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Research Article

Biological Evaluation and SAR Analysis of *O*-Methylated Analogs of Quercetin as Inhibitors of Cancer Cell Proliferation

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Strategy, Management and Health Policy				
Enabling Technology, Genomics, Proteomics	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT Using a high-throughout screening approach, the anticancer activities of 16 *O*-methylated (OMe) analogs of quercetin were assessed. The structure–activity relationships showed that OMe moieties at the 4' and/or 7 positions were important for maintaining inhibitory activities against the 16 cancer cell lines. Furthermore, when the OH groups at the 3' and 4' positions were both replaced by OMe moieties, anticancer activity was enhanced. Drug Dev Res 75 : 455–462, 2014.

Key words: structure–activity relationship; quercetin; methylation; analog; anticancer

INTRODUCTION

Cancer, characterized by the uncontrolled growth of abnormal cells is projected as the primary cause of death in the future [Sashidhara et al., 2010] with growth in lung [Lv et al., 2012; Bejjanki et al., 2013], head and neck [Mannelli and Gallo, 2012], melanoma [Choi et al., 2011], breast [Labrie et al., 1999], and cervical [Parkin, 2006] cancer incidence rising globally. There is thus an urgent need to develop more effective drugs.

Quercetin is found in abundance in onions, tea [Scalbert and Williamson, 2000], apples, broccoli, berries [Nijveldt et al., 2001], and red wine [Ramos, 2008] and is of interest due to its variety of biological properties. Predominant among these are its anticancer activities that have been shown in vitro in a variety of cancer cell lines including U138MG (glioma) [Braganhol et al., 2006], U2.US/MTX300 (osteosarcoma) [Xie et al., 2011], HeLa (cervical cancer) [Vidya et al., 2010], CWR22Rv1 (prostate cancer) [Hsieh and Wu, 2009], MDA-MB-453 (breast cancer) [Choi et al., 2008], HT-29 (colorectal) [Priego et al., 2008], myeloid leukemia [Duraj et al., 2005], and oral cancer [Kang et al., 2010]. The doses of quercetin that exhibit

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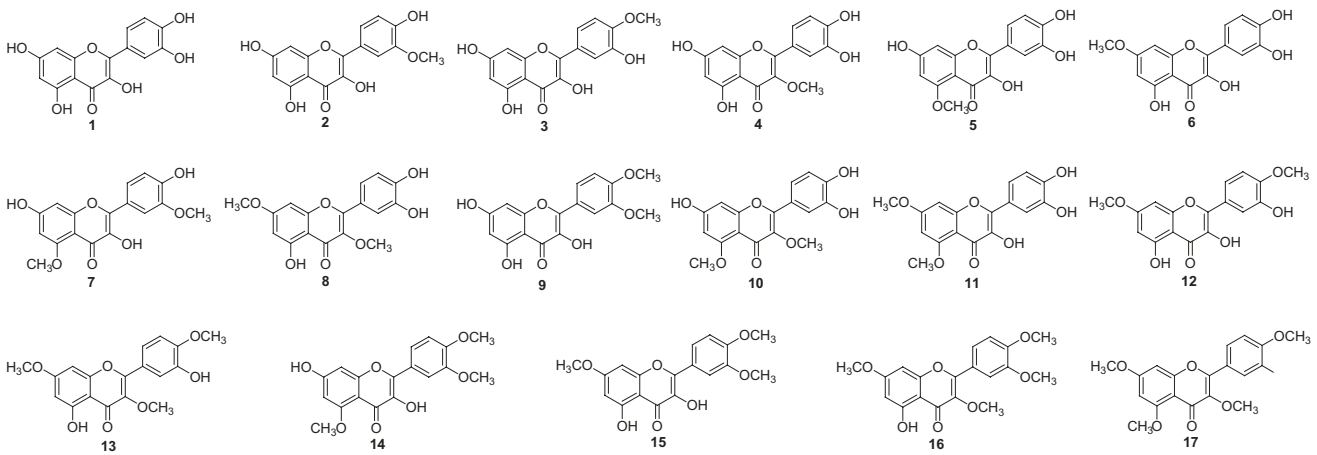

Fig. 1. Structures of quercetin (1) and series of methylated quercetin analogs (2–17).

antiproliferative effects in vitro range from 3 to 50 mM [Lamson and Brignall, 2000; Gibellini et al., 2011].

