are polyphenolic antioxidants that are widely distributed in the plant kingdom, and dietary intakes of certain foods rich in flavonoids are known to be related with potential health benefits. Along with the

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well-known safety feature, various bioactivities associated with flavonoids such as antioxidant, anti-inflammatory, anticancer, and antiviral activities allowed large amounts of daily human consumption in the forms of antioxidant supplements and complementary cancer therapy. Even though the antioxidant activity is the representative bioactivity of the flavonoids, we and others have reported that the flavonoids or flavonoid-rich plant extracts are able to alter cellular functions independently of their antioxidant potential [21]. For example, as the A- (5-hydroxyl group) and C-ring (4-carbonyl functionality) moiety of flavonoids are supposed to mimic the adenine moiety of ATP [Fig. 1], some flavonoids are known to serve as inhibitors of the ATP-binding proteins such as protein kinases [22 – 29] and ATPases [30 – 33] via ATP-competitive binding.

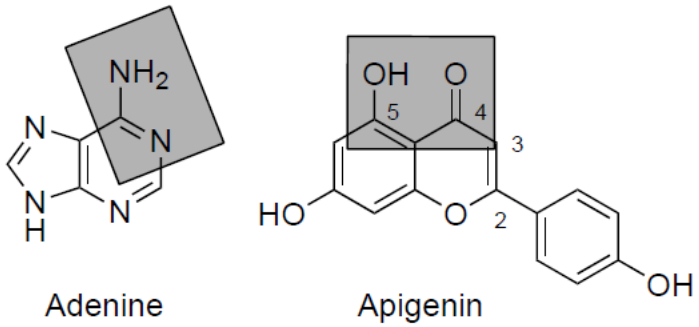


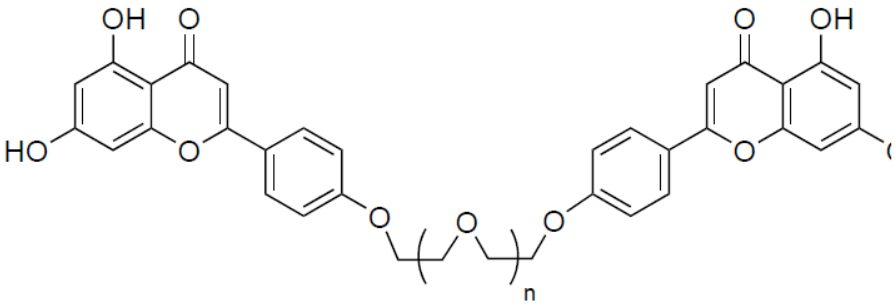
Fig. 1. Hydrophobic interactions between cobicistat and residues of 6vhn.pdb analyzed using the LigPlot program.

By the same mechanism, the flavonoids are also expected to bind to the ATP-binding site of the ABC transporters to inhibit efflux of the drugs and/or drug conjugates [34]. In this context, it is not surprising that there have been a number of attempts to evaluate MDR modulating activity of the flavonoids, which resulted in identification of flavonoids such as chrysin, quercetin, kaempferol, and dehydrosilybin as potential safe MDR modulators [35 – 41].

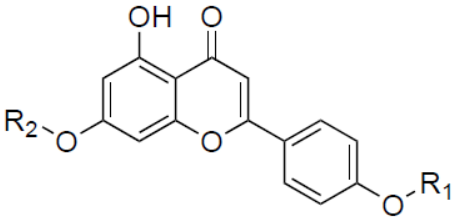
By far the most successful approach to develop flavonoids with MDR-modulatory activity was to use flavonoid dimers [42 – 44]. Even though the mechanism of MDR modulatory activity of the flavonoid dimers has yet to be elucidated, the structure-activity relationship study [42 – 44] revealed the possible binding role of the flavonoid core structure to the target transporters as well as the importance of the bivalent binding of the two flavonoid monomers connected by poly(ethylene glycol) linkers. Apigenin homodimers [Fig. 2] are representative flavonoid dimers which have shown remarkable bivalent modulatory effect for both Pgp-mediated [42] and MRP1-mediated [45] multidrug resistance.

However, due to the highly hydrophobic nature, the flavonoid dimers were shown to be barely soluble in water, which did not allow in vivo animal model study [44]. In addition, relatively high molecular weight (~ 700) as well as loss of natural flavonoid structure due to incorporation of an uncleavable covalent spacer might raise other issues during development of this class of MDR modulators.

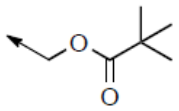
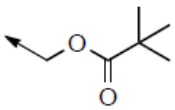
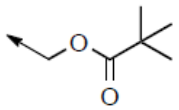
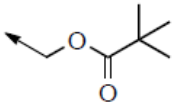
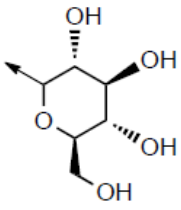
Based on these findings, in this study, we intended to derivatize the apigenin structure through conjugation with various promoieties such as pivaloxymethyl (POM), glucose, and amino acids via cleavable linkages [Fig. 2]. Herein, we report preparation and evaluation of MDR-reversal activity of novel apigenin conjugates.



Apigenin Dimers



Apigenin Conjugates

	R_1	R_2
1a	H	
1b		
1c		H
1d		H

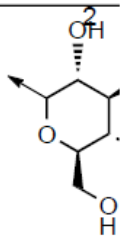
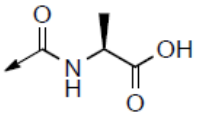
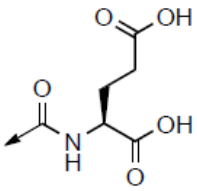
	R_1	R
1e	H	
1f		H
1g		H

Fig. 2. Hydrophobic interactions between cobicistat and residues of 6vhn.pdb analyzed using the LigPlot program.

