J. Pharm. Pharmacol. 1997, 49: 1150–1156
Received May 15, 1997
Accepted July 30, 1997

Cirsimarin and Cirsimaritin, Flavonoids of *Microtea debilis* (Phytolaccaceae) with Adenosine Antagonistic Properties in Rats: Leads for New Therapeutics in Acute Renal Failure

J. A. HASRAT, T. DE BRUYNE, J.-P. DE BACKER*, G. VAUQUELIN* AND A. J VLIETINCK

*Department of Pharmaceutical Sciences, University of Antwerp, Universiteitsplein 1, B-2610, Antwerp and *Department of Protein Chemistry, Free University of Brussels, Paardenstraat 65, B-1640, St Genesius Rode, Belgium*

Abstract

In traditional medicine *Microtea debilis* is used against proteinuria. In ligand-binding studies extracts of *Microtea debilis* have been shown to inhibit the binding of [^{3}H]1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) to adenosine-A_1 receptors in rat forebrain membranes. Subsequently, cirsimarin, a flavonoid, was isolated as the active component and was shown to function as adenosine antagonist at the adenosine-A_1 receptor in-vitro. In this study we have investigated the adenosine-A_2 receptor activity of cirsimarin the in-vivo inhibition of the effects of adenosine by cirsimarin in rats, the absorption of cirsimarin and the inhibition of the binding of [^{3}H]DPCPX to the adenosine-A_1 receptor by urine samples obtained after oral administration of crude extract of *Microtea debilis*, cirsimarin or cirsimaritin to rats.

Cirsimarin inhibited the binding of [^{3}H]5′-*N*-ethylcarboxamidoadenosine ([^{3}H]NECA) to adenosine-A_2 receptors in rat striatum with an inhibition constant, K_i, of $6{\cdot}5 \pm 0{\cdot}3$ μM. The decrease of heart rate and blood pressure induced by adenosine was significantly inhibited by cirsimarin. After oral administration of 8 and 80 mg kg^{-1} cirsimarin, the compound could not be detected in either plasma or urine, but the presence of cirsimaritin was established. By use of β-glucuronidase, glucuronides of cirsimaritin were also detected in the urine. The concentrations of cirsimaritin in the plasma were $0{\cdot}126 \pm 0{\cdot}04$, $0{\cdot}138 \pm 0{\cdot}015$, and $0{\cdot}120 \pm 0{\cdot}022$ μM, respectively, 2, 5 and 12 h after administration of 8 mg kg^{-1} cirsimarin. The concentrations of cirsimaritin in the urine at the same times after administration of the same dose were $2{\cdot}05 \pm 1{\cdot}86$, $5{\cdot}05 \pm 2{\cdot}6$ and $2{\cdot}06 \pm 0{\cdot}09$ μM, respectively. The inhibition of the binding of [^{3}H]DPCPX to the adenosine-A_1 receptor by urine samples collected 2, 5 and 12 h after oral administration of 8 mg kg^{-1} cirsimarin or a crude extract of *Microtea debilis* containing approximately 8 mg kg^{-1} cirsimarin and 2·8 mg kg^{-1} cirsimaritin, or 6·8 mg kg^{-1} cirsimaritin, was not significantly different from that of urine samples collected from untreated rats, in contrast with urine samples collected 1 and 2 days after oral administration of 80 mg kg^{-1} cirsimarin. Approximately 3% of the cirsimarin was excreted in the urine as cirsimaritin.

The results indicate that in the kidney and urinary tract the concentrations of cirsimaritin produced after ingestion of more than 8 mg kg^{-1} cirsimarin can be high enough to inhibit the interaction of adenosine with its receptors; this might explain the effectiveness of *Microtea debilis* preparations against proteinuria in traditional medicine.

Flavonoids have many pharmacological activities which have mainly been investigated in-vitro. However, evidence exists that the in-vivo beneficial effects of many natural products (e.g. cardiovascular, liver and gastrointestinal protective activity) are a result of the presence of flavonoids (Middleton & Kandaswami 1994; Formica & Regelson 1995) yet despite all this evidence very little scientific research has been conducted to confirm that the effects observed in organisms can be attributed to the presence of flavonoids. The wide distribution and the relatively large amounts of flavonoids in plants provide arguments for their systemic effects. In addition, because little is known about the fate of flavonoids, it is not possible to designate any effect observed after use of natural products to flavonoids or their metabolites. In recent years many efforts have been made to rationalize the bioactivity and the importance of flavonoids (Middleton & Kandaswami 1994). It is obvious that the more data that are available the better will be the understanding of the in-vivo activity of the flavonoids.