In vivo quercetin can prevent induced carcinogenesis, particularly in the colon [Murakami et al., 2008], and can inhibit melanoma growth, invasion, and metastatic potential [Caltagirone et al., 2000]. When administered in the diet, quercetin inhibited the initiation, growth, and/or dissemination of induced tumors in animal models [Yang et al., 2001].

The anticancer effects of quercetin have been attributed to its ability to interfere with tumor vascularization via inhibition of endothelial cell growth and migration [Igura et al., 2001; Tan et al., 2003]. Quercetin reduces the expression and activity of matrix metalloproteinase-2, and inhibits constitutive endothelial nitric oxide synthase [Chiesi and Schwaller, 1995; Tan et al., 2003]. Replacement of OH groups with *O*-methylated (OMe) moieties can enhance the metabolic stability of flavones while retaining antiproliferative potency [Cai et al., 2009]. We have reported the synthesis of a series of monomethylated (**2–6**), dimethylated (**7–12**), trimethylated (**13–15**), tetramethylated (**16**), and pentamethylated (**17**) quercetins [Li et al., 2009, 2011; Shi et al., 2012]. In the present study, the potential anticancer activity of these 16 OMe quercetin analogs (Fig. 1) were determined in an in vitro human disease-oriented cancer cell line using high throughout screening (HTS) method [Li et al., 2012], including human lung cancers, melanoma, cervical, neck and head, and human breast cancer cells.

MATERIALS AND METHODS

Synthesis

The 16 OMe analogs of quercetin were synthesized as previously described [Li et al., 2009, 2011; Shi et al., 2012]. The preparation of 3'-*O*-methylquercetin (**2**) is shown in Figure 2. Based on the different reactivity of the five hydroxy groups in quercetin following a specific sequence: 4' > 7 > 3 > 3' > 5 [Bouktaib et al., 2002], benzylation of quercetin with 3.0 equivalent of benzyl bromide produced the tribenzylated product **18**, then partial methylation of the free phenolic function at C-3' position in **18** with iodomethane (the C-5 position is less reactive) afforded **19** in 92% yield. Finally, deprotection of the benzyl groups gave 3'-*O*-methylquercetin (**2**) in 90% yield.

4'-*O*-Methylquercetin (**3**) was hemisynthesized from quercetin (**1**) (Fig. 2) relying on selective and successive protections of the different quercetin phenolic functions [Li et al., 2009]. Treatment of **1** with dichlorodiphenylmethane in diphenyl ether at 175°C [Li et al. 2007] afforded the desired product **20** in 86% yield. Subsequently, reaction of **20** with an excess of chloromethyl methyl ether and K_2CO_3 in acetone afforded **21** with a free phenolic hydroxy group at the C-5 position. Under hydrogen conditions using 10% palladium on carbon as a catalyst, the benzophenone ketal was cleaved to selectively afford **22** in 95% yield with no side products detected by thin-layer chromatography (TLC) analysis. Treatment of **22** with 1.2 equivalent. of iodomethane led selectively to **23** in 92% yield with a methyl group at C-4' position. Finally, hydrochloric acid–catalyzed removing of the methoxymethyl-protecting group afforded 4'-*O*-methylquercetin (**3**) in 90% yield.

3-*O*-Methylquercetin (**4**) [Li et al. 2004] was synthesized as summarized in Figure 2. Selective benzylation of rutin (**24**) with benzyl bromide and hydrolysis of the glycosidic bond with HCl led mainly to the formation of the tribenzylated product **25**, then regioselective methylation of C_3-OH afforded **26** in

