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Inhibitory effects of flavonoids on phosphodiesterase isozymes from guinea pig and their structure-activity relationships

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Abbreviations: PDE, phosphodiesterase; cAMP, adenosine 3',5' cyclic monophosphate; cGMP, guanosine 3',5' cyclic monophosphate; EGTA, ethylene glycol-bis(β-aminoethyl ether),*N*,*N*',*N*'-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; PMSF, phenylmethanesulfonyl fluoride; EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone.

Abstract

The structure-activity relationships of flavonoids with regard to their inhibitory effects on phosphodiesterase (PDE) isozymes are little known. The activities of PDE1~5 were measured by a two-step procedure using cAMP with [^3H]-cAMP or cGMP with [^3H]-cGMP as substrates. In the present results, PDE1, 5, 2, and 4 isozymes were partially purified from guinea pig lungs in that order, and PDE3 was from the heart. The IC_{50} values of PDE 1~5 were greater than those reported previously for the reference drugs, vinpocetin, EHNA, milrinone, Ro 20-1724, and zaprinast, by 5-, 5-, 7-, 5-, and 3-fold, respectively. As shown in Table 2, luteolin revealed non-selective inhibition of PDE1~5 with IC_{50} values in a range of 10~20 μM, as did genistein except with a low potency on PDE5. Daidzein, an inactive analogue of genistein in tyrosine kinase inhibition, showed selective inhibition of PDE3 with an IC_{50} value of around 30 μM, as did eriodictyol with an IC_{50} value of around 50 μM. Hesperetin and prunetin exhibited more-selective inhibition of PDE4 with IC_{50} values of around 30 and 60 μM, respectively. Luteolin-7-glucoside exhibited dual inhibition of PDE2/PDE4 with an IC_{50} value of around 40 μM. Diosmetin more-selectively inhibited PDE2 (IC_{50} of 4.8 μM) than PDE1, PDE4, or PDE5. However, biochanin A more-selectively inhibited PDE4 (IC_{50} of 8.5 μM) than PDE1 or PDE2. Apigenin inhibited PDE1~3 with IC_{50} values of around 10~25 μM. Myricetin inhibited PDE1~4 with IC_{50} values of around 10~40 μM. The same was true for quercetin, but we rather consider that it more-selectively inhibited PDE3 and PDE4 (IC_{50} of < 10 μM). In conclusion, it is possible to synthesize useful drugs through elucidating the structure-activity relationships of flavonoids with respect to inhibition of

PDE isozymes at concentrations used in this *in vitro* study.

Keywords: Flavonoids; guinea pig; lung; heart; phosphodiesterase isozymes; structure-activity relationships

Introduction

Phosphodiesterases (PDEs) have been classified according to their primary protein and cDNA sequences, co-factor and substrate specificities, and pharmacological roles. It is now known that PDEs comprise at least 12 distinct enzyme families that hydrolyze cAMP and/or cGMP [1]. PDE1~5 isozymes, which are calcium/calmodulin-dependent (PDE1), cGMP-stimulated (PDE2), cGMP-inhibited (PDE3), cAMP-specific (PDE4), and cGMP-specific (PDE5), have been found to be present in canine trachea [2], guinea pig lung [3], and human bronchi [4]. In the guinea pig airway, PDE3 and 4 have been identified [5], but other isozymes might also be present. PDE3, 4, or dual 3/4 inhibitors were reported to have potential in the treatment of asthma [6].

Flavonoids are naturally occurring polyphenolic compounds with a wide distribution in the plant kingdom. It is thought that the average Westerner consumes approximately 1 g of flavonoids per day in the diet [7]. These plant flavonoids are reported to have therapeutic potentials because of their scavenging, antioxidant [8], anti-inflammatory [9], anticancer [10], antimutagenic [11], and antispasmodic properties [12]. Also, flavonoids have been reported to inhibit xanthine oxidase [13], protein kinase C [14], and PDE [15]. However, little is known about the structure-activity relationships between flavonoids and their inhibitory effects on PDE isozymes. In the present study, we attempted to determine these relationships, which may be helpful for understanding the active moieties of these isozymes.

2. Materials and Methods

2.1. Reagents and drugs

Apigenin, chrysin, quercetin, myricetin, hesperetin, daidzein, biochanin A, genistein, Bistris base, Trizma base, d,l-dithiothreitol, benzamidine, zaprinase, EDTA, EGTA, PMSF, BSA, cyclic AMP, cyclic GMP, calmodulin, Dowex resin, DMSO, *Crotalus atrox* snake venom, etc. were purchased from Sigma Chemical. Luteolin, luteolin-7-glucoside, and diosmetin were purchased from Indofine Chemical. Eriodictyol and prunetin were purchased from Fluka Chemie. Vinpocetin, EHNA, Ro 20-1724, and milrinone were purchased from Biomol. [^3H]cAMP, [^3H]cGMP, Q-sepharose, and calmodulin-agarose were purchased from Amersham Pharmacia Biotech. Ethyleneglycol was purchased from Merck. Other reagents, such as $CaCl_2$, $MgCl_2$, and NaCl, were of analytical grade.

Vinpocetin, EHNA, Ro 20-1724, and PMSF were dissolved in ethyl alcohol. Milrinone and zaprinast were dissolved in DMSO. EGTA was dissolved in 3 N NaOH. All flavonoids were dissolved in a mixture of DMSO and ethyl alcohol (1: 1). Other drugs were dissolved in distilled water. The final concentration of ethyl alcohol or DMSO was less 0.1% and did not significantly affect the activities of the PDE isozymes.

