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Structure–Activity Relationships of Methylquercetin on Anti-migration and Anti-proliferation Activity in B16 Melanoma Cells

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Abstract. *Background: Melanoma is a malignant skin tumor and quercetin has been reported to inhibit metastasis. A quercetin glycoside and 7 methylquercetins were synthesized from rutin to investigate structure-activity relationships. Materials and Methods: We evaluated the anti-proliferative and anti-migration activity of quercetin glycoside and 7 methylquercetins in murine B16 melanoma cells by commercially available kits. We also examined the effect of these compounds on growth of human fibroblast cells to evaluate cytotoxicity. Results: 3-O-methylquercetin (**2**) exhibited 30, 38, 45% migration activity at 25, 12.5, 6.25 μM respectively. Furthermore, 3,4',7-O-trimethylquercetin (**5**) and 3,4'-O-dimethylquercetin (**3**) inhibited migration more potent than 2 with no cytotoxicity. 3-hydroxymethylquercetin and quercetin glycoside exhibited no anti-migration activity. Furthermore, the **3** and **4** inhibited melanoma proliferation with no cytotoxicity. Conclusion: The methoxyl group at the C-3 position plays an important role in inhibiting the migration of B16 melanoma cells.*

Melanoma is a malignant skin tumor caused by ultraviolet (UV) damage to melanocytes, that are pigment-producing cells distributed on the surface of the skin, causing 75% of deaths related to skin cancer (1-3). Melanoma causes metastasis to the lymph nodes, bone, and lungs (4, 5). Numerous studies have reported anti-metastasis agents for melanoma. For example, CCT129254 and AT13148, Rho kinase inhibitors, dramatically reduce melanoma invasion (6). Galangin, a flavonoid isolated from the root of *Alpinia officinarum*, inhibits proliferation and metastasis of B16F10 melanoma through the suppression of Focal Adhesion Kinase (FAK), a cytoplasmic tyrosine kinase that is related to cellular processes such as proliferation, adhesion, and invasion (7).

Quercetin is a flavonoid present as a glycoside in various fruits and vegetables (8-10). Numerous studies have demonstrated that quercetin exhibits a variety of pharmacological effects, including antioxidant, anti-inflammatory, and anti-cancer properties (11-13). Quercetin has been reported to inhibit migration and invasion of prostate cancer cells, and human hepatoma HepG2 cells (14, 15). It also inhibits migration and invasion of human oral cancer cells by reducing the protein levels of matrix metalloproteinase (MMP)-2, -7, -9 and -10, vascular endothelial growth factor (VEGF), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) p65 (16). Furthermore, quercetin inhibits growth and metastasis in melanoma by suppressing the phosphorylation of c-Met downstream molecules including GRB2-associated-binding protein 1 (Gab1), FAK, and p21-activated kinases relating to the metastasis (17-21). However, the structure-activity relationships using quercetin derivatives on anti-proliferation and anti-migration activity in B16 melanoma cells have not yet been elucidated. In our previous study, we evaluated the effect of two novel quercetin glycosides, 4'-*O*-β-D-glucopyranosyl-quercetin-3-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside (**9**) and 4'-*O*-β-D–glucopyra-nosyl-(1→2)-β-D-glucopyranosyl-quercetin-3-*O*-β-D-glucopyranosyl-(1→4)-β-D-glucopyranoside isolated from *Helminthostachys zeylanica* root extract on melanogenesis (23). Furthermore, 19 quercetin derivatives have also been synthesized from rutin similarly (23, 24). The anti-migration and anti-proliferation activities of the 7

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methylquercetin (**2-8**) and a quercetin glycoside (**9**) on B16 melanoma cells were investigated in this study (Figure 1). Furthermore, we investigated the effect of the quercetin derivatives on cell viability using human fibroblast cells to evaluate its application in humans.