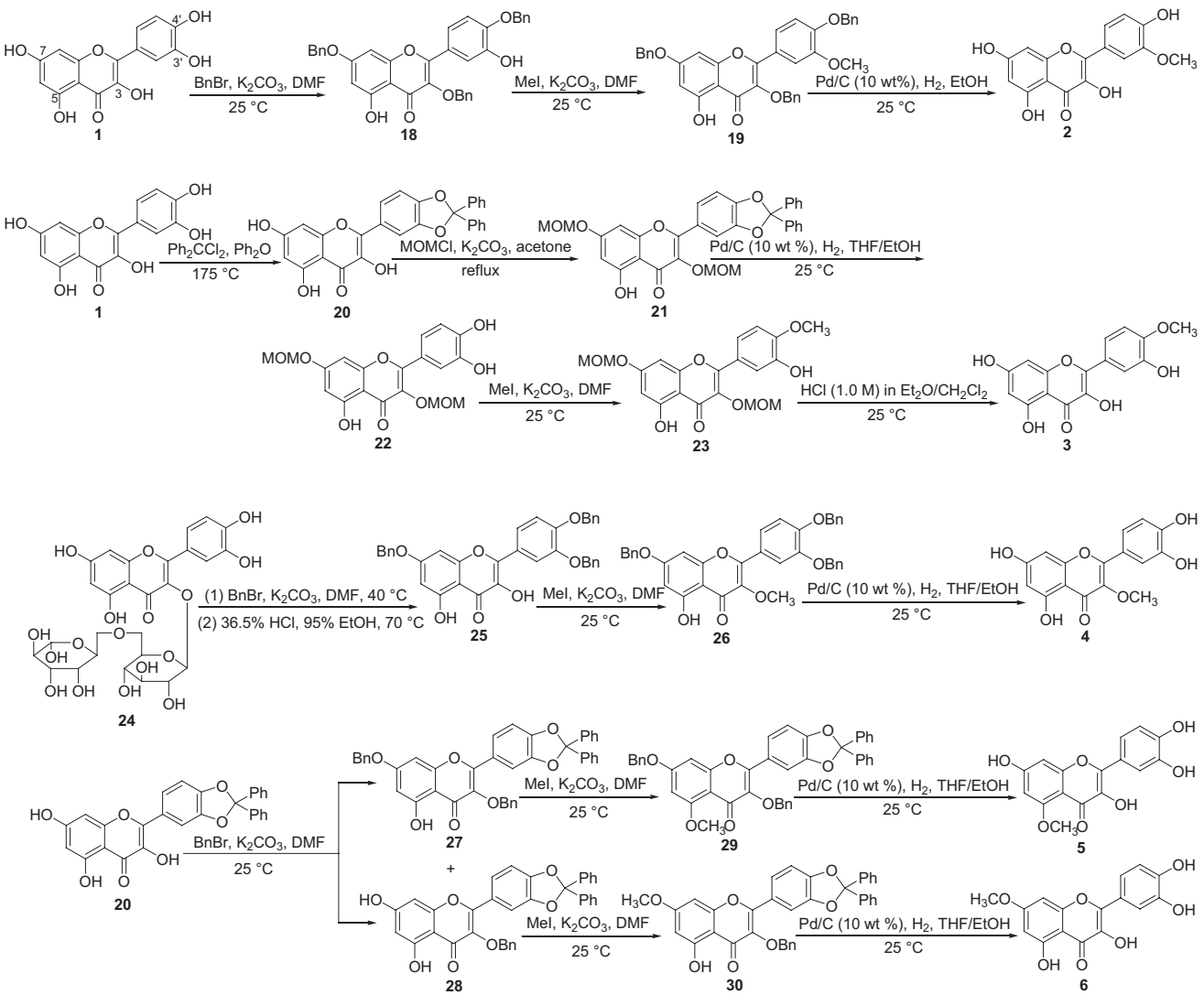

Fig. 2. Synthesis of **1** to **6**.

95% yield; subsequently the cleavage of the benzyl group by hydrogenolysis gave 3-*O*-methylquercetin (**4**) in 91% yield.

5-*O*-Methylquercetin (**5**) and 7-*O*-methylquercetin (**6**) were synthesized from **20** as shown in Figure 2. Treatment of **20** with benzyl bromide and K_2CO_3 afforded two easily separated products: the 3,7-dibenzyl (**27**) and the 3-benzyl (**28**) isomers. Reaction of **27** with iodomethane led to **29** in 94% yield, then the benzophenone ketal and benzyl groups were deprotected with 10% palladium on carbon as the catalyst afforded **5** in 92% yield. Treatment of **28** with iodomethane led selectively to **30** in 93% yield with the desired methyl group at C-7 position, then the deprotection of benzophenone ketal and benzyl groups under hydrogenation conditions using 10% palladium on carbon as the catalyst afforded **6** in 93% yield.