2.2. Separation of cyclic nucleotide PDE isozymes

Under a protocol approved by the Animal Care and Use Committee of Taipei Medical University, five male guinea pigs (Hartley), weighing 500~600 g, were sacrificed. According to the method described by Ko et al. [16], their lungs (15 g) or hearts (4 g)

were chopped into small pieces and homogenized with a glass/teflon homogenizer (Glas-Col) in 10 volumes of cold medium (pH 7.5) containing 20 mM Tris-HCl, 2 mM benzamidine, 2 mM EDTA, 50 mM sodium chloride, 0.1 mM PMSF, and 1 mM dithiothreitol. At 4 ¢J, the homogenate was centrifuged at 170 *g* for 5 min, and the supernatant was then re-centrifuged at 40,000 *g* for 30 min. The final supernatant fraction was filtered through 0.22-μm filters and applied to a Q-sepharose fast flow column (2.2 × 28 cm) pre-equilibrated in homogenization buffer. The column was washed with two bed volumes of homogenate buffer to remove unbound material. Proteins bound to the Q-sepharose beads were eluted with various concentrations (0.23, 0.34, 0.44, 0.69, and 1.00 M) of NaCl in homogenate buffer (40 mL each concentration) at a flow rate of 30 mL/h. Fractions (3 mL) were collected, ethylene glycol was added to a final concentration of 30% (v/v), and then the samples were frozen at –70 ¢J. Under these conditions, the enzyme activity was stable for at least 3 months. In order to eliminate possible contamination of PDE5 by PDE1, the second peak of activity was further purified on a calmodulin-agarose column. The column (1.6 × 4 cm) was pre-equilibrated by a buffer containing 20 mM Bistris, 1 mM dithiothreitol, 2 mM benzamidine, 50 mM NaCl, 3 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.1 mM PMSF, pH 6.5 (buffer A). The sample, with a concentration of $CaCl_2$ adjusted to 2 mM, was loaded and allowed to be absorbed on the gel for 30 min. The PDE isoezymes were eluted by stepwise elution using 20 mL of buffer A followed by 20 mL of buffer A with 1 M NaCl and 20 mL of buffer A with 1 M NaCl plus 1 mM EGTA. PDE5 was collected first followed by PDE1.

2.3. Cyclic nucleotide PDE assay

The activities of PDE1~5 in the homogenate were measured with a two-step procedure according to the method of Thompson and Appleman [17], using cAMP with [^3H]-cAMP or cGMP with [^3H]-cGMP as substrates. The enzyme preparation (25 μL) was incubated for 30 min at 37 ¢J in a total assay volume of 100 μL containing 50 mM Tris/HCl (pH 7.4), 3 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% BSA, 1 μM cAMP with 0.2 μCi [^3H]-cAMP as a substrate alone or in the presence of 0.1 unit calmodulin with 10 μM $CaCl_2$ or 5 μM cGMP, and 1 μM cGMP with 0.2 μCi [^3H]-cGMP as another substrate alone or in the presence of 0.1 unit calmodulin with 10 μM $CaCl_2$. In tests of enzyme inhibition, the reaction mixture contained 10 μL of vehicle or inhibitors, at various concentrations of flavonoids or selective PDE1~5 inhibitors, such as vinpocetin [18], EHNA [19], milrinone [20], Ro 20-1724 [21], and zaprinast [22], as reference drugs, respectively. The reagents and homogenate were mixed on ice, and the reaction was initiated by transferring the mixture to a water bath at 37 ¢J. Following a 30-min incubation, the reaction was stopped by transferring the reaction vessel to a bath of boiling water for 3 min. After cooling on ice, 20 μL of a 1 mg/mL solution of *Crotalus atrox* venom was added to the reaction mixture, and the mixture was incubated at 37 ^oC for 10 min. Unreacted [^3H]-cAMP or [^3H]-cGMP was removed by the addition of 500 μL of a 1-in-1 Tris-HCl (40 mM) buffer suspension of Dowex resin (1 × 8─200) with incubation on ice for 30 min. Each tube was then centrifuged for 2 min at 6000 rpm, and 150 μL of the supernatant was removed for liquid scintillation counting. Less than 10% of the tritiated cyclic nucleotide was hydrolyzed in this assay.

2.4. Statistical analysis

Concentrations of flavonoids at which 50% of maximum activity (IC_{50} value) was produced were compared to each other. The IC_{50} values of flavonoids and various reference drugs were calculated using non-linear regression analysis by the software SigmaPlot 4.0 (Sigma). All values are shown as the mean ± SEM. Differences among these values were statistically calculated by one-way analysis of variance (ANOVA), and then determined by the least significant difference (LSD). The difference between two values, however, was determined by use of Student′s unpaired *t*-test. Differences were considered statistically significant if the *P* value was less than 0.05.

3. **Results**

3.1. Separation of PDE isozymes and their inhibition by flavonoids

As in our previous report [16], PDE subtype 1 (fraction 10~14), 5 (fraction 22~28), 2 (fraction 34~36), and 4 (fraction 42~50) isozymes were partially purified from guinea pig lungs in that order, while subtype 3 (fraction 44~46) isozyme was from the heart. The IC_{50} values of the reference drugs of vinpocetin, EHNA, milrinone, Ro 20-1724, and zaprinast for PDE 1~5 were respectively determined (Table 2). In the present results, the IC_{50} values were greater than those previously reported [18-22] by 5-, 5-, 7-, 5-, and 3-fold, respectively. Thirteen natural and synthetic flavonoids, divided into the four classes of flavones, flavonols, flavanones, and isoflavones (Table 1), were tested for their inhibitory effects on PDE 1~5; their IC_{50} values were determined and are listed in Table 2.

