Adenosine-1 Active Ligands: Cirsimarin, a Flavone Glycoside from *Microtea debilis*[1]

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Several plants collected through different approaches were screened on distinct receptors using ligand-binding studies as bioassay. Extracts of *Microtea debilis* showed high activity on adenosine A_1 receptors. Bioassay-guided fractionation using ligand-binding studies resulted in the isolation of an adenosine A_1 active ligand, cirsimarin (cirsimaritin 4′-O-glucoside). GTP did not influence the radioligand inhibition curve of cirsimarin, indicating that this compound is acting as an antagonist at the adenosine-A_1 receptors. The use of this plant against "proteinuria" in traditional medicine in Suriname (South America) may be explained by the adenosine A_1 antagonistic action of cirsimarin. A series of flavonoids was tested in the same assay, but they were less active. No structure−activity relationship could be observed.

Introduction

Plants are still a main source of bioactive compounds for the development of new therapeutics or as tools to examine biological processes. The collection of plants can occur in different ways, i.e., based on the basis of traditional medicine, observation, the interaction between organisms, chemotaxonomy, or literature data of investigated plants, or at random.[2] Plants from Suriname, collected through different approaches, were screened on a number of receptors using ligand-binding studies as the bioassay.[3] Extracts from *Bellucia grossularioides* Triana (Melastomaceae), *Irlbarchia purpurascens* Aublet (Maas) (Gentianaceae), *Mimosa myriadena* Benth (Mimosaceae), and *Scoparia dulcis* L. (Scrophulariaceae) showed moderate activity, whereas extracts of *Microtea debilis* L. (Phytolaccaceae) showed powerful activity as inhibitor of the binding of [^{3}H]-1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) to adenosine A_1 receptors, and therefore, *M. debilis* was selected for further examination. Extracts of *M. debilis* are used in Suriname against proteinuria and elsewhere against cough and stomach-ache. Evidence exists for the involvement of adenosine in renal physiology and pathophysiology via interactions with adenosine receptors in the kidney.[4,5] In addition, it has been observed that in certain types of acute renal failure adenosine A_1 receptor blockade could be beneficial.[6−9]

The existence of adenosine A_1 ligands in *M. debilis* could explain the traditional use against proteinuria.

Results and Discussion

Bioassay-guided fractionation of the crude extract of *M. debilis* using inhibition of the binding of [^{3}H]DPCPX to adenosine A_1 receptors resulted in the isolation and characterization of compound **1** as an adenosine A_1 active ligand. The plant material collected in different parts from Suriname showed no difference in activity. Compound **1** could be obtained from the concentrated 80% ethanol extract of *M. debilis* by washing the aqueous solution with petroleum ether and chloroform followed by precipitation from the water layer. Compound **1** reacted with Draggendorf reagent according to Munier for alkaloids and Neu reagent. UV spectroscopy of compound **1** pointed toward a flavone. Subsequent MS and NMR analysis identified compound **1** as cirsimaritin 4′-*O*-glucoside (cirsimarin).

Although it could be demonstrated by thin layer chromatography that the aglycon **2** is present in the crude extract, it was obtained by hydrolysis of compound **1**. UV spectroscopy yielded a typical flavone spectrum, and subsequent MS and NMR analysis identified compound **2** as cirsimaritin.

The FAB mass spectrum of compound **1** showed a $[M + H]^+$ ion at *m/z 477*, indicating a molecular weight of 476, and an intense peak at *m/z* 315 corresponding to the loss of one hexose unit and suggesting an *O*-glycoside.[10] In order to characterize the aglycon part tandem mass spectrometry was performed; i.e., the protonated aglycon ion at *m/z* 315 was subjected to low-energy collision-induced dissociation.[11] The low-energy CID spectrum was characterized by a relatively abundant ion at *m/z* 300 (70%), which is due to the loss of a $CH_3^{\bullet}$ radical and is typical of a methylated flavone. The ions at *m/z* 119 and 121 correspond to B ring fragments, are characteristic of a flavone derivative, and indicate that the hydroxyl group in the B ring is not methylated. In the upper mass region the intense ions at *m/z* 282 and 254 correspond to the combined loss of $CH_3^{\bullet}$ and H_2O and of $CH_3^{\bullet}$, H_2O, and CO, respectively. The ion at *m/z* 270 is due to the combined loss of $CH_3^{\bullet}$ and formaldehyde and is in agreement with a second methoxyl substituent in the A ring. The even-mass product ions at *m/z* 152 and 136 are attributed to A ring fragments and can only be explained by fragmentation following loss of a $CH_3^{\bullet}$ radical.

