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Original article

Synthesis of methylated quercetin derivatives and their reversal activities on P-gp- and BCRP-mediated multidrug resistance tumour cells

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ABSTRACT

Three methylated quercetins and a series of O-3 substituted 5,7,3′,4′-tetra-O-methylated quercetin derivatives have been synthesized and evaluated on the modulating activity of P-gp, BCRP and MRP1 in cancer cell lines. Compound **17** (with a 2-((4-methoxybenzoyl)oxy)ethyl at O-3) is the most potent P-gp modulator. Three derivatives, compound **9** (3,7,3′,4′-tetra-O-methylated quercetin), compound **14** (with a 2-((3-oxo-3-(3,4,5trimethoxyphenyl)prop-1-en-1-yl)oxy)ethyl at O-3) and compound **17**, consistently exhibited promising BCRP-modulating activity. Interestingly, compound **17** was found to be equipotent against both P-gp and BCRP. Importantly, these synthetic quercetin derivatives did not exhibit any inherent cytotoxicity to cancer cell lines or normal mouse fibroblast cell lines. These quercetin derivatives can be employed as safe and effective modulators of P-gp- or BCRP-mediated drug resistance in cancer.

1. Introduction

Multidrug resistance (MDR) in cancer has been a major obstacle to successful cancer chemotherapy. An important mechanism for MDR is the enhanced cellular efflux of anticancer agents due to overexpression of ATP-binding cassette (ABC) transporter proteins [1]. Among the 48 ABC transporters identified so far, P-glycoprotein (P-gp, ABCB1), multidrug resistance-related protein 1 (MRP1, ABCC1) and breast cancer resistance protein (BCRP, ABCG2) are three main efflux transporters that have been shown to be associated with MDR [2]. The three ABC proteins consist of transmembrane domains (TMDs) and nucleotide-binding domains (NBDs) [3–10]. Despite their structural similarities, the substrate binding modes and binding sites of the three proteins are not well characterized. There is no common "pharmacophore" that can be identified as an inhibitor of these three ABC transporters [3]. Nevertheless, different inhibitors or modulators of ABC multidrug efflux pumps have been identified either by serendipitous discovery, combinatorial chemistry and rational drug design or based on the known structure of these transporters [11–13]. There are three generations of P-gp inhibitors including calcium channel blockers [14–16], calmodulin antagonists [17,18], cyclic peptides, steroids, and some synthetic compounds such as elacridar, tariquidar, biricodar, S-9788 (shown in Fig. 1), ontogen, zosuquidar, and purine derivatives [3,19–23]. Pantoprazole, fumitremorgin C and its derivatives (eg. Ko143) are specific ABCG2 inhibitors [3,24]. The third generation P-gp inhibitors of elacridar and tariquidar also modulate ABCG2 activity [25,26]. Fewer MRP1 inhibitors have been identified. The Leukotriene C4 (LTC4) analogue, MK571, S-decylglutathione and probenecid have been described as MRP1 modulators [3,27,28]. All of these three ABC drug transporters can interact with the modulator biricodar [3].

It has been demonstrated that non-toxic natural flavonoids such as kaempferol, quercetin, baicalein, myricetin, fisetin, morin and

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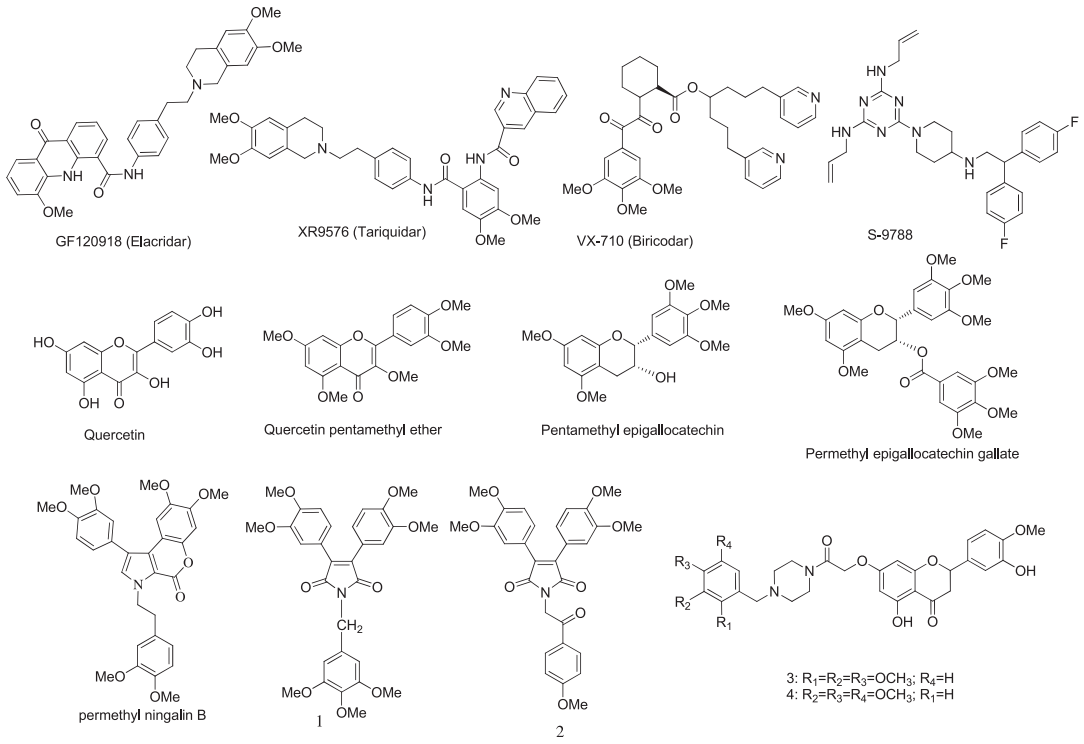


Fig. 1. Inhibitors of ABC transporters.

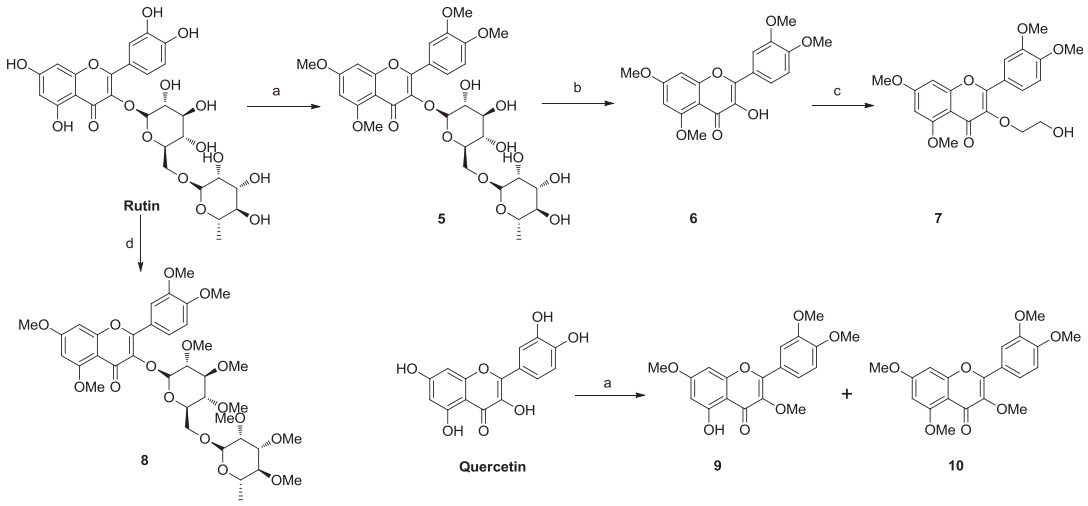
epigallocatechin gallate may reverse MDR [29]. Quercetin (Fig. 1) can modulate P-gp-, BCRP-, or MRP1-mediated drug resistance [30–34], and preliminary SAR studies of flavonoids as MDR modulating agents were carried out by Conseil et al. [35]. However, the concentration of quercetin in the experiment was still high. For example, in one study, addition of 100 μM of quercetin could result in an increased daunomycin accumulation (201.8 ± 16.4%) in the P-gp overexpressed resistant cell line MCF-7/ADR, which was comparable to 100 μM of verapamil (229.4 ± 17.6%). Interestingly, quercetin pentamethyl ether (at 20 μM) was found to have shown potent P-gp modulating activity [31,36]. Other methylated phenolic compounds like permethyl epigallocatechin gallate (Fig. 1) [37], permethyl ningalin B (Fig. 1) [38] and its synthetic analogues (eg. compounds **1** and **2** in Fig. 1) [39] also displayed potent P-gp-modulating activities. These results suggest that methylation of some natural phenolic compounds could improve P-gp modulating activity, and multi-methoxy substituted phenyl rings may be an important pharmacophore for reversing MDR. Moreover, we introduce a basic nitrogen on flavonoids in order to increase their lipophilic properties, Ferte et al. synthesized a series of flavonoid (including diosmetin, diosmin and the flavanone hesperetin) derivatives containing a *N*-benzylpiperazine side chain at O-5, O-7, or O-3′, and investigated their anti-MDR ability [40]. At 5 μM, it was found that most compounds (such as compounds **3** and **4** shown in Fig. 1) potentiated doxorubicin cytotoxicity on resistant K562/DOX MDR cells [40]. These compounds were also able to increase the intracellular accumulation of JC-1, a fluorescent molecule recently described as a probe of P-glycoprotein-mediated MDR [40]. In the present work, we aimed to synthesize a novel series of methylated quercetin derivatives and 5,7,3′,4′-tetra-O-methylated quercetin derivatives containing a heterocycle or a multi-methoxyphenyl group in the O-3 substituents, and to investigate their anti-MDR activity affected by different side chain positions from the literature [40]. The modulating selectivity of the synthesized quercetin derivatives on the three main MDR-associated ABC efflux transporters, P-gp, BCRP, and MRP1, was also investigated.