3.2. Inhibitory effects of flavonoids on PDE1

The IC_{50} value of diosmetin on PDE1 inhibition did not significantly differ from that of luteolin, suggesting that methylation of the C-4′ hydroxyl group of flavones does not alter the potency of PDE1 inhibition. This suggestion is supported by the fact that neither eriodictyol nor hesperetin had an effect (IC_{50} >100 μM) on PDE1 inhibition, as shown in Table 2. By comparisons of the inhibition of PDE1 between luteolin and eriodictyol, and between diosmetin and hesperetin, therefore, it was concluded that the presence of a

double bond between C-2 and C-3 may be very important for PDE1 inhibition. This conclusion has also been suggested for a guinea pig tracheal relaxant [23], and for lipid peroxidation-inhibiting [24], and NO production-inhibiting activities in LPS-activated RAW 264.7 cells [25]. However, deletion of the C-4′ hydroxyl group of apigenin to form chrysin resulted in no effect on PDE1 inhibition, suggesting that the presence of the C-4′ hydroxyl group of flavones is very important. Contrarily, the presence of the C-3′ hydroxyl group of flavones is not important, because the IC_{50} value of luteolin did not significantly differ from that of apigenin. Glycosylation of the C-7 hydroxyl group of luteolin may greatly reduce its potency, suggesting that the bulky glycosyl residues may hinder its binding to the moiety of the isozyme, and that the hydroxyl group is very important for PDE1 inhibition. The IC_{50} value of luteolin for PDE1 inhibition did not significantly differ from that of quercetin, suggesting that flavones may be similar to flavonols with regard to PDE isozyme inhibition. Also, the IC_{50} value of apigenin for PDE1 inhibition did not significantly differ from that of genistein, a specific tyrosine kinase inhibitor [27], suggesting that flavones may be similar to isoflavones in their inhibition of PDE isozymes. The C-5′ hydroxyl group of flavonols might not be important for PDE1 inhibition, because the IC_{50} value of myricetin for PDE1 inhibition did not significantly differ from that of quercetin. The IC_{50} value of genistein was significantly lower than that of biochanin A, suggesting that the hydroxyl group at position C-4′ of isoflavones is important for PDE1 inhibition. Substitution of the C-7 hydroxyl group of genistein by a methoxy group to form prunetin may have abolished the effect on PDE1 inhibition. Deletion of the C-5 hydroxyl group of genistein to form daidzein, an inactive analogue of genistein in tyrosine kinase inhibition [26], resulted in

no effect on PDE1 inhibition, suggesting that either the C-7 or C-5 hydroxyl group is very important for PDE1 inhibition.

3.3. Inhibitory effects of flavonoids on PDE2

The IC_{50} value of diosmetin on PDE2 inhibition was significantly less than that of luteolin, which suggests that methylation of the C-4′ hydroxyl group of flavones may enhance their potency for PDE2 inhibition. However, methylation occurring at the same position of flavanones does not change their potency for PDE2 inhibition, based on the fact that both eriodictyol and hesperetin had no effect (IC_{50} > 100 μM) on PDE isozyme inhibition. By comparing PDE2 inhibition between luteolin and eriodictyol, and between diosmetin and hesperetin, therefore, it was concluded that the presence of a double bond between C-2 and C-3 may be very important for PDE2 inhibition. The C-4′ hydroxyl group of flavones is also important for isozyme inhibition, because chrysin, which lacks a hydroxyl group at the position from apigenin, showed no effect on PDE2 inhibition. However, the C-3′ hydroxyl group of flavones is not important for isozyme inhibition, because the IC_{50} value of luteolin did not significantly differ from that of apigenin. The IC_{50} value of luteolin was significantly lower than that of luteolin-7-glucoside, suggesting the glycosylation of the C-7 hydroxyl group of flavones hinders their binding to the moiety of the PDE isozyme. The IC_{50} value of luteolin for PDE2 inhibition did not significantly differ from that of quercetin, suggesting that inhibition of PDE isozymes by flavones may be similar to that by flavonols. Also, the IC_{50} value of apigenin for PDE2 inhibition did not significantly differ from that of genistein, suggesting that inhibition of

PDE isozymes by flavones may be similar to that by isoflavones. The C-5′ hydroxyl group of flavonols is not important for PDE2 inhibition because the IC_{50} value of myricetin for PDE2 inhibition did not significantly differ from that of quercetin. The IC_{50} value of genistein was significantly lower than that of biochanin A, suggesting that the C-4′ hydroxyl group of isoflavones is important for PDE2 inhibition, because substitution of a hydroxyl group by a methoxy group may reduce their potency for PDE2 inhibition. Substitution of the C-7 hydroxyl group of genistein by a methoxy group to form prunetin may have abolished the effect for PDE2 inhibition. Deletion of the C-5 hydroxyl group of genistein to form daidzein resulted in no effect on PDE2 inhibition, suggesting that either the C-7 or C-5 hydroxyl group is very important for PDE2 inhibition.

3.4. Inhibitory effects of flavonoids on PDE3

The C-4′ hydroxyl group of flavones is very important for PDE3 inhibition. For example, if the hydroxyl group of luteolin is methylated to form diosmetin, or that of apigenin is deleted to form chrysin, the resultants lose their inhibitory effects on PDE isozyme activities. This conclusion was also supported by a comparison between the inhibition of PDE3 by eriodictyol and hesperetin. However, the C-3′ hydroxyl group of flavones does not seem important for PDE3 inhibition, because the IC_{50} value of luteolin did not significantly differ from that of apigenin which lacks a hydroxyl group at that position. The C-7 hydroxyl group of luteolin is very important for PDE3 inhibition, because lutolin-7-glucoside had no effect on the inhibition of PDE3 isozyme. It is possible that the bulky glycosyl residues hinder its binding to the moiety of the isozyme.

The IC_{50} value of luteolin for PDE3 inhibition did not significantly differ from that of quercetin, suggesting that inhibition of PDE3 by flavones may be similar to that by flavonols. Also, the IC_{50} value of apigenin for PDE3 inhibition did not significantly differ from that of genistein, suggesting that inhibition of PDE3 by flavones may be similar to that by isoflavones. The C-5′ hydroxyl group of flavonols is not important for PDE3 inhibition because the IC_{50} value of myricetin for PDE3 inhibition did not significantly differ from that of quercetin. Comparing the inhibition of PDE3 between diosmetin and hesperetin, and between luteolin and eriodictyol suggests that the importance of the presence of a double bond between C-2 and C-3 for isozyme inhibition is unclear. Substitution of the C-7 or C-4′ hydroxyl group of genistein with a methoxy group to respectively form prunetin or biochanin A may have abolished the effect on PDE3 inhibition. Interestingly, the IC_{50} value of PDE3 inhibition by genistein did not significantly differ from that of daidzein, which lacks the C-5 hydroxyl group and has been reported to be inactive in tyrosine kinase inhibition [26], suggesting that the hydroxyl group is not important for the inhibition of PDE3.

