Arch Pharm Res Vol 29, No 7, 582-586, 2006

Archives of Pharmacal Research
http://apr.psk.or.kr

Relationships Between the Larval Growth Inhibition of *Caenorhabditis elegans* by Apigenin Derivatives and Their Structures

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(Received May 23, 2006)

Due to consumer reluctance to take synthetic drugs for nematode infections and the appearance of resistance to anthelminthic drugs, new drugs from natural products must be developed. *Caenorhabditis elegans* is one of the freely living nematodes and serves as a good model system for screening anthelminthic drugs. In this study, thirteen flavonoid derivatives were tested for anthelminthic activity and the relationships between their activities and structures were investigated. The structural information combined with the data for the larval growth inhibition of *C. elegans* provided meaningful structural insights in the search for new anthelminthic drugs.

Key words: *Caenorhabditis elegans*, Flavonoids, Structure-activity relationships, Larval growth inhibition, Apigenin

INTRODUCTION

Although nematode infections cause high morbidity, they have low mortality because of the long evolutionary history between host and parasite. Many chemotherapeutic drugs against nematode infections have been developed. However, due to consumer rejection of chemical synthetic drugs, the development of new drugs is required. In addition, the appearance of resistance to anthelminthic drugs and the side effects compel discovery of new drugs from natural products. Since *Caenorhabditis elegans* (*C. elegans*) is one of the freely living nematodes, it serves as a good model system in the search for new anthelminthic drugs. Even though *C. elegans* is not a parasite, its developmental mechanism is similar to that of parasitic nematodes, so that it can be used for screening anthelminthic drugs. In addition, *C. elegans* is an ideal model organism with convenient features for examining phenotypic changes induced by drugs. It has a 3-day reproductive cycle, producing more than 300 progeny (hermaphrodite) each cycle, and a short life span (2-3 weeks) that can easily be observed in the laboratory (Wood, 1988). Its complete genome sequence is available, and numerous genetic mutants as well as information regarding gene functions are accessible. In addition to such convenient features, the simplicity of this organism that consists of 959 somatic cells and has well-defined phenotypes is advantageous for *in vivo* studies.

Among the many natural products, one of the flavonoids, apigenin, has a chemopreventive effect against ultraviolet radiation, as well as an inhibitory effect on the growth of human leukemia cells via apoptosis (Torkin *et al.*, 2005). The flavonoids are the group of benzo-g-pyran derivatives with C6-C3-C6 skeletons. Based on the oxidation state of the pyran ring, the flavonoids are subdivided into flavonols, flavanols, flavones, isoflavones, and anthocyanidins (Cook and Samman, 1996). Apigenin belongs to the flavones and is found in vegetables such as celery, parsley, and artichoke (Hempel *et al.*, 1999; Mihean and Mohamed, 2001). Since the biochemical activities of these compounds are dependent on their chemical structures, the relationships between their structures and activities have been investigated. In order to determine which flavone skeleton is required for the physiological effects of apigenin, the activities of apigenin derivatives were tested in *C. elegans*. Larval growth inhibition of *C. elegans* by flavonoids has not been reported previously, and the authors report the structure-activity relationships based on computational structural modeling methods here.

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MATERIALS AND METHODS

Nematode strain and general methods

Methods for maintenance and handling of *C. elegans* were as previously described (Brenner, 1974). Strain N2 was used as a wild-type strain for most analyses. This strain was provided by the *Caenorhabditis* Genetics Center. Wild-type N2 worms were grown at 20°C on NGM (nematode growth medium) agar plates containing *E. coli* (OP50).

Larval growth inhibition of *C. elegans*

Wild type N2 worms were synchronized at L1 stage by hatching embryos in M9 buffer (42 mM of KH_2PO_4, 2 mM of Na_2HPO_4, 0.86 M of NaCl, 1 mM of $MgSO_4$) and 50 worms were transferred to NGM agar plates containing OP50 *E. Coli* and 1 mM of apigenin. Worms were grown at 20°C until they reached the gravid adult stage. Every 24 h, worms were observed for their growth by examining any morphological changes. Since the apigenin was dissolved in DMSO, worms were also grown on plates containing 1% DMSO as a control.