^{1}H NMR indicated a glycosylated flavonoid with 4′ monosubstitution in the B ring and two methoxyl groups. ^{13}C NMR of **1** and **2** (Table 1) led to the identification of the sugar moiety as glucose and the 4′-

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Table 1. ^{13}C NMR Assignments for Cirsimarin (**1**) and Cirsimaritin (**2**) (50 MHz, DMSO-d_6)[a]

C	cirsimarin (**1**)	cirsimaritin (**2**)	D
2	163.4	164.0	
3	99.9	102.6	
4	182.3	182.1	
5	152.1*	152.0*	
6	132.0	131.9	
7	158.7	158.6	
8	91.7	91.5	
9	152.7*	152.6*	
10	105.2	105.1	
1′	123.9	121.0	-2.9
2′,6′	128.2	128.4	+0.2
3′,5′	116.6	115.9	−0.7
4′	160.4	161.3	+0.9
1″	103.7		
2″	73.2		
3″	77.2		
4″	69.8		
5″	76.6		
6″	60.7		
6-OCH_3	60.1	60.0	
7-OCH_3	56.5	56.4	

[a] Assignments marked with an asterisk may be interchanged.

OH as the glycosylation site. ^1H and ^{13}C NMR data of the aglycon **2** were in agreeement with published data for cirsimaritin (or 6-methoxygenkwanin).[12] These data indicate that compound **1** is the glucoside of compound **2**. A downfield shift of the para-related carbon signal in the range of 1.7−4 ppm appears to be a reliable indication of the site of glycosylation.[13] Therefore, compound **1** was identified as cirsimaritin 4′-*O*-glucoside, a known compound isolated previously from *Cirsium maritimum*.[14] ^1H and ^{13}C NMR spectral data of cirsimarin have not been published before.

Cirsimarin, cirsimaritin, and flavonoids obtained commercially and isolated in our laboratory (Table 2) were tested for their potency to inhibit the binding of the radiolabeled adenosine-A_1 receptor antagonist [^3H]-DPCX to its receptors in rat forebrain membranes. Some of the flavonoids tested show an interesting activity, but no structure−activity relationship could be observed. At a concentration of 100 μg/mL cirsimarin was the most active one. It produced an almost complete inhibition of the binding of [^3H]DPCX to adenosine A_1 receptors, whereas its aglycon cirsimaritin was almost 25 times less active. Flavonoid glycosides (e.g., compound **8** and **11**) and aglycons (e.g., compound **4**, **6**, **10**, and **12**) were less active than cirsimarin.

The K_i-value of cirsimarin, calculated according to Cheng and Prusoff,[15] is in the micromolar range (3.2 μM). The [^3H]DPCX-inhibition curve for cirsimarin is shown in Figure 1.

Flavonoids have already been reported to inhibit the binding of adenosine to the different types of adenosine receptors, but the interaction of cirsimarin with adenosine A_1 receptors was not described before. Genistein, an isoflavone, was the first flavonoid reported to be an adenosine A_1 ligand.[16] More recently, the interactions of some other flavonoids with adenosine receptors have also been reported.[17] In this context, it is noteworthy that in the present paper apigenin, cirsimaritin, and quercetin show a lower activity than in previous reports. These differences could be related to differences in the incubation temperature, i.e., 25 °C in our experiments compared to 0 °C, since it has been shown that a decrease of the incubation temperature increases the affinity of antagonists for A_1 receptors and decreases that of agonists.[18]