One activity recently discovered is the influence on the actions of adenosine (Okajima et al 1994; Ji et al 1996; Hasrat et al 1997a). Besides interactions of flavonoids with adenosine deaminase and tyrosine kinase, direct interaction with adenosine receptors has been reported (Koch et al 1992; Okajima et al 1994). Moreover, other activities of flavonoids such as the scavenging property and calcium-complex formation (Middleton & Kandaswami 1994) might contribute to the influence of flavonoids on the actions of adenosine. The cardiovascular effects of flavonoids might in part be explained by these properties.

One application in traditional medicine that provides evidence for the influence of flavonoids on adenosine receptors is the use of extracts of *Microtea debilis* against proteinuria. There are enough data available to establish the role of adenosine in renal processes. It has been established that adenosine can induce many effects in the kidney through interaction with several adenosine receptors (McCoy et al 1993). More-

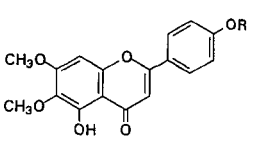
Correspondence: A. J Vlietinck, Department of Pharmaceutical Sciences, University of Antwerp, Universiteitsplein 1, B-2610, Antwerp, Belgium.

over, adenosine-A_1 antagonists have been shown to attenuate the effects of adenosine in acute renal failure (Schnermann et al 1990; Knight et al 1991; Mizumoto et al 1993; Schnermann & Briggs 1993; Osswald et al 1995). The effects of adenosine on the kidney, reduction of glomerular filtration rate and filtration fraction, imply that adenosine might cause proteinuria. The proteinuria (myoglobinuria), as in rhabdomyolysis, against which adenosine antagonists are effective precedes acute renal failure, in which process adenosine plays an essential role.

Because *Microtea debilis* preparations are used against proteinuria in traditional medicine in Surinam (Hasrat et al 1997b), extracts of the plant were screened on different receptors and shown to interact potently with adenosine receptors (Hasrat et al 1997b). The flavonoid cirsimarin was isolated as the active component inhibiting the binding of [^{3}H]DPCPX to the adenosine-A_1 receptors in rat forebrain (Hasrat et al 1997a). Subsequently it was shown that cirsimarin has adenosine antagonistic properties at the adenosine-A_1 receptor (Hasrat et al 1997a). These findings might yet explain the use of *Microtea debilis* preparations in proteinuria. However, because cirsimarin is a glycoside, it is unlikely it will be absorbed after oral administration, as was found for other flavonoid glycosides (Formica & Regelson 1995; Fuhr & Kummert 1995). Therefore, an explanation of the efficacy of cirsimarin against proteinuria might be found in the formation of active metabolites, one of which could be its aglycone, cirsimaritin (Fig. 1). For this reason the presence of both cirsimarin and cirsimaritin was determined in urine and plasma after oral administration of the crude extract of *Microtea debilis* and cirsimarin. In addition, it was necessary to establish whether concentrations of both compounds in body fluids, especially in the kidney, were sufficient to inhibit the action of adenosine. It has been established that flavonoids are rapidly excreted, mostly as glucuronides in the urine, and so the possibility that these or other metabolites might produce adenosine receptor inhibitory activity cannot be excluded. Consequently, biological analyses were also performed on urine treated with β-glucuronidase. To assess whether there is any inhibition of adenosine receptors after administration of cirsimarin in ligand-binding-studies, the inhibition of the binding of [^{3}H]DPCPX to adenosine-A_1 receptors in rat forebrain membranes by collected urine samples was investigated.

Although, flavonoids showed no selectivity towards adenosine-receptor subtypes (Ji et al 1996; Karton et al 1996), the inhibition by cirsimarin of the binding of [^{3}H]5′-*N*-ethylcarboxamidoadenosine ([^{3}H]NECA) to adenosine-A_2 receptors in rat striatum was examined to establish the adenosine-A_2 receptor activity of cirsimarin. Moreover, cirsimarin or its metabolites might also influence the effects of adenosine by interaction with adenosine-A_2 receptors present in the kidney.

To confirm the observed in-vitro adenosine receptor activity of cirsimarin, in-vivo experiments were performed to determine the adenosine antagonistic activity of the product. The effects of adenosine on the cardiovascular system are reductions of heart rate and blood pressure via the adenosine-A_1 and adenosine-A_2 receptors, respectively (Mullane & Williams 1990; Barret et al 1992). Adenosine was therefore administered to anaesthetized rats under certain conditions immediately after administration of cirsimarin to measure the inhibition of the adenosine-induced decrease of blood pressure and heart rate.



R = H Cirsimaritin
R = glucosyl Cirsimarin

FIG. 1. The structures of cirsimarin and cirsimaritin.

Materials and Methods

R-N^6-(2-phenyl-1-methylethyl)adenosine, N^6-cyclopentyladenosine, adenosine and β-glucuronidase were purchased from Boehringer Mannheim (Germany), isoproteronol bitartrate from Sigma, atenolol from ICN and genistein from Roth (Germany); [^{3}H]NECA (36 Ci mmol^{-1}) and [^{3}H]DPCPX (107 Ci mmol^{-1}) were from Amersham, UK.