2. Results and discussion

2.1. Chemistry

We have previously found that permethyl epigallocatechin gallate was more potent than pentamethyl epigallocatechin (Fig. 1) in modulating P-gp in LCC6MDR cells [37], suggesting that substitutions at C-3 position of quercetin may be important in P-gp modulating activity. Here, we tested such hypothesis by synthesizing and evaluating a series of 3-substitued 5,7,3′,4′-tetra-O-methylated quercetins, such as compounds **7**–**17**, **19**, **21**, and **23** (shown in Schemes 1–3 respectively) for their P-gp modulating activities. Based on the published method [39], rutin was methylated by methyl iodide to generate tetramethylated rutin **5**, followed by acidolysis of **5** to give 5,7,3′,4′-tetra-O-methylated quercetin **6** (Scheme 1). Spectroscopic data of compound **6** was identical to that reported in literature [39]. Compound **6** was refluxed with 2-bromoethanol in the presence of potassium carbonate to give compound **7** which was also used as intermediate in the preparation of 3-substitued 5,7,3′,4′-tetra-O-methylated quercetins. Rutin was also methylated by methyl iodide in the presence of NaH to produce permethylated rutin **8** (Scheme 1) [41]. Compounds **9** and **10** were effectively obtained via the reaction of quercetin with methyl iodide in DMF in the presence of potassium carbonate (Scheme 1). The reaction conditions were milder than reported [42]. When four equivalents of methyl iodide were used in the reaction, it afforded 21.2% of **9** and 35.6% of **10**. If methyl iodide was increased to ten equivalents, it produced trace of **9** and 57.6% of **10**.

Further modifications of 5,7,3′,4′-tetra-O-methylated quercetin **6** at O-3 were described in Scheme 2. Esterification of 5,7,3′,4′-tetra-O-methylated quercetin **6** with 3,4,5-trimethoxybenzoic acid catalyzed by EDCI afforded compound **11**. Intermediate **6** was coupled



Scheme 1. Synthetic routes of compounds **6**, **7**, **8**, **9** and **10**. Reagents and conditions: a) CH_3I, K_2CO_3, DMF, rt, 2 d; b) HCl, 95% ethanol, reflux, 2 h; c) $BrCH_2CH_2OH$, acetone, K_2CO_3, reflux, 12 h; d) CH_3I, NaH, DMF, rt, 15 h.

with 3,4,5-trimethoxybenzyl methanesulfonate to give compound **12**, and reacted with 2-bromo-1-(3,4-dimethoxyphenyl)ethanone to produce compound **13**. As shown in Scheme 2, esterification of intermediate **7** with (*E*)-3-(3,4,5-trimethoxyphenyl)acrylic acid, 3,4,5-trimethoxybenzoic acid, 3,4-dimethoxybenzoic acid, or 4-methoxybenzoic acid provided compounds **14**, **15**, **16**, and **17** respectively.

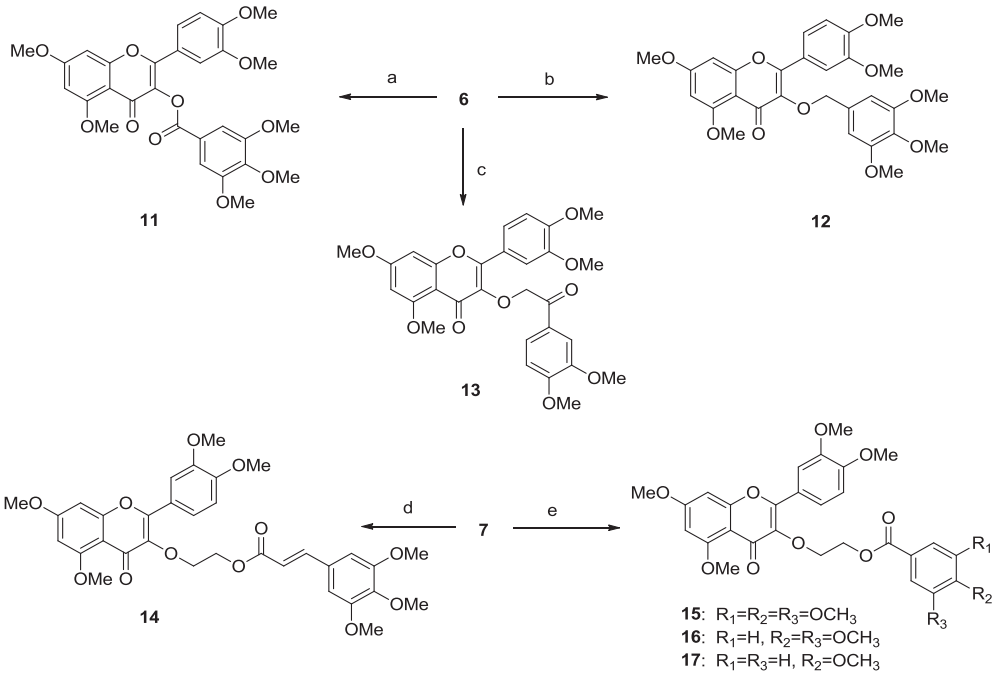
In order to investigate heterocycle-containing quercetin derivatives, three new compounds were prepared and the synthetic routes were shown in Scheme 3. Coupling of compound **6** with 9-(2-bromoethyl)-9*H*-purin-6-amine **18** [43], 9-(2-bromoethyl)-1,3-dimethyl-1*H*-purine-2,6(3*H*,9*H*)-dione **20** [44], or 6-(2-bromoethoxy)-*N*-(3-chloro-4-fluorophenyl)-7-methoxy-3,4-dihydroquinazolin-4-amine **22** [45] provided compounds **19**, **21**, and **23** respectively.

2.2. Biological evaluation

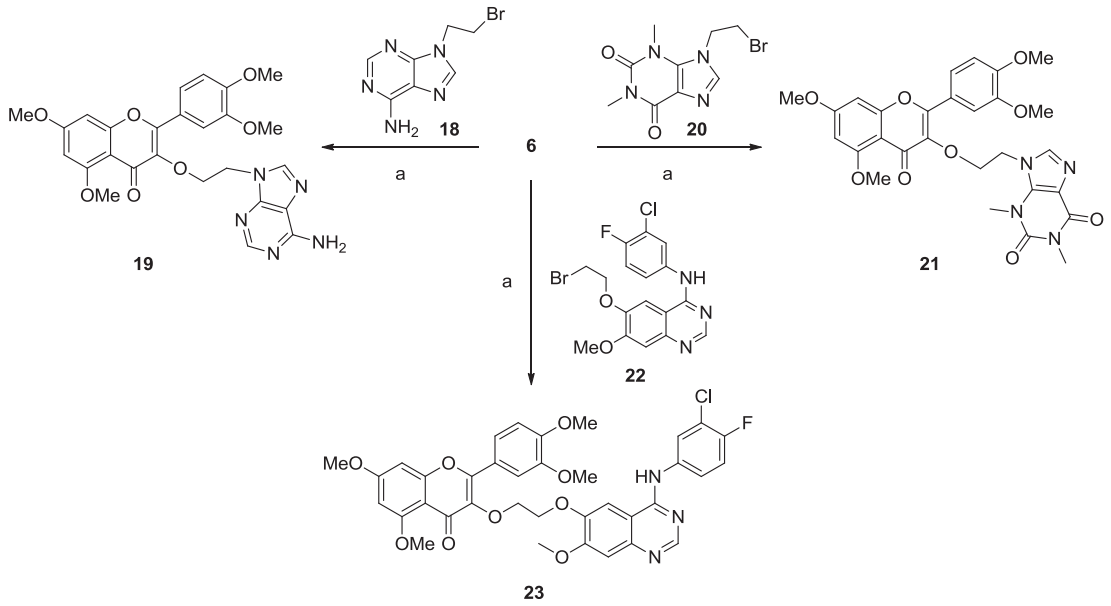
2.2.1. P-Gp-, BCRP- and MRP1-modulating activities of quercetin derivatives