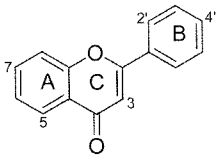
Structures of flavonoids

Ten flavone derivatives including apigenin, were used in this experiment. Two flavonols and one flavanone were tested as well. Their nomenclatures and structures are listed in Table I. Because three-dimensional structures were not known, molecular modeling calculations were carried out on a Pentium IV 3.2 GHz PC with the Linux OS (Red Hat Enterprise WS) with Sybyl 7.2 (Tripos, St. Louis, MO). The Tripos forcefield was used for molecular dynamics (MD) and energy minimization. A conjugate gradient was carried out until a maximum derivative of 0.5 kcal/molÅ was reached for energy minimization. After energy minimization, MD was performed at 300K for 1,000 fsec in 1-fsec increments. The output conformers were collected every 5 fsec. Two hundred conformers were saved in the history file. The conformers showing the lowest total energy were selected for the structure-activity relationship calculations (Yang *et al.*, 2003).

Relationships between larval growth inhibition and structures of flavonoids

Structure-activity relationships (SARs) were performed on a Pentium IV 3.0 GHz PC with the Linux OS (Red Hat Enterprise 3 release) with Sybyl 7.2/QSAR module (Tripos, St. Louis, MO). Thirteen compounds shown in Table I were used for the training set of SAR. For the alignments, a maximum common subgraph method was applied to all 13 compounds, with apigenin used as a template.

Table I. Nomenclature and structures of flavonoids used in this experiment



Compound	3	5	6	7	8	2'	3'	4'	5'
Acacetin	H	OH	H	OH	H	H	H	OCH_3	H
Apigenin	H	OH	H	OH	H	H	H	OH	H
Chrysin	H	OH	H	OH	H	H	H	H	H
6,4'-Dihydroxyflavone	H	H	OH	H	H	H	H	OH	H
4'-Hydroxyflavone	H	H	H	H	H	H	H	OH	H
Luteolin	H	OH	H	OH	H	H	OH	H	H
5,7,3',4',5''-Pentahydroxyflavone	H	OH	H	OH	H	H	OH	OH	OH
5,7,3',4'-Tetrahydroxyflavone	H	OH	H	OH	H	H	OH	OH	H
5,7,2'-Trihydroxyflavone	H	OH	H	OH	H	OH	H	H	H
7,8,4'-Trihydroxyflavone	H	H	H	OH	OH	H	H	OH	H
Kaempferol	OH	OH	H	OH	H	H	H	OH	H
Quercetin	OH	OH	H	OH	H	H	OH	OH	H
Naringenin*	H	OH	H	OH	H	H	H	OH	H

Naringenin has a single bond between C-2 and C-3.

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Statistical analysis

The data are reported as the mean of three independent experiments, and were evaluated by Student's *t*-test. Values of $P<0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

As shown in Fig. 1, while *C. elegans* reached the L4 stage after 48 h incubation at 20°C from the hatched L1 stage, worms treated with apigenin showed growth inhibition. When apigenin was added to the growth media, worms developed to the L4 stage after 48 h incubation of their parental generation (P0) with slightly reduced body size as shown in Fig. 1. Larval development was severely impaired when the F1 generation was continuously treated with apigenin. Among the flavonoids tested in this study, only apigenin affected the larval development of *C. elegans*. To further understand the structural information of apigenin that gives this specific activity *in vivo*, we also investigated other flavonoids. Flavones including apigenin are composed of three rings that are named the A, B, and C rings, where the A ring and the C ring of the flavone are not in the same plane. The dihedral angle of the A ring and the C ring is about 3.2. In the case of flavonols such as quercetin, the dihedral angle is 8.1, and in flavanones such as naringenin, 23.3. In order to obtain information about SAR, therefore, the three-dimensional structures of the compounds tested in this experiment were determined first. As mentioned in the materials and methods section, all three-dimensional structures of the compounds used for SAR were calculated using molecular modeling.