3.5. Inhibitory effects of flavonoids on PDE4

The IC_{50} value of luteolin on PDE4 activity did not differ from that of diosmetin. Substitution of the C-4′ hydroxyl group of eriodictyol with a methoxy group to form hesperetin even enhanced the potency of PDE4 inhibition. Moreover, deletion of the C-4′ hydroxyl group of apigenin to form chrysin resulted in no change in PDE4 inhibition. These results suggest that the C-4′ hydroxyl group of flavones or flavanones is not

important. In contrast, the C-3′ hydroxyl group of flavones is very important because luteolin was more potent than apigenin for PDE4 inhibition. The C-7 hydroxyl group of luteolin is important for PDE4 inhibition, because the IC_{50} value of lutolin-7-glucoside was significantly greater than that of luteolin. It is possible that the bulky glycosyl residues at that position may hinder its binding to the moiety of the isozyme. However, luteolin was less potent than quercetin, and apigenin was less potent than genistein for PDE4 inhibition, suggesting that flavonols and isoflavones are more potent than flavones for isozyme inhibition. In flavonols, the IC_{50} value of myricetin for PDE4 inhibition was significantly greater than that of quercetin, suggesting that the C-5′ hydroxyl group may hinder quercetin's binding to the moiety of the isozyme. The IC_{50} value of diosmetin for PDE4 inhibition was significantly greater than that of hesperetin, suggesting that the presence of a double bond between C-2 and C-3 is important for isozyme inhibition. This conclusion was strongly supported by a comparison between the inhibitory effects of luteolin and eriodictyol, which almost had no effect (IC_{50} > 100 μM) on PDE4 inhibition. The IC_{50} value of genistein did not significantly differ from that of biochanin A, suggesting that the hydroxyl group at position C-4′ of isoflavones is not important for PDE4 inhibition. However, the IC_{50} value of prunetin was significantly greater than that of genistein, suggesting that the hydroxyl group at position C-7 of isoflavones may be important for PDE4 inhibition. Deletion of the C-5 hydroxyl group of genistein to form daidzein resulted in no effect on PDE4 inhibition, suggesting that the hydroxyl group is very important for PDE4 inhibition.

3.6. Inhibitory effects of flavonoids on PDE5

Among flavones, only luteolin and diosmetin inhibited PDE5 activities with IC_{50} values of 19.3 and 15.3 μM, respectively, which did not significantly differ from each other. This suggests that the C-4′ hydroxyl group is not important for PDE5 inhibition, because substitution of the hydroxyl group with a methoxy group did not alter its inhibitory effect. This conclusion was supported by the present results that the potency of eriodictyol did not differ from that of hesperetin and neither showed any effect on PDE5 inhibition. Also, deletion of the C-4′ hydroxyl group of apigenin to form chrysin resulted in no change of PDE5 inhibition. In contrast, the C-3′ hydroxyl group of luteolin is very important for PDE5 inhibition, because deletion of the hydroxyl group of luteolin to form apigenin resulted in no effect on PDE5 inhibition. However, adding a C-5′ hydroxyl group to quercetin resulted in no change in PDE5 inhibition, suggesting that the hydroxyl group is not important. The C-7 hydroxyl group of luteolin is also very important for PDE5 inhibition, because lutolin-7-glucoside showed no inhibition of this isozyme. It is possible that the bulky glycosyl residues may hinder its binding to the moiety of the isozyme. Comparing the inhibition of PDE5 between quercetin and luteolin suggests that the C-3 hydroxyl group of flavonols may hinder the binding of flavones to the moiety of the isozyme. Luteolin was more potent than eriodictyol, and diosmetin was more potent than hesperetin for PDE5 inhibition, suggesting that the presence of a double bond between C-2 and C-3 is important for PDE5 inhibition. Comparing the inhibition of PDE5 between apigenin and genistein suggests that it may be easier for isoflavones than flavones to bind to the moiety of this isozyme. Although genistein only had low potency of PDE5 inhibition among these PDE isozyme subtypes 1~5, its IC_{50} value was

significantly lower than that of biochanin A or prunetin, suggesting that the hydroxyl group at position C-4′ or C-7 of genistein is relatively important for PDE5 inhibition. Deletion of the C-5 hydroxyl group of genistein to form daidzein resulted in no effect on PDE5 inhibition, suggesting that the hydroxyl group is important for PDE5 inhibition.

4. Discussion

As shown in Table 2, luteolin effectively inhibited PDE1~5. Replacement of the C-4′ hydroxyl group of luteolin with a methoxy group, as in diosmetin, enhanced the potency on PDE2 inhibition, but abolished the inhibitory effect of PDE3 activity. Deletion of the C-3′ hydroxyl group of luteolin to form apigenin caused the loss of the inhibitory effects on PDE4 and PDE5 activities. Moreover, deletion of the C-3′ and C-4′ hydroxyl groups of luteolin to form chrysin caused the loss of all inhibitory effects on the activities of PDE1~5. Glycosylation of the C-7 hydroxyl group of luteolin reduced the potency of inhibiting the activities of PDE2 and PDE4, and furthermore abolished the inhibitory effects on the activities of PDE1, PDE3, and PDE5. Replacement of the C-3 hydrogen of luteolin with a hydroxyl group to form quercetin enhanced the potency of PDE4 inhibition, but caused the loss of inhibitory effect on PDE5 activity. Replacement of the C-5′ hydrogen of quercetin with a hydroxyl group to form myricetin reduced the potency of PDE4 inhibition, but had no effect on the inhibition of PDE5. Saturation of the double bond between C-2 and C-3 of luteolin to form eriodictyol greatly reduced the potency of PDE3 inhibition and abolished the inhibitory effects on all other isozyme activities. Similarly, saturation of the double bond between C-2 and C-3 of diosmetin to form