Materials and Methods

Quercetin derivatives were synthesized using methods presented in our previous publication (23, 24). Murine melanoma B16-F0 cells (DS Pharma Biomedical, Osaka, Japan) were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100,000 unit/L penicillin, and 100 mg/l streptomycin. Human fibroblast cells (NB1RGB) were grown in Minimum Essential Medium (MEM) α supplemented with 10% FBS, 100,000 unit/L penicillin, and 100 mg/l streptomycin. Cells were cultured at 37˚C in humidified atmosphere of 5% CO_2. The migration activity was performed using migration assay kits (CBA-100, Cell Biolabs, San Diego, CA, USA). Cell suspension containing 5.0×10^5 cells/well in 0.5% FBS DMEM was prepared. DMSO (control) and quercetin derivatives were added to the inside of each insert. A 500 μl of DMEM including 10% FBS and quercetin derivative was added to the lower well of the migration plate and 300 μl of cell suspension solution was loaded into a cell culture inserts placed in 24 well plate. The cells were incubated with compounds at 37˚C for 24 h. Cells migrating to other sides of the inserts were stained, and the absorbance of extracted solutions was measured at 590 nm using a microplate reader.

Measurement of proliferation activity was performed according to a previously described method, using the microculture tetrazolium technique (MTT) (24). Following incubation with compounds for 72 h, 50 μl of MTT reagent (5 mg/mL of 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide in PBS) was added to each well. The plates were incubated in a humidified atmosphere of 5% CO_2 at 37˚C for 4 h. After the medium was removed, 1.0 ml of isopropyl alcohol (containing 0.04 N HCl) was added to each well, and a 150 μL sample was withdrawn and transferred to a 96-well plate. Absorbance was measured at 590 nm by using a microplate reader.

B16 melanoma cells and human fibroblast cells were used to determine cytotoxicity of quercetin derivatives. Following incubation with compounds for 24 h, 50 μl of MTT reagent was added to each well. The plates were incubated in a humidified atmosphere of 5% CO_2 at 37˚C for 4 h. After the medium was removed, 600 μl of isopropyl alcohol (containing 0.04 N HCl) was added to each well, and a 150 μl sample was withdrawn and transferred to a 96-well plate. Absorbance was measured at 590 nm by using a microplate reader.

Statistical method. Data from methylquercetin-treated *in vitro* experiments were compared to those from basal DMSO-treated controls using the Student's *t*-test with repeated measures design as methylquercetin dosages are varied within each experiment. Experiments were repeated 5 times to produce the replicates. Student's *t*-test was used to detect differences between the different methylquercetin dosages. All data were expressed as means±SD. A *p*-value of less than 0.05 was considered significant.

Table I. *Migration of quercetin **1** and synthesized quercetin derivatives **2-9** in B16 melanoma cells.*

	Migration activity (%)		
	25 μM	12.5 μM	6.25 μM
1	43.5±9.7*	66.5±6.4*	75.1±1.8**
2	30.4±1.8**	37.8±2.6**	45.0±1.8**
3	27.5±2.4**	32.9±1.4**	59.1±2.8**
4	44.5±8.1*	70.8±10.3	99.1±0.4
5	22.0±6.9**	30.6±2.8**	34.7±2.4**
6	55.8±21.0	96.3±9.6	102.0±3.8
7	71.3.±19.7	95.1±11.6	96.0±12.2
8	100.7±4.3	99.0±8.1	130.9±18.5
9	100.0±9.5	104.5±52.4	116.8±0.9

Data are expressed as means±SD (n=2). *$p\leq0.05$ and **$p\leq0.01$ compared to respective control values.

Table II. *Cell viability of quercetin **1** and synthesized quercetin derivatives **2-9** in B16 melanoma cells. B16 melanoma cells were treated with the compounds using MEMα including 0.5% FBS.*

	Cell viability (%)		
	25 μM	12.5 μM	6.25 μM
1	92.7±8.1	102.4±0.3	82.9±1.1
2	107.2±3.2	106.9±0.8	81.7±5.5
3	111.1±1.4	114.2±4.8*	106.8±3.0
4	115.3±0.1	115.3±1.4	100.5±1.2
5	94.5±0.7	105.8±0.0	99.6±5.
6	100.4±0.4	86.7±2.8	90.2±1.5
7	86.5.±0.7	85.1±4.1	76.3±7.4
8	92.9±3.5	47.8±1.3**	64.4±3.7*
9	93.7±1.9*	79.0±0.3	78.4±0.4

Data are expressed as means±SD (n=2). *$p\leq0.05$ and **$p\leq0.01$ compared to respective control values.