No structure−activity relationship for the flavonoids investigated could be established (Table 2). The activity was not restricted to aglycons or to some classes of flavonoids. According to Ji et al.,[17] the carbonyl group at the 4-position had no effect on or decreased adenosine A_1 receptor affinity. Glycosidation diminished affinity for adenosine receptors. However, from our work it appeared that the glycoside cirsimarin probably is one of the most powerful adenosine A_1 receptor-binding flavonoids. Cirsimarin is slightly more active than theophylline, a known adenosine receptor antagonist.[19]

It is now accepted that adenosine plays a vital role in the activity of cells in living organisms.[19] On a renal level it is probably crucial in many processes.[4] There is evidence that A_1 antagonists could protect the kidney against some forms of proteinuria, although the exact mechanism is not known.[5−9] Other subtypes of adenosine receptors, A_2 and A_3, have been found in the kidney, which have obviously distinct locations.[20,21]

Several studies have indicated the ability of adenosine antagonists to reduce renal effects of some types of acute renal failure.[6−9] The glycerol-induced acute renal failure in rats is one of the most applied models of acute renal trauma.[22] Intramuscular injection of 50% glycerol causes rapid myoglobinuria, oliguria, and a rapid reduction in glomerular filtration rate. Although the pathophysiology of this model is not yet completely understood, there is evidence that the decrease in renal blood flow and ischemia, due to the contribution of endogenous adenosine, are responsible for kidney damage.[22]

Apart from the inhibition of adenosine binding to adenosine receptors reported for some flavonoids, genistein was also shown to be an inhibitor of protein tyrosine kinase, and some compounds were reported to inhibit adenosine deaminase activity.[16,23]

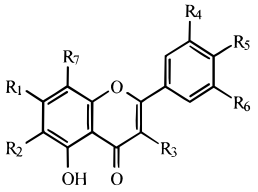
In conclusion, our results provide a scientific explanation of the use of *M. debilis* in traditional medicine against proteinuria especially because of the relative high amount of cirsimarin in the plant (1%). Although the number of plants investigated is rather small, these results again prove that the ethnopharmacological approach for the search of bioactive compounds produces the best results.

Experimental Section

Plant Material. *M. debilis* (whole plant) was collected from several regions in Suriname, very different in soil structure, to investigate if this influences the activity. The plant material was identified by the Herbarium of the University of Suriname where a voucher specimen is deposited.

General Experimental Procedures. The FAB mass spectral data of compound **1** were obtained in the positive ion mode on a VG70SEQ instrument (Micromass, Manchester, U.K.) using glycerol as matrix. Low-energy collision-induced dissociation (CID) was performed with argon at a gas pressure of 8×10^{-6} mbar and a collision energy of 30 eV. ^1H and ^{13}C NMR spectra were recorded on a JEOL FX 200 instrument operating at 200 MHz for ^1H in DMSO-d_6. Vitexin, quercitrin dihydrate, hyperoside, apigenin, kaempferol, and myricetin were purchased from Roth (Germany);

Table 2. Structural−Activity Relationship between Flavonoids[a]



	compd	R_1	R_2	R_3	R_4	R_5	R_6	R_7	% bound [^{3}H]DPCPX
1	cirsimarin	OCH_3	OCH_3	H	H	*O*-glucosyl	H	H	2 ± 2
2	cirsimaritin	OCH_3	OCH_3	H	H	OH	H	H	48 ± 1
3	chrysoeriol 7-*O*-neohesperidose	*O*-neohesperidosyl	H	H	H	OH	OCH_3	H	94 ± 5
4	apigenin	OH	H	H	H	OH	H	H	50 ± 2
5	vitexin	OH	H	H	H	OH	H	glucosyl	103 ± 3
6	quercitrin	OH	H	*O*-rhamnosyl	H	OH	OH	H	50 ± 2
7	hyperoside	OH	H	*O*-galactosyl	H	OH	OH	H	86 ± 4
8	quercetin 3-*O*-rhamnose	OH	H	*O*-rhamnosyl	H	OH	OH	H	58 ± 2
9	quercetin 3-*O*-rutinose	OH	H	*O*-rutinosyl	H	OH	OH	H	103 ± 9
10	kaempferol	OH	H	OH	H	OH	H	H	18 ± 1
11	kaempferol 3-*O*-rutinose	OH	H	*O*-rutinosyl	H	OH	H	H	37 ± 2
12	myricetin	OH	H	OH	OH	OH	OH	H	24 ± 1

[a] The concentration of the products was 100 μg/mL; rat forebrain membranes were incubated with 0.4 nM [3*H*]-1,3-dipropyl-8-cyclopentylxanthine and competitor at 25 °C in 50 mM Tris−HCl (pH 7.4); nonspecific binding was obtained in the presence of 5 μM (*R*)-*N*^{6}-(2-phenyl-1-methylethyl)adenosine. The values, average and standard error, of the compounds are the results of two experiments (both in triplicate) and are expressed as the percentage of the radioligand still bound to the receptor.