The synthesis of the dimethylated quercetins (**7**–**11**) is shown in Figure 3. Methylation of the free hydroxy function at C-3' and C-5 positions in **18** with an excess of iodomethane gave **31**, then the cleavage of the benzyl groups by hydrogenolysis on 10% palladium on carbon afforded **7**. **20** reacted with iodomethane in the presence of K_2CO_3 afforded **32**, then the 3,7-*O*-dimethylquercetin **8** was directly obtained in 93% yield after hydrogenolysis of the benzophenone ketal using 10% palladium on carbon. Compound **22** reacted with iodomethane leading to **33** in 92% yield with two methyl group at C-3' and C-4' positions, then the

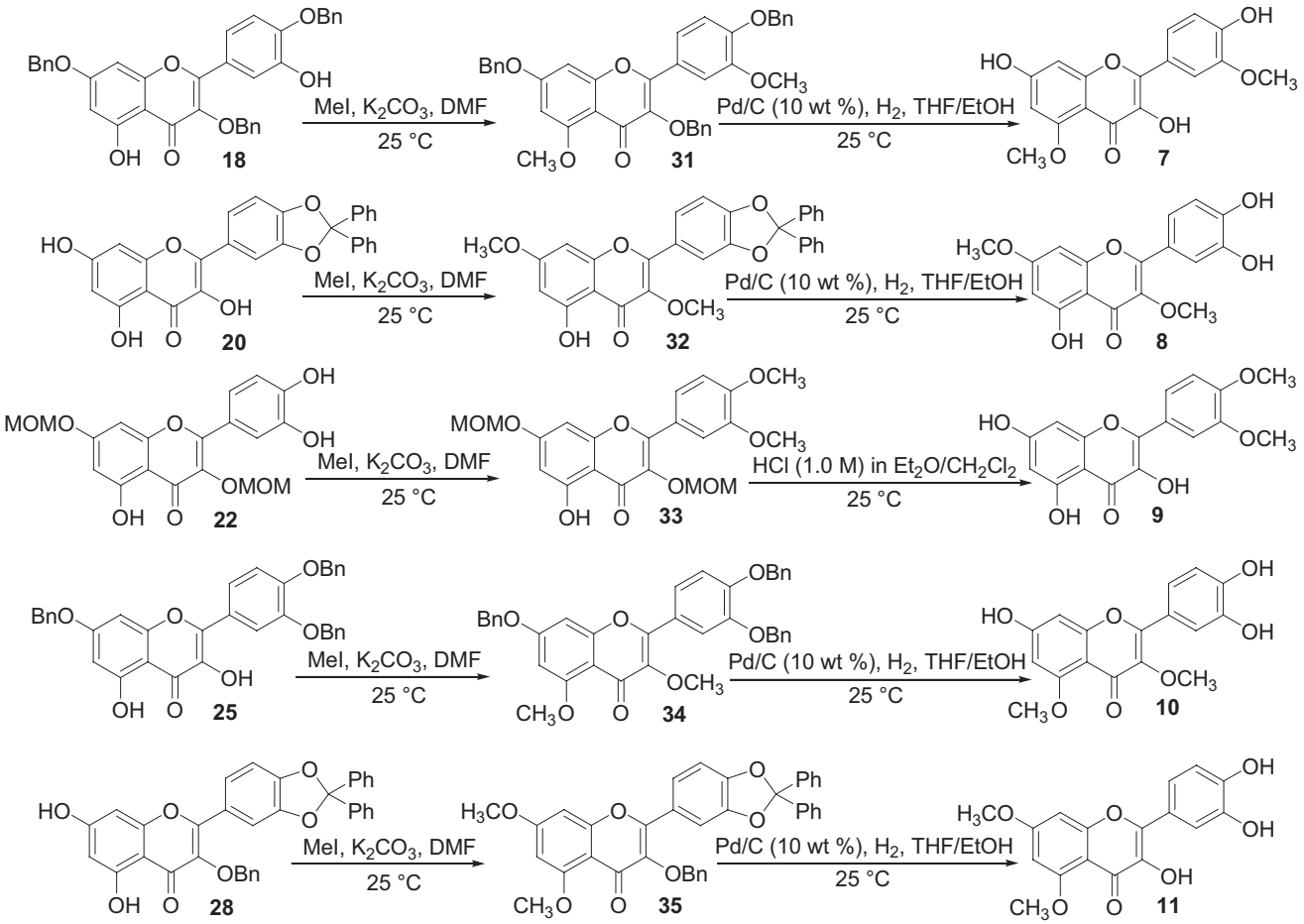

Fig. 3. Synthesis of **7** to **11**.

hydrolysis of the methoxymethyl group with HCl gave **9** in 91% yield. Methylation of the free hydroxy groups at C-3 and C-5 positions in **25** with an excess of iodomethane and K_2CO_3 gave **34** in 91%, and the deprotection of the benzyl groups by hydrogenolysis on 10% palladium on carbon afforded **10** in 91% yield. An excess of iodomethane and K_2CO_3 reacted with **28** afforded **35**, then the deprotection of the two kinds of protecting group in **35** by hydrogenolysis on 10% palladium on carbon afforded **11** in 92% yield.

