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Structural characteristics of flavanones and flavones from *Cudrania tricuspidata* for neuraminidase inhibition

Young Bae Ryu [b], Marcus J. Curtis-Long [c], Ji Won Lee [a], Hyung Won Ryu [a], Jun Young Kim [a], Woo Song Lee [b,*], Ki Hun Park [a,*]

[a] *Division of Applied Life Science (BK21 program), EB-NCRC, Institute of Agriculture and Life Science, Graduate School of Gyeongsang National University, Jinju 660-701, Republic of Korea*
[b] *Bioindustry Technology and AI Control Biomaterial Research Center, KRIBB, Jeongeup 580-185, Republic of Korea*
[c] *12 New Road, Nafferton, Driffield, East Yorkshire YO25 4JP, UK*

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ABSTRACT

The structural characteristics of flavonoids (**1**–**3** and **6**–**8**) from the root of *Cudrania tricuspidata* required for neuraminidase inhibition were studied and compared with commercially available flavonoids (**4**, **5**, and **9**–**12**). Alkylated flavanones (**1**–**3**) display better inhibition than the corresponding parent compound **4**. Importantly, flavanone **1** bearing a C-8 hydrated prenyl group showed extremely high inhibition with IC_{50} of 380 nM. On the other hand, the parent flavone **5** was more effective than alkylated analogues (**6**–**8**). Isolated inhibitors (**1**–**3** and **6**–**8**) showed noncompetitive inhibition in kinetic studies. The binding affinity of flavanones (**1**–**4**) for neuraminidase in in silico docking experiments correlated well with their IC_{50} values and noncompetitive inhibition mode.

Neuraminidase (sialidase, EC 3.2.1.18, NA) belongs to a class of glycosyl hydrolases that release terminal *N*-acetylneuraminic acid residues from glycoproteins, glycolipids, and polysaccharides.[1] Its most commonly known form is viral neuraminidase, a target for the prevention of influenza infection. Indeed, viral NAs are frequently used as antigenic determinants found on the surface of the influenza virus, giving rise to the infamous H_xN_x notation. Influenza remains the most likely cause of a pandemic among the human population. Historically numerous bouts of this disease have caused widespread human deaths, most notably the 1918 'Spanish flu'[2,3] outbreak which killed between 50 and 100 million people, a large percentage of which were healthy *young* adults. For this reason considerable research efforts have been devoted by numerous groups to unearth drugs to combat its severe threat. Given the rapid spread of the most recent outbreak of H1N1 flu in Mexico, there is a sustained need for readily available neuraminidase inhibitors.

Despite its strong association with viruses, homologs have been detected in a number of bacteria and vertebrates.[4–7] This enzyme is important as a key enzyme for sialic acid metabolism, hydrolyzing the glycosidic linkage between sialic acid molecules and the penultimate sugar of the carbohydrate chains of oligosaccharides and oligoconjugates.

Polyphenols, the most abundant organic compounds in plants, have emerged to be a rich source of NA inhibitors. Surprisingly, given the huge importance of understanding structure–activity relationships (SAR) of these compounds, very few publications have reported any investigations into flavanoids. The most notable insights have come from Du and co-workers, who suggested the order of potency for NA inhibition in flavonoids decreases in the following order: aurones > flavon(ol)es > isoflavones > flavanon(ol)es and flavan(ol)es.[8] Thus it is of utmost importance to establish the key functions required for potent NA inhibitory activity.

Ongoing research efforts in our laboratory have been directed at developing xanthone-derived glycosidase inhibitors from *Cudrania tricuspidata*,[9] a deciduous tree which grows in Korea, China, and Japan.

The cortex and root bark of this plant have been used for the treatment of gonorrhea, jaundice, hepatitis, neuritis, and inflammation in the Orient.[10] Herein, we isolate six highly potent NA inhibitory flavanones and flavones from the root of this plant all of which share a resorcinol as a common chemotype. This striking similarity encouraged us to investigate their SAR. To firm up our conclusions we extended the study to include a number of commercially available flavonoids, which are the parent compounds of the isolated inhibitors. Most importantly we were able to show that flavanone **1**, which possesses a hydrated prenyl group, showed the most potent NA inhibitory activity (IC_{50} = 380 nM) reported so far. We also analyzed the kinetic mechanism of these species. The latter was found to be consistent with in silico docking analysis

* Corresponding authors. Tel.: +82 63 570 5170 (W.S.L.), tel.: +82 55 751 5472; fax: +82 55 757 0178 (K.H.P.).
E-mail addresses: wslee@kribb.re.kr (W.S. Lee), khpark@gsnu.ac.kr (K.H. Park).