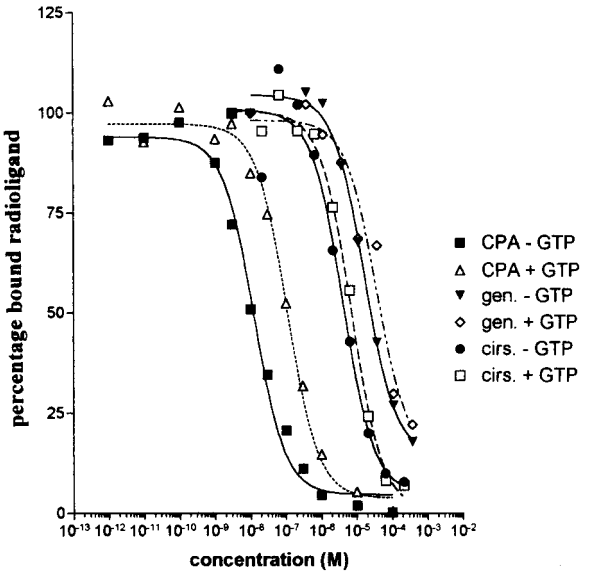


Figure 1. Adenosine-A_1 activity. The radioligand-inhibition curves of cirsimarin, genistein, and *N*^{6}-cyclopentyladenosine (CPA) were obtained by incubation of rat forebrain membranes for 60 min with 0.4 nM [^{3}H]-1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) and an increasing concentration of the compounds at 25 °C in 50 mM Tris−HCl (pH 7.4); nonspecific binding was obtained in the presence of 5 μM (*R*)-*N*^{6}-(2-phenyl-1-methylethyl)adenosine. K_i was calculated according to Cheng and Prusoff.[15] GTP displaces the inhibition curve of agonists, i.e., CPA, significantly, whereas those from antagonists are not influenced, i.e., genistein, an isoflavone.

other flavonoids included in Table 1 were isolated in our laboratory.[24,25] [^{3}H]-1,3-Dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) (107 Ci/mmol) was from Amesham (U.K.) and (*R*)-*N*^{6}-(2-phenyl-1-methylethyl) adenosine ((*R*)-PIA) from Boehringer Mannheim (Germany).

Isolation and Identification. Bioguided fractionation was performed using the inhibition of [^{3}H]DPCPX to adenosine A_1 receptors as the bioassay. The crude extract of sun-dried whole plant of *M. debilis* was prepared by maceration in and percolation with 80% ethanol. In preliminary fractionation procedures with a small amount (<5 g) of plant material it was observed that after evaporation of the ethanol layer under reduced pressure at 40 °C and subsequent partition of the crude extract between water and chloroform, both fractions were active. The chloroform fraction was subsequently fractionated by column chromatography on silica gel using a chloroform−methanol gradient of increasing polarity. Elution with 20% methanol resulted in the collection of one highly active fraction. Compound(s) in this fraction reacted with Draggendorf reagent according to Munier for alkaloids and Neu reagent for α and γ pyrones. The latter reagent is frequently used for identifying flavonoids in samples. When large amounts of plant material (100 g) were used to obtain the active compound(s), precipitation occurred in the water layer after evaporation of ethanol from the crude extract and subsquent washing with chloroform. The precipitate was washed with methanol/water and dissolved in hot methanol. Chromatographically (TLC on silica gel) pure **1** (about 1 g) crystallized from the methanol solution after cooling, giving compound **1**. In the bioassay, this compound was highly active and reacted in the same manner with the reagents described above. Washing the crude extract, after evaporation of ethanol, first with petroleum ether and next with chloroform in the purification procedure yielded a cleaner precipitate from the water layer.