2. Materials and Methods

2.1. chemicals

All chemicals were purchased from Sigma-Aldrich. RPMI-1640 medium, penicillin, streptomycin and fetal bovine serum (FBS) were purchased from Invitrogen. Thin layer chromatography (TLC) was performed on Silica gel 60 F254 purchaded from Merck. Column chromatography was performed using Silica Gel. Nuclear magnetic resonace NMR spectra were recorded on a bruker 400 AMX spectrometer at 400 MHz for ^{1}H-NMR and at 100 MHz for ^{13}C-NMR with tetramethylsilane as the 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). The chemical shifts were reported as parts per million (δ) relative to the solvent peak. Mass spectrometric data (MS) were obtained by electron spray ionization (ESI). High resolution mass spectra (HRMS) were obtained at Korea Basic Science Institute (Daegu, Korea) and reported in the form of m/z (intensity relative to base peak=100).

*2.2. (5-Hydroxy-2-(4-hydroxyphenyl)-4-oxo-4H-chromen-7-yloxy)methyl pivalate (1a) & (5-hydroxy-4-oxo-2-(4-(pivaloyloxymethoxy)phenyl)-4H-chromen-7-yloxy)methyl pivalate (**1b**)*

To a solution of 4,5,7-trihydroxychromone (**2**) (100.0 mg, 0.4 mmol) in acetone (5 ml) was added K2CO3 (130.0 mg, 0.9 mmol) and POM iodide (0.2 ml, 0.5 mmol). The reaction mixture was stirred for 2 h at rt, filtered and concentrated under reduced pressure to give yellow powder. Purification by column chromatography (SiO2, hexane:ethyl aceate = 2:1) provided **1a** (35.0 mg, 0.1 mmol) and **1b** (40.0 mg, 0.2 mmol) in 32% and 49% yields, respectively: For **1a**, ^{1}H NMR (400 MHz, Acetone-d6) δ (ppm) 8.10 (d, J = 8.9 Hz, 2H), 7.29 (d, J = 8.9 Hz, 2H), 6.85 (d, J = 2.1 Hz, 1H), 6.79 (s, 1H), 6.49 (d, J = 2.1 Hz, 1H), 5.93 (s, 4H), 2.82 (d, J = 13.2 Hz, 4H), 1.19-1.20 (m, 18H); ^{13}C NMR (100 MHz, acetone-d6) δ (ppm) 184.6, 184.5, 178.5, 178.5, 166.2, 164.7, 164.5, 164.2, 162.1, 159.8, 127.4, 108.4, 106.8, 106.7, 101.3, 96.7, 96.6, 86.9, 86.6, 40.8, 31.9. For **1b**, ^{1}H NMR (400 MHz, MeOD) δ (ppm) 7.87 (d, J = 8.8 Hz, 2H), 6.93 (d, J = 8.8 Hz, 2H), 6.76 (d, J = 2.2 Hz, 1H), 6.65 (s, 1H), 6.46 (d, J = 2.2 Hz, 1H), 5.87 (s, 2H), 1.19-1.23 (m, 9H); ^{13}C NMR (100 MHz, DMSO-d6) δ (ppm) 182.3, 176.6, 164.7, 162.0, 161.8, 161.6, 157.2, 128.9, 121.2, 116.3, 106.0, 103.4, 99.3, 94.8, 84.7, 38.7, 26.8.

*2.3. 7-(Benzyloxy)-5-hydroxy-2-(4-hydroxyphenyl)-4H-chromen-4-one (**3**)*

To a solution of 4,5,7-trihydroxychromone (**2**) (2.0 g, 7.4 mmol) and K2CO3 (1.0 g, 7.4 mmol) in DMF (15 ml) was added benzyl bromide (0.9 ml, 7.4 mmol). The reaction mixture was stirred at rt and the progress of reaction was monitored by TLC. After stirring for 10 h at rt, DMF was removed under reduced pressure. The residue was purified by column chromatography (SiO2, hexane:ethyl aceate = 4 : 1) to afford **3** (1.6 g, 4.3 mmol, 59%) as yellow powder: ^{1}H NMR (400 MHz, Acetone-d6) δ (ppm) 13.0 (s, 1H), 7.95 (d, J = 8.8 Hz, 2H), 7.53 (d, J = 7.22 Hz, 2H), 7.43 (t, J = 7.4 Hz, 2H), 7.35-7.38 (m, 1H), 7.03 (d, J = 8.8 Hz, 2H), 6.79 (d, J = 2.0 Hz, 1H), 6.67 (d, J = 1.3 Hz, 1H), 6.43 (d, J = 3.4 Hz, 1H), 5.28 (s, 2H); ^{13}C NMR (100 MHz, DMSO-d6) δ (ppm) 180.5, 162.7, 159.9, 159.8, 155.7, 134.7, 127.1, 126.7, 126.6, 126.4, 119.6, 114.6, 113.4, 103.4, 101.6, 97.1, 92.0, 68.5.

*2.4. (4-(5,7-Dihydroxy-4-oxo-4H-chromen-2-yl)phenoxy)methyl pivalate (**1c**)*

To a solution of **3** (126.7 mg, 0.4 mmol) in acetone (3 ml) was added K2CO3 (72.6 mg, 0.5 mmol) and POM iodie (0.1 ml, 0.4 mmol). The reaction mixture was stirred for 4 h at rt, filtered and concentrated under reduced pressure to give yellow powder, which was purified by column chromatography (SiO_2, hexane: ethyl aceate = 6:1) to give (4-(7-(benzyloxy)-5-hydroxy-4-oxo-4H-chromen-2-yl)phenoxy)methyl pivalate (123.4 mg, 0.3 mmol), 86% yields: ^{1}H NMR (400 MHz, Acetone-d6) δ (ppm) 8.08 (d, J = 9.0 Hz, 2H), 7.53 (d, J = 8.9 Hz, 2H), 7.36-7.45 (m, 3H), 7.26-7.29 (m, 2H), 6.81 (t, J = 1.9 Hz, 1H), 6.75 (d, J = 1.1 Hz, 1H), 6.44 (t, J = 2.2 Hz, 1H), 5.93 (s, 2H), 5.28 (t, J = 4.9 Hz, 2H), 1.18-1.20 (m, 9H).

