Published in final edited form as:

Adv Exp Med Biol. 2002 ; 505: 163–171.

INTERACTIONS OF FLAVONES AND OTHER PHYTOCHEMICALS WITH ADENOSINE RECEPTORS

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Abstract

Dietary flavonoids have varied effects on animal cells, such as inhibition of platelet binding and aggregation, inhibition of inflammation, and anticancer properties, but the mechanisms of these effects remain largely unexplained. Adenosine receptors are involved in the homeostasis of the immune, cardiovascular, and central nervous systems, and adenosine agonists/antagonists exert many similar effects. The affinity of flavonoids and other phytochemicals to adenosine receptors suggests that a wide range of natural substances in the diet may potentially block the effects of endogenous adenosine. We used competitive radioligand binding assays to screen flavonoid libraries for affinity and a computational CoMFA analysis of flavonoids to compare steric and electrostatic requirements for ligand recognition at three subtypes of adenosine receptors. Flavone derivatives, such as galangin, were found to bind to three subtypes of adenosine receptors in the μM range. Pentamethylmorin (K_i 2.65 μM) was 14- to 17-fold selective for human A_3 receptors than for A_1 and A_{2A} receptors. An isoflavone, genistein, was found to bind to A_1 receptors. Aurones, such as hispidol (K_i 350 nM) are selective A_1 receptor antagonists, and, like genistein, are present in soy. The flavones, chemically optimized for receptor binding, have led to the antagonist, MRS 1067 (3,6-dichloro-2'-(isopropoxy)-4'-methylflavone), which is 200-fold more selective for human A_3 than A_1 receptors. Adenosine receptor antagonism, therefore, may be important in the spectrum of biological activities reported for the flavonoids.

2. INTRODUCTION

Flavonoids are ubiquitous phenolic compounds occurring throughout the plant kingdom. Ingested flavonoids have recently been recognized to have many surprising and exciting implications when taken as dietary supplements. Flavonoids have been linked to many beneficial effects in humans, such as inhibition of platelet binding and aggregation (for review see Beretz and Cazenave, 1988), inhibition of inflammation (for a review see Middleton and Ferriola, 1988; Manthey, 2000), and anticancer properties (for a review see Wang et al., 1998). Some flavonoids also have estrogen-like effects and/or inhibit tyrosine kinases (Barnes et al., 1999). The mechanisms of many of these effects, however, remain largely unexplained (Karton et al., 1996).

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Flavonoids from plant sources comprise an important component of the human diet. Concentrations of these compounds are especially high in legumes, such as soybeans. Soy proteins are included in many foods and have been found to contain concentrations of 0.1-3.0 mg of flavonoids per gram (Coward et al., 1993). Data indicates that consuming “modest” quantities of these flavonoids can result in high levels in the circulatory system (Setchell, 1996).

Adenosine receptors are involved in the homeostasis of the immune, cardiovascular, and central nervous systems. Activation of adenosine receptors is associated with cerebroprotective (von Lubitz et al., 1994) and cardioprotective (Stickler et al., 1996) properties, and also with effects on the immune and inflammatory systems (Sajjadi et al., 1996). A₃ receptor-selective antagonists have been proposed to have anti-asthmatic (Beaven et al., 1994) and possibly cerebroprotective (Jacobson et al., 1995) properties. We have noted that some of the observed effects for flavonoids are similar to *in vivo* effects related to activation or antagonism of adenosine receptors. Therefore, we have explored the interactions between purine receptors and certain flavonoids as a possible mechanism for the observed effects of flavonoids in humans.

A wide variety of non-purine ligands that bind selectively to adenosine receptors have been described (Jacobson et al., 1997; Müller, 1997). The availability of selective ligands has facilitated studies of the physiological roles of particular subtypes of adenosine receptors. The A₃ receptors have already been implicated in vascular effects, inflammation and cancer, *i.e.* three areas in which flavonoids have been considered biologically active. One example is the common effect that both flavonoids and adenosine have on histamine release.

A broad screening of phytochemicals, using competition for specific radioligand binding to human A₃ receptors as an assay, demonstrated moderate affinities, with K_i values in the micromolar range (Moro et al., 1998). This suggests, that if blood levels reach this range following ingestion of flavonoids, the flavonoids may antagonize the activity of these receptors (Karton et al., 1996; Ji et al., 1995). These findings might ultimately help to elucidate a mechanism for the effects that have been attributed to ingested flavonoids, and warrant further screening of flavonoids.

3. RESULTS AND LITERATURE REVIEW

3.1. flavonoids in Chemical Library Identified as Adenosine Antagonists

The primary screening assay consisted of single point competition at fixed flavonoid concentration (10 μ M) for specific binding of ¹²⁵I-AB-MECA by recombinant human A₃ receptors expressed in HEK-293 cells, and promising candidates were further examined for concentration dependence in binding (Ji et al., 1995).

