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Design, synthesis and inhibitory activities of naringenin derivatives on human colon cancer cells

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ABSTRACT

Based on the previous result, several naringenin derivatives modified at position 7 with bulky substituents were designed and synthesized, and their inhibitory effects on HCT116 human colon cancer cells were tested using a clonogenic assay. The half maximal inhibitory concentrations (IC_{50}) of five naringenin derivatives ranged between 1.20 μM and 20.01 μM which are much better than naringenin used as a control. In addition, new structural modification at C-4 of flavanone results in improving both the anti-cancer effect and anti-oxidative effect. In vitro cyclin dependent kinase 2 (CDK2) binding assay was carried out based on the previous results. To elucidate the possible interaction between naringenin derivatives and CDK2, in silico docking study was performed. This result demonstrates the rationale for the different inhibitory activities of the naringenin derivatives. These findings could be used for designing cancer therapeutic or preventive flavanone-derived agents.

Colon cancer is the fourth most commonly diagnosed malignant disease and is prevalent where the people have adopted western diets and also among the elderly. Its symptom is worsening constipation or bloody stool. When such symptom arises, complete treatment is late and 5 years survival is <60%, and thus, a periodic colonoscopy is desired.[1] Main treatment for colon cancer is surgery, radiation, or chemotherapy which is applied as adjuvant therapy in many cases. Chemotherapeutic agents such as oxaliplatin, leucovorin, and irinotecan are known, however, they are associated with severe adverse effects. Therefore, there is a need for more potent and less toxic drugs.[2]

Flavanones, 2-phenylchroman-4-one, belong to the family of flavonoids, many of which are produced as secondary metabolites in the plant kingdom. They have three-ring skeletons, C6-C3-C6, and the rings are referred to as A-, C-, and B-rings, respectively. Their functional groups are attached to the main skeleton through oxygen or carbon linkages.[3] The biological activities of flavonoids depend on the degree of condensation in their structures and the position and number of substitutions, such as hydroxy groups, glucosides, isoprenyl units, homodimers, and heterodimers.[4] Flavanones compared to flavones, 2-phenyl-4*H*-chromen-4-one, have more molecular flexibility due to the absence of a carbon–carbon double bond in the C-ring. In our previous studies, we found that a methoxy or hydroxy substituent at C-7 position of flavanone resulted in better inhibitory effects on HCT116 human colon cancer cell lines.[5] Based on this result, we tried to design flavanone derivatives with bulkier substituents at C-7 position and elucidate their inhibitory effects.

Since the anti-cancer activity of naringenin, 4′,5,7-trihydroxyflavanone against colon cancer cells has been reported, several flavanone derivatives were designed from naringenin.[6] Besides, naringenin plays a key role as an estrogenic substance in humans and as an endogenous regulator in plants.[7] Various flavonoid derivatives modified at C-7 position have been reported, but in flavanone, especially naringenin, derivatives modified at position 7 have rarely been studied.[8,9] Naringenin derivatives designed in this study have a common moiety, so that they may result in small changes in their biological activities. Therefore, one of long-term survival assays, clonogenic assay, was applied here.[10] As a control, naringenin was used.

Naringenin contains three hydroxy groups (Fig. 1). *O*-substitutions can be easily at carried out at the 4′- and 7-hydroxy groups, however, the 5-hydroxy group forms a hydrogen bond (H-bond) with the ketone at C-4, making it less accessible. The treatment of cancer cell lines such as breast cancer cell line Michigan Cancer Foundation 7 (MCF7) with flavanone derivatives shows different effects according to the variation in functional groups on C-4′

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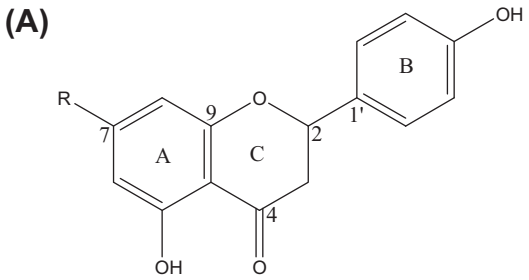
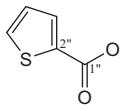
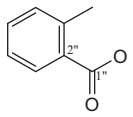
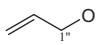
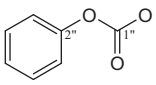


derivative	R
naringenin	OH
N1	
N2	
N3	
N4	
N5	

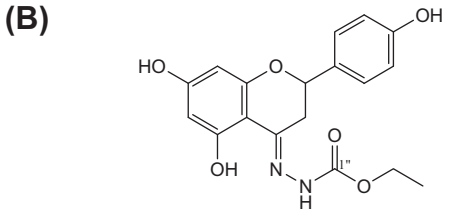


Figure 1. (A) Structures of synthetic naringenin derivatives **N1**–**N5** and (B) the structure of derivative **N6**.

and C-7.[11] In this study, five naringenin derivatives modified at position 7 with thiophenecarboxylate (**N1**), methylbenzoate (**N2**), isobutyrate (**N3**), allyloxy (**N4**), and phenyl carbonate (**N5**) groups were synthesized. All derivatives except 7-allyl substituted naringenin are novel.[12] The aim of this research is to discover potent anti-cancer agents showing better inhibitory effects on human colon cancer cell lines than naringenin whose anti-cancer activity on colon cancer has already been reported.