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that we carried out. This analysis also suggested a role for the hydrated prenyl group in binding to the enzyme.

Activity guided fractionation of the root of *C. tricuspidiata* yielded six potent NA inhibitory flavonoids (**1**–**3**, **6**–**8**). These compounds were identified as 2′,5,7-trihydroxy-4′,5′-(2,2-dimethylchromeno)-8-(3-hydroxy-3-methylbutyl) flavanone (**1**), cudraflavanone A (**2**), cudraflavanone D (**3**), cycloartocarpetin (**6**), cudraflavone B (**7**), and cudraflavone A (**8**) using spectroscopic data including 2D NMR (Fig. 1).[11] The logic behind the structural identification of compound **1** will be described as an example because this inhibitor proved to be the most potent NA inhibitor and we believe that the location of its hydrated prenyl group may play a critical role in its high activity. The presence of the latter was confirmed by an obvious HMBC correlation between H-2″ (δ_H 1.64) and C-3″ (δ_C 72.7) and C-4″ (δ_C 29.4). This group was placed at A ring C-8 because of a HMBC correlation between H-1″ (δ_H 2.61) and C-8 (δ_C 110.7).

All the isolated compounds were tested for their inhibitory activities against neuraminidase from *C. welchii*. The assay was carried out according to the supplied manual with modifications:[12] activity was assessed by measuring the concentration required to effect 50% inhibition (IC_{50}). Initial velocity (v_i) was recorded over a range of concentrations and the data were analyzed using a non-linear regression program [Sigma Plot (SPCC Inc., Chicago, IL)]. To supplement our results and to allow a basis for our SAR investigations we also performed similar analyses on a range of commercially available compounds, which were similar in structure although less elaborate than our isolated species and importantly some of these (**9–12**) lacked a resorcinol chemotype in the B ring, a common feature in the isolated species.

As shown in Figures 2 and 3A, all tested compounds (**1**–**12**) showed a dose-dependent inhibitory effect toward neuraminidase activity. The inhibition was reversible because increasing the inhibitor concentration rapidly decreased enzyme activity [line gradient decreased (Fig. 3B for compound **1** representatively)]. Importantly, compound **1** emerged to have an IC_{50} value in the high nanomolar range showing it to be an excellent inhibitor of NA. We progressed to analyze the mode of inhibition using Lineweaver–Burk and Dixon plots (Fig. 3C and D), which revealed that all isolated compounds exhibited noncompetitive inhibition because

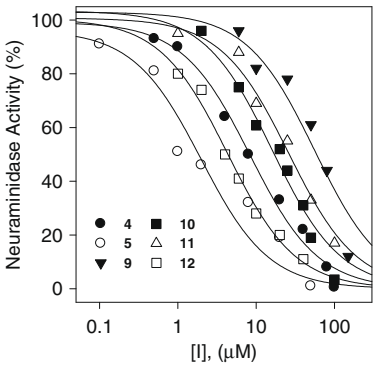

Figure 2. Effects of commercially available compounds (**4**, **5**, and **9**–**12**) on the activity of neuraminidase.

increasing concentrations of substrate resulted in a family of lines which intersected at a non-zero point on the *x*-axis (-K_i) (Fig. 3D).