Flavone derivatives, such as galangin, were found to bind to three subtypes of adenosine receptors in the μ M range. Galangin displayed K_i values of 1 μ M for both rat A₁ and A_{2A} receptors and 3 μ M for human A₃ receptors. Methylation but not acetylation of the hydroxyl groups of galangin enhanced A₃ affinity. Triethyl- and tripropyl-galangin displayed K_i values of 0.3-0.4 μ M at human A₃ receptors (Karton et al., 1996). Pentamethylmorin showed 4- to 17-fold selectivity for human A₃ receptors vs. rat A₁ and A_{2A} receptors, with a K_i value of 2.65 μ M. Several polymethoxylated flavones from citrus, tetramethylscutellarein and tetramethyl-kaempferol, also showed high affinities for the A₁ receptor. Reduction of the 2,3-Oisoflavan bond, as in ($\pm$)-dihydroquercetin, or glycosidation, as in robinin, greatly diminished affinity. α -Naphthoflavone had greater receptor affinity (0.79 μ M to A₁ receptors) than the β -isomer. Other natural products of plant origin, including oxogalanthine lactam, hematoxylin, and arborinine were found to bind to At adenosine receptors with K_i

values of 3-13 μM . These findings indicate that flavonoids and other phytochemicals may provide leads for the development of novel adenosine antagonists.

A naturally occurring isoflavone, genistein, was found to bind with moderate affinity to A_1 receptors (Okajima et al., 1994), but only very weakly to A_3 receptors (Ji et al., 1995). We have examined the affinity of other isoflavones to A_1 and A_{2A} receptors and found that genistein is the most potent (Table 1).

The principle of adenosine antagonism as a mechanism for the observed biological effects of flavonoids has been demonstrated. Cirsimaritin and cirsimaritin, flavonoids of *Microtea debilis* (Phytolaccaceae), were found to have adenosine antagonistic properties in rats (Hasrat, 1997). In traditional medicine *Microtea debilis* is used against proteinuria. Cirsimaritin was isolated as the active component and was shown to be an A_1 adenosine antagonist. The decreased heart rate and blood pressure induced by adenosine was inhibited by cirsimaritin. The concentration of cirsimaritin in urine was $\sim 2 \mu\text{M}$ after administration of 8 mg kg^{-1} cirsimaritin. In the kidney and urinary tract the concentration of cirsimaritin produced after cirsimaritin ingestion was sufficient to inhibit adenosine receptors and might explain the clinical effectiveness of *Microtea debilis*.

3.2. Chemical Optimization of Affinity of Flavonoids to Adenosine Receptors

The initial finding that flavonoids bind to adenosine receptors (Ji et al., 1995; Ares et al., 1995) has been exploited synthetically to increase receptor selectivity and potency. The structure activity relationships of flavonoids in binding to adenosine receptors were further explored (Karton et al., 1996). The flavone structure may be treated as a molecular template for design of receptor ligands. MRS 1067 (3,6-dichloro-2'-(isopropoxy)-4'-methylflavone; $K_i=0.56 \mu\text{M}$ at human A_3 receptors) was identified through chemical optimization of flavones for receptor binding. MRS 1067 is both relatively potent and highly selective (200-fold) for human A_3 vs human A_1 receptors. This derivative effectively blocked the effects of an agonist in the inhibition of adenylyl cyclase in Chinese hamster ovary (CHO) cells expressing either cloned rat or human A_3 receptors (Karton et al., 1996; Jacobson, 1997). MRS 1067 antagonized the effects of the A_3 receptor-selective agonist CI-IB-MECA to induce a rise in intracellular $[\text{Ca}^{2+}]$ in RBL-2H3 rat mast cells (Shin et al., 1996). MRS 1067 is approximately 20-fold selective for rat A_3 receptors.

3.3. Computer Modeling of Flavonoids as Adenosine Antagonists

A quantitative study of the structure activity relationships (SAR) for binding of flavonoids to adenosine A_1 , A_{2A} , and A_3 receptors has been conducted using comparative molecular field analysis (CoMFA), which addresses the steric and electrostatic requirements for ligand binding. The relatively rigid ring structure of flavonoids compared to other A_3 receptor antagonists, makes them particularly amenable to CoMFA analysis. Correlation coefficients (cross-validated r^2) of 0.605, 0.595, and 0.583 were obtained for the three sub-types, respectively.