(±)-Naringenin was purchased from INDOFINE chemical company (Hillsborough, NJ, USA). All the *O*-alkylated naringenin derivatives **N1**–**N5** were efficiently synthesized from the coupling reaction of naringenin and the corresponding acid halides (derivatives **N1**, **N2**, **N3**, and **N5**) or alkyl halide (derivative **N4**) as shown in Scheme 1. Because of the hydrogen bond between hydroxy group at 5 position and ketone group at 4 position, alkylation at 7-position was very selective and gave the 7-*O*-alkylated naringenin products in good yield. Derivative **N6** was obtained from the reaction of naringenin and ethyl carbazate at 110–120 °C in a reasonable yield. The compounds used for synthesis were supplied by a local company in Korea.

All nuclear magnetic resonance (NMR) measurements were performed according to the methods published previously (Supplementary data). [13,14]

HCT116 human colon cancer cells (Health Protection Culture Collections, Salisbury, UK) were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT). HCT116 cells (5×10^3 cells/well) were seeded onto 24-well tissue culture plates (BD Falcon, Franklin Lakes, NJ) in the absence or presence of different concentrations of six naringenin derivatives and naringenin, and incubated for 7 days.[10] HCT116 cells were tested on four plates treated with 0, 10, 20, and 40 μM of samples (Fig. 2). The colonies that formed were fixed with 6% glutaraldehyde and stained with 0.1% crystal violet, as described previously.[15] The clonogenic survival densities were measured using the densitometry (Multi-Guage, Fujifilm, Japan) and their half maximal inhibitory concentrations (IC_{50}) were calculated using SigmaPlot software (SYSTAT, Chicago, IL).

The 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay was used to screen for anti-oxidative effects. DPPH radical-scavenging effects were tested according to the method reported previously.[16]

In order to confirm whether naringenin derivatives including naringenin inhibit cyclin dependent kinase 2 (CDK2), in vitro CDK2 binding assay was performed using EMD Millipore's KinaseProfiler service assay protocol (EMD Millipore Corporation, Billerica, MA).

All docking experiments were carried out on an Intel Core 2 Quad Q6600 (2.4 GHz) Linux PC with Sybyl 7.3 (Tripos, St. Louis, MO).[17] Of many crystallographic structures of CDK2 deposited in protein data bank, 2r3j.pdb was selected because its crystal structure was determined with an inhibitor with bicyclic cores and the structure of the ligand, 3-bromo-5-phenyl-*N*-(pyridin-3-ylmethyl)pyrazolo[1,5-a]pyrimidin-7-amine, was similar to naringenin derivatives modified at position 4 or 7 (Supplementary data Fig. 1).[18] It was originated from *Homo sapiens* and its resolution was 1.65 Å. Its apo-protein without its ligand was obtained from energy minimization using the Sybyl/FlexX Single Receptor Module. Since the Sybyl program provides flexible docking procedure, the binding pocket was defined first. The docking radius was set to 6.5 Å and the residues for docking were selected; Ile10, Ala31, Glu81, Phe82, Leu83, His84, Gln131, and Leu134. Naringenin derivatives including naringenin were used as ligands. Their three dimensional (3D) structures were constructed based on the X-ray crystallographic structure of naringenin contained in chalcone isomerase as a ligand, 1eyq.pdb deposited in the protein data bank and subjected to energy minimization using the molecular mechanics algorithms provided by Sybyl 7.3.[19] Minimization was stopped upon convergence of the total energy (0.05 kcal/mol Å). Systematic conformational searches were performed because rotational bonds existed in the compounds.[15] The conformers with the lowest energy were selected for the docking. They were docked into apo-protein CDK2. The docking process was iterated 30 times and 30 docking poses were obtained. Among them, the results showing the similar docking pose comparing to the ligand contained in 2r3j.pdb were selected. The residues surrounding the derivatives were analyzed using LigPlot provided by the European Bioinformatics Institute.[20] All 3D images were constructed using PyMOL program (The PyMOL Molecular Graphics System, Version 1.0r1, Schrödinger, LLC.).

The naringenin derivatives synthesized here were characterized using NMR spectroscopy and mass spectrometry (MS). The NMR data of naringenin, which have been reported previously, could be applied to the derivative **N1**, 5-hydroxy-2-(4-hydroxyphenyl)-4-oxochroman-7-yl thiophene-2-carboxylate, since the structure is similar with the exception of the thiophene-2-carboxylate moiety.[21] The ^{13}C peaks in the B- and C-rings were easily assigned (Supplementary data Fig. 2). Previous reports did not show the