To a solution of the benzyl ether obtained above (123.4 mg, 0.3 mmol) in a mixture of methanol (2 ml) and THF (1 ml) was added palladium on activated carbon (10% w/w, 12.3 mg) and the reaction flask was charged with hydrogen (balloon, 1 atm). The reaction mixture was stirred at rt, and the progress of reaction was monitored by TLC. After stirring for 12 h at rt, volatiles were removed under reduced pressure to give a crude product, which was purified by column chromatography (SiO_2, hexane:ethyl aceate = 2: 1) to give **1c** (85.1 mg, 0.2 mmol) in 72% yield: ^{1}H NMR (400 MHz, Acetone-d6) δ (ppm) 8.08 (d, J = 8.9 Hz, 2H), 7.28 (d, J = 8.9 Hz, 2H), 6.72 (s, 1H), 6.57 (d, J = 1.42 Hz, 1H), 6.27 (s, 1H), 5.93 (s, 2H), 1.19 (s, 9H); ^{13}C NMR (100 MHz, DMSO-d6) δ (ppm) 181.6, 176.1, 164.2, 162.6, 161.2, 158.9, 157.2, 128.2, 124.5, 116.1, 103.9, 103.6, 98.8, 93.9, 84.8, 38.2, 26.3.

*2.5. 5,7-Dihydroxy-2-(4-((3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yloxy)phenyl)-4H-chromen-4-one (**1d**)*

To a solution of glycosyl acceptor **3** (238.2 mg, 0.7 mmol) and donor **5** (543.6 mg, 1.3 mmol) in a mixture of chloroform (7 ml) and H_2O (3 ml) were added K_2CO_3 (182.7 mg, 1.3 mmol) and Aliquat 336 (0.1 ml). The

reaction mixture was stirred at 50 ºC. After 18 h, TCL showed most of **3** was consumed, and the reaction was quenched by addition of 2 N HCl. The solution was diluted with CH_2Cl_2 and washed by brine. The organic layer was dried by $MgSO_4$ and, after evaporation of the solvent under reduced pressure, the crude product was purified by column chromatography (SiO_2, hexane:acetone = 2:1) to give apigenin-7-O-benzyl-4′-O-glucoside pentaacetate (176 mg, 0.3 mmol, 36% yield): ^{1}H NMR (400 MHz, Acetone-d6) δ (ppm) 8.06 (d, J = 8.8 Hz, 2H), 7.27 (d, J = 8.8 Hz, 2H), 6.72 (s, 1H), 6.56 (d, J = 1.7 Hz, 1H), 6.27 (d, J = 1.7 Hz, 1H), 5.63 (d, J =7.9 Hz, 1H), 5.43 (dd, J = 9.6, 19.1 Hz, 1H), 5.25 (dd, J = 8.0, 9.7 Hz, 1H), 5.16 (dd, J = 9.5, 18.9 Hz, 1H), 4.32 (m, 2H), 4.22 (dd, J = 4.1, 14.0 Hz, 1H), 2.07 (m, 18H); ^{13}C NMR (100 MHz, DMSO-d6) δ (ppm) 182.0, 170.0, 169.6, 169.3, 169.1, 164.2, 163.1, 161.2, 159.0, 157.2, 136.1, 128.5, 128.4, 128.1, 127.8, 124.9, 116.6, 104.9, 104.4, 96.4, 93.6, 71.9, 71.0, 70.8, 70.6, 70.0, 68.5, 67.9, 62.1, 61.6, 30.6, 20.5, 20.3, 20.2.

The compound obtained above (176 mg, 0.3 mmol) was dissolved in a mixture of methanol (4 ml) and THF (2 ml) and the solution was treated with palladium on activated carbon (10%w/w, 17.6 mg). After charging the reaction flask with hydrogen (balloon, 1 atm), the reaction mixture was stirred at rt and the progress of the reaction was monitored by TLC. After stirring for 12 h at rt, solvent was removed under reduced pressure to give a crude product which was purified by column chromatography (SiO_2, hexane:acetone= 2:1) to give apigenin-4′-O-glucoside pentaacetate (122 mg, 0.2 mmol, 80% yield): ^{1}H NMR (400 MHz, Acetone-d6) δ (ppm) 8.06 (d, J = 8.8 Hz, 2H), 7.27 (d, J = 8.8 Hz, 2H), 6.72 (s, 1H), 6.56 (d, J = 1.7 Hz, 1H), 6.27 (d, J = 1.7 Hz, 1H), 5.63 (d, J =7.9 Hz, 1H), 5.43 (dd, J = 9.6, 19.1 Hz, 1H), 5.25 (dd, J = 8.0, 9.7 Hz, 1H), 5.16 (dd, J = 9.5, 18.9 Hz, 1H), 4.32 (m, 2H), 4.22 (dd, J = 4.1, 14.0 Hz, 1H), 2.07 (m, 12H); ^{13}C NMR (100 MHz, DMSO-d6) δ (ppm) 183.9, 171.6, 171.2, 170.9, 170.6, 165.9, 165.0, 164.1, 161.3, 159.6, 129.9, 127.5, 118.6, 106.2, 106.1, 100.7, 99.5, 95.7, 74.1, 73.6, 72.69, 70.00, 63.5, 21.5, 21.4, 21.4, 21.4.