hesperetin reduced the potency of PDE4 inhibition and abolished the inhibitory effects on all other isozyme activities. Isoflavones, which shift the B-ring from C-2 to C-3 of flavones, generally produced little or no inhibition of PDE5 activity. For example, genistein had only weak potency for PDE5 inhibition. However, genistein had considerable inhibitory effects of other PDE isozyme activities. In particular, it highly effectively inhibited the activities of PDE2 and PDE4. Daidzein, an inactive analogue of genistein in tyrosine kinase inhibition [26], selectively inhibited PDE3 activity, although it has been reported to inhibit both PDE1 and PDE3 activities [27]. The reason for this contradiction is not clear, but may have resulted from the different sources of PDE1 used. We separated PDE1 from guinea pig lung, while it came from bovine heart in the other study. Our result for PDE3 inhibition was consistent with what they reported. It is possible that the source of PDE3 was from the same organ (heart), although we separated ours from guinea pig, while theirs was from a bovid. Biochanin A effectively inhibited PDE4 activity and moderately inhibited PDE1 activity, although it has been reported to have no effect (IC_{50} > 100 μM) on PDE1 activity [27]. This contradiction may also have resulted from the same reason, different sources of PDE1. Prunetin moderately but selectively inhibited PDE4 activity in our present results, although it has been reported to have no effect (IC_{50} > 100 μM) on PDE4 inhibition [27].

Recently, increasing evidence suggests that the more-selective inhibition of these PDEs there is, the more useful these compounds will be in the clinic. For eaxample, selective PDE5 inhibitors, such as sildenafil, tadalafil, and vardenafil, are successfully being used in the treatment of erectile dysfunction [28]. Unfortunately none of the tested flavonoids was a selective inhibitor of the PDE5 isozyme. Selective PDE3 inhibitors, such as

milrinone, vesnarinone, and enoximone, are believed to have a positive effect in the treatment of chronic congestive heart failure. Although morbidity and mortality of patients with severe chronic heart failure increased after long-term oral administration of milrinone, short-term clinical use of this compound has been approved for the treatment of patients with acute decompensated heart failure [28]. Daidzein, an inactive analogue of genistein in tyrosine kinase inhibition [26], and eriodictyol revealed selective inhibition of PDE3, in the present results (Table 2). PDE4 is the most important isozymes in airway smooth muscles, pulmonary nerves, and almost all pro-inflammatory and immune cells relevant to the pathogenesis of asthma [29]. Molecular studies have provided evidence that at least 4 human genes encode PDE4 isozymes, and therefore there are at least 4 human PDE4 subtypes, PDE4A, PDE4B, PDE4C, and PDE4D [30]. Rolipram, an archetypal inhibitor of PDE4, has been reported to have potential in the treatment of asthma, although nausea and vomiting were found in clinical trials [29]. It seems that the anti-inflammatory and bronchodilating effects of rolipram are due to its inhibition of PDE4D and the adverse effects are due to PDE4C [29]. Alternatively, rolipram has high and low affinities for $PDE4_H$ and $PDE4_L$, respectively. In general, it is believed that the inhibitions of $PDE4_H$ and $PDE4_L$ are associated with an adverse response and with anti-inflammatory and bronchodilating effects, respectively [29]. Therefore, this has provided a rational basis for designing new compounds with high $PDE4_H/PDE4_L$ ratios. Indeed, some compounds, such as CDP-840, SB-207499, and piclamilast, with higher $PDE4_H/PDE4_L$ ratios compared to rolipram are under investigation and being used in clinical trials [29]. The present results reveal that hesperetin and prunetin more-selectively inhibited PDE4 (Table 2). Determing whether hesperetin or prunetin has

high $PDE4_H/PDE4_L$ ratios requires further evaluation. It has been proposed that compounds that inhibit both PDE3 and PDE4 would be less likely to produce adverse effects than would selective PDE4 inhibitors, since the effectiveness could be expected at lower doses [29]. However, zardaverine, a dual PDE3/PDE4 inhibitor, demonstrated modest bronchodilator activity in patients with asthma when given by inhalation, but was inactive in a group of patients with chronic obstructive pulmonary disease [29]. Although cardiovascular complications of a PDE3 inhibitor may preclude the development of this hybrid inhibitors for asthma, the knowledge that the effect of PDE3 in cardiac muscle (PDE3A) differs from that of the isoform (PDE3B) expressed by pro-inflammatory cells, such as T lymphocytes, provides an opportunity to synthesize compounds with reduced activity against PDE3A [29]. Theoretically, an inhibitor with more-selective action against PDE3B can be synthesized, because a new cardiotonic agent, vesnarinone, which is 10-fold more potent against PDE3A than PDE3B, has been synthesized [31]. In the present results, none of the tested flavonoids revealed this hybrid inhibitory property. In addition to its anti-asthma property, it has been reported that the inhibition of PDE3B enhances PDE4 inhibitor-induced apoptosis in a subset of patients with chronic lymphocytic leukemia [32]. Exisulind, a dual PDE2/PDE5 inhibitor [33], has been reported to induce apoptosis in several cancer cell lines [34-36]. Rolipram, a selective PDE4 inhibitor [37], promotes apoptosis in HL60 promyelocytic leukemic cells [38], malignant murine carcinoma cells [39], and chronic lymphocytic leukemia [40, 41]. Genistein has also been reported to inhibit the growth of human tumor cells by inhibition of proteasome activity [42]. In our present results, genistein very potently inhibited PDE2 (IC_{50} of 1.7 μM) and PDE4 (IC_{50} of 9.5 μM). Interestingly, flavopiridol, a semisynthetic

flavone, is currently in clinical trials for the treatment of different cancers [43-45]. The mechanisms of the anticancer property of flavopiridol have been reported to be its inhibition of cyclin-dependent kinases [46]. However, determining whether it selectively inhibits PDE isozymes requires further investigation.