Chromatography was performed with two model 303 pumps, an 802 C manometric module, a model 811 dynamic mixer and a model 621 data module, all from Gilson (Middleton, USA), a Rainin Instruments Company (USA) Dynamax model UV-1 absorbance detector and a Rheodyne (USA) Model 7125 injector. The chromatographic system was controlled by means of a Gilson 714 V1.1 HPLC System Controller.

Inhibition of the binding of [^{3}H]5′-N-ethylcarboxamidoadenosine ([^{3}H]NECA) to adenosine-A_2 receptors

Rat striata dissected by a neuroanatomist were used in these experiments. Membranes from the striata were prepared by homogenizing the tissue in Tris-Mg buffer (a mixture of Tris-HCl (50 mM; pH 7·7) and $MgCl_2$ (10 mM)) with an Ultra Turrax for 15 s. The homogenate was centrifuged at 30 000 *g* for 10 min and the pellet then re-suspended in Tris-Mg buffer with an Ultra Turrax for 15 s and then centrifuged once again at 30 000 *g* for 10 min. The pellet was then washed twice (by re-suspension and centrifugation as above) with Tris-Mg buffer and the final pellet re-suspended in Tris-Mg buffer and incubated for 30 min with 2 units mL^{-1} adenosine deaminase at 37°C. Membrane suspensions were frozen in liquid nitrogen and stored at −80°C until used.

Protein concentrations were determined as described previously (Hasrat et al 1997a).

Ligand-binding adenosine-A_2 assays were performed according to Bruns et al (1986) with minor modifications. Briefly, rat striatum membranes were incubated for 60 min at 25°C with 4 nM [^{3}H]NECA and competitor in a final volume of 200 μL Tris HCl (50 mM; pH 7·4). At the end of the incubation the samples were filtered under reduced pressure through a glass-fibre filter and rapidly washed with ice-cold buffer. Specific binding to the A_2 receptors was calculated by subtracting non-specific binding, obtained in the presence of 0·1 mM N^6-cyclopentyladenosine, from total binding.

The amount of radioligand remaining on the filters was determined by liquid-scintillation counting.

Radioligand-inhibition curve

Radioligand-inhibition curves were obtained by the use of increasing doses of cirsimarin. The inhibition constant (K_i)

value was calculated according to Cheng & Prusoff (1973).

Dimethylsulphoxide (DMSO) was used to increase the solubility of the compounds. The DMSO concentration did not exceed 1% in the test tube.

In-vivo experiments: antagonism of cardiovascular effects of adenosine

Preparation of animals. Two Wistar rats, 294 *g* (female) and 276 *g* (male), were anaesthetized with sodium pentobarbital (60 mg kg^{-1}, i.p.); anaesthesia was maintained by bolus administration (3 mg kg^{-1}, i.v.) when required. A tracheotomy was performed and a tracheal tube inserted, through which the animals breathed room air spontaneously. Catheters containing heparinized (50 units mL^{-1}) saline were inserted into the right carotid artery and the right external jugular vein for blood-pressure measurement and intravenous administration of drugs, respectively. The arterial catheter was connected to a Gold P23 ID pressure transducer and mean arterial blood pressure was measured directly. The heart rate was captured from the blood pressure. The cervical vagal nerves were isolated and bilaterally sectioned. The recording of heart rate and blood pressure was started when both were stable.

Experimental protocol. Section of the vagal nerves was followed by a period of stabilization. After one administration the next was performed when both heart rate and blood pressure returned to approximately baseline values and after a period of stabilization. First isoproterenol (0·07 nMol i.v.) was administered, then atenolol (37·5 μmol kg^{-1}; i.v.) followed by the same dose of isoproterenol. Hereafter, in every rat adenosine (360 μg) was administered three times, followed by administration of cirsimarin (3 mg) plus adenosine (360 μg). The volume administered did not exceed 200 μL. Except for cirsimarin, which was dissolved in 15% DMSO in 0·1 M NaOH, the drugs were dissolved in sterile saline.

Determination of cirsimarin and cirsimaritin. In the first experiment cirsimarin (20 mg in 1 mL 15% DMSO and 0·1 M NaOH) or vehicle (1 mL 15% DMSO and 0·1 M NaOH) was administered into the stomach via the mouth by use of a syringe. Rats were individually housed in metabolic cages for the collection of urine and faeces for four successive days.