To apigenin-4′-O-glucoside pentaacetate (122 mg, 0.2 mmol) obtained above was added excess NH_3 in methanol at 0 ºC. The resulting mixture was stirred for 10 h at rt, and the volatiles were removed under reduced pressure. The residue was triturated with ethyl acetate to give the desired product 1d (72 mg, 0.13 mmol, 66% yield); ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm) 12.9 (s, 1H), 8.03 (d, J = 8.8 Hz, 2H), 7.19 (d, J = 8.8 Hz, 2H), 6.88 (s, 1H), 6.50 (s, 1H), 6.19 (s, 1H), 5.40 (s, 1H), 5.13 (s, 1H), 5.06 (s, 1H), 5.02 (d, J = 7.0 Hz, 1H), 4.60 (s, 1H), 3.70 (d, J = 11.0 Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d6) δ (ppm) 181.2, 171.0, 162.5, 160.9, 159.7, 156.9, 127.6, 123.5, 116.1, 103.3, 99.3, 98.4, 93.7, 76.6, 76.0, 72.6, 69.1, 60.1, 22.0.

*2.6. 4-(5-Acetoxy-7-hydroxy-4-oxo-4H-chromen-2-yl)phenyl acetate (**4**)*

To a solution of 4′,5,7-trihydroxychromone (**2**) (2.0 g, 7.4 mmol) in pyridine (20 ml) was added acetic anhydride (3.0 ml, 29.6 mmol). The reaction mixture was stirred at 60 ºC and the progress of reaction was monitored by TLC. After stirring for 4 h at 60 ºC, the reaction mixture was cooled to rt and evaporated to dryness. The residue was purified by column chromatography (SiO2, hexane:acetone = 4:1) to afford 2-(4-acetoxyphenyl)-4-oxo-4H-chromene-5,7-diyltriacetate (2.6 g, 7.3 mmol, 97% yield) as yellow powder: ^{1}H NMR (400 MHz, Acetone-d6) δ (ppm) 8.13 (dd, J = 2.7, 9.4 Hz, 2H), 7.50 (d, J = 2.2 Hz, 1H), 7.35 (dd, J = 2.7, 9.5 Hz, 2H), 6.96 (d, J = 2.2 Hz, 1H), 6.75 (d, J = 2.5 Hz, 1H), 2.32 (s, 3H), 2.32 (s, 3H), 2.31 (s, 3H).

To a solution of 2-(4-acetoxyphenyl)-4-oxo-4H-chromene-5,7-diyltriacetate (2.6 g, 7.3 mmol) and imidazole (510 mg, 7.3 mmol) in N-methyl-2-pyrrolidone (20 ml) was added thiophenol (0.6 ml, 5.8 mmol) at 60 ºC in dropwise fashion. The reaction mixture was stirred at rt and the progress of reaction was monitored by TLC. After stirring for 2 h, the reaction mixture was quenched with 2 N HCl and washed with ethyl acetate. The organic layer was dried over MgSO4 and concentrated under reduced pressure. The residue was purified by column chromatography (SiO2, hexane:acetone = 2:1) to afford 4 (1.9 g, 5.3 mmol, 72% yield) as yellow powder: ^{1}H NMR (400 MHz, Acetone-d6) δ (ppm) 8.08 (dd, J = 2.1, 6.9 Hz, 2H), 7.33 (dd, J = 2.0, 6.9 Hz, 2H), 6.98 (d, J = 2.4 Hz, 1H), 6.60 (d, J = 3.3 Hz, 2H), 2.30 (s, 6H); ^{13}C NMR (100 MHz, DMSO-d6) δ (ppm) 174.4, 168.1, 168.0, 161.4, 159.4, 157.4, 152.1, 149.3, 127.5, 126.8, 121.7, 108.6, 107.9, 106.6, 100.1, 29.8, 20.1.

*2.7. 5-Hydroxy-2-(4-hydroxyphenyl)-7-((3S,4R,5R,6S)-3,4,5-trihydroxy-6-(hydroxymethyl) tetrahydro-2H-pyran-2-yloxy)-4H-chromen-4-one (**1e**)*

To a stirred solution of 4 (238 mg, 0.7 mmol) and K_2CO_3 (182.7 mg, 1.3 mmol) in anhydrous DMF (5 ml) was added **5** (544 mg, 1.3 mmol) at rt. The reaction mixture was stirred for 12 h at 70 ºC, cooled to rt, and filtered. The filtrate was evaporated, and the residue was purified by column chromatography (SiO2, hexane:acetone = 2:1) to afford 4-(5-acetoxy-4-oxo-7-sugar-4H-chromen-2-yl)phenyl acetate (88 mg, 0.2 mmol, 19% yield) as brown powder: ^{1}H NMR (400 MHz, Acetone-d6) δ (ppm) 8.14 (d, J = 7.0 Hz, 2H), 7.37

(d, J = 8.8 Hz, 2H), 6.87 (s, 1H), 6.83 (d, J = 2.1 Hz, 1H), 6.51 (d, J = 2.2 Hz, 1H), 5.44 (d, J = 9.6 Hz, 1H), 5.25 (dd, J = 7.9, 9.7 Hz, 1H), 5.14 (dd, J = 9.5, 18.9 Hz, 1H), 4.32-4.37 (m, 1H), 4.27-4.28 (m, 1H), 4.21-4.25 (m, 1H), 2.30 (s, 3H), 2.08-2.09 (m, 18H).