The synthesis of dimethylated quercetin **12**, trimethylated quercetins (**13**–**15**), tetramethylated quercetin (**16**), and pentamethylated quercetin (**17**) are shown in Figure 4. Treatment of **1** with iodomethane in the presence of K_2CO_3 afforded a mixture of two main products: the 4',7-dimethyl isomer **12** and the 3,4',7-trimethyl isomer **13** (Fig. 4) that were easily separated by chromatography on silica gel. Treatment of **22** with excess iodomethane afforded **36** in 90% yield with the desired methyl groups at C-3', C-4' and C-5 positions. Then hydrolysis of the methoxymethyl group with hydrochloric acid gave **14** in 92% yield (Fig. 4). The trimethylquercetin **15** was synthesized from rutin (**24**) in two steps including methylation and hydrolysis of the glycosidic bond. Treatment of **1** with excess iodomethane in the presence of K_2CO_3 afforded a mixture of three products: the trimethylated quercetin **13**, the tetramethylated quercetin **16** and the pentamethylated quercetin **17** (Fig. 4), which were easily separated by chromatography on silica gel.

Biological Screening

Human lung cancers (A549, H157, H460, 1792, H266, Hop62, 1299, 292G, and Calu1), melanoma (LOX-IMVI and M14), cervical (Hela), neck and head (M4E), and human breast cancer (SKBR) were from American Type Culture Collection (ATCC, Manassas, Virginia) grown in RPMI 1640 containing 10% fetal calf serum (FCS), 100 UI/mL penicillin G, and 100 mg/mL streptomycin. Dimethyl sulphoxide (DMSO) was