Since apigenin showed the larval growth inhibition of *C. elegans*, three apigenin (5,7,4'-trihydroxyflavone) derivatives with different A rings, 4'-hydroxyflavone, 6,4'-dihydroxyflavone, and 7,8,4'-trihydroxyflavone, were selected. These three derivatives did not exhibit the larval growth inhibition. When comparing the structures of all four compounds including apigenin (Fig. 2), 4'-hydroxyflavone does not contain any hydroxyl groups in the A ring. Even 6,4'-dihydroxyflavone and 7,8,4'-trihydroxyflavone contain only one or two hydroxyl groups in the A ring, and these compounds do not display activities. Therefore, it was supected that the positioning of these two hydroxyl groups on the A ring is important for the activity.

Apigenin contains a total of three hydroxyl groups. Among these, the 4'-hydroxyl group is attached to the B ring. In order to know the effect of the 4'-hydroxyl group on the activity, six more apigenin derivatives (Fig. 3) were tested. They contain a 2'-hydroxylated B ring (5,7,2'-

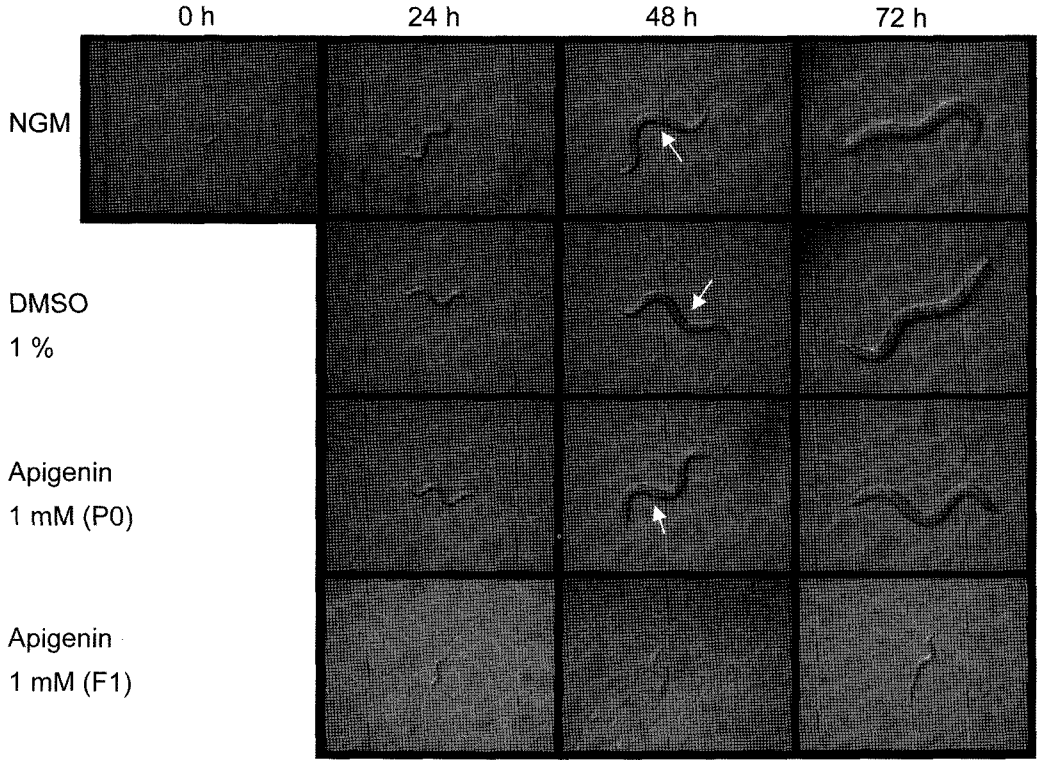

Fig. 1. Apigenin-induced growth inhibition of *C. elegans*. Worms were grown at 20°C on NGM plates or NGM plates with 1% DMSO or 1 mM apigenin as indicated. Every 24 h, worms were photographed with original magnification, X70. Worms reached the L4 stage after 48 h incubation from the hatched L1 stage as indicated with white arrows at their vulval structures. Two generations of worms, parental (P0) and F1 generation were observed.