To a compound obtained above (88 mg, 0.2 mmol) was added excess NH_3 in methanol at 0 ºC, and the resulting mixture was stirred for 10 h at rt. After removal of the volatiles under reduced pressure, the residue was triturated with ethyl acetate to give the desired product **1e** (44 mg, 0.1 mmol, 48% yield); ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm) 7.96 (d, J = 7.0 Hz, 2H), 6.93 (d, J = 8.8 Hz, 2H), 6.87 (s, 1H), 6.83 (d, J = 2.1 Hz, 1H), 6.44 (d, J = 2.1 Hz, 1H), 5.40 (d, J = 4.3 Hz, 1H), 5.13 (d, J = 4.1 Hz, 1H), 5.06 (d, J = 7.1 Hz, 2H), 4.82-4.90 (m, 1H), 4.60 (d, J = 5.7 Hz, 1H), 3.65-3.76 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d6) δ (ppm) 172.0, 168.6, 165.9, 133.4, 133.2, 122.7, 104.8, 101.2, 96.5, 78.1, 68.7, 63.0, 62.3, 61.5, 50.3, 26.8, 22.4, 15.8, 15.5.

*2.8. general procedure for preparation (S)-2-((4-(5,7-dihydroxy-4-oxo-4H-chromen-2-yl)phenoxy)carbonylamino)propanoic acid (**1f**) and (S)-2-((4-(5,7-dihydroxy-4-oxo-4H-chromen-2-yl)phenoxy)carbonylamino)pentanedioic acid (**1g**). Preparation 1f is representative*

A solution of 4′,5,7-trihydroxychromone (2) (660 mg, 2.4 mmol) in THF (20 ml) was added to a mixture of **9** (3.1 g, 7.3 mmol) and DIPEA (940 mg, 7.3 mmol). The reaction mixture was stirred at rt, and the reaction was monitored by TLC. Upon disappearance of the starting material, the reaction mixture was concentrated under reduced pressure and the residue was purified by column chromatography on silica gel (hexane:acetone = 4:1) to give the deacetylated product in 61% yield (780 mg, 1.4 mmol). Finally, deprotection of the tert-butyl group was accomplished upon treatment with excess TFA to furnish the desired compound **1f** in 86% yield (532 mg, 1.2 mmol): ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm) 8.10 (d, J = 8.6 Hz, 2H), 7.32 (d, J = 8.6 Hz, 2H), 6.95 (s, 1H), 6.52 (s, 1H), 6.23 (s, 1H), 4.09 (q, J = 7.4 Hz, 1H), 1.36 (d, J = 7.4 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d6) δ (ppm) 182.4, 164.8, 164.4, 161.8, 158.0, 128.5, 124.9, 122.7, 121.8, 116.6, 104.3, 103.5, 99.5, 94.6, 53.5, 50.4, 17.4.

*2.9. (S)-2-((4-(5,7-dihydroxy-4-oxo-4H-chromen-2-yl)phenoxy)carbonylamino)pentanedioic acid (**1g**)*

The desired product was obtained as a yellow powder in 51% overall yield starting from **10**: ^{1}H NMR (400 MHz, DMSO-d6) δ (ppm) 8.29 (d, J = 8.0 Hz, 1H), 8.11 (d, J = 8.7 Hz, 2H), 7.32 (d, J = 8.7 Hz, 2H), 6.96 (s, 1H), 6.53 (d, J = 1.8 Hz, 1H), 6.23 (d, J = 1.8 Hz, 1H), 4.06-4.11 (m, 1H), 2.33-2.26 (m, 2H), 1.82-2.11 (m, 2H); ^{13}C NMR (100 MHz, DMSO-d6) δ (ppm) 179.6, 171.6, 171.0, 162.2, 160.4, 159.2, 155.2, 151.6, 126.2, 125.6, 125.2, 119.9, 102.7, 101.7, 96.8, 91.9, 51.1, 27.9, 23.9.

*2.10. (2R,3R,4S,5R)-2-(Acetoxymethyl)-6-bromotetrahydro-2H-pyran-3,4,5-triyl triacetate (**5**)*

To a solution of β-D-glucose pentaacetate (**4**) (300 mg, 0.8 mmol) in dichloromethane (5 ml) was added hydrogen bromide (0.6 ml, 3.5 mmol) at 0 ºC. The reaction mixture was stirred at rt and the progress of reaction was monitored by TLC. After stirring for 3 h, the reaction mixture was washed with sat'd aqueous $NaHCO_3$ solution. The organic layer was dried over $MgSO_4$ and concentrated under reduced pressure to give the desired product (253 mg, 0.6 mmol, 78% yield), which was used for the next step without further purification: ^{1}H NMR (400 MHz, Acetone-d6) δ (ppm) 6.61 (d, J = 3.8 Hz, 1H), 5.64 (dd, J = 8.5, 13.2 Hz, 1H), 5.12 (m, 2H), 4.28 (m, 2H), 4.14 (d, J = 6.7 Hz, 1H), 2.81 (s, 1H), 2.78 (s, 1H), 2.07 (m, 12H).

*2.11. general procedure for preparation of (S)-tert-butyl 2-((4-nitrophenoxy) carbonylamino)propanoate (**9**) and (S)-5-tert-butoxy-4-((4-nitrophenoxy)carbonylamino)-5-oxopentanoic acid (10). Preparation of **9** is representative*

To a solution of compound **7** (1.5 g, 5.1 mmol) and bis-4-nitrophenylcarbonate (1.5 g, 5.1 mmol) in THF (15.0 ml) was added DIPEA (1.3 g, 10.1 mmol) at 0 ºC. The resulting mixture was stirred for 2 h at rt. After concentration of the reaction mixture under reduced pressure, the residue was purified by column chromatography on silica gel (hexane:ethyl acetate = 4:1) to give the desired product **9** in 83% yield (1.8 g, 4.2 mmol): ^{1}H NMR (400 MHz, Acetone-d6) δ (ppm) 8.30 (dd, J = 2.2, 7.0 Hz, 2H), 7.42 (dd, J = 2.2, 4.9 Hz, 2H), 4.18-4.21 (m, 1H), 1.44-1.49 (m, 9H), 1.18-1.22 (m, 3H).

2.12. cell line

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).