Here, we designed and synthesized a novel series of methylated quercetin derivatives (containing various side chains at O-3) and tested for their P-gp-, BCRP- and MRP1-modulating activities. Almost all quercetin derivatives exhibited no cytotoxicity towards LCC6, LCC6MDR or normal mouse fibroblast cells (L929) with IC_{50} higher than 100 μM (Table 1). The only slightly toxic compound is compound **12**, with IC_{50} towards L929 at 17.6 μM.

We employed four drug-resistant cell lines in this study. These include (1) a P-gp-transfected human breast cancer cell line, LCC6MDR which displayed 90.7-fold resistance to paclitaxel



Scheme 2. Synthetic routes of compounds **11**–**17**. Reagents and conditions: a) EDCI, DMAP, CH_2Cl_2, 3,4,5-trimethoxybenzoic acid, rt, overnight; b) K_2CO_3, DMF, 3,4,5-trimethoxybenzyl methanesulfonate, rt, 12 h; c) K_2CO_3, DMF, 2-bromo-1-(3,4-dimethoxyphenyl)ethanone, rt, 12 h; d)EDCI, DMAP, CH_2Cl_2, (*E*)-3-(3,4,5-trimethoxy-phenyl)acrylic acid, rt, overnight; e) EDCI, DMAP, CH_2Cl_2, 3,4,5-trimethoxybenzoic acid, 3,4-dimethoxybenzoic acid, or 4-methoxybenzoic acid, rt, overnight.



Scheme 3. Synthetic routes of compounds **19**, **21**, and **23**. Reagents and conditions: a) K_2CO_3, DMF, 60 °C, 12 h.

(IC_{50} = 145.1 ± 8.2 nM) compared with parental LCC6 cells (IC_{50} = 1.6 ± 0.3 nM) (Table 2), (2) a BCRP-transfected human embryonic kidney cell line, HEK293/R2, which was 30.8-fold more resistant to topotecan (IC_{50} = 486.9 ± 34.6 nM) than parental HEK293/pcDNA3.1 cell line (IC_{50} = 15.8 ± 1.5 nM) (Table 2), (3) a mitoxantrone-selected breast cancer cell line MCF7-MX100 in which BCRP was found to be overexpressed and exhibited 117.5-fold resistance to topotecan (IC_{50} = 37.6 ± 2.7 μM) compared to wild-type MCF7 (IC_{50} = 0.32 ± 0.07 μM) (Table 2), (4) a MRP1-transfected ovarian cancer cell line, 2008/MRP1 which was 6.7-fold more resistant to DOX (IC_{50} = 417.8 ± 65.4 nM) than parental 2008/P cells (IC_{50} = 62.5 ± 5.1 nM) (Table 2). Verapamil is a known P-gp and MRP1 inhibitor, whereas Ko143 was a BCRP-specific modulator. Here, we included these positive controls in the cell proliferation assay.

Table 1
In vitro cytotoxicity IC_{50} values (μM) of quercetin derivatives to LCC6, LCC6MDR and L929 cells.

Cpds[a]	LCC6	LCC6MDR	L929
6	>100	>100	>100
7	>100	>100	>100
8	>100	>100	>100
9	>100	>100	>100
10	>100	>100	>100
11	>79	87.4 ± 6.0	74.1 ± 10.4
12	14.5 ± 3.8	46.6 ± 20.3	17.6 ± 1.6
13	>100	>100	>100
14	>100	>100	>100
15	>100	>100	>100
16	>100	>100	>100
17	>100	>100	>100
19	>100	>100	>100
21	>100	>100	>100
23	>100	>100	>100
26	>100	>100	>100
Quercetin	>100	>100	>100
Rutin	>100	>100	>100

IC_{50} were determined by treating LCC6, LCC6MDR or L929 cells with different concentrations of quercetin derivatives. L929 is a mouse fibroblast cell line. Each experiment has been repeated 3 times with the data presented as mean ± standard error of mean.3.

[a] All compounds were dissolved in DMSO and the highest percentage of DMSO used was 1% at which no toxicity towards cancer cell lines and L929 was observed.

The MDR reversal activity of quercetin derivatives were listed in Table 3.

Quercetin and its derivatives were categorized into five subgroups depending on their structures (Table 3 and Fig. 2). In group A, quercetin and rutin (at 1.0 μM) did not exhibit any P-gp- (RF of quercetin = 0.8 and RF of rutin = 0.9) or MRP1-modulating activity (RF of quercetin = 1.1 and RF of rutin = 1.2). They also displayed no BCRP-reversal activity in HEK293/R2 (RF of quercetin = 1.3 and RF of rutin = 1.3) or very weak BCRP-reversal activity in MCF7-MX100 cell lines (RF of quercetin = 4.7 and RF of rutin = 4.3). These data suggest that quercetin and rutin are poor chemosensitizers and are not specific for the ABC transporters. Permethylated rutin **8**, being more hydrophobic than rutin, displayed no P-gp- (RF of **8** = 1.0) or MRP1-modulating activity (RF of **8** = 1.4) at 1.0 μM. With respect to the BCRP-modulating activity, compound **8** displayed similar potency (RF = 1.5 in HEK293/R2 and RF = 5.3 in MCF7-MX100) as rutin (RF = 1.3 in HEK293/R2 and RF = 4.3 in MCF7-MX100). These data indicate that methylation of

Table 2
Cells lines used to study P-gp-, BCRP- and MRP1-modulating activities.

Cell lines	IC_{50} towards	
	Paclitaxel (*nM*)	*RF*
LCCMDR	145.1 ± 8.2	1.0
LCC6MDR + DMSO	148.0 ± 13.9	1.0
LCC6	1.6 ± 0.3	90.7
	Topotecan (*nM*)	*RF*
HEK293/R2	486.9 ± 34.6	1.0
HEK293/R2 +DMSO	534.7 ± 25.7	0.9
HEK293/pcDNA3.1	15.8 ± 1.5	30.8
	Topotecan (*μM*)	
MCF7-MX100	37.60 ± 2.7	1.0
MCF7-MX100 + DMSO	33.00 ± 4.0	1.1
MCF7	0.32 ± 0.07	117.5
	DOX (*nM*)	*RF*
2008/MRP1	417.8 ± 65.4	1.0
2008/MRP1 + DMSO	420.0 ± 24.0	1.0
2008/P	62.5 ± 5.1	6.7

Four cell lines were used in this study (refer to text for details). 0.1% DMSO was added to control for the solvent effect. All modulators used in this study were dissolved in DMSO and used at a final concentration of 1.0 μM, which was equivalent to 0.1% DMSO.

Table 3
Structural characteristics and MDR reversal activity of quercetin derivatives.