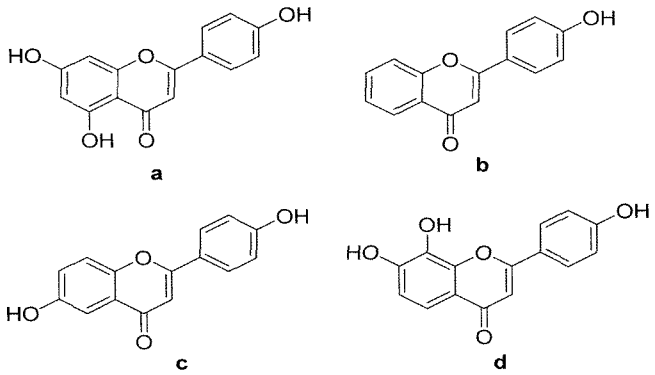

Fig. 2. The structures of apigenin (5,7,4'-trihydroxyflavone) derivatives with different A rings: apigenin (a), 4'-hydroxyflavone (b), 6,4'-dihydroxyflavone (c), and 7,8,4'-trihydroxyflavone (d).

trihydroxyflavone), a 3'-hydroxylated B ring (luteolin), a 3',4'-dihydroxylated B ring (5,7,3',4'-tetrahydroxyflavone), a 3',4',5'-trihydroxylated B ring (5,7,3',4',5'-pentahydroxyflavone), a B ring with no substituents (chrysin), and a 4'-methoxylated B ring (acacetin), instead of the 4'-hydroxyl group like apigenin. Of these derivatives, only the 3',4',5'-trihydroxylated B ring showed weak activity, while the others did not exhibit any larval growth inhibition activity. 5,7,2'-trihydroxyflavone and luteolin do not have a 4'-hydroxyl group. Even though 5,7,3',4'-tetrahydroxyflavone contains a 4'-hydroxyl group, it does not show any activity. In the case of acacetin, aside from the 4'-methoxyl group, its structure is identical to apigenin. However, it does not induce the growth inhibition of worms, suggesting that methoxylation at the 4'-position in place of the hydroxyl group may cause the failure to inhibit worm growth. In chrysin, there is no 4-hydroxyl group and no inhibition. Taken together, these results demonstrate that the 4'-hydroxyl group is required for larval inhibition, and the substituted positions of the B ring should be symmetrical.

In order to test the effect of the double bond between C-2 and C-3 in apigenin, naringenin was selected. Its structure is the same as apigenin except for a single bond between C-2 and C-3. The lack of activity for naringenin suggests that the double bond is required for the activity. As mentioned above, in flavones, the dihedral angle of the A ring and the C ring is about 3.2. However, that of flavanones is twisted by 23.3. The activity difference between apigenin and naringenin may also be caused from the difference in their dihedral angles.

Kaempferol has the same structure as apigenin except that it has a 3-hydroxyl group. Since kaempferol did not exhibit any activity, the 3-hydroxyl group must be an obstacle to the activity. Quercetin contains two more hydroxyl groups, at C-3 and C-3', than apigenin. As expected, because quercetin does not contain symmetrical substituents in the B ring and contains a 3-hydroxyl group, it does not display any activity.