2.13. cyotoxicity

Cytotoxicity was determined by the 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% CO2). Apigenin (**2**) and apigenin conjugates (**1a** ~ **1g**) dissolved in DMSO were serially diluted to 6 concentrations (100 μM, 50 μM, 10 μM, 1 μM, 0.1 μM, 0.01 μM), and the resulting solutions were added to media. After 24 h, cell viability was estimated by MTT assay. Every assay was repeated three times.

2.14. determining MDR-modulatory effect of the apigenin conjugates on the sensitivities of MES-SA and MES-SA/Dx5 to doxorubicin

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, 50 μM, 10 μM, 1 μM, 0.1 μM, 0.01 μM) of doxorubicin with or without apigenin conjugates (5 μM, 1% DMSO) or verapamil (5 μM, 1% DMSO) in the complete RPMI-1640 medium were added and the cells were incubated for 24 h. To the control well, 1% DMSO in 100 μL of the growth medium was added and the cells were incubated for 24 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 an ELISA microplate reader. 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, doxorubicin and modulator, the Mcontrol presents light absorption values of cell and growth medium, and M_{blank} presents the light absorption values of growth medium alone. The survival and concentrations of doxorubicin were plotted to define the IC_{50} of doxorubicin against both MES-SA cells and MES-SA/Dx5 cells.

3. Results and Discussion

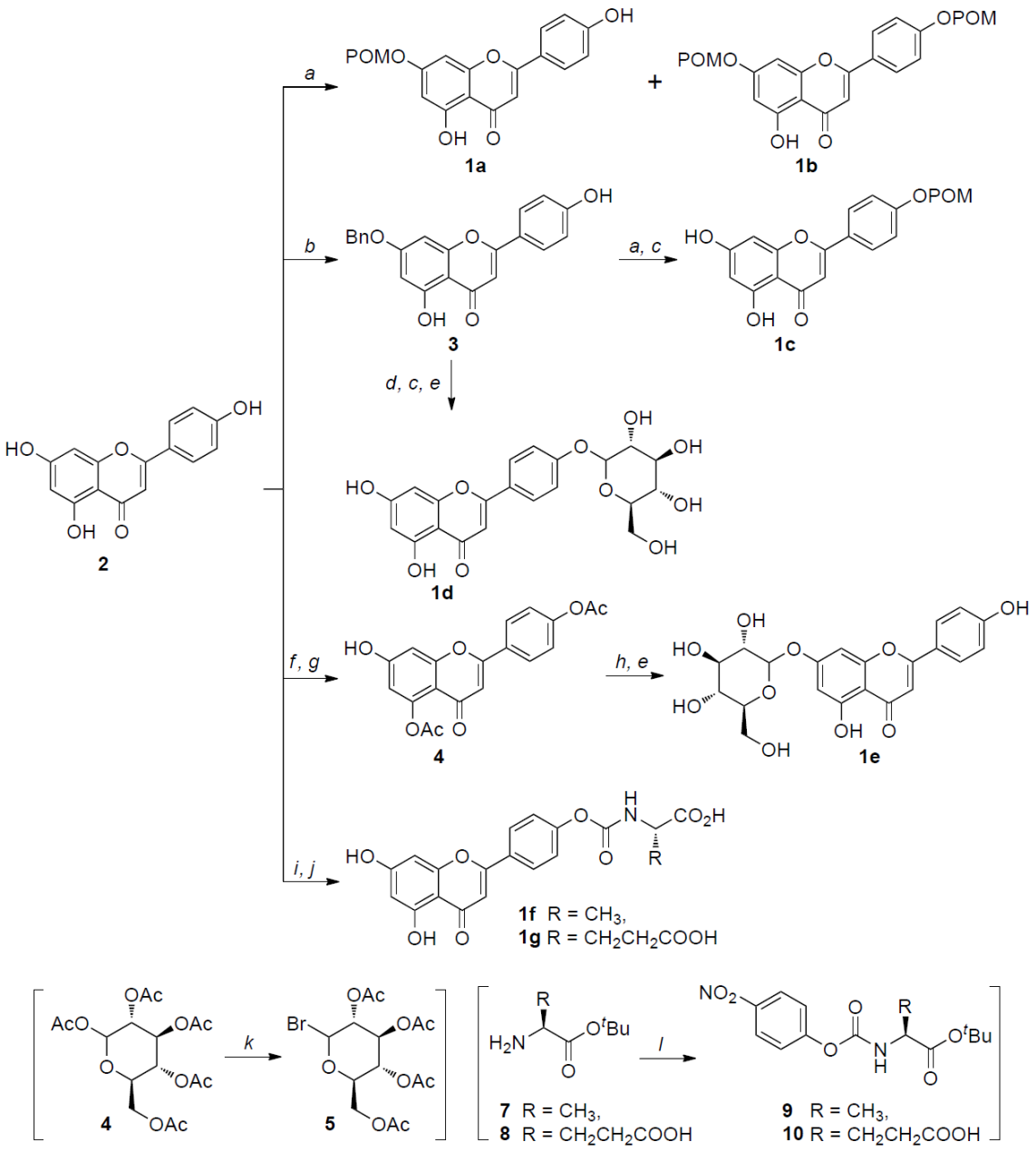
3.1. chemistry

Preparation of the apigenin conjugates (**1a** ~ **1g**) is summarized in Scheme 1. Treatment of apigenin (**2**) with 1.5 equiv. of pivaloxymethyl iodide (POM-I) in acetone gave a readily separable mixture of 7-O-POM-apigenin (1a, 32% yield) and 4′,7-bis-O-POM-apigenin (**1b**, 49% yield). 4′-O-POM-apigenin (**1c**), on the other hand, was obtained via protection of 7-OH of apigenin (**2**) with a benzyl group followed by alkylation with POM-I and deprotection of the benzyl group. Thus, reaction of apigenin (**2**) with 1.0 equiv. of BnBr in DMF provided the corresponding 7-O-benzyl ether **3** in 58% yield, which, upon treatment with POM-I, underwent regioselective alkylation 4′-O position. Deprotection of the benzyl group under hydrogenolysis conditions afforded the desired 4′-O-POM-apigenin (**1c**) in 72% yield.