Cirsimarin (1). UV λ_{max} (MeOH) 325 nm, 278 nm; (2 M NaOH) 325 nm (sh), 296 nm; (5%$AlCl_3$/MeOH) 350 nm, 300 nm; ($AlCl_3$/HCl) 350 nm, 300 nm. FAB/MS: *m*/*z* [M + H]$^+$ 477 (100), 315 (92). FAB/MS/MS (precursor *m*/*z* 315): *m*/*z* 315 (100), 300 (70), 282 (74), 270 (18), 254 (100), 152 (22), 139 (26), 136 (48), 121 (17), 119 (17), 108 (17). ^{13}C NMR: see Table 1. ^{1}H NMR (200 MHz, DMSO-d_6) δ: 3.0−3.5 glucosyl; δ 3.74, 3H, s, $-OCH_3$; δ 3.93, 3H, s, $-OCH_3$; δ 5.04, 1H, d, J = 6.8 Hz, H-1″; δ 6.97, 2H, 2 × s, H-3, H-8; δ 7.20, 2H, d, J = 8.8 Hz, H-3′, H-5′; δ 8.08, 2H, d, J = 8.8 Hz, H- 2′, H- 6′ (5-OH signal not observed).

Compound **2** was prepared from **1** by the method described by Harborne.[26]

Cirsimaritin (2). UV λ_{max} (MeOH) 334 nm, 276 nm; (2 M NaOH) 367 nm, 280 nm; (5% $AlCl_3$/MeOH) 361 nm, 302 nm; ($AlCl_3$/HCl) 353 nm, 301 nm. FAB/MS: m/z $[M + H]^+$ 315. ^{13}C NMR: see Table 1. ^1H NMR (200 MHz, DMSO-d_6) δ: 3.74, 3H, s, $-OCH_3$; δ 3.93, 3H, s, $-OCH_3$; δ 6.93, 6.85, 2 × 1H, 2 × s, H-8, H-3; δ 6.94, 2H, d, J = 8.8 Hz, H-3′, H-5′; δ 7.96, 2H, d, J = 8.8 Hz, H-2′, H-6′; δ 10.40, 1H, 4′-OH; δ 12.93, 1H, 5-OH.

Biological Activity. Membranes from rat forebrain (without striatum) for adenosine-1 receptor assays were prepared by homogenizing the tissue in 0.32 M sucrose solution as above for 30 s at 500*g*. The homogenate was first centrifuged at 1000*g* for 10 min and the supernatant centrifuged at 30000*g* for 30 min. The pellet was then suspended in H_2O, kept on ice for 30 min, and centrifuged once more at 30000*g* for 30 min. The pellet was then washed twice (by resuspension and centrifugation as above) with 50 mM Tris−HCl (pH 7.4), and the final pellet was resuspended in 50 mM Tris−HCl (pH 7.4) and incubated for 30 min with 2 U/mL of adenosine deaminase at 37 °C. The membrane suspension was stored in liquid nitrogen.

Ligand binding assays were performed according to Lohse et al.[27] with minor modifications. In summary, rat forebrain membranes were incubated with 0.4 nM [^{3}H](DPCPX) and competitor (100 μg/mL) in a final volume of 200 μL for 60 min at 25 °C in 50 mM Tris−HCl (pH 7.4). At the end of the incubation, the samples were filtered under reduced pressure through a glass fiber filter and rapidly washed with ice-cold buffer. The amount of radioligand remaining on the filters was then determined by liquid scintillation counting. Specific binding to the A_1 receptors is calculated by subtracting nonspecific binding, obtained in the presence of 5 μM R-PIA, from total binding.

Radioligand-Inhibition Curve. Radioligand-inhibition curves were obtained by increasing doses of cirsimarin. The K_i value was calculated according to the method of Cheng and Prusoff.[15] Dimethyl sulfoxide (DMSO) was used to increase the solubility of the compounds. The DMSO concentration did not exceed 1% in the test tube.

The results presented in Table 2 are expressed as the fraction (percentage) of the radioligand still bound to the receptor.

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