In conclusion, it is possible to synthesize useful drugs through the elucidation of structure-activity relationships of flavonoids on inhibition of PDE isozymes at concentrations (1~100 μM) used in this study, although these concentrations are not easily reached under normal physiological conditions. The presence of a double bond between C-2 and C-3 of these flavonoids is very important for inhibition of PDE1~5 except PDE3, for which the situation remains unclear. Also, the C-5 and C-7 hydroxyl groups are important to very important for the inhibition of PDE1~5, except for the C-5 hydroxyl group for PDE3 inhibition. The C-3′ hydroxyl group is very important for the inhibition of PDE4 and PDE5, but is not important for the inhibition of PDE1~3. The C-3 hydroxyl group is not important for the inhibition of PDE1~5, except it is important for PDE5 inhibition. Isoflavones, with a C-3 B ring shifted from flavones, have greatly increased potency of PDE4 inhibition, but only slightly increased PDE5 inhibition. The C-5′ hydroxyl group is not important for the inhibition of PDE1~5, and even decreased the potency of PDE4 inhibition. The C-4′ hydroxyl group of flavones is very important for the inhibition of PDE3, but not for the inhibition of PDE1, PDE2, PDE4, or PDE5. The C-4′ hydroxyl group of isoflavones is very important for the inhibition of PDE3, important for that of PDE1, PDE2, and PDE5, but not important for that of PDE4. The C-4′ hydroxyl group of flavanones is important for the inhibition of PDE3, but not important for that of PDE1, PDE2, PDE4, or PDE5.

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References

[1] Lee ME, Markowitz J, Lee JO, Lee H. Crystal structure of phosphodiesterase 4D and inhibitor complex. FEBS Lett 2002;530:53-8.

[2] Torphy TJ, Cieslinski LB. Characterization and selective inhibition of cyclic nucleotide phosphodiesterase isozymes in canine tracheal smooth muscle. Mol Pharmacol 1990;37:206-14.

[3] Kapui Z, Schaeffer P, Mikus EG, Boronkay E, Gyürky J, Herbert JM, Pascal M. Experimental studies on guanosine 3',5'-cyclic monophosphate levels and airway responsiveness of the novel phosphodiesterase type 5 inhibitor SR 265579 in guinea-pigs. Drug Res 1999;49:685-93.

[4] De Boer J, Philpott AJ, van Amsterdam RG, Shahid M, Zaagsma J, Nicholson CD. Human bronchial cyclic nucleotide phosphodiesterase isoenzymes: biochemical and pharmacological analysis using selective inhibitors. Br J Pharmacol 1992;106:1028-34.

[5] Silver PJ, Hamel LT, Perrone MH, Bentley RG, Bushover CR, Evans DB. Differential pharmacologic sensitivity of cyclic nucleotide phosphodiesterase isozymes isolated from cardiac muscle, arterial and airway smooth muscle. Eur J Pharmacol

1988;150:85-94.

[6] Underwood DC, Kotzer CJ, Bochnowicz S, Osborn RR, Luttmann MA, Hay DWP, Torphy TJ. Comparison of phosphodiesterase III, IV and dual III/IV inhibitors on bronchospasm and pulmonary eosinophil influx in guinea pigs. J Pharmacol Exp Ther 1994;270:250-9.

[7] Kuhnau J. The flavonoids: a class of semi-essential food components: their role in human nutrition. World Rev Nut Diet 1976; 24:117-91.

[8] Chen Y, Zheng R, Jia Z, Ju Y. Flavonoids as superoxide scavengers and antioxidants. Free Radic Biol Med 1990;9:19-21.

[9] Ferrandiz ML, Alcaraz MJ. Anti-inflammatory activity and inhibition of arachidonic acid metabolism by flavonoids. Agent Act 1991;32:283-8.

[10] Menon LG, Kuttan R, Kuttan G. Inhibition of lung metastasis in mice induced by B16F10 melanoma cells by polyphenolic compounds. Cancer Lett 1995;95:221-5.

[11] Edenhardder R, von Petersdorff I, Rauscher R. Antimutagenic effects of flavonoids, chalcones and structurally related compounds on the activity of 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and heterocyclic amine mutagens from cooked food. Mut Res 1993;287:261-74.

[12] Duarte J, Vizcaíno FP, Utrilla P, Jiménez J, Tamargo J, Zarzuelo A. Vasodilatory effects of flavonoids in rat aortic smooth muscle. Structure-activity relationships. Gen Pharmacol 1993;24:857-62.

[13] Hayashi T, Sawa K, Kawasaki M, Arisawa M, Shimizu M, Morita N. Inhibition of cow's milk xanthine oxidase by flavonoids. J Nat Prod 1988;51:345-8.

[14] Ferriola PC, Cody V, Middleton Jr E. Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure-activity relationships. Biochem Pharmacol

1989;38:1617-24.

[15] Kuppusamy UR, Das NP. Effects of flavonoids on cyclic AMP phosphodiesterase and lipid mobilization in rat adipocytes. Biochem Pharmacol 1992;44:1307-15.

[16] Ko WC, Chen MC, Wang SH, Lai YH, Chen JH, Lin CN. 3-*O*-Methylquercetin more selectively inhibits phosphodiesterase subtype 3. Planta Med 2003;69:310-5.

[17] Thompson WJ, Appleman MM. Multiple cyclic nucleotide phosphodiesterase activities from rat brain. Biochemistry 1971;10:311-6.

[18] Ahn HS, Crim W, Romano M, Sybertz E, Pitts B. Effects of selective inhibitors on cyclic nucleotide phosphodiesterases of rabbit aorta. Biochem Pharmacol 1989;38:3331-9.

[19] Mery PF, Pavoine C, Pecker F, Fischmeister R. *Erythro*-9-(2-hydroxy-3-nonyl)adenine inhibits cyclic GMP-stimulated phosphodiesterase in isolated cardiac myocytes. Mol Pharmacol 1995;48:121-30.