The glucose functionality was introduced to the apigenin 4′-O or 7-O positions via glycosylation with 1-bromo-glucose-pentaacetate (**5**) which was obtained by bromination of the anomeric carbon of the commercially available β-D-glucose-pentaacetate (**4**) in 87% yield. Glycosylation of the apigenin 7-O-benzyl ether (**3**) with 1-bromo-glucose-pentaacetate (**5**) using Aliquat 336 as the phase-transfer-catalyst [46, 47] provided the apigenin-glucose conjugate in 56% yield, which, after a series of deprotection, was smoothly converted to the desired apigenin-4′-O-glucoside (**1d**) in 71% combined yield. Regioselective introduction of the glucose functionality at 7-O position was accomplished after selective deprotection of 7-OAc group [48] of the 4′,5,7-tri-O-acetyl-apigenin which was obtained by peracetylation of apigenin (**2**). The 4′,5′-di-O-acetyl-apigenin (**4**), thus obtained, was treated with 1-bromo-glucose-pentaacetate (**5**) in the presence of K2CO3 to give the apigenin 7-O-glucoside as a β-anomer in 60% yield. Interestingly, glycosylation under the phase-

transfer-catalysis conditions (Aliquat 336) did not produce the desired product. Deprotection of the acetyl protecting groups was attempted with saturated methanolic ammonia to provide the desired apigenin-7-O-glucoside (**1e**) in 84% yield.

Amino acid tert-butyl esters such as H-Ala-OtBu·HCl (**7**) and H-Glu(OtBu)-OtBu·HCl (**8**) were treated with bis(4-nitrophenyl) carbonate and DIPEA in THF to provide the activated 4-nitrophenyl carbamates of amino acid esters, 9 and 10, in 83% yield and 61% yield, respectively. Condensation of apigenin (**2**) with the carbamates of amino acid esters 9 and 10 in the presence of DIPEA in THF smoothly underwent to give the corresponding amino acid conjugates, of which tert-butyl groups were removed upon treatment with TFA to furnish the desired apigenin 7-O-carbamates conjugated with alanine (**1f**) and glutamic acid (**1g**), in 86% and 42% combined yields, respectively.



Reagent & Conditions : (a) POMl, K_2CO_3, Acetone, rt; (b) BnBr, Pyridine, rt; (c) H_2, Pd/C, MeOH, THF, rt; (d) **5**, K_2CO_3, Aliquat 336, $CHCl_3/H_2O$ (2:1), 50 °C; (e) NH_3, MeOH, rt; (f) Ac_2O, Pyridine, 50 °C; (g) Thiophenol, imidazole, NMP, rt; (h) **5**, K_2CO_3, DMF, 80 ℃; (i) **9** or **10**, DIPEA, THF, rt; (j) TFA, rt; (k) HBr,CH_2Cl_2, rt; (l) Bis-4-nitrophenylcarbonate, DIPEA, THF, rt.

Scheme 1. Hydrophobic interactions between cobicistat and residues of 6vhn.pdb analyzed using the LigPlot program.

3.2. biological activity

We studied sensitivities to doxorubicin in human MDR cancer cells and then evaluated the reversing activities of the apigenin (**2**) and its conjugates (**1a** ~ **1g**) against doxorubicin resistance in vitro.

*3.3. intrinsic cytotoxicity of the apigenin conjugates (**1a** ~ **1g**)*

The cell viability curves of apigenin (**2**) and its conjugates (**1a** ~ **1g**) showed clear-cut distinction between the active and inactive concentrations with a sudden drop of cytotoxicity below 50 μM (data not shown). As a result, at concentrations below 50 μM, neither apigenin (**2**) nor apigenin conjugates (**1a** ~ **1g**) showed growth-inhibiting effect on drug-sensitive MES-SA and multidrug-resistant MES-SA/Dx5 cells (survival rates of tumor cells were more than 90%).

*3.4. in vitro MDR reversal effect of the apigenin (2) and apigenin conjugates (**1a** ~ **1g**)*

When the drug-sensitive cancer cell line (MES-SA) was treated with anticancer drug doxorubicin, the cell viability was almost completely decreased with the increased concentration of doxorubicin [Fig. 3a]. In contrast, viability of the drug-resistant cell line (MES-SA/Dx5) was shown to be unaffected by doxorubicin concentration [Fig. 3b].

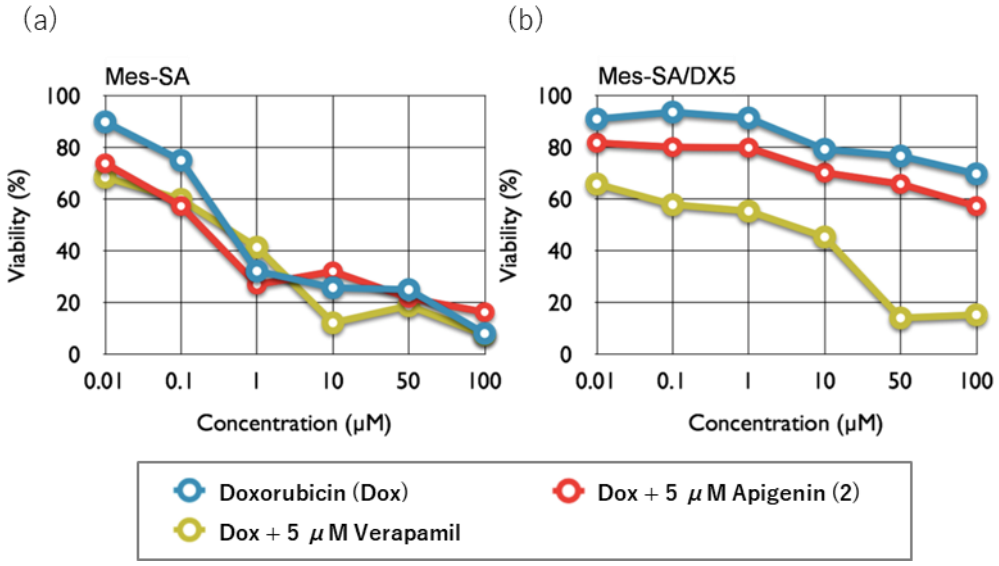


Fig. 3. Hydrophobic interactions between cobicistat and residues of 6vhn.pdb analyzed using the LigPlot program.