Results

A quercetin glycoside and 7 methylquercetins were synthesized from rutin as a starting material. As shown in Table I, 3-*O*-methylquercetin (2) exhibited 30.4, 37.8, 45% migration activity at 25, 12.5, 6.25 μM respectively that is more potent than that of quercetin as the positive control. 3,7-*O*-dimethylquercetin (4) also inhibited migration to 44.5, 70.8, 99.1% at the same concentrations. Furthermore, 3,4',7-*O*-trimethylquercetin (5) and 3,4'-*O*-dimethylquercetin (3) potently inhibited migration to 22.0, 30.6, 34.7% and 27.5, 32.9, 59.1% respectively at the same concentrations that is more potent than 2 and quercetin. 3-hydroxy methylquercetin and quercetin glycoside exhibited none or weak anti-migration activity.

Compound **1** R_1=R_2=R_3=OH (quercetin)
2 R_1=OCH_3, R_2=R_3=OH
3 R_1=R_2=OCH_3, R_3=OH
4 R_1=R_3=OCH_3, R_2=OH
5 R_1=R_2=R_3=OCH_3
6 R_2=OCH_3, R_1=R_3=OH
7 R_3=OCH_3, R_1=R_2=OH
8 R_1=OH, R_2=R_3=OCH_3
9 R_1=cellobiose, R_2=glucose, R_3=OH

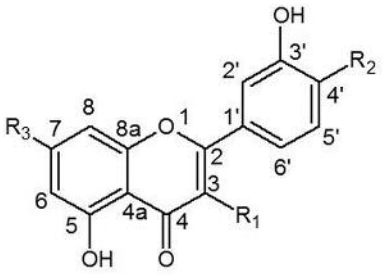

Figure 1. *Structures of synthesized quercetin derivatives.*

Cell viability of quercetin derivatives in B16 melanoma cells cultured with DMEM including 10% FBS were described in the previous paper (24). Compounds **3** and **4** inhibited proliferation to 63.2, 50.0, 75.6% and 54.1, 60.4, 80.4% at 25, 12.5, 6.25 μM, respectively. In contrast, they exhibited no cytotoxicity in B16 melanoma cells cultured with DMEM containing 0.5% FBS (Table II). Compounds **1-5** showing anti-migration activity did not exhibit cytotoxicity. The proliferation activity of quercetin derivatives in human fibroblast cells are shown in Table III. Quercetin showed no effect on the proliferation in human fibroblast cells. Compound **2** slightly inhibited proliferation with no cytotoxicity. Compound **4** stimulated proliferation on 0.5% FBS MEMα to 161.3, 153.3, 130.2% at 25, 12.5, 6.25 μM, respectively, even though it did not increase on 10% FBS MEMα. Compounds **5, 6, 7** and **8** also significantly increased cell numbers cultured with MEMα containing 0.5% FBS, without further increasing the number of cells in 10% FBS medium.

Table III. *Cell viability of quercetin **1** and synthesized quercetin derivatives **2-9** in human fibroblast cells. Fibroblast cells were treated with the compounds using MEMα including 10% or 0.5% FBS.*

	Cell viability (%)		
	25 μM	12.5 μM	6.25 μM
1			
10% FBS MEMα	96.1±5.2	97.7±1.4	107.2±6.1
0.5% FBS MEMα	100.9±0.7	94.9±0.3*	82.9±1.1**
2			
10% FBS MEMα	74.8±4.0*	74.8±0.0**	75.3±0.0**
0.5% FBS MEMα	119.9±1.7**	104.5±1.0*	87.5±4.2
3			
10% FBS MEMα	83.0±3.6*	84.0±3.0*	92.9±8.7
0.5% FBS MEMα	114.6±2.5**	105.8±4.7	99.7±0.3
4			
10% FBS MEMα	78.3±7.5	80.9±1.8**	73,9±7.3
0.5% FBS MEMα	161.3±1.4**	153.3±1.3**	130.2±1.4**
5			
10% FBS MEMα	101.3±4.8	103.0±1.6	103.1±0.2
0.5% FBS MEMα	134.1±0.8**	129.1±1.0**	120.8±0.8**
6			
10% FBS MEMα	100.4±1.2	104.1±0.4	103.4±1.0
0.5% FBS MEMα	135.9±0.6**	107.5±0.9**	101.1±2.1
7			
10% FBS MEMα	89.2±0.6	100.9±1.0	103.3±4.4
0.5% FBS MEMα	138.0±3.5**	132.2±1.5**	119.0±2.0**
8			
10% FBS MEMα	110.8±0.6*	108.3±1.8*	113.1±1.4*
0.5% FBS MEMα	125.6±13.3	122.5±2.8**	101.7±5.9
9			
10% FBS MEMα	102.7±1.6	106.7±4.4	109.8±1.2*
0.5% FBS MEMα	96.6±3.1	98.5±1.6	91.9±9.1

Data are expressed as means±SD (n=2). *$p \leq 0.05$ and **$p \leq 0.01$ compared to respective control values.