Principal conclusions from the CoMFA analysis are:

- a. a) All three CoMFA models have similar average steric and electrostatic contributions, implying that A_1 , A_{2A} and A_3 have the same relative contribution of steric and electrostatic factors inside the binding cavity. However, the specific distribution of steric and electrostatic interactions for each receptor is different.
- b. b) Similarities were seen in the topology of steric and electrostatic regions with A_1 and A_3 receptors (Fig. 1), but not the A_{2A} receptor. This is in accord with the

structure-activity relationship data in which similarity between A₁ and A₃ receptors has been demonstrated.

- c. c) C-2 chromone phenyl ring substituents are considered important for the binding affinity for all adenosine receptors. This phenyl ring may interact in a similar hydrophobic pocket inside the receptor binding-site.
- d. d) An interesting consideration about A₁/A₃ selectivity can be deduced from CoMFA contour map analysis. An important region of favorable steric bulk interaction is located around the 2'-position of the phenyl ring, in the A₃ model. This is consistent with the experimental data for MRS 1067, which is among the most selective flavonoid antagonists of human A₃ receptors.
- e. e) The presence of a C-6 substituent in the chromone moiety is well tolerated, and increases the A₁/A₃ selectivity.

3.4. Identification of Additional Classes of Flavonoids as Adenosine Antagonists

Aurones contain fused 5- and 6-membered rings, and like genistein, naturally occur in soy. We have screened a number of aurones in binding to adenosine receptors (Table 2) and found considerable affinity. Hispidol was found to be a selective A₁ receptor antagonist, with a K_i value of 350 nM. Antagonism of the A₁ receptor by hispidol was demonstrated in a functional assay consisting of agonist-induced stimulation of binding of [³⁵]GTP- γ -S to the associated G-proteins (Fig. 2).

The earlier observation that methylation of flavones increased affinities for adenosine receptors led us to also analyze the binding properties of a number of the polymethoxylated flavones in citrus. As shown in Table 3, most polymethoxylated flavones exhibited affinities to the A₁ and A_{2A} receptors in the micromolar range, but showed little selectivity. The several compounds exhibiting greater selectivity had unsubstituted hydroxyl groups at the 3 position. 3-Hydroxy-5,7,3',4'-tetramethoxyflavone had the highest affinity to A₁, (0.3 μ M) and a greater than 100-fold selectivity for the A₁ vs the A_{2A} subtype. Tight binding to the human A₃ receptors expressed in CHO cells was also observed for sinensetin and 5,7,3',4'-tetramethyl quercetin (K_i=0.34 and 0.31 μ M, respectively). The affinity of sinensetin at the human A₃ receptor is the highest exhibited by a naturally occurring flavone thus far examined.

4. DISCUSSION AND CONCLUSIONS

A broad screening of phytochemicals in competitive radioligand binding assays vs the high affinity agonist [¹²⁵I]AB-MECA (N⁶-(4-amino-3-iodobenzyl)adenosine-5'-N-methyluronamide) has demonstrated that certain naturally occurring flavonoids show affinity at micromolar levels 10 cloned human brain A₃-adenosine receptors (Ji et al., 1995). A later study (Karton et al., 1996) concluded that the affinity of common phytochemicals to adenosine receptors suggests that a wide range of natural substances in the diet may potentially antagonize the effects of endogenous adenosine. Many flavonoids described above appear to be selective antagonists for the A₁ or A₃ receptors. Thus, adenosine receptor antagonism may be important in the range of biological activities reported for such flavonoids.

We have used CoMFA analysis to distinguish steric and electrostatic requirements for binding of flavones to A₁, A_{2A}, and A₃ receptors. In this manner, the design or discovery of more potent and selective flavonoid antagonists may be accomplished. All three CoMFA models have the same steric and electrostatic contributions, implying similar requirements inside the binding cavity. Similarities were seen in the topology of steric and electrostatic

regions with the A₁ and A₃ receptors, but not the A_{2A}. Substitutions on the phenyl ring at the C-2 position of the chromone moiety may be considered important for binding to all adenosine receptors. In the A₃ model a region of favorable bulk interaction is located around the 2'-position of the phenyl ring. The presence of a C-6 substituent in the chromone moiety is well tolerated and increases the A₁/A₃ selectivity. The CoMFA coefficient contour plots provide a self-consistent picture of the main chemical features responsible for the pK_i variations and also result in predictions that agree with experimental values.

Additional SAR studies will be necessary to provide flavonoids that are truly selective adenosine antagonists. Flavonoids are known to bind to a wide range of enzymes (Middleton, 1988; Limasset, 1993; Raghavan, 1995; Dai, 1997), thus the true selectivity of substances that antagonize subtypes of adenosine receptors requires extensive characterization at many binding sites. These non-adenosine-related actions may interfere with their use as pharmacological probes at adenosine receptors. For example, MRS 1067 was found to inhibit (~50% at 28 ~M) rat liver cytochrome P450-2CII (Dai, 1997).