Gp.	Cpds	Substituents at O-3	Types of linker	LCC6MDR		HEK293/R2		MCF7-MX100		2008/MRP1	
				Paclitaxel IC_{50} (nM)	RF	Topotecan IC_{50} (nM)	RF	Topotecan IC_{50} (μM)	RF	DOX IC_{50} (nM)	RF
	Verapamil			43.9 ± 5.2	3.3					77.0 ± 3.0	5.4
	Ko143					24.0 ± 1.9	20.3	0.5 ± 0.1	75.2		
A	Quercetin	OH	None	174.8 ± 25.9	0.8	368.8 ± 152.9	1.3	8.1 ± 1.6	4.7	367.0 ± 54.8	1.1
	Rutin	Dissacharide	None	167.2 ± 33.9	0.9	361.9 ± 137.5	1.3	8.7 ± 2.3	4.3	345.1 ± 61.8	1.2
	8	Methylated dissacharide	None	149.4 ± 43.8	1.0	318.2 ± 133.7	1.5	7.1 ± 0.6	5.3	298.6 ± 29.7	1.4
B	**6**	OH	None	83.0 ± 6.7	1.7	146.8 ± 85.1	3.3	6.3 ± 3.0	6.0	608.4 ± 70.6	0.7
	7	2-Hydroxyethyl	None	105.6 ± 9.9	1.4	124.2 ± 8.6	3.9	25.6 ± 13.2	1.5	497.1 ± 87.9	0.8
	9	Methoxy	None	39.6 ± 1.7	3.7	37.3 ± 2.0	13.0	2.0 ± 0.6	19.3	575.0 ± 123.1	0.7
	10	Methoxy	None	76.8 ± 2.6	1.9	55.9 ± 4.1	8.7	4.8 ± 0.8	7.9	554.0 ± 100.5	0.8
C	**11**	Trimethoxybenzoyl	Carbonyl	67.7 ± 6.4	2.1	84.5 ± 27.2	5.8	3.9 ± 0.8	9.6	465.9 ± 60.9	0.9
	12	Trimethoxybenzyl	Methylene	15.1 ± 1.3	9.6	43.0 ± 6.2	11.3	7.9 ± 1.3	4.7	345.5 ± 60.4	1.2
	13	Dimethoxyphenyl-2-oxo-ethyl	Methylenecarbonyl	115.1 ± 15.1	1.3	349.4 ± 68.3	1.4	27.7 ± 9.0	1.4	481.4 ± 75.0	0.9
D	**14**	(*E*)-2-((3-oxo-3-(3,4,5-Trimethoxy phenyl)prop-1-en-1-yl)oxy)ethyl	Ethyleneoxycarbonylvinyl	24.2 ± 2.2	6.0	34.7 ± 3.4	14.0	4.1 ± 0.6	9.1	287.0 ± 1.0	1.5
	15	2-((3,4,5-Trimethoxybenzoyl)oxy) ethyl	Ethyleneoxycarbonyl	46.0 ± 10.5	3.2	102.7 ± 12.3	4.7	18.9 ± 5.8	2.0	376.5 ± 73.2	1.1
	16	2-((3,4-Dimethoxybenzoyl)oxy) ethyl	Ethyleneoxycarbonyl	22.8 ± 2.1	6.4	105.5 ± 2.8	4.6	5.6 ± 0.8	6.7	359.0 ± 9.6	1.2
	17	2-((4-Methoxybenzoyl)oxy)ethyl	Ethyleneoxycarbonyl	12.9 ± 1.2	11.3	40.2 ± 8.2	12.1	5.2 ± 0.6	7.3	272.4 ± 18.4	1.5
E	**19**	Ethyl×ene linked heterocycle	ethyleneoxy	129.1 ± 19.7	1.1	410.9 ± 72.6	1.2	32.9 ± 7.1	1.2	509.8 ± 91.4	0.8
	21	Ethylene linked heterocycle	ethyleneoxy	122.2 ± 12.8	1.2	355.4 ± 55.0	1.4	27.0 ± 3.0	1.4	408.4 ± 32.7	1.0
	23	Ethylene linked heterocycle	ethyleneoxy	99.7 ± 9.9	1.5	113.2 ± 18.1	4.3	31.5 ± 6.8	1.2	438.3 ± 22.3	1.0

IC_{50} value was determined for paclitaxel, topotecan and DOX with 1.0 μM of quercetin derivatives using LCC6MDR, HEK293/R2, MCF7-MX100 and 2008/MRP1 cells. At such a low concentration of quercetin derivatives, no cytotoxic effect on the cell lines was observed. Relative fold (RF) = (IC_{50} without modulator)/(IC_{50} with 1.0 μM modulator). $N = 2–4$ independent experiment and values were presented as mean ± standard error of mean. Verapamil was used at 20 μM for testing MRP1-modulating activity.

carbohydrate side chain has no impact on P-gp-, MRP1- and BCRP-modulating activities.

Among the alkylated quercetins in group B, compound **9** (3,7,3′,4′-tetra-O-methylated quercetin) at 1.0 μM showed a relatively higher P-gp (RF = 3.7) and BCRP- (RF = 13.0 in HEK293/R2 and RF = 19.3 in MCF7-MX100) modulating activities than **6** (5,7,3′,4′-tetra-O-methylated quercetin) (RF = 1.7 in LCC6MDR, RF = 3.3 in HEK293/R2 and RF = 6.0 in MCF7-MX100) and **10** (quercetin pentamethyl ether) (RF = 1.9 in LCC6MDR, RF = 8.7 in HEK293/R2 and RF = 7.9 in MCF7-MX100). It demonstrates that 5-O-non-methylation and 3-O-methylation contribute significantly to P-gp- and BCRP-modulation activities. Compound **7**, having a 2-hydroxyethyl substitution at O-3 of compound **6**, also has weak P-gp- (RF = 1.4) and BCRP- (RF = 3.9 in HEK293/R2 and RF = 1.5 in MCF7-MX100) modulating activities. None of the compounds in group B displayed MRP1-modulating activity as all RF values were close to 1.0.

In group C, 5,7,3′,4′-tetra-O-methylated quercetin **6** was modified at O-3 to give the three compounds **11**, **12**, and **13**. Compound **12** (RF = 9.6), having a 3,4,5-trimethoxyphenyl group linked by a methylene at O-3, displayed very potent P-gp-modulating activity than the parent compound **6** (RF = 1.7). Unlike compound **12**, both **11** and **13**, which possessed a carbonyl group in their linkers, gave low P-gp-modulating activity (RF of **11** = 2.1 and RF of **13** = 1.3). These data indicate that phenyl ring connected to the quercetin at O-3 with a methylene linker but not carbonyl linker can enhance P-gp-modulating activity.

Consistently, an improved BCRP-modulating activity of compound **12** (RF = 11.3 in HEK293/R2) was also noted in HEK293/R2 as compared to compound **6** (RF = 3.3 in HEK293/R2). Compound **13** with a methylenecarbonyl linker has no BCRP-modulating activity in both HEK293/R2 (RF = 1.4) and MCF7-MX100 (RF = 1.4). Compound **11**, with a carbonyl group in the linker, can retain a moderate BCRP-modulating activity in both HEK293/R2 (RF = 5.8) and MCF7-MX100 (RF = 9.6) as compared to the compound **6** (RF = 3.3 in HEK293/R2 and RF = 6.0 in MCF7-MX100). These data suggest that the type of linkers between the terminal phenyl ring and methylated quercetin at O-3 position is an important factor for determining the BCRP-modulating activities of quercetin derivatives. No MRP1-modulating activity was found in compounds **11**, **12** or **13**.

In group D, compounds **14** (RF = 6.0), **15** (RF = 3.2), **16** (RF = 6.4), and **17** (RF = 11.3) all possessed promising P-gp modulating activities. Compounds **15**, **16** and **17** have mono, di and tri methoxy groups on the terminal phenyls respectively. This result suggests that the compound containing fewer methoxy groups on the terminal phenyl ring would exhibit stronger P-gp-modulating activity. Compound **14** (RF = 6.0), containing a longer ethyleneoxycarbonylvinyl linker and an extra C=C bond, was found to display a stronger P-gp-modulating activity than compound **15** (RF = 3.2) which possessed only the ethyleneoxycarbonyl linker.

Consistent with P-gp-modulation, the number of methoxy groups on terminal phenyl ring also has a strong effect on BCRP modulating activity. Compound **17** with the monomethoxy group (RF = 12.1 in HEK293/R2 and RF = 7.3 in MCF7-MX100) exhibited stronger BCRP-activity than compound **15** with trimethoxy groups (RF = 4.7 in HEK293/R2 and RF = 2.0 in MCF7-MX100). Here, compound **14** with longer linker and an extra C=C bond (RF = 14.0 in HEK293/R2 and RF = 9.1 in MCF7-MX100) showed a higher BCRP1-modulating activity than compound **15** (RF = 4.7 in HEK293/R2 and RF = 2.0 in MCF7-MX100) possessing a shorter linker. Once again, we found that linker length between the terminal phenyl ring and the methylated quercetin plays an important role in P-gp- and BCRP-modulating activity. None of quercetin derivatives in this series can modulate MRP1.

In group E, compounds **19**, **21**, and **23** contained various nitrogen-containing heterocycles were designed and synthesized in order to investigate varied side rings. These three compounds, however, exhibited no significant P-gp-, BCRP- or MRP1-modulating activities. Only compound **23** displayed a moderate RF of 4.3 in BCRP-overexpressed HEK293/R2 cell line.