In the second experiment cirsimarin (2 mg in 1 mL 5% DMSO and 0·1 M NaOH), crude extract of *Microtea debilis* (1 mL in water, containing approximately 2 mg cirsimarin and 0·7 mg cirsimaritin) or cirsimaritin (1·7 mg in 1 mL 15% DMSO and 0·1 M NaOH) were administered as described in the first experiment. Rats were individually housed in metabolic cages for the collection of body specimens. Two and five hours after administration of 2 mg cirsimarin, urine and blood (renal; after an abdominal midline incision under diethyl ether anaesthesia) were collected from three rats on each occasion. The stomach and the small and large intestines were dissected and the faeces present were collected. Twelve hours after receiving cirsimarin, crude extract of *Microtea debilis* or cirsimaritin urine, blood and faeces were collected from two rats for each administration.

Quantification and measurement of cirsimarin and cirsimaritin. Detection of cirsimarin and cirsimaritin was performed in the crude extract of *Microtea debilis*, in urine, in urine after pre-treatment with β-glucuronidase, and in plasma, faeces, stomach, and small and large intestine.

Crude extract of *Microtea debilis* was prepared by adding the air-dried whole plant (5 *g*) to boiling water, boiling 80% ethanol and room temperature 80% ethanol for 30 min. Urine samples were prepared both by evaporation of the water to dryness under reduced pressure at 40°C and re-dissolving the residue in the same volume of methanol, and by extraction with ethyl acetate. Plasma samples were prepared by extraction with ethyl acetate. Faeces and the contents of the stomach, and small and large intestine were prepared by suspension in hot methanol.

Urine was pre-treated for 24 h at room temperature with an equal volume of a solution of β-glucuronidase in 1 M NaH_2PO_4 (pH 5) with an enzyme activity of 20 000 units mL^{-1}.

Calibration curves were constructed by dissolving appropriate amounts of cirsimarin and cirsimaritin in both methanol and blank samples. For measurement of concentration by HPLC, 50 μL of redissolved sample was injected on to a reversed-phase semi-preparative Nucleosil-100 AB 7-μm column which was eluted with a gradient from 10 to 70% methanol. Detection was performed at 330 nM. Typical chromatograms are shown in Fig. 2. A linear correlation between detector response and concentration was observed between 0·1 μM and 370 μM for cirsimarin and cirsimaritin dissolved in methanol and for cirsimaritin dissolved in urine samples

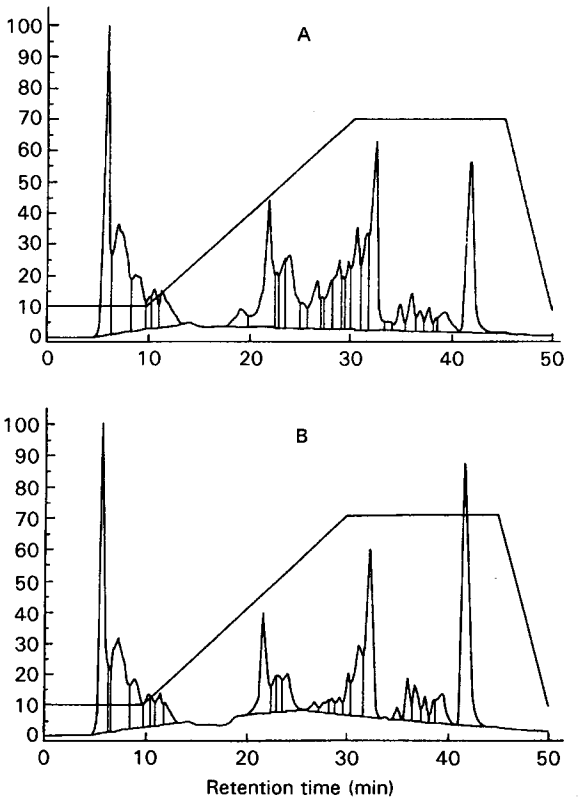


FIG. 2. HPLC determination of cirsimarin and cirsimaritin in urine samples (A) without and (B) with β-glucuronidase treatment. Cirsimarin retention time 41·4 min; cirsimarin glucuronides retention times 25 to 32 min.

($r > 0.99$ for all calibration curves). The minimum concentration detected was 30 nM.

Total amount of cirsimaritin excreted in the urine. The total amount of cirsimaritin excreted in the urine was determined as the sum of the calculated amount in the urine during the four successive days of the experiment described above after oral administration of 80 mg kg^{-1} cirsimarin.

Inhibition of the binding of [^{3}H]DPCPX by urine samples. Ligand-binding studies were performed as described elsewhere (Hasrat et al 1997a). Where possible, two concentrations of each urine sample were used in the ligand-binding studies. The first concentration was that of the unModified urine and the second was prepared by concentrating the collected urine threefold. Methanol and ethyl acetate fractions were subsequently treated with β-glucuronidase.

Results

Inhibition of the binding of [^{3}H]5′-N-ethylcarboxamidoadenosine ([^{3}H]NECA) to adenosine-A_2 receptors

Cirsimarin interacts with adenosine-A_2 receptors, because the product inhibited the binding of [^{3}H]NECA to adenosine-A_2 receptors in striata (Fig. 3). In these experiments the IC50s of cirsimarin and genistein, a flavonoid with adenosine antagonistic properties (Okajima et al 1994), were 6.5 ± 0.3 and 11 ± 0.7 μM, respectively.