[20] Harrison SA, Reifsnyder DH, Gallis B, Cadd GG, Beavo JA. Isolation and characterization of bovine cardiac muscle cGMP-inhibited phosphodiesterase: a receptor for new cardiotonic drugs. Mol Pharmacol 1986;29:506-14.

[21] Sheppard H, Wiggan G, Tsien WH. Structure-activity relationships for inhibitors of phosphodiesterase from erythrocytes and other tissues. Adv Cyclic Nucleotide Res 1972;1:103-12.

[22] Gillespie PG, Beavo JA. Inhibition and stimulation of photoreceptor phosphodiesterases by dipyridamole and M&B 22,948. Mol Pharmacol 1989;36:773-81.

[23] Ko WC, Liu PY, Chen JL, Leu IJ, Shih CM. Relaxant effects of flavonoids in

isolated guinea pig trachea and their structure-activity relationships. Planta Med 2003;69:1086-90.

[24] Cos P, Calomme M, Sindambiwe JB, Bruyne TD, Cimanga K, Pieters L, Vlietinck AJ, Berghe DV. Cytotoxicity and lipid peroxidation-inhibiting activity of flavonoids. Planta Med 2001;67:515-9.

[25] Kim HK, Cheon BS, Kim YH, Kim SY, Kim HP. Effects of naturally occurring flavonoids on nitric oxide production in the macrophage cell line RAW 264.7 and their structure-activity relationships. Biochem Pharmacol 1999;58:759-65.

[26] Akiyama T, Ishida J, Nakagawa S, Ogawara H, Watanabe SI, Itoh N, Shibuya M, Fukami Y. Genistein a specific inhibitor of tyrosine-specific protein kinase. J Biol Chem 1987;262:5592-5.

[27] Nichols MR, Morimoto BH. Differential inhibition of multiple cAMP phosphodiesterase isozymes by isoflavones and tyrphostins. Mol Pharmacol 2000;57:738-45.

[28] Rybalkin SD, Yan C, Bornfeldt KE, Beavo JA. Cyclic GMP phosphodiesterases and regulation of smooth muscle function. Circ Res 2003;93:280-91.

[29] Giembycz MA. Phosphodiesterase 4 inhibitors and the treatment of asthma. Where are we now and where do we go from here? Drug 2000;59:193-212.

[30] Torphy TJ. Phosphodiesterase isozymes, molecular targets for novel antiasthma agents. Am J Respir Crit Care Med 1998;157;351-70.

[31] Masuoka H, Ito M, Sugioka M, Kozeki H, Konishi T, Tanaka T, Nakano T. Two isoforms of cGMP-inhibited cyclic nucleotide phosphodiesterases in human tissues distinguished by their responses to vesnarinone, a new cardiotonic agent. Biochem

Biophys Res Commun 1993;190:412-7.

[32] Moon E, Lee R, Near R, Weintraub L, Wolda S, Lerner A. Inhibition of PDE3B augments PDE4 inhibitor-induced apoptosis in a subset of patients with chronic lymphocytic leukemia. Clin Cancer Res 2002;8:589-95.

[33] Thompson WJ, Piazza GA, Li H, Liu L, Fetter J, Zhu B, Sperl G, Ahnen D, Pamukcu R. Exisulind induction of apoptosis involves guanosine 3',5'-cyclic monophosphate phosphodiesterase inhibition, protein kinase G activation, and attenuated beta-catenin. Cancer Res 2000;60:3338-42.

[34] Thompson HJ, Jiang C, Lu J, Mehta RG, Piazza GA, Paranka NS, Pamukcu R, Ahnen DJ. Sulfone metabolite of sulindac inhibits mammary carcinogenesis. Cancer Res 1997;57:267-71.

[35] Lim JT, Piazza GA, Han EK, Delohery TM, Li H, Finn TS, Buttyan R, Yamamoto H, Sperl GJ, Brendel K, Gross PH, Pamukcu R, Weinstein IB. Sulindac derivatives inhibit growth and induce apoptosis in human prostate cancer cell lines. Biochem Pharmacol 1999;58:1097-107.

[36] Li H, Liu L, David ML, Whitehead CM, Chen M, Fetter JR, Sperl GJ, Pamukcu R, Thompson WJ. Pro-apoptotic actions of exisulind and CP461 in SW480 colon tumor cells involve beta-catenin and cyclin D1 down-regulation. Biochem Pharmacol 2002;64:1325-36.

[37] Schneider HH, Schmiechen R, Brezinski M, Seidler J. Stereospecific binding of the antidepressant rolipram to brain protein structures. Eur J Pharmacol 1986;127:105-15.

[38] Zhu WH, Majluf-Cruz A, Omburo GA. Cyclic AMP-specific phosphodiesterase inhibitor rolipram and Ro 20-1724 promoted apoptosis in HL60 promyelocytic

leukemic cells via cyclic AMP-independent mechanism. Life Sci 1998;63:265-74.

[39] Marko D, Romanakis K, Zankl H, Furstenberger G, Steinbauer B, Eisenbrand G. Induction of apoptosis by an inhibitor of cAMP-specific PDE in malignant murine carcinoma cells overexpressing PDE activity in comparison to their nonmalignant counterparts. Cell Biochem Biophys 1998;28:75-101.

[40] Kim DH, Lerner A. Type 4 cyclic adenosine monophosphate phosphodiesterase as a therapeutic target in chronic lymphocytic leukemia. Blood 1998;92:2484-94.

[41] Siegmund B, Welsch J, Loher F, Meinhardt G, Emmerich B, Endres S, Eigler A. Phosphodiesterase type 4 inhibitor suppresses expression of anti-apoptotic members of the Bcl-2 family in B-CLL cells and induces caspase-dependent apoptosis. Leukemia 2001;15:1564-71.

[42] Kazi A, Daniel KG, Smith DM, Kumar NB, Dou QP. Inhibition of the proteasome activity, a novel mechanism associated with the tumor cell apoptosis-inducing ability of genistein. Biochem Pharmacol 2003;66:965-76.