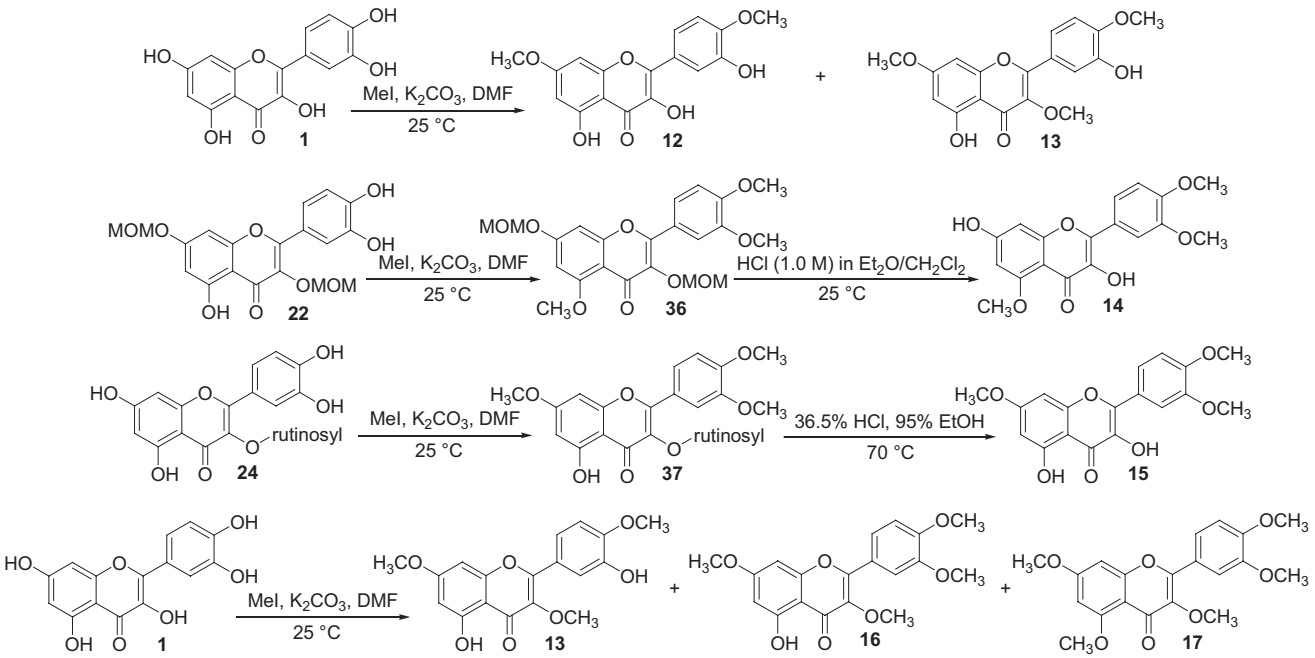

Fig. 4. Synthesis of **12** to **17**.

purchased from Sigma Chemical Co. (St. Louis, MO), and Alamar blue was from Promega (Madison, Wisconsin). Cells were seeded into 384-well plates (Costar# 3712) (800–1000 cell/well or 20–25 cells/μL, 45 μL medium/well) using a liquid dispenser (Thermo Fisher Multidrop Combi, Waltham, Massachusetts) in a biosafety cabinet. Plates were placed in an incubator overnight to allow for attachment and recovery. Compound plates were utilized and prepared to yield 10 mM of compound in DMSO (stock) by robot (Sciclone software, San Francisco, California) to generate eight concentrations with serial dilution; wells were reserved on each plate for background and vehicle control (0.5% DMSO). With the use of the liquid handling system, the following day the cells were treated with compounds for 72 h, at final concentrations of 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, and 0.39 μM in triplicate. A volume of 5 μL/well Alamar blue was transferred into the assay plates for a final concentration of 10%. The plates were exposed to an excitation wavelength of 530 nm, and the emission at 560 nm was recorded to determine whether any of the test compounds fluoresced at the emission wavelength and thus interfere with the assay.

Plates were returned to the incubator, and the fluorescence was read at 4 h. The percent viability was expressed as fluorescence counts in the presence of test compound as a percentage of that in the vehicle control. The mean value and standard error for each treatment were determined, and the percentage of cell viability relative to control was calculated. The resulting solutions were measured at optical density 560 (OD_{560}) and the values used to calculate the inhibitory rates using the equation (OD_{560} [100%] – OD_{560} [compound]) / (OD_{560}[100%] – OD_{560}[blank]) ×100%, where the 100% group contained no compound and the blank group contained only the cells. The IC_{50} value was defined as the concentration of compound that killed 50% of the total cell population as compared with control cells at the end of the incubation period and was derived using OriginPro 7.5 software (OriginLab, Hampton, MA).

RESULTS AND DISCUSSION

The results of anticancer activities of the 16 quercetin analogs against the growth of 16 human cancer cell lines are shown in Table 1.

5-*O*-Methylquercetin (**5**) generally had reduced inhibitory activities against the 16 cancer cell lines as compared with quercetin (**1**). 4'-*O*-methylquercetin (**3**) and 7-*O*-methylquercetin (**6**) were more potent in inhibiting cancer cell growth inhibition than quercetin (**1**), indicating that the methylation at the 4' or 7 positions improved activity. The 4',7-dimethoxyquercetin (**12**), where the OH groups at the 4',7 positions were methylated, had stronger antiproliferative activity compared to quercetin (**1**), with its IC_{50} values against A549, H157, H460, 1792, 1944, M14, SKBR and Hela were

TABLE 1. In Vitro Anticancer Activities in Human Cancer Cell Lines (IC_{50} Values* in μM) for *O*-Methylated Analogs of Quercetin