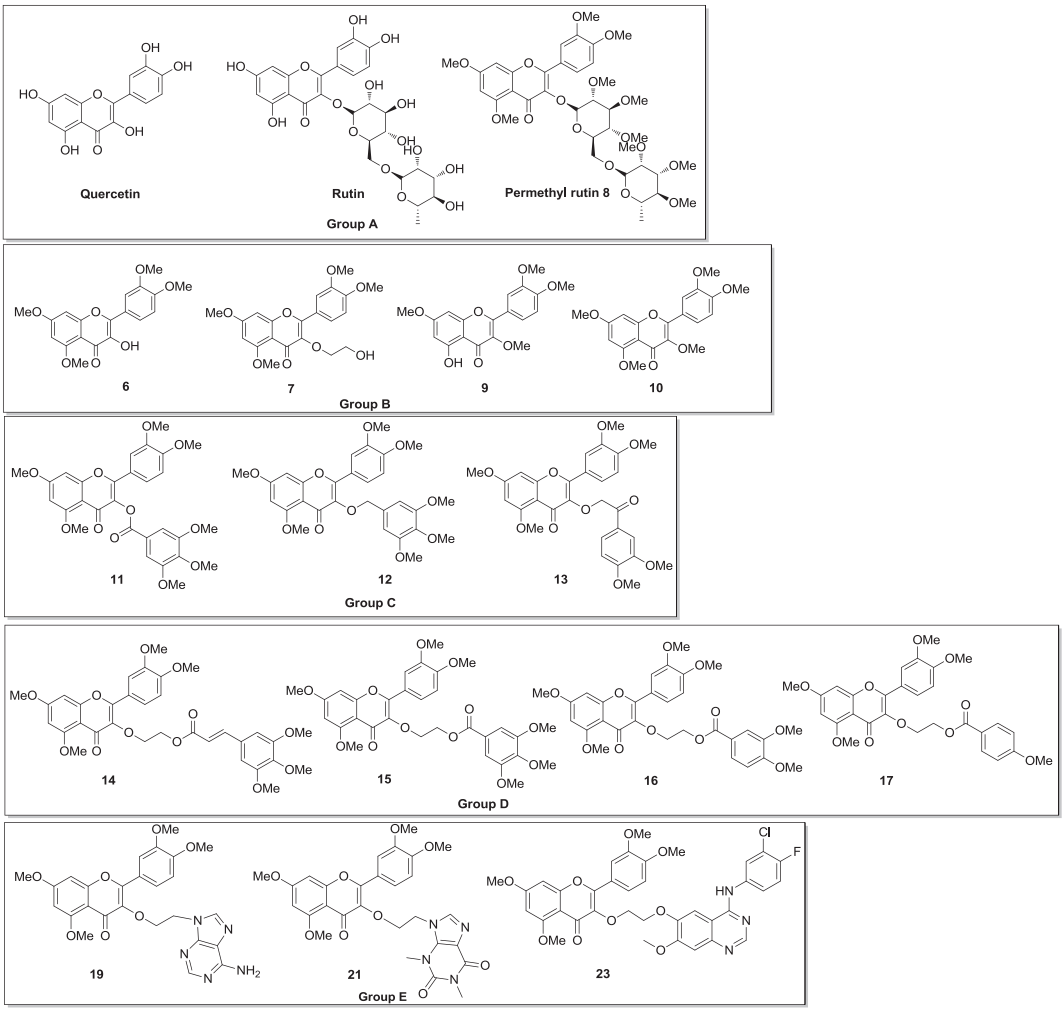


Fig. 2. Structure of synthetic quercetin derivatives.

Of all quercetin derivatives tested, compound **17** (containing a monomethoxy substituted phenyl linked by an ethyleneoxycarbonyl group) displayed the highest P-gp-modulating activity (RF = 11.3). This activity was about 3.4-fold higher than that of verapamil (RF = 3.3). With respect to BCRP modulation, three quercetin derivatives (compound **9**, **14** and **17**) displayed the highest BCRP-modulating activity with RF ranging from 7.3 to 19.3. These activities are still relatively low compared to Ko143.

None of the quercetin derivatives displayed any MRP1-modulating activity at 1.0 μM, suggesting that our quercetin derivatives are less specific for MRP1 transporter as compared to the P-gp and BCRP transporters. Verapamil is a weak MRP1 modulator. At a high concentration (20 μM), verapamil completely reversed DOX resistance in 2008/MRP1 (RF = 5.4 in Table 3). It is likely that MRP1 modulator might have its own special pharmacophore. The structure of MRP1 is highly similar to P-gp, but with an extra transmembrane domain (TM_0) at its N-terminal [3,5,7]. It is known that substrate selectivity of the ABC transporters markedly differs. P-gp substrates are neutral or mildly positive lipophilic compounds, whereas MRP1 is able to transport lipophilic anions. MRP1 has a broad substrate specificity including glutathione S-conjugates, glucuronide conjugates, sulphate conjugates, anticancer drugs, heavy metals and organic anions etc [46–50]. This wide difference in substrate specificity of various ABC transporters may explain why our quercetin derivatives markedly exhibit different potencies against P-gp, BCRP and MRP1. It seems that our quercetin derivative exhibit higher specificity for P-gp and BCRP than the MRP1.

3. Conclusions

In summary, we demonstrated that synthetic quercetin derivatives exhibited promising P-gp- and BCRP-mediated MDR reversal activities without any inherent cytotoxicity to cancer cell lines or normal mouse fibroblast cell lines. The number of methoxy substituents on the terminal phenyl ring and the type of linkers at O-3 side chain are two key structural features for determining P-gp-modulating activity of quercetin derivatives. Compound **17** with a 3-methoxybenzoyloxyethyl at O-3 showed the highest P-gp-modulating activity. Compound **9** (3,7,3′,4′-tetra-O-methylated quercetin) or compound **14** (with a 3,4,5-trimethoxyphenylacryloyloxyethyl at O-3) or compound **17** displayed promising BCRP-modulating activity. Interestingly, compound **17** was found to be equipotent against P-gp and BCRP. It might be a good MDR reversal agent because of its dual-modulating activities. The present study demonstrates that synthetic quercetin derivatives can be employed as safe and effective modulators of P-gp- or BCRP-mediated drug resistance in cancer cells. However, none of the synthesized quercetin derivatives displayed any MRP1-modulating activity at 1.0 μM. The reason of less specific for MRP1

transporter as compared to the P-gp and BCRP transporters is under investigated.

4. Experimental section

4.1. General

Starting materials and reagents were purchased from commercial suppliers and were used without further purification. 9-(2-Bromoethyl)-9*H*-purin-6-amine (**18**) [43], 9-(2-bromoethyl)-1,3-dimethyl-1*H*-purine-2,6(3*H*,9*H*)dione (**20**) [44], or 6-(2-bromoethoxy)-*N*-(3-chloro-4-fluorophenyl)-7-methoxy-3,4-dihydroquinazolin-4-amine (**22**) [45] were prepared following the methods in literature. Anhydrous methylene chloride was distilled under nitrogen from CaH_2. Anhydrous DMF was distilled under vacuum from CaH_2. Reaction flasks were flame-dried under a stream of N_2. All reactions were monitored by thin-layer chromatography (TLC), on aluminium sheets (Silica gel 60-F254, E. Merck). Compounds were visualized by UV light. Flash chromatography was carried out using silica-gel 60 (200–300 mesh). Silica gel chromatography solvents were of analytical grade. Melting points were recorded on a micro melting point apparatus MP-500D and were uncorrected. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra were measured with TMS as an internal standard when $CDCl_3$ or CD_3OD were used as a solvent. Chemical shifts are expressed in δ (ppm) and coupling constants (J) in Hz. High-resolution (ESI) MS spectra were recorded using a QTOF-2 Micromass spectrometer. In addition to NMR and High-resolution (ESI) MS, HPLC analysis was used to determine the purity (>95%) of the compounds. Compounds were dissolved in methanol (1.5 mL). A reversed phase Diamonsil C18 (2) (4.6 × 150 mm) column attached to a Gilson 322 pump coupled to a Gilson UV–vis-152 detector was used. Each sample was injected at a volume of 20 μL and eluted with methanol and the flow rate was 1 mL/min.