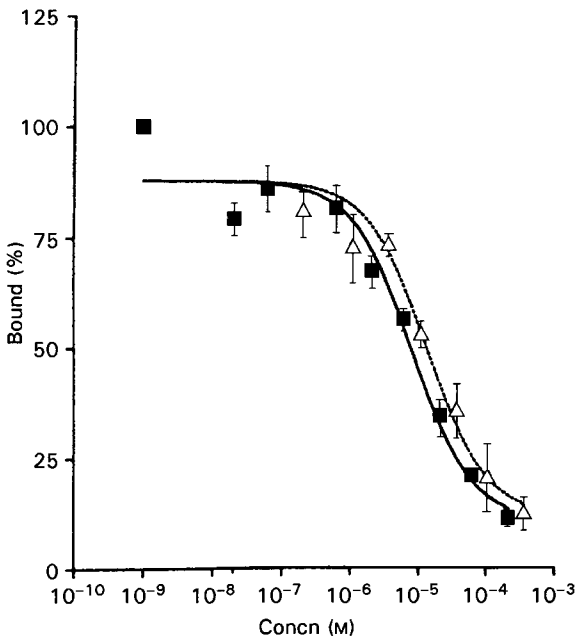


FIG. 3. Adenosine-A_2 activity of cirsimarin. The radioligand-inhibition curves of cirsimarin (■) and genistein (△), a flavonoid with established adenosine-A_2 activity, were obtained by incubation of rat striatum membranes for 60 min with [^{3}H]5′-*N*-ethylcarboxamidoadenosine and increasing concentrations of the compounds at 25°C in a mixture of 50 mM Tris HCl (pH 7.4) and 10 mmol $MgCl_2$; nonspecific binding was measured in the presence of 0.1 mM N^6-cyclopentyladenosine. K_i was calculated according to the method of Cheng & Prusoff (1973).

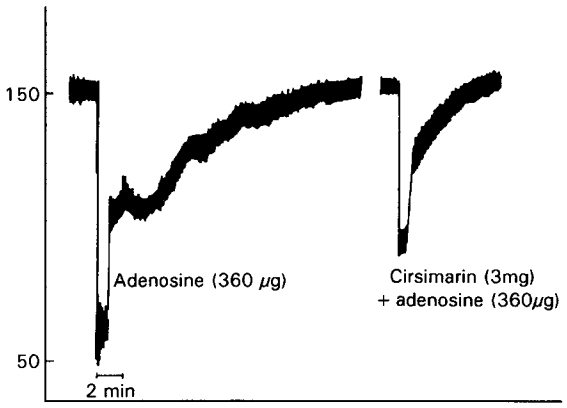


FIG. 4. Reduction by cirsimarin of the blood pressure decrease induced by adenosine. The decrease in blood pressure induced by adenosine, administered immediately after cirsimarin, was significantly inhibited and the blood pressure returned more quickly to baseline values with the absence of the slight secondary decrease.

Antagonism of cardiovascular effects of adenosine

Determination of the antagonistic action of cirsimarin on the binding of adenosine to adenosine receptors in-vivo showed that both blood-pressure and heart-rate effects of adenosine were reduced, indicating interaction of cirsimarin with adenosine-A_2 and -A_1 receptors respectively (Fig. 4). In both rats the effect of adenosine on blood pressure was characterized by a rapid, short decrease followed by a rapid increase, then a slight decrease and finally a slower increase to baseline values. Administration of cirsimarin immediately before that of adenosine resulted in a significant reduction of the decrease in blood pressure and heart rate induced by adenosine (Fig. 4 and Table 1). The influence of cirsimarin showed typical features. When the blood pressure returned to the starting value, the slight decrease was not observed and the increase thereafter was much faster.

Determination of cirsimarin and cirsimaritin

The results of the determination of plasma concentrations of cirsimarin and cirsimaritin showed that cirsimarin was never detectable whereas cirsimaritin plasma concentrations 2, 5 and 12 h after administration of 8 mg kg^{-1} cirsimarin were 0.126 ± 0.04, 0.138 ± 0.015 and 0.120 ± 0.022 μM, respectively. Cirsimarin was also not detectable in the urine. The recorded HPLC retention times of cirsimarin and cirsimaritin dissolved in methanol were 34.7 ± 0.1 and 41.4 ± 0.1 min,

Table 1. Antagonism by cirsimarin of the cardiovascular effects of adenosine.