		Lung cancer								Neck and head	Melanoma		Breast	Cervical
No.	A549	H157	H460	1792	1944	H266	H522	Hop62	1299	292G	Calu1	M4E	LOX-IMVI	M14	SKBR	HeLa
1	6.20 ± 0.51	6.00 ± 0.47	9.62 ± 0.89	3.85 ± 0.45	10.18 ± 1.11	16.87 ± 1.12	5.77 ± 0.038	7.52 ± 0.58	13.60 ± 1.24	>50†	23.58 ± 1.82	21.71 ± 1.87	4.65 ± 0.28	12.77 ± 1.08	16.71 ± 1.21	3.56 ± 0.28
2	10.78 ± 1.03	20.52 ± 1.18	12.48 ± 1.14	33.36 ± 2.79	>50†	42.37 ± 3.99	>50†	23.34 ± 1.15	>50†	>50†	>50†	>50†	>50†	>50†	>50†	17.93 ± 1.32
3	2.63 ± 0.19	3.04 ± 0.02	4.45 ± 0.02	4.06 ± 0.03	3.86 ± 0.02	7.57 ± 0.05	>50†	9.93 ± 0.78	21.97 ± 1.78	12.54 ± 1.10	24.95 ± 1.89	19.91 ± 1.95	>50†	14.85 ± 1.26	7.97 ± 1.01	1.81 ± 0.01
4	8.14 ± 0.74	18.67 ± 1.91	9.91 ± 1.03	24.09 ± 2.17	23.64 ± 2.79	>50†	>50†	35.54 ± 3.03	46.81 ± 3.89	49.48 ± 3.69	>50†	38.65 ± 4.01	>50†	>50†	13.87 ± 1.32	6.09 ± 0.36
5	6.26 ± 0.23	23.00 ± 1.83	29.21 ± 2.01	>50†	>50†	>50†	>50†	37.91 ± 2.79	>50†	>50†	>50†	>50†	>50†	>50†	>50†	7.74 ± 0.34
6	3.08 ± 0.10	3.31 ± 0.01	3.32 ± 0.02	14.73 ± 1.08	4.25 ± 0.02	13.87 ± 1.16	24.97 ± 2.21	6.87 ± 0.04	10.25 ± 0.88	27.95 ± 2.17	23.43 ± 1.76	10.82 ± 0.98	>50†	13.32 ± 1.76	6.25 ± 0.03	4.26 ± 0.02
7	3.14 ± 0.08	2.93 ± 0.02	4.90 ± 0.04	16.11 ± 1.10	>50†	38.20 ± 2.24	>50†	21.41 ± 1.19	13.68 ± 1.65	>50†	29.12 ± 2.01	>50†	12.33 ± 1.73	23.36 ± 2.43	6.59 ± 0.04	26.48 ± 1.45
8	5.40 ± 0.06	12.97 ± 1.05	7.02 ± 0.03	19.67 ± 1.94	0.46 ± 0.01	>50	>50†	13.18 ± 1.02	15.57 ± 1.23	23.96 ± 1.43	39.57 ± 3.12	21.32 ± 1.76	24.10 ± 1.92	21.66 ± 2.13	5.28 ± 0.02	11.12 ± 0.85
9	6.29 ± 0.09	2.36 ± 0.01	2.04 ± 0.01	14.32 ± 1.13	9.12 ± 0.98	17.32 ± 1.17	6.80 ± 0.05	13.04 ± 1.06	8.22 ± 0.78	5.08 ± 0.45	14.76 ± 1.04	5.40 ± 0.04	14.08 ± 0.97	14.17 ± 1.94	6.26 ± 0.04	24.37 ± 1.78
10	10.32 ± 0.92	>50†	23.83 ± 2.89	>50†	>50†	>50†	>50†	>50†	3.28 ± 0.02	>50†	0.39 ± 0.01	>50†	13.07 ± 0.99	>50†	39.53 ± 3.02	7.79 ± 0.03
11	6.67 ± 0.04	14.42 ± 1.13	>50†	>50†	>50†	>50†	>50†	>50†	>50†	>50†	>50†	>50†	>50†	>50†	>50†	11.76 ± 0.85
12	3.07 ± 0.02	3.45 ± 0.02	2.75 ± 0.01	3.36 ± 0.02	2.86 ± 0.01	>50†	17.37 ± 1.11	12.11 ± 1.11	34.82 ± 3.21	40.72 ± 3.79	>50†	30.69 ± 2.98	>50†	4.29 ± 0.02	8.30 ± 0.54	1.49 ± 0.01
13	20.79 ± 1.86	18.64 ± 1.08	12.79 ± 1.11	>50†	21.92 ± 1.99	>50†	20.21 ± 1.23	20.06 ± 1.02	>50†	>50†	>50†	16.23 ± 1.36	45.97 ± 3.85	37.07 ± 3.02	10.84 ± 0.74	19.47 ± 0.21
14	4.66 ± 0.05	5.15 ± 0.03	5.57 ± 0.55	29.85 ± 2.97	4.86 ± 0.03	>50†	29.05 ± 3.01	25.70 ± 1.89	6.44 ± 0.05	>50†	32.58 ± 2.69	>50†	7.34 ± 0.65	>50†	5.24 ± 0.02	3.99 ± 0.01
15	5.90 ± 0.03	1.27 ± 0.01	0.63 ± 0.01	6.32 ± 0.04	39.87 ± 2.97	5.70 ± 0.06	2.00 ± 0.01	4.37 ± 0.01	3.17 ± 0.01	>50†	6.12 ± 0.03	5.54 ± 0.31	5.06 ± 0.32	4.54 ± 0.53	4.06 ± 0.01	4.29 ± 0.02
16	>50†	>50†	>50†	>50†	>50†	>50†	>50†	>50†	10.46 ± 1.06	>50†	6.53 ± 0.02	>50†	0.39 ± 0.01	0.38 ± 0.01	>50†	46.37 ± 3.21
17	23.72 ± 2.12	30.55 ± 2.89	20.95 ± 1.94	45.69 ± 3.85	14.48 ± 0.98	28.35 ± 1.19	42.33 ± 3.78	48.21 ± 4.02	14.91 ± 0.99	26.49 ± 2.11	>50†	38.10 ± 3.01	28.12 ± 1.27	42.97 ± 3.24	10.64 ± 0.65	36.17 ± 2.01