Discussion

Quercetin has been reported to inhibit migration and invasion of several types of cancer cells such as prostate cancer cells, human hepatoma HepG2 cells, human oral cancer cells, and melanoma (14-21). As seen in Table I, quercetin inhibits migration in a dose-dependent manner at 25-6.25 μM in B16 melanoma cells. In the present study, we evaluated the anti-migration activity of quercetin derivatives that synthesized from rutin reported in our previous study. Compounds **6**, **7**, **8**, and **9** did not inhibit or only gradually inhibited the migration. This anti-migratory activity is less potent compared to quercetin as a positive control. In contrast, **4** decreased the migration activity at the same level as quercetin, and **2**, **3**, and **5** that include 3-methoxyl group, inhibited the migration more potently than quercetin, suggesting that the 3-methoxyl group in methylquercetin is necessary to increase the anti-migration activity of quercetin. The activity of **4**, 7, 3-*O*-dimethylquercetin, was less than **2**, which indicates that 7-methoxyl group did not increase the anti-migratory activity. While 3, 4', 7-*O*-trimethylquercetin **5**

and 3, 4'-*O*-dimethylquercetin **3** inhibited migration more than **2** that suggests that 4'-methoxyl group stimulates the activity.

Cell viability in B16 melanoma cells of quercetin derivatives using DMEM including 10% FBS was determined in our previous report (24). This study investigated the cytotoxicity using DMEM including 0.5% FBS in B16 melanoma cells, which inhibited cell division and determined the proliferation activity using human fibroblast cells to estimate human application. Compounds **3** and **4** inhibited proliferation in B16 melanoma cells. However, they exhibited no effect in melanoma cells cultured with 0.5% FBS DMEM, suggesting the compounds inhibited proliferation without any cytotoxicity. Compounds **2**, **3**, and **5** elicited potent anti-migration activity without affecting the cell viability in both 10% and 0.5% FBS DMEM. This data suggesting that the compounds directly inhibiting migration of B16 melanoma cells without reducing cell viability.

Table III illustrates quercetin derivatives **4**, **5**, **7**, and **8**, all of which possess the 7-methoxyl group increased fibroblast cell numbers cultured with 0.5% FBS medium in a dose dependent manner, however they did not increase cell viability on 10% MEMα. Alternatively, quercetin derivatives **1**, **2**, **3**, and **6** showed little to no effect in comparison with **4**, **5**, **7**, and **8**. The 7-methoxyl group of methlquercetin may play an important role in human fibroblasts proliferation in the absence of FBS. Fibroblast cells are known to play an important role in wound-healing and cosmetics. Dermal fibroblasts lose their proliferative capacity with aging (25). Dermal fibroblasts have been reported to decrease mitogenic responsiveness *via* decline of epidermal growth factor receptor in aging (26-28). Interestingly, **4** increased the number of human fibroblast in the absence of the growth factor; however, it inhibited proliferation in B16 melanoma cells. Compound **5** possesses 3 and 4-methoxyl groups that are involved in stimulation of anti-migration activity of B16 melanoma cells and 7-methoxyl group increasing human fibroblast proliferation. We could suggest that compound **5** exhibited both beneficial effects in the inhibition of migration, as well as an increase in fibroblast proliferation. These data strongly suggest that **5** and **4** might be used as a cosmetic tool and for wound healing purposes, as well an anticancer agent.

Conclusion

Quercetin and 8-series of quercetin derivatives were used to determine their anti-proliferation and anti-migration activity in B16 melanoma cells, in order to improve our understanding of their structure-activity relationships. Among the quercetin derivatives, **2**, **3**, and **5** potently inhibited migration more than quercetin, with no cytotoxicity observed. This result suggested that 3-methoxyl group in the methylquercetin plays an important role in the anti-migration activity and 4'-methoxyl group stimulates the activity. The discovery of the novel anti-migration, anti-proliferation and fibroblast growth promotion activity of methylquercetin supports further research into its potential anticancer applications, and into its possible uses in cosmetic products.

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