	Blood pressure (mm Hg)	Heart rate (beats min^{-1})
Adenosine (360 μg)	102 ± 18	180 ± 45
Cirsimarin (3 mg) + adenosine (360 μg)	57 ± 6*	84 ± 16*

Rats under ether anaesthezia, vagotomized and treated with atenolol (37.5 μmol kg^{-1}; i.v.), received intravenous adenosine and cirsimarin followed immediately by administration of adenosine. In each rat this procedure was repeated three times. The values, mean ± s.e. are the results from experiments on two rats. Statistical analysis: Student's *t*-test, paired two samples for means. *$P < 0.05$, significantly different compared with result for adenosine alone.

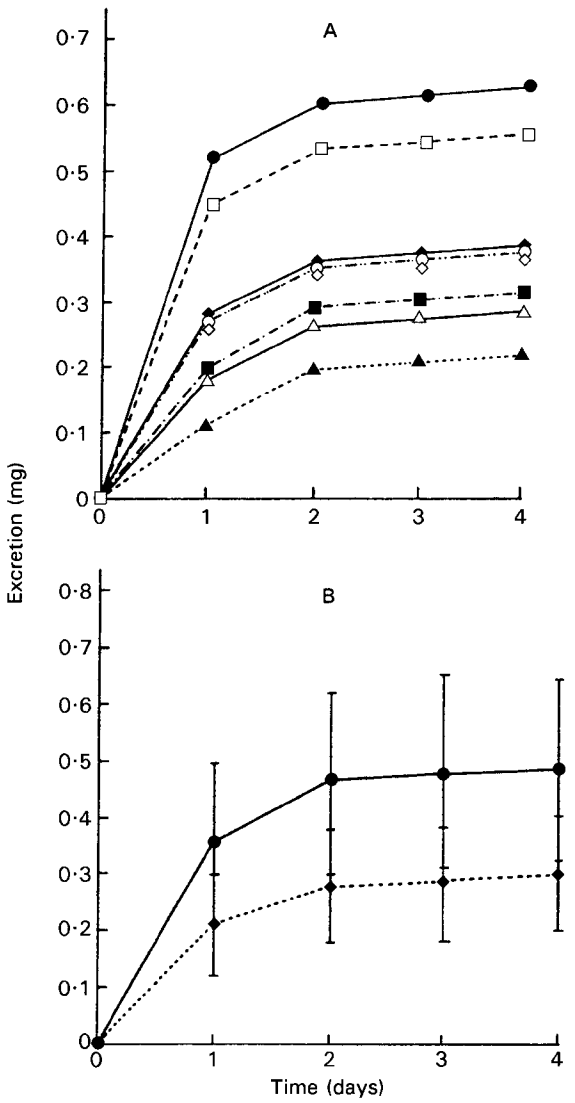


FIG. 5. The amount of cirsimaritin excreted in the urine of individual rats after administration of 80 mg kg^{-1} cirsimarin. Urine was collected for four successive days and samples (filled symbols) were treated with β-glucuronidase. A. Individual data; B. Mean ± s.e.

respectively. After HPLC of urine of untreated and treated rats, a compound with the retention time of cirsimarin was detected, whereas no compounds were recorded with the retention time of cirsimaritin. Although treatment of urine with β-glucuronidase did not change the amount of compound with the same retention time as cirsimarin, the amount of cirsimaritin recorded changed significantly (Fig. 2). The concentrations of cirsimaritin in the urine 2, 5 and 12 h after oral administration of 8 mg kg^{-1} cirsimarin were 2·05 ± 1·86, 5·05 ± 2·6 and 2·06 ± 0·09 μM respectively.

Of the administered cirsimarin (80 mg kg^{-1}) approximately 3% only could be recovered from the urine as cirsimaritin (Fig. 5). In distinct urine samples collected until two days after administration of cirsimarin, the mean cirsimaritin concentrations in urine samples treated with β-glucuronidase were not significantly different from those in untreated urine samples.