In conclusion, dietary flavonoids make up a significant portion of the human diet. Many of the effects attributed to flavonoids have unexplained mechanisms, but have many similarities to biological effects observed from adenosine receptor antagonism. Through competitive radioligand binding and functional assays, flavonoids were found to be moderately potent antagonists for adenosine receptors, identifying a new class of adenosine receptor ligands, *i.e.* aurones. The aurone hispidol displays selectivity as an A₁ adenosine receptor antagonist. Computer modeling of the interactions of flavonoids and A₁, A_{2A}, and A₃ adenosine receptors defined the steric and electronic features of this recognition. These findings suggest antagonism of adenosine receptors, and potentially other G protein-coupled receptors, might be linked to a significant number of the effects proposed for flavonoids.

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Figure 1.

Representation of the steric and electrostatic factors leading to binding of a representative flavone (triethylgalangin) to human A₃ adenosine receptors obtained through CoMFA (Comparative Molecular Field Analysis). + region of positive charge; - region of negative charge; elliptical object = region of tolerance of steric bulk.

Figure 2.

Demonstration of adenosine antagonism by an aurone: Displacement GTP- γ -S binding by hispidol binding 10 rat A₁ receptors.

Table 1 Affinities (K_i , μM) of isoflavone derivatives in a radioligand binding assay of rat A_1 receptors

Compound	R ₁	R ₂	R ₃	A ₁ ^a
Daidzein	H	H	H	23.4±4.7
Genistein	H	H	5-OH	12.6±1.4
Biochanin A	H	CH ₃	5-OH	34.5±4.7
Prunetin	CH ₃	H	5-OH	>100

^a Other compounds (R₁, R₂, R₃) showed <10% displacement at 100 μM : (CH₃, CH₃, 5-OH), (H, CH₃, H), (CH₃, CH₃, H), (H, H, 6-OH), (H, H, 6-OCH₃), and 7,3',4'-trihydroxyisoflavone; ($\pm$)-equol (7,4'-isoflavandiol) displaced 36±2% at 100 μM .

Table 2

Affinities (K_i , μM) of aurone derivatives in a radioligand binding assay of rat adenosine receptors

Compound	R ₁	R ₂	R ₃	A ₁	A _{2A}
Sulfuretin	H	H	OH	4.44±1.22	28.1±10.1
Aureusidin	OH	H	OH	13.2±4.8	>100
Hispidol	H	H	H	0.352±0.080	52.7±9.1
Maritimetin	H	OH	OH	3.47±0.47	9.35±2.51

Table 3

Affinities (K_i , μM) of Polymethoxylated Flavones in Radioligand Binding Assay to Rat Adenosine Receptors

Compound	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇	A ₁	A _{2A}
3,5,6,7,8,3',4'-heptamethoxyflavone	OCH ₃	OCH ₃	OCH ₃	OCH ₃	OCH ₃	OCH ₃	OCH ₃	15.8±3.4	39.4±5.4
nobiletin (5,6,7,8,3',4'-hexamethoxyflavone)	OCH ₃	OCH ₃	OCH ₃	OCH ₃	OCH ₃	OCH ₃	H	3.74±0.89	21.5±5.3
tangeretin (5,6,7,8,4'-pentamethoxyflavone)	OCH ₃	OCH ₃	OCH ₃	OCH ₃	H	OCH ₃	H	4.45±0.82	23.7±3.8
tetramethylscutellarein (5,6,7,4'-tetramethoxyflavone)	OCH ₃	OCH ₃	OCH ₃	H	H	OCH ₃	H	1.46±0.14	14.3±2.1
sinenstin (5,6,7,3',4'-pentamethoxyflavone)	OCH ₃	OCH ₃	OCH ₃	H	OCH ₃	OCH ₃	H	0.88±0.13	11.4±0.6
5-desmethylnobiletin 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone	OH	OCH ₃	OCH ₃	OCH ₃	OCH ₃	OCH ₃	H	4.93±0.14	11.4±3.6
3,5,7,3',4'-pentamethoxyflavone	OCH ₃	H	OCH ₃	H	OCH ₃	OCH ₃	OCH ₃	17.3±0.4	45.3±6.4
5-hydroxy-3,7,8,3',4'-pentamethoxyflavone	OH	H	OCH ₃	OCH ₃	OCH ₃	OCH ₃	OCH ₃	10.2±2.0	12.6±6.1
5,8-dihydroxy-3,7,3',4'-tetramethoxyflavone	OH	H	OCH ₃	OH	OCH ₃	OCH ₃	OCH ₃	15.4±3.2	>(10 ⁴)
3-hydroxy-5,7,3',4'-tetramethoxyflavone	OCH ₃	H	OCH ₃	H	OCH ₃	OCH ₃	OH	0.298±0.03	34.2±6.5