4.1.1. 2-(3,4-Dimethoxyphenyl)-3-(2-hydroxyethoxy)-5,7-dimethoxy-4H-chromen-4-one (*7*)

A mixture of compound **6** (1.00 g, 2.80 mmol), 2-bromoethanol (600 mg, 4.20 mmol), anhydrous K_2CO_3 (773 mg, 5.60 mmol) and KI (100 mg, 0.60 mmol) in DMF (20 mL) was heated with stirring at 60 °C for overnight. Then H_2O (100 mL) was added and the resulting mixture was extracted with ethyl acetate. The organic layer was dried over anhydrous $MgSO_4$. The solvent was evaporated to dryness, and the resulting residue was purified by flash chromatography to afford the title compound **7** (974 mg, 86.5% yield). Mp 120–122 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 7.71–7.69 (m, 2H), 6.98 (d, J = 8.8 Hz, 1H, H), 6.52 (d, J = 2.2 Hz, 1H), 6.37 (d, J = 2.2 Hz, 1H), 4.65 (br s, 1H, –OH), 3.98 (t, J = 3.8 Hz, 2H), 3.97, 3.96, 3.95, 3.91 (4 s, 12H, 4 × OCH_3), 3.79 (t, J = 3.8 Hz, 2H); ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 174.9, 164.3, 161.0, 158.9, 153.7, 151.1, 148.9, 139.8, 129.9, 121.8, 111.1, 110.9, 109.0, 96.1, 92.5, 75.0, 61.7, 56.5, 55.9; HRMS calcd for $(C_{21}H_{22}O_8 + H)^+$ 403.1393, found 403.1372.

4.1.2. 2-(3,4-Dimethoxyphenyl)-5,7-dimethoxy-3-(((2R,3R,4S,5R,6R)-3,4,5-trimethoxy-6-((((2S,3R,4R,5S,6S)-3,4,5-trimethoxy-6-methyltetrahydro-2H-pyran-2-yl)oxy)-methyl)tetrahydro-2H-pyran-2-yl)oxy)-4H-chromen-4-one (*8*)

Under an N_2 atmosphere, to a solution of rutin (1.00 g, 1.60 mmol) and sodium hydride (0.58 g, 24.00 mmol) in DMF (20 mL) methyl iodide (4.50 g, 32.00 mmol) was added at 0 °C. The solution was stirred for 6 h. The solution was poured into ice-water (100 mL), and the resulting mixture was extracted with chloroform. The organic layer was dried over anhydrous $MgSO_4$. The solvent was evaporated to dryness, and the resulting residue was purified by flash chromatography to afford the title compound **8** (651 mg, 44.2% yield). Mp 65–67 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 7.73 (d, J = 1.6 Hz, 1H), 7.59 (dd, J = 8.2, 1.6 Hz, 1H), 6.85 (d, J = 8.8 Hz, 1H), 6.37 (d, J = 2.2 Hz, 1H), 6.24 (d, J = 2.2 Hz, 1H), 5.66 (d, J = 7.14 Hz, 1H), 4.56 (s, 1H), 3.88, 3.86, 3.85, 3.78, 3.56, 3.53, 3.41, 3.32, 3.24, 3.12 (10s, 30H, 10 × OCH_3), 3.66 (q, J = 4.9 Hz, 1H), 3.31–3.25 (m, 3H), 3.22–3.21 (m, 2H), 3.17–3.13 (m, 2H), 3.90–3.84 (m, 2H), 2.89–2.84 (m, 2H), 0.98 (d, J = 6.1 Hz, 3H); ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 173.1, 163.9, 160.9, 158.6, 153.8, 150.7, 148.1, 135.9, 123.2, 121.8, 112.1, 110.4, 109.1, 100.3, 97.3, 95.7, 92.2, 85.9, 84.3, 81.8, 80.7, 79.8, 74.3, 67.6, 66.6, 60.8, 60.5, 59.7, 58.5, 57.4, 56.2, 56.0, 55.8, 55.7, 17.5; HRMS calcd for $(C_{37}H_{50}O_{16} + H)^+$ 751.3177, found 751.3201.

4.1.3. 2-(3,4-Dimethoxyphenyl)-5-hydroxy-3,7-dimethoxy-4H-chromen-4-one (*9*) and 2-(3,4-dimethoxyphenyl)-3,5,7-trimethoxy-4H-chromen-4-one (*10*)

Method 1: A solution of quercetin (100 mg, 0.33 mmol) in DMF (10 mL) and anhydrous potassium carbonate (69 mg, 0.50 mmol) was treated with methyl iodide (187 mg, 1.32 mmol) at 0 °C, and the solution was stirred at room temperature for 12 h until TLC showed that the reaction had been completed. The mixture was poured into ice-water (30 mL), and the solution was extracted with ethyl acetate, and then the extract was washed with saturated aqueous $NaHCO_3$ and brine, and then was dried over anhydrous $MgSO_4$. The solvent was evaporated and the residue was purified by flash chromatography on silica gel to afford the desired compounds **9** (25.1 mg, 21.2% yield) and **10** (43.7 mg, 35.6% yield).

Method 2: When 10 equivalents methyl iodide were used in the above reaction, trace of compound **9** and 57.6% of **10** were obtained.

The melting point of compounds **9** and **10** were identical to the literature [32].

Compound **9**: mp 156–157 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 12.65 (s, 1H), 7.74 (dd, J = 8.7, 2.3 Hz), 7.69 (d, J = 1.8 Hz, 1H), 6.99 (d, J = 8.7 Hz, 1H), 6.45 (d, J = 1.9 Hz, 1H), 6.36 (d, 2.3 Hz, 1H), 3.98, 3.97, 3.88, and 3.86 (4s, 12H); ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 178.8, 165.5, 162.1, 156.8, 155.9, 151.4, 148.8, 139.0, 123.0, 122.3, 111.3, 110.9, 106.1, 97.9, 92.3, 60.3, 56.1, 56.0, 55.9; HRMS calcd for $(C_{19}H_{18}O_7 + H)^+$ 359.1131, found 359.1145. Compound **10**: mp 150–152 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 7.68–7.66 (m, 2H), 6.94 (d, J = 8.8 Hz, 1H), 6.46 (d, J = 2.2 Hz, 1H), 6.30 (d, J = 1.6 Hz, 1H), 3.93–3.92 (m, 9H), 3.87 (s, 3H) 3.84 (s, 3H); ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 178.9, 165.5, 162.1, 156.8, 155.7, 148.8, 145.6, 139.3, 123.7, 121.7, 114.5, 110.5, 106.2, 97.9, 92.2, 60.3, 56.1, 55.9; HRMS calcd for $(C_{20}H_{20}O_7 + H)^+$ 373.1287, found 373.1264.

4.1.4. 2-(3,4-Dimethoxyphenyl)-5,7-dimethoxy-4-oxo-4H-chromen-3-yl 3,4,5-trime-thoxybenzoate (*11*)

A mixture of compound **6** (200 mg, 0.56 mmol), 3,4,5-trimethoxycinnamic acid (178 mg, 0.84 mmol), 1-ethyl-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl, 161 mg, 0.84 mmol), 4-dimethylaminopyridine (DMAP, 102 mg, 0.84 mmol) in anhydrous CH_2Cl_2 (20 mL) was stirred at room temperature for overnight. Then the solution was washed by saturated aqueous Na_2CO_3, saturated aqueous NH_4Cl, and brine in sequence. The organic layer was dried by anhydrous $MgSO_4$. The solvent was evaporated to dryness, and the residue was purified by flash chromatography to afford the title compound **11** (264 mg, 85.4% yield). Mp 108–110 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 7.52 (dd, J = 2.22, 8.82 Hz, 1H), 7.45 (s, 1H), 7.41 (d, J = 1.68 Hz, 1H), 6.91 (d, J = 8.82 Hz, 1H), 6.54 (d, J = 2.22 Hz, 1H), 6.35 (d, J = 2.22 Hz, 1H), 3.92, 3.90, 3.89, 3.88, 3.79 (5s, 21H, 7 × OCH_3); ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 170.6, 164.4, 163.9, 161.3, 159.2, 153.3, 153.0, 151.3, 148.8, 142.9, 134.0, 123.9, 122.4, 121.7, 110.9, 110.7, 108.8, 107.8, 96.1, 92.7, 61.0, 56.3, 56.0, 55.9; HRMS calcd for $(C_{29}H_{28}O_{11} + H)^+$ 553.1710, found 553.1701.