Inhibition of the binding of [^{3}H]DPCPX by urine samples

Experiments performed to determine the influence of urine

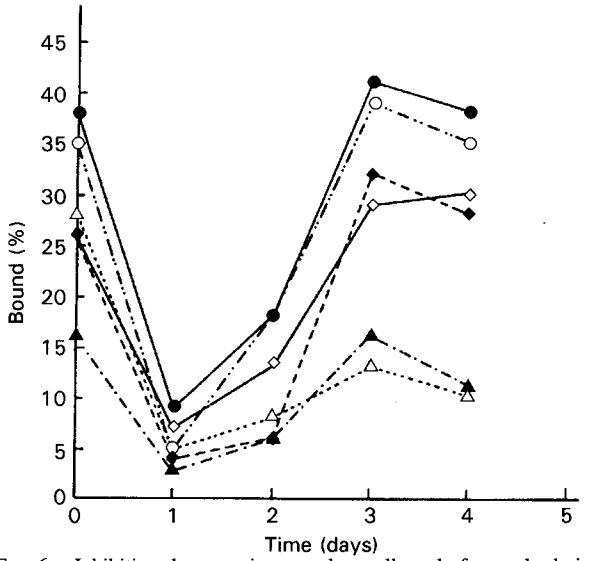


FIG. 6. Inhibition, by rat urine samples, collected after oral administration of 80 mg kg^{-1} cirsimarin, of the binding of [^{3}H]1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) to the adenosine-A_1 receptor in rat forebrain membranes. Where possible two concentrations of each urine sample was used. The first concentration (◇, ◆) was the unmodified urine; the second concentration (△, ▲, ○, ●) was prepared by threefold concentration of the urine. Methanol (○, ●) and ethyl acetate (◇, ◆, △, ▲) fractions were subsequently treated with β-glucuronidase. The methanol and ethyl acetate fractions were not tested in the same experiments. The mean values of results from two experiments (both in triplicate), expressed as the percentage of the radioligand still bound to the receptor, are plotted. (The mean values ± s.e. are listed in Table 2.)

samples, collected after administration of 80 mg kg^{-1} cirsimarin, on the binding of [^{3}H]DPCPX to adenosine-A_1 receptors in rat forebrain membranes showed that a significant increase of inhibition occurred (Fig. 6 and Table 2). The inhibition of binding of [^{3}H]DPCPX to adenosine-A_1 receptors by urine samples collected after administration of 8 mg kg^{-1} cirsimarin, a crude extract of *Microtea debilis*, and 6·8 mg kg^{-1} cirsimaritin is shown in Table 3. The increased inhibition measured for urine samples treated with β-glucuronidase was not significant.

Discussion

The inhibition by cirsimarin of the binding of [^{3}H]NECA to adenosine-A_2 receptors in rat striatum indicates interaction with these receptors with an affinity (6·5 μM) slightly less than that for the adenosine-A_1 receptor (3·2 μM). Adenosine-A_2 receptors are present in the kidney and mediate many of the effects of adenosine, for example vasodilation of the postglomerular arterioles and calcium re-absorption (Edlund & Sollevi 1993; McCoy et al 1993); this might be important in the pathophysiology of acute renal failure. Adenosine antagonists at adenosine-A_2 receptors might attenuate the noxious effects of adenosine in acute renal failure.

The in-vivo attenuation by cirsimarin of adenosine-induced decreases of blood pressure and heart rate provided evidence that cirsimarin can have adenosine-antagonistic effects after absorption. However, after oral administration of cirsimarin and crude extract of *Microtea debilis*, cirsimarin was not detected in plasma or in urine. It has already been reported that flavonoid glycosides cannot be detected after oral administra-

Table 2. Inhibition, by rat urine samples, collected after oral administration of 80 mg kg^{-1} cirsimarin, of the binding of [^{3}H]1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX) to rat forebrain membranes.

Procedure	Day 0	Day 1	Day 2	Day 3	Day 4
Ethyl acetate extract of dilute urine without β-glucuronidase treatment	26±8	7±0	13±2	29±7	30±0
Ethyl acetate extract of dilute urine with β-glucuronidase treatment	26±10	4±1	6±1	32±1	28±2
Ethyl acetate extract of concentrated urine without β-glucuronidase treatment	28±6	5±0	8±0	13±2	10±0
Ethyl acetate extract of concentrated urine with β-glucuronidase treatment	16±1	3±0	6±1	16±0	11±0
Methanol extract of concentrated urine without β-glucuronidase treatment	35±3	9±1	18±2	39±3	38±2
Methanol extract of concentrated urine with β-glucuronidase treatment	38±2	5±1	18±2	41±3	35±2

Membranes were incubated with 0·4 nM [^{3}H]DPCPX and the urine preparation at 25°C in 50 mM Tris HCl (pH 7·4); non-specific binding was measured in the presence of 5 μM *R*-N^6-(2-phenyl-1-methylethyl)adenosine. Urine was collected for four successive days and urine samples were either dried and redissolved in methanol or extracted with ethyl acetate. Where possible two concentrations of each urine sample were used, unmodified urine and urine concentrated threefold. Methanol and ethyl acetate fractions were subsequently treated with β-glucuronidase. The methanol and ethyl acetate fractions were not tested in the same experiments. The values are means ± s.e. of results from two experiments (both performed in triplicate) and are expressed as the percentage of the radioligand still bound to the receptor.

Table 3. Inhibition, by rat urine samples collected after oral administration of cirsimarin (8 mg kg^{-1}), crude extract (8 mg kg^{-1}) or cirsimaritin (6·8 mg kg^{-1}), of the binding of [^{3}H]1,3-dipropyl-8-cyclopentylxanthine to rat forebrain membranes.