*IC_{50} values of compounds to inhibit human cancer cell lines shown as mean ± SD of three determinations.
†>50 means that the data were not applicable.

3.07, 3.45, 2.75, 3.36, 2.86, 4.29, 8.30 and 1.49 μM respectively.

Although 3-*O*-methylquercetin (**4**) showed less antiproliferative potency compared with quercetin (**1**), methylation of the OH groups at the 3 and 7 positions resulted in the most potent compound **8** with an IC_{50} value of 0.46 μM against 1944; this compound also showed potent cancer cell growth inhibition for A549, H157, H460, 1792, Hop62, and 1299 with IC_{50} values of 5.40, 12.97, 7.02, 19.67, 13.18, and 15.57 μM, respectively. Methylation of the 3' OH (**2**) reduced antiproliferative potency with this compound only showing potent cancer cell growth inhibition for A549, H157, H460, 1792, H266, Hop62, and Hela with IC_{50} values of 10.78, 20.52, 12.48, 33.36, 42.37, 23.34, and 17.93 μM, respectively, its IC_{50} values being greater 50 μM against the other cell lines. Methylation of the 3' and 4' OH resulted in 3',4'-dimethoxyquercetin (**9**), which had potent cancer cell growth inhibition with IC_{50} values of less than 10 μM against A549, H157, H460, 1944, H522, 1299, 292G, M4E and SKBR, IC_{50} values of 14.32, 17.32, 13.04, 14.76, 14.08, 14.17 μM against 1792, H266, Hop62, Calu1, LOX-IMVI, and M14, respectively, and an IC_{50} value of 24.4 μM against HeLa. These results indicate that the 3',4'-arrangement of OMe residues is important for inhibiting cancer growth and that the OMe analogs may be superior to their OH counterparts. Furthermore, introduction of OMe moiety at the 5 position in **9** was important for growth arrest of these cells; compound **14** showed comparable cancer cell growth inhibition with IC_{50} values of less than 10 μM against A549, H157, H460, 1944, 1299, LOX-IMVI, Hela, and SKBR. Compound **15**, where the OH groups at 3', 4', 7 positions were methylated, showed more potent cancer cell growth inhibition compared with 3',4'-dimethoxyquercetin (**9**), with IC_{50} values of less than 10 μM except for the 1944 and 292G cell lines.

Methylation of the OH group at position 3 in compound **15** yielded tetramethylated quercetin **16** with IC_{50} values of 0.39 and 0.38 μM against LOX-IMVI and M14, respectively, which warrant further investigation. Pentamethylated quercetin (**17**) had reduced antiproliferative activity compared with quercetin (**1**) except for the cancer cell lines 1299, 292G, and SKBR where it was more active.

CONCLUSION

The present study provides an initial assessment of the structure–activity relationships of methylated quercetins as inhibitors of cancer proliferation. Methylation at the 4' or (and) 7 positions was important in maintaining inhibitory activities against the 16

cancer cell lines while dimethylation enhanced activity. These findings suggest that methylation of the OH groups quercetin warrants further evaluation for compounds as potential lead compounds for novel anticancer agents.

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