From analysis of the parent compounds, it is clear that a resorcinol moiety in the B ring is very important for activity: resorcinolic compounds (**4**, **5**) showed much improved activities compared with their respective phenol (**9**, **11**) and catechol (**10**, **12**) derived counterparts (Fig. 2 and Table 1). Although this effect was more pronounced for flavones (**5**, **11**, **12**) rather than flavanones (**4**, **9**, **10**) it was none-the-less present in each group. The fact that the former group was more active than the latter is in accord with the findings of Du and co-workers.[8]

The flavanones examined showed enhanced NA inhibitory potencies when prenylated in the A and B rings. This is particularly pertinent to compound **1** which has a hydrated prenyl group on C8 and proved to be 20-fold more effective than parent compound **4**. When the three flavanones (**1**–**3**) are compared together, the chromenon moiety also appears to contribute to enhanced activity because compound **2** (IC_{50} = 1.5 μM) is almost 10-fold more effective than compound **3** (IC_{50} = 10.7 μM). On the other hand, the parent flavone **5** (IC_{50} = 1.1 μM) was more effective at neuraminidase inhibition than alkylated flavones (**6**–**8**) in Table 1.

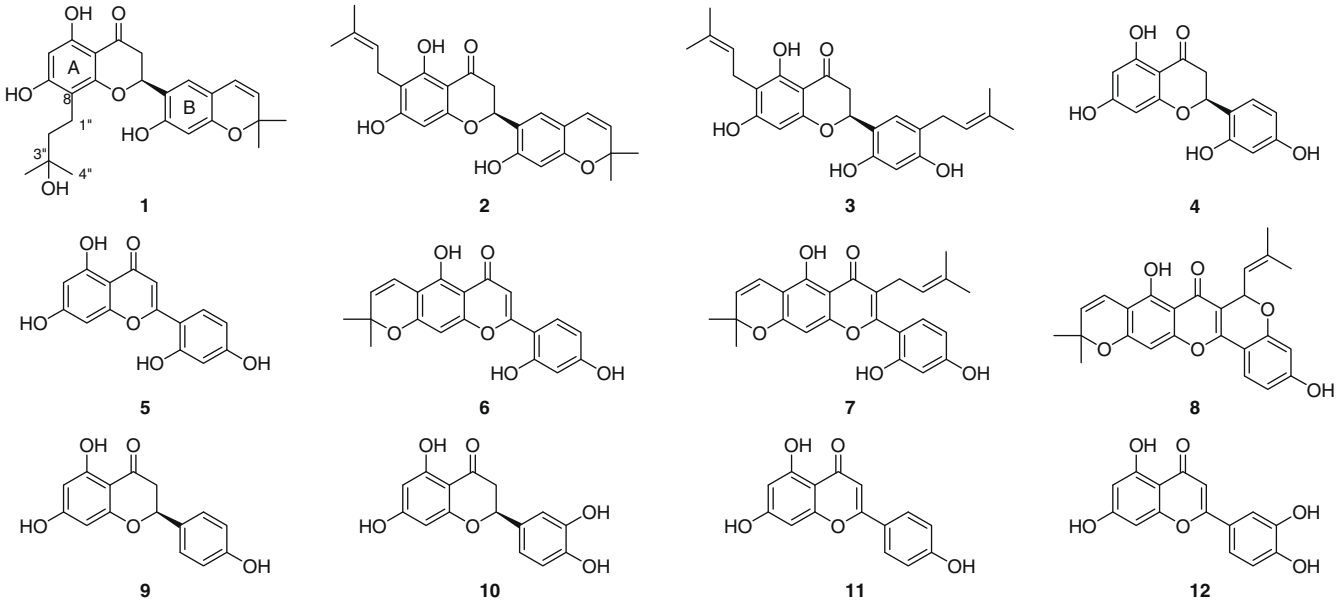

Figure 1. Chemical structures of isolated compounds (**1**–**3** and **6**–**9**) from *C. tricuspidata* and tested compounds (**4**, **5**, and **9**–**12**).[13]