Superposition of the 13 compounds tested in this experiment gives structural information about *C. elegans* F1 larval growth inhibition. The colored CoMFA contours in the map (Fig. 4) show the analysis of the electrostatic contours, displaying where one red contour (electronegative substituents favored) and three blue contours (elec-

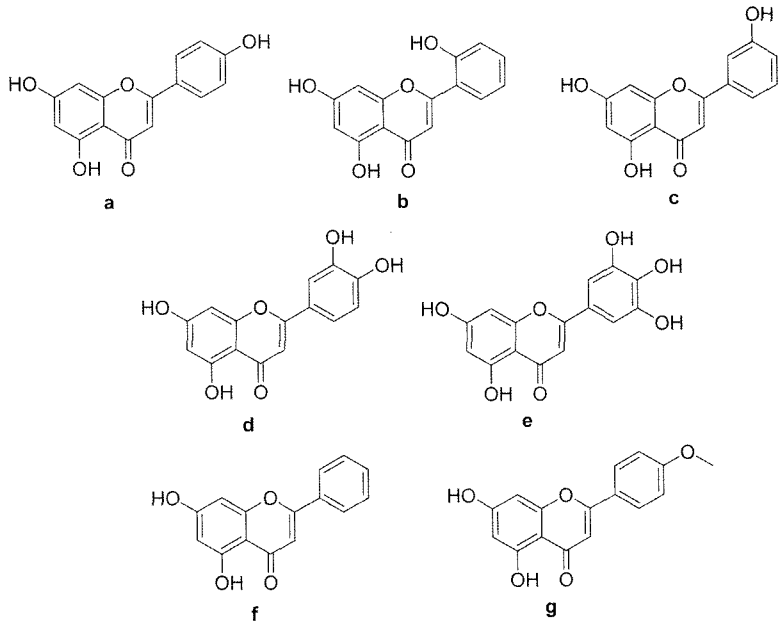

Fig. 3. The structures of apigenin derivatives with different B rings: apigenin (a), 5,7,2'-trihydroxyflavone (b), luteolin (c), 5,7,3',4'-tetrahydroxyflavone (d), 5,7,3',4',5'-pentahydroxyflavone (e), chrysin (f), and acacetin (g).

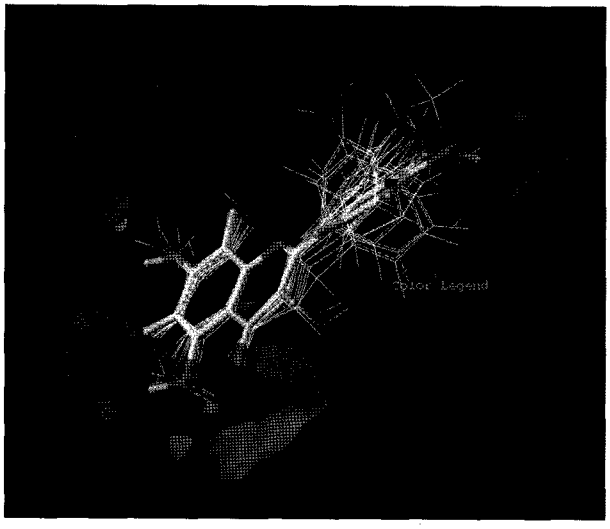

Fig. 4. The colored CoMFA contours in the map show the electrostatic contours, illustrating where one red contour (electronegative substituents favored) and three blue contours (electropositive substituents favored) exist.

tropositive substituents favored) exist. The red contour was near the double bond between C-2 and C-3 in the C ring, which means that the compounds with electronegative substituents could exhibit good activity. The blue contours near the C-5 and C-7 positions in the A ring and the C-4' position in the C ring revealed that electropositive substituents would be favored.

As a result, we can summarize the reasons why apigenin and 5,7,3',4',5'-pentahydroxyflavone exhibit larval growth inhibition activity in *C. elegans*. Two hydroxyl groups at C-5 and C-7 of the A ring, and a double bond between C-2 and C-3 of the C ring are required for F1 larval growth inhibition. In the B ring, the existence of a 4'-hydroxyl group and the symmetry of hydroxylated substitutions are necessary for the activity. As a basis for determining the larval growth inhibition in an animal model, *C. elegans* provided meaningful structural insights into the search for new anthelminthic drugs that can be applied to future studies.

ACKNOWLEDGEMENTS

This study was supported by KRF2004-F00019 (KRF) and the second BK21 (MOE). Yoon and Kim contributed equally to this work.

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