4.1.5. 2-(3,4-Dimethoxyphenyl)-5,7-dimethoxy-3-((3,4,5-trimethoxybenzyl)oxy)-4H-chromen-4-one (**12**)

A solution of compound **6** (200 mg, 0.54 mmol), 3,4,5-trimethoxybenzyl methanesulfonate (232 mg, 0.81 mmol), K_2CO_3 (149 mg, 1.1 mmol) in DMF (10 mL) was stirred in room temperature for 12 h. The solution was poured into ice-water, and the precipitate was filtrated, and purified by flash chromatography to afford the title compound **12** (252 mg, 86.7%). Mp 90–92 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 7.64 (d, J = 2.22 Hz, 1H), 7.58 (dd, J = 2.16, 8.76 Hz, 1H), 6.89 (d, J = 8.82 Hz, 1H), 6.57 (s, 2H), 6.46 (d, J = 2.22 Hz, 1H), 6.31 (d, J = 2.16 Hz, 1H), 4.99 (s, 2H), 3.94, 3.90, 3.86, 3.77, 3.75, 3.71 (6s, 21H, 7 × OCH_3); ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 174.1, 163.9, 158.9, 153.3, 153.0, 150.7, 148.4, 139.5, 137.7, 132.7, 123.5, 121.8, 111.9, 110.5, 109.4, 106.1, 106.0, 95.8, 92.5, 74.2, 60.8, 56.5, 56.0, 55.8; HRMS calcd for $(C_{29}H_{30}O_{10} + H)^+$ 539.1917, found 539.1931.

4.1.6. 2-(3,4-Dimethoxyphenyl)-3-(2-(3,4-dimethoxyphenyl)-2-oxoethoxy)-5,7-dime- thoxy-4H-chromen-4-one (**13**)

A solution of compound **6** (200 mg, 0.56 mmol), 2-bromo-1-(3,4-dimethoxy- phenyl)ethanone (217 mg, 0.84 mmol), K_2CO_3 (154.6 mg, 1.12 mmol) in DMF (10 mL) was stirred at room temperature for overnight, the solution was poured into ice-water (30 mL). The precipitate was filtrated, and purified by flash chromatography to afford the title compound **13** (231 mg, 76.8% yield). Mp 222–224 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 7.88 (s, 1H), 7.76 (d, J = 8.3 Hz, 1H), 7.73 (d, J = 8.3 Hz, 1H), 7.54 (s, 1H), 6.92 (d, J = 8.22 Hz, 1H), 6.84 (d, J = 8.82 Hz, 1H), 6.50 (s, 1H), 6.33 (s, 1H), 5.44 (s, 2H), 3.94, 3.91, 3.89 (3s, 18H, 6 × OCH_3); ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 193.1, 173.9, 164.0, 160.9, 158.8, 153.6, 152.7, 151.0, 149.1, 148.6, 139.5, 127.9, 123.3, 123.0, 121.7, 111.8, 110.6, 110.2, 110.1, 109.2, 95.9, 92.5, 73.9, 56.5, 56.2, 56.1, 55.9, 55.9; HRMS calcd for $(C_{29}H_{28}O_{10} + H)^+$ 537.1760, found 537.1777.

4.1.7. (E)-2-((2-(3,4-Dimethoxyphenyl)-5,7-dimethoxy-4-oxo-4H-chromen-3-yl)oxy)-ethyl 3-(3,4,5-trimethoxyphenyl)acrylate (**14**)

A mixture of compound **7** (200 mg, 0.48 mmol), 3,4,5-trimethoxycinnamic acid (229 mg, 0.96 mmol), 1-ethyl-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl, 184 mg, 0.96 mmol), 4-dimethylaminopyridine (DMAP, 117 mg, 0.96 mmol) in anhydrous CH_2Cl_2 (20 mL) was stirred at room temperature for overnight. Then the solution was washed by saturated aqueous Na_2CO_3, saturated aqueous NH_4Cl, and brine in sequence. The organic layer was dried by anhydrous $MgSO_4$. The solvent was evaporated to dryness, and the residue was purified by flash chromatography to afford the title compound **14** (261 mg, 87.5% yield). Mp 152–154 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 7.72 (d, J = 2.22 Hz, 1H), 7.66 (dd, J = 2.22, 8.82 Hz, 1H), 7.44 (d, J = 16.96, 1H), 6.83 (d, J = 8.76 Hz, 1H), 6.71 (s, 2H), 6.49 (d, J = 2.16 Hz, 1H), 6.33 (d, J = 2.22 Hz, 1H), 6.16 (d, J = 15.96, 1H), 4.42 (s, 4H), 3.95, 3.92, 3.90, 3.88, 3.87, 3.71 (6s, 21H, 7 × OCH_3); ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 173.9, 166.7, 164.0, 161.0, 158.9, 153.4, 153.1, 150.9, 148.4, 144.8, 140.1, 139.5, 129.9, 123.2, 121.9, 116.9, 112.0, 110.6, 109.3, 105.3, 95.9, 92.5, 70.10, 63.68, 61.0, 56.4, 56.2, 56.1; HRMS calcd for $(C_{33}H_{34}O_{12} + H)^+$ 623.2128, found 623.2106.

4.1.8. 2-((2-(3,4-Dimethoxyphenyl)-5,7-dimethoxy-4-oxo-4H-chromen-3-yl)oxy)ethyl 3,4,5-trimethoxybenzoate (**15**)

Following the procedure for the preparation of compound **14**, but with 3,4,5-trimethoxybenzoic acid as starting material, compound **15** was obtained. Yield 85.0%; mp 80–82 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 7.63 (d, J = 8.28 Hz, 1H), 7.53 (s, 1H), 7.17 (s, 2H), 6.61 (d, J = 8.82 Hz, 1H), 6.43 (s, 1H), 6.28 (s, 1H), 4.51 (s, 2H), 4.43 (s, 2H), 3.89, 3.85, 3.84, 3.82, 3.81, 3.74 (6s, 21H, 7 × OCH_3); ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 173.8, 165.9, 164.0, 160.9, 158.7, 152.8, 152.8, 150.8, 148.4, 142.1, 139.7, 125.0, 123.0, 122.0, 111.1, 110.5, 109.3, 106.9, 95.8, 92.4, 69.9, 64.3, 60.9, 56.4, 56.2, 55.9, 55.8, 55.7; HRMS calcd for $(C_{31}H_{32}O_{12} + H)^+$ 597.1972, found 597.1994.

4.1.9. 2-((2-(3,4-Dimethoxyphenyl)-5,7-dimethoxy-4-oxo-4H-chromen-3-yl)oxy)ethyl 3,4-dimethoxybenzoate (**16**)

Using the same procedure for the preparation of compound **14**, but with 3,4-dimethoxybenzoic acid as the starting material, the titled compound **16** was prepared. Yield 84.2%; mp 181–183 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 7.67 (dd, J = 1.62, 8.22 Hz, 1H), 7.58 (d, 2.16 Hz, 1H), 7.45–7.43 (m, 2H), 6.78 (d, J = 8.82 Hz, 1H), 6.66 (d, J = 8.76 Hz, 1H), 6.47 (d, J = 2.22 Hz, 1H), 6.33 (d, J = 2.22 Hz, 1H), 4.52–4.47 (AB, J = 14.88, 5.52 Hz, 4H), 3.95, 3.92, 3.89, 3.87, 3.85, 3.78 (6s, 18H, 6 × OCH_3); ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 173.9, 166.1, 164.0, 161.0, 158.8, 152.9, 150.7, 148.5, 139.7, 123.7, 123.2, 122.5, 122.1, 112.0, 111.3, 110.5, 110.1, 95.8, 92.5, 70.0, 64.0, 56.4, 56.1, 56.0, 55.8, 55.7; HRMS calcd for $(C_{30}H_{30}O_{11} + H)^+$ 567.1866, found 567.1860.

4.1.10. 2-((2-(3,4-Dimethoxyphenyl)-5,7-dimethoxy-4-oxo-4H-chromen-3-yl)oxy)ethyl 4-methoxybenzoate (**17**)

Using the same procedure for the preparation of compound **14**, but with 4-methoxybenzoic acid as the starting material, the titled compound **17** was prepared. Yield 83.4%; mp 107–109 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 7.76 (d, J = 8.76, 2H), 7.68 (dd, J = 1.62, 8.22 Hz, 1H), 7.58 (d, J = 1.62 Hz, 1H), 6.81 (d, J = 8.82 Hz, 2H), 6.67 (d, J = 8.82 Hz, 1H), 6.47 (d, J = 2.22 Hz, 1H), 6.33 (d, J = 2.16 Hz, 1H), 4.48 (s, 4H), 3.95, 3.88, 3.85, 3.83, 3.78 (5s, 15H, 5 × OCH_3); ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 174.0, 166.1, 164.0, 163.3, 161.0, 158.8, 150.7, 148.5, 139.6, 131.6, 123.2, 122.3, 122.2, 113.4, 111.3, 110.5, 109.4, 95.8, 92.5, 70.1, 63.8, 56.4, 56.0, 55.8, 55.7, 55.4; HRMS calcd for $(C_{29}H_{28}O_{10} + H)^+$ 537.1760, found 537.1772.