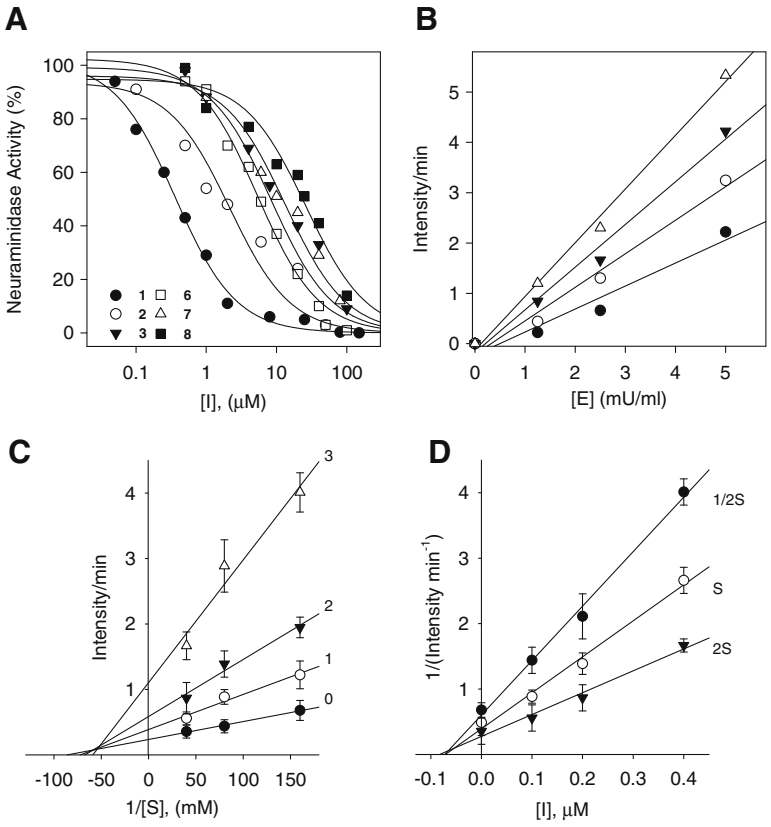

Figure 3. (A) Effects of isolated compounds (**1**–**3** and **6**–**8**) on the activity of neuraminidase. (B) Relationship between hydrolytic activity of neuraminidase and enzyme concentration at different concentrations of compound **1**. Concentrations of inhibitor for curves 0–3 were 0, 0.1, 0.4, and 1.0 μM, respectively. (C) Lineweaver–Burk plots for neuraminidase inhibition by compound **1**. Concentrations of compound **1** for curves 0–3 were 0, 0.1, 0.2, and 0.4 μM, respectively. (D) Dixon plot for compound **1** determining the inhibition constant K_i.

Table 1
Inhibitory effects of tested compounds **1**–**12** on neuraminidase activities

Compds	IC_{50}[a] (μM)	Inhibition type (K_i, μM)
1	0.38 ± 0.1	Noncompetitive (0.19 ± 0.11)
2	1.53 ± 0.8	Noncompetitive (2.01 ± 1.77)
3	10.74 ± 2.4	Noncompetitive (7.16 ± 3.86)
4	8.14 ± 3.9	Noncompetitive (7.20 ± 1.61)
5	1.13 ± 0.2	Noncompetitive (1.96 ± 0.01)
6	6.01 ± 0.2	ND[b]
7	10.14 ± 3.0	Noncompetitive (10.82 ± 0.23)
8	29.01 ± 1.9	Noncompetitive (44.37 ± 2.10)
9	57.92 ± 5.0	ND
10	15.97 ± 1.9	ND
11	22.11 ± 3.9	ND
12	4.60 ± 1.0	ND
Mangiferin[c]	11.20 ± 2.1	ND

[a] All compounds were examined in a set of duplicated experiments; IC_{50} values of compounds represent the concentration that caused 50% enzyme activity loss.
[b] Not determined.
[c] These compounds were used as a positive control.

Table 2
The calculated chemical parameters (IC_{50} values, ligscore2 values, and *C* log *P* values) of flavanones (**1**–**4**)

Compds	IC_{50} (μM)	Ligscore2[a]	*C* Log *P*[b]
1	0.38 ± 0.1	6.42	4.20
2	1.53 ± 0.8	6.10	5.87
3	10.74 ± 2.4	5.90	5.58
4	8.14 ± 3.9	5.55	1.73

[a] The ligscore2 values reflect docking stability (high values indicates high stability).
[b] The *C* log *P* values indicate molecular hydrophobic properties; the *C* log *P* were calculated by using ChemDraw Ultra version 9.0.

We proceeded to investigate interaction of neuraminidase with most potent inhibitor **1** using an in silico docking simulation. The X-ray structure of neuraminidase determined by Newstead et al.[14] was obtained from the protein data bank (PDB code: 2vk6). For the initial exploration, various sites were searched and analyzed by docking of compound **1** to the enzyme.