	Cirsimarin					
	0 h	2 h	5 h	12 h	Crude extract 12 h	Cirsimaritin 12 h
Ethyl acetate extract of dilute urine without β-glucuronidase treatment	26±8	28±2	29±9	24±1	–	–
Ethyl acetate extract of dilute urine with β-glucuronidase treatment	26±10	18±1	17±5	20±4	–	–
Ethyl acetate extract of concentrated urine without β-glucuronidase treatment	28±6	–	–	39±2	31±3	28±7
Ethyl acetate extract of concentrated urine with β-glucuronidase treatment	16±1	–	–	11±1	12±3	10±2

Urine, collected 2, 5 and 12 h after administration of cirsimarin, and 12 h after administration of crude extract or cirsimaritin, was extracted with ethyl acetate. Where possible two concentrations of each urine sample were used, unmodified urine and urine concentrated threefold. Ethyl acetate fractions were subsequently treated with β-glucuronidase. The values are the mean ± s.e. of results from two experiments (both performed in triplicate) and are expressed as the percentage of the radioligand still bound to the receptor.

tion (Formica & Regelson 1995; Fuhr & Kummert 1995), and that only the aglycones can be absorbed; these are then rapidly metabolized in the liver. Determination of cirsimaritin, the aglycone of cirsimarin, has shown that it was present in plasma and urine. However, the plasma concentrations of cirsimaritin obtained after administration of a low dose of cirsimarin (8 mg kg^{-1}) were not high enough ($<10^{-6}$ μM) to produce systemic effects, because to produce adenosine antagonistic effects a much higher concentration of cirsimaritin ($>5\times10^{-6}$ μM) is needed. Of the cirsimarin ingested only 3–5% was excreted in the urine as cirsimaritin or glucuronides of cirsimaritin; similar results have been obtained for other flavonoids (Fuhr & Kummert 1995).

The conversion of cirsimarin to cirsimaritin is assumed to take place in the gastrointestinal tract, as was found for other glycosides (Formica & Regelson 1995; Fuhr & Kummert 1995). Bacteria in the intestine might be responsible for this conversion, although in a pilot study (results not shown) it was observed that after oral administration of cirsimarin cirsimaritin and cirsimarin were detected in the stomach 2 h later. Therefore, it seems that the conversion of cirsimarin into cirsimaritin occurs even in the stomach.

Although, with a low dose of cirsimarin effective concentrations might not occur in the plasma, it is possible that during passage of the filtrate in the nephron higher concentrations might build up in the kidney, resulting in adenosine-antagonistic effects, especially when higher doses are used. The inhibition of the binding of [^{3}H]DPCPX to the adenosine-A_1 receptor by urine samples collected after administration of a high dose of cirsimarin indeed indicated that adenosine-antagonistic effects can occur in the kidney.

The ethyl acetate and methanol extracts of urine of untreated rats induced relatively high inhibition of the binding of

[^{3}H]DPCPX to the adenosine-A_1 receptor. This inhibition should not be ascribed to excreted adenosine, because the incubation was performed in the presence of adenosine deaminase.

In summary, these results demonstrate that cirsimarin is not absorbed from the gastrointestinal tract and that it must first be converted to cirsimaritin to produce systemic effects. It is, however, hard to envisage the occurrence of adenosine-antagonistic effects with preparations containing low amounts of this flavonoid, i.e. amounts comparable with the lower dose used in this study.

It might still be argued that for several reasons extracts of *Microtea debilis* containing these amounts of cirsimarin might show adenosine antagonistic effects in the kidney and consequently against (some types of) proteinuria. Because flavonoids generally show no adenosine-receptor subtype selectivity and, moreover, cirsimaritin is active at both adenosine-A_1 and -A_2 receptors (Ji et al 1996), in the kidney adenosine-mediated tubuloglomerular feedback and vasodilation of efferent arteries might be inhibited. In addition, interaction of cirsimaritin with both receptors might lead to inhibition of adenosine-induced sodium and calcium transport, respectively, both of which might subsequently attenuate the tubuloglomerular feedback response (Schnermann et al 1990; Knight et al 1991; Edlund & Sollevi 1993; McCoy et al 1993; Schnermann & Briggs 1993; Haysleft et al 1995). Because both calcium and adenosine are involved in acute renal failure (Deray et al 1990), cirsimaritin might have a beneficial effect on this disorder. Moreover, cirsimaritin–calcium complex formation might further attenuate the effects of calcium.

In conclusion, potentiation of adenosine-antagonistic effects might occur through these distinct mechanisms of action of cirsimaritin, thus explaining the effectiveness of *Microtea debilis* preparations against proteinuria.

Acknowledgements

J. A. Hasrat is a recipient of a grant of the Algemeen Bestuur voor Ontwikkelings-samenwerking (ABOS, Belgium). G. Vauquelin is Research Director and T. De Bruyne is a post-doctoral Researcher of the Fund for Scientific Research, Flanders-Belgium. This work was supported by grants