As shown above, at the concentration used to modulate MDR (5 μM), apigenin (**2**) and its conjugates (**1a** ~ **1g**) were not cytotoxic at all, and addition of these compounds in combination with doxorubicin did not change the cytotoxic profile of the anticancer drug [Figs. 3a and 4a]. The well-known MDR-modulating agent verapamil did not affect the viability of the wild-type cell line, either [Fig. 3a].

In the drug-resistant MES-SA/Dx5 cell line, however, the positive control (verapamil) restored the sensitivity of the resistant MES-SA/Dx5 cells to doxorubicin to a significant degree, and a concentration-dependent sensitization of MES-SA/Dx5 cells to anticancer drug doxorubicin by verapamil is shown in Fig. 3b.

In case of apigenin (**2**), addition of apigenin (**2**) slightly improved the cytotoxic effect of doxorubicin in the drug-resistant cell line, but its effect was not significant [Fig. 3b]. Among the synthesized apigenin conjugates (**1a** ~ **1g**), two compounds (**1b** and **1c**) showed increased MDR-reversal activity compared with apigenin (**2**) [Fig. 4b]. In particular, 7,4′-bis-O-POM-apigenin (**1b**), the purple line in Fig. 4b, showed MDR-reversal activity comparable to that of verapamil at doxorubicin concentrations below 10 μM. Unfortunately, however, the apigenin conjugate **1b** failed to show concentration-dependent MDR-reversal activity at doxorubicin concentrations higher than 10 μM [Fig. 4b].

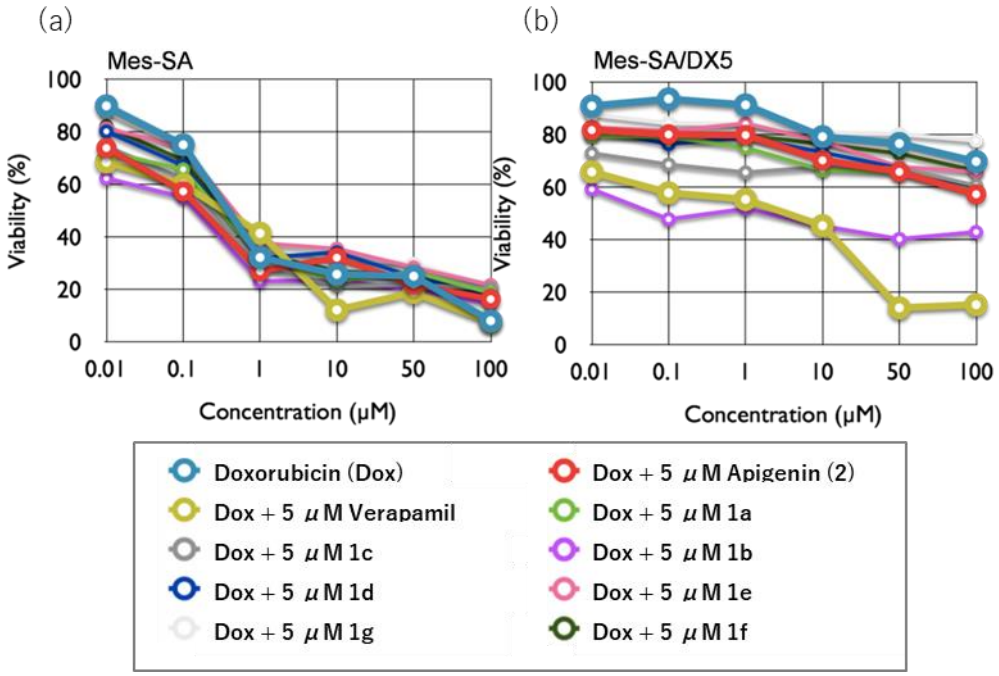


Fig. 4. Hydrophobic interactions between cobicistat and residues of 6vhn.pdb analyzed using the LigPlot program.

MDR is a serious problem in cancer chemotherapy, but MDR-modulators developed to date suffer from side effects as well as unpredictable pharmacokinetic properties. In this study, as a part of our ongoing efforts to develop a new MDR-modulator with reduced side effect, we attempted to use conjugates of the natural flavonoid, apigenin. A series of apigenin conjugates with a pivaloxymethyl, glucose, alanine, and glutamic acid promoieties attached at either or both 7 and 4′-O position of apigenin were prepared and their MDR-modulatory effects were evaluated. Among those, 7,4′-bis-O-POM-apigenin (**1b**) showed promising MDR-modulating activity particularly at low concentrations of the anticancer drug, doxorubicin. Even though the doxorubicin concentration-independent MDR-modulatory activity of **1b** needs further investigation regarding its mode of action, the proven safety of the natural flavonoid along with interesting MDR-modulating activity found in some of the apigenin conjugates warrant extensive structure-activity relationship study of the flavonoid conjugates as novel and safe MDR-modulating agents.

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Conflicts of Interest: The author has no conflict of interest related to this study to disclosure.

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