[43] Karp JE, Ross DD, Yang W, Tidwell ML, Wei Y, Greer J, Mann DL, Nakanishi T, Wright JJ, Colevas AD. Timed sequential therapy of acute leukemia with flavopiridol: in vitro model for a phase I clinical trial. Clin Cancer Res 2003;9:307-15.

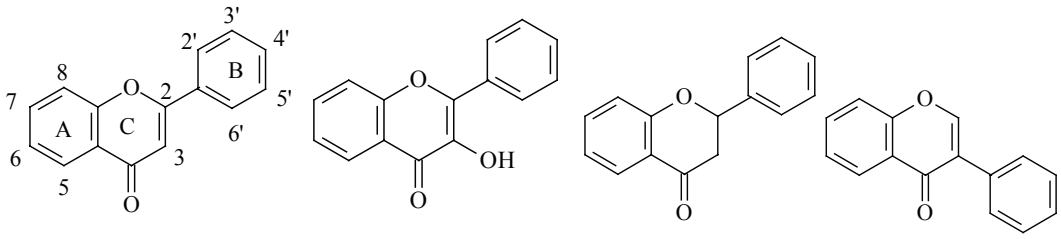
[44] Aklilu M, Kindler HL, Donehower RC, Mani S, Vokes EE. Phase II study of flavopiridol in patients with advanced colorectal cancer. Ann Oncol 2003;14:1270-3.

[45] Senderowicz AM, Sausville EA. Preclinical and clinical development of cyclin-dependent kinase modulators. J Natl Cancer Inst 2000;92:376-87.

[46] Senderowicz AM. Small-molecule cyclin-dependent kinase modulators. Oncogene 2003;22:6609-20.

Table 1

Structures of flavonoids investigated for the inhibition of the activities of PDE 1~5



Flavones Flavonols Flavanones Isoflavones

Class	Name	Substitution				
		5	7	3’	4’	5’
Flavones	Luteolin	OH	OH	OH	OH	
	Luteolin-7-glucoside	OH	O-glu	OH	OH	
	Diosmetin	OH	OH	OH	OCH_3	
	Apigenin	OH	OH		OH	
	Chrysin	OH	OH			
Flavonols	Quercetin	OH	OH	OH	OH	
	Myricetin	OH	OH	OH	OH	OH
Flavanones	Eriodictyol	OH	OH	OH	OH	
	Hesperetin	OH	OH	OH	OCH_3	
Isoflavones	Genistein	OH	OH		OH	
	Daidzein		OH		OH	
	Biochanin A	OH	OH		OCH_3	
	Prunetin	OH	OCH_3		OH	

Glu: glucose

Table 2

IC_{50} (μM) values of flavonoids on phosphodiesterase isozymes

Class	Name	PDE isozymes				
		1	2	3	4	5
Flavones	Luteolin	21.5 ± 2.9 (3)	13.3 ± 0.8 (3)	10.1 ± 1.8 (5)	19.1 ± 2.4 (6)	19.3 ± 3.2 (4)
	Luteolin-7-glucoside	>100 (3)	35.1 ± 0.2 (3)*	>100 (3)	43.0 ± 5.3 (4)*	>100 (3)
	Diosmetin	14.4 ± 6.2 (3)	4.8 ± 0.8 (4)*,#	>100 (3)	20.2 ± 2.4 (3)	15.3 ± 3.6 (3)
	Apigenin	25.4 ± 3.7 (3)#	16.7 ± 6.3 (5)#	10.5 ± 3.5 (4)#	>100 (3)	>100 (3)
	Chrysin	>100 (3)	>100 (4)	>100 (3)	>100 (4)	>100 (4)
Flavonols	Quercetin	27.8 ± 5.7(3)#	17.9 ± 3.4 (4)	5.6 ± 1.0 (4)	9.9 ± 2.5 (3)*	>100 (3)
	Myricetin	24.9 ± 3.6 (3)#	12.8 ± 0.6 (4)#	12.4 ± 3.3 (4)#	39.8 ± 2.1(6)*,&	>100 (3)#
Flavanones	Eriodictyol	>100 (3)	>100 (3)	52.5 ± 17.7 (4)*,#	>100 (3)	>100 (3)
	Hesperetin	>100 (3)#	>100 (3)#	>100 (3)#	28.2 ± 1.1(3)$	>100 (3)#
Isoflavones	Genistein	16.8 ± 2.3 (3)#	1.7 ± 0.2 (4)*,#	12.9 ± 5.2 (3)	9.5 ± 1.9 (4)*,+	73.9 ± 7.1 (3)*,#,+
	Daidzein	>100 (3)*,!	>100 (4)*,!	28.6 ± 8.5 (3)*,#	>100 (4)*,!	>100 (3)*,!
	Biochanin A	29.1 ± 0.3 (3)#,*,!	27.9 ± 4.1(4)#,*,!	>100 (3)#,*,!	8.5 ± 0.1 (4)*	>100 (3)#,*,!
	Prunetin	>100 (3)*,!	>100 (4)*,!	>100 (3)*,!	61.9 ± 17.3 (4)*,!	>100 (3)*,!
	Reference drugs[a]	122.8 ± 44.9 (3)	4.4 ± 1.0 (5)	2.4 ± 0.5 (3)	11.4 ± 1.6 (8)	3.3 ± 0.9 (7)

All values are expressed as the mean ± SEM (n), where n is the number of experiments.

[a] Reference drugs for PDE isozymes 1, 2, 3, 4, and 5 were vinpocetine, EHNA, milrinone, Ro 20-1724, and zaprinast, respectively. * $p < 0.05$ when compared with the corresponding value of luteolin. # $p < 0.05$ when compared with the corresponding value of PDE4. $ $p < 0.05$ when compared with the corresponding value of diosmetin. & $p < 0.05$ when compared with the corresponding value of quercetin. + $p < 0.05$ when compared with the corresponding value of apigenin. ! $p < 0.05$ when compared with the corresponding value of genistein.