Our modeling calculations unveiled two potential binding sites one of which is the substrate binding site, while the other is on the opposite side of the protein (Fig. 4A). Figure 4A shows compound **1** occupying its preferred site, which is the latter (ligscore2 = 6.42). The modeling calculations unveiled H-bond interactions in the preferred site between **1** and: Arg1467, Glu1470, Lys1272, and Lys1472 (Fig. 4B). Importantly the hydroxy group within the hydrated prenyl appendage in the A ring of **1**, which our SAR studies implicated as one of the key chemotypes in this inhibitor, interacted with Lys1472. The other three flavanones (**2**–**4**) also preferred the same binding site over the active site (Fig. 4). Pleasingly the potency of inhibitors **1**–**4** correlates well with their ligand score (Table 2). The noncompetitive inhibition mode of all four flavanones (**1**–**4**) observed in the kinetic study is consistent with the inhibitors not interacting with the active site.

In conclusion, through this study we have started a process, which we hope will lead to a profound understanding of the SAR of important NA inhibitors. We have been able to isolate six flavones/flavanones, one of which, **1**, emerged to have exemplary potency.

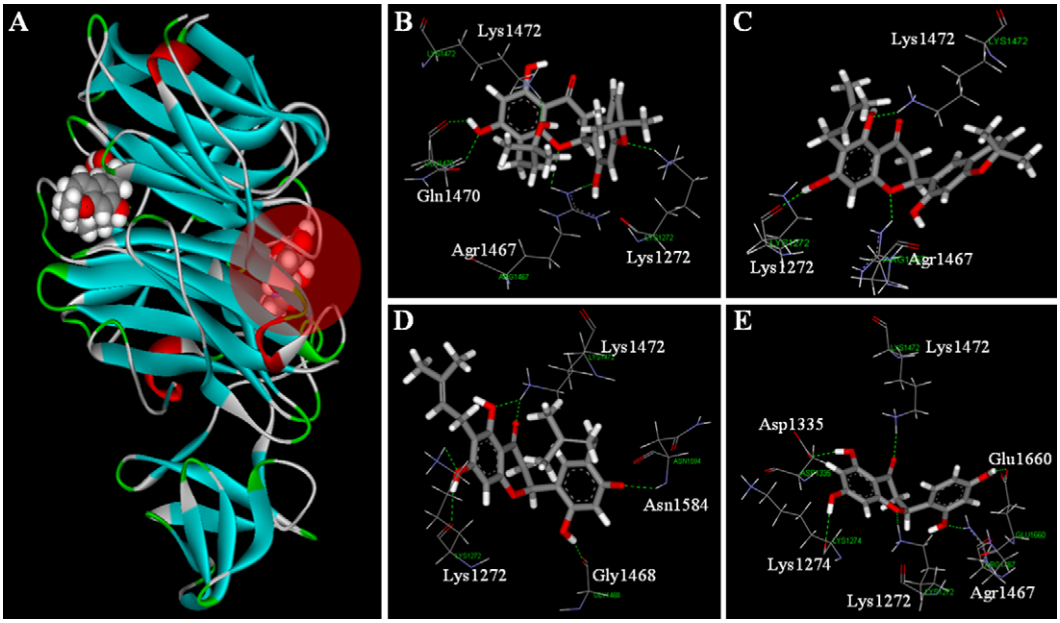

Figure 4. Comparison of in silico docking arrangements for four flavanones (**1**–**4**). (A) Ribbon plots of compound **1** complexed to neuraminidase, representatively (red cloud, original active site). (B–E) Hydrogen bond interactions between flavanones (**1**–**4**) and neuraminidase residues are presented as green dots.

By extending our study to parent compounds similar in chemical structure to the isolated species, we were able to uncover some of the key aspects of the interaction of these compounds with the enzyme. This emerged to be a resorcinol moiety in the B ring, and additional H-bonding elements in the A ring (hydrated prenyl group). Our results were backed up by molecular docking analysis, which in turn produced a result entirely consistent with our kinetic analysis.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.07.098.

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