4.1.11. 3-(2-(6-Amino-9H-purin-9-yl)ethoxy)-2-(3,4-dimethoxyphenyl)-5,7-dimethoxy-4H-chromen-4-one (**19**)

A mixture of compound **6** (200 mg, 0.48 mmol), 9-(2-bromoethyl)-9*H*-purin-6-amine **18** (140 mg, 0.58 mmol), K_2CO_3 (133 mg, 0.96 mmol) in anhydrous DMF (20 mL) was stirred at 60 °C for overnight. The solution was poured into ice-water, and the precipitate was filtrated, and purified by flash chromatography to afford the title compound **19** (203 mg, 81.4%). Mp 267–269 °C; ^{1}H NMR (CD_3COOD, 600 MHz) δ: 8.47 (s, 1H), 8.34 (s, 1H), 7.32 (d, J = 2.22 Hz, 1H), 7.29 (dd, J = 2.16, 8.76 Hz, 1H), 6.73 (d, J = 8.82 Hz, 1H), 6.63 (d, J = 2.16 Hz, 1H), 6.44 (d, J = 2.22 Hz, 1H), 4.61–4.59 (m, 2H), 4.56–4.54 (m, 2H), 3.90, 3.88, 3.86, 3.78 (4s, 12H, 4 × OCH_3); ^{13}C NMR (CD_3COOD, 150 MHz) δ: 174.7, 165.3, 160.8, 155.1, 152.8, 151.5, 148.6, 148.0, 143.6, 138.8, 122.2, 122.1, 111.2, 110.6, 108.4, 96.2, 92.7, 68.9, 55.7, 55.6, 55.4, 55.3, 44.7; HRMS calcd for $(C_{25}H_{25}N_5O_7 + H)^+$ 520.1832, found 520.1817.

4.1.12. 9-(2-((2-(3,4-Dimethoxyphenyl)-5,7-dimethoxy-4-H-chromen-3-yl)oxy)ethyl)-1,3-dimethyl-1H-purine-2,6(3H,9H)-dione (**21**)

Following the procedure for the preparation of compound **19**, but with 9-(2-bromo-ethyl)-1,3-dimethyl-1*H*-purine-2,6(3*H*,9*H*)-dione **20** as starting material, the titled compound **21** was prepared. Yield 84.6%; mp 263–265 °C; ^{1}H NMR ($CDCl_3$, 600 MHz) δ: 7.87 (s, 1H), 7.38 (d, J = 1.62 Hz, 1H), 7.31 (s, 1H), 6.79 (d, J = 8.28 Hz, 1H), 6.48 (s, 1H), 6.36 (s, 1H), 4.70 (t, J = 4.83 Hz, 2H), 4.35 (t, J = 4.38 Hz, 2H), 3.97, 3.91, 3.89, 3.80, 3.58, 3.35 (6s, 18H, 6 × OCH_3); ^{13}C NMR ($CDCl_3$, 150 MHz) δ: 173.8, 164.2, 161.0, 158.9, 155.2, 153.6, 151.6, 151.0, 148.9, 148.6, 142.5, 139.7, 122.8, 121.6, 111.1, 110.4, 106.6, 96.0, 92.62, 69.95, 56.5, 56.0, 55.9, 55.9, 47.6, 29.8, 27.9; HRMS calcd for $(C_{28}H_{28}N_4O_9 + H)^+$ 565.1934, found 565.1941.

*4.1.13. 3-(2-((4-((3-Chloro-4-fluorophenyl)amino)-7-methoxy quinazolin-6-yl)oxy)-ethoxy)-2-(3,4-dimethoxyphenyl)-5,7-dimethoxy-4H-chromen-4-one (**23**)*

Following the procedure for the preparation of compound **19**, but with 6-(2-bromoethoxy)-*N*-(3-chloro-4-fluorophenyl)-7-methoxy-3,4-dihydroquinazolin-4-amine **22** as starting material, the titled compound **23** was prepared. Yield 64.5%; mp 127–129 °C; ^{1}H NMR (DMSO-d_6, 600 MHz) δ: 9.45 (s, 1H), 8.49 (s, 1H), 8.10 (dd, J = 2.76, 6.60 Hz, 1H), 7.79–7.77 (m, 1H), 7.68 (d, J = 2.16 Hz, 1H), 7.66 (s, 1H), 7.62 (d, J = 1.68 Hz, 1H), 7.43 (t, J = 9.36 Hz, 1H), 7.14 (s, 1H), 6.84 (d, J = 8.76 Hz, 1H), 6.69 (d, J = 2.22 Hz, 1H), 6.49 (d, J = 2.22 Hz, 1H), 4.48 (s, 2H), 4.34 (s, 2H), 3.89, 3.84, 3.73, 3.69 (4s, 15H, 5 × OCH_3); ^{13}C NMR (DMSO-d_6, 150 MHz) δ: 172.7, 164.2, 160.8, 158.7, 156.5, 154.9, 153.1, 152.8, 152.7, 151.0, 148.6, 148.4, 147.4, 139.5, 137.3, 123.9, 122.9, 122.7, 122.1, 117.1, 117.0, 111.8, 111.3, 109.1, 109.04, 107.6, 102.6, 96.4, 93.5, 70.1, 68.7, 56.6, 56.6, 56.2, 55.8, 55.8; HRMS calcd for $(C_{36}H_{31}ClFN_3O_9 + H)^+$ 704.1811, found 704.1820.

4.2. Materials for biological studies

Dimethyl sulfoxide (DMSO), paclitaxel, DOX, verapamil, topotecan and phenazine methosulfate (PMS) were purchased from Sigma–Aldrich. Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 medium, trypsin-ethylenediaminetetraacetic acid (EDTA) and penicillin/streptomycin were purchased from Gibco BRL. The foetal bovine serum (FBS) was purchased from HyClone Laboratories. 3-(4,5-Dimethylthiazol-2-yl)-5-[3-(carboxymethoxy)phenyl]-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) was purchased from Promega. The human breast cancer cell lines MDA435/LCC6 and MDA435/LCC6MDR were kindly provided by Dr. Robert Clarke (Georgetown University, United States). The human ovarian carcinoma cell lines 2008/P and 2008/MRP1 were generous gifts from Prof. P. Borst (The Netherlands Cancer Institute, Amsterdam, Netherlands). The human embryonic kidney (HEK) 293 cell lines, HEK293/pcDNA3.1 (empty vector-transfected) and HEK293/R2 (BCRP-transfected) and MCF7-MX100 mitoxantrone selected cell lines were kindly provided by Dr. Kenneth To (The Chinese University of Hong Kong, Hong Kong).

4.3. Cell culture

MDA435/LCC6, MDA435/LCC6MDR cell lines were cultured in supplemented DMEM media with 10% heat inactivated FBS and 100 U/mL penicillin and 100 μg/mL of streptomycin. 2008/P and 2008/MRP1 cells or HEK293/pcDNA3.1 and HEK293/R2 or MCF7 and MCF7-MX100 were cultured in RPMI 1640 medium containing heat inactivated 10% FBS and 100 U/mL penicillin and 100 μg/mL of streptomycin. They were maintained at 37 °C in a humidified atmosphere with 5% CO_2. The cells were split constantly after a confluent monolayer has been formed. To split cells, the plate was washed briefly with phosphate-buffered saline (PBS), treated with 0.05% trypsin-EDTA and harvested by centrifugation.

4.4. Cell proliferation assay

6000 cells of LCC6 or LCC6MDR and paclitaxel were mixed with or without 1 μM modulator to a final volume of 200 μL in each well of 96-well plates. 4000 Cells of 2008/P or 2008/MRP1 and DOX were co-incubated with or without 1 μM modulator to a final volume of 200 μL. 6500 Cells of HEK293/pcDNA3.1 or HEK293/R2 and topotecan were co-incubated with or without 1 μM modulator to a final volume of 200 μL. 7500 Cells of MCF7 or MCF7-MX100 and topotecan were co-incubated with or without 1 μM modulator to a final volume of 200 μL. The plates were then incubated for 5 days at 37 °C. After 5 days, the % of survival or viability was determined by MTS according to procedures reported previously [51]. These results were represented as mean ± standard error of mean. IC_{50} values were calculated from the dose–response curves of MTS assays (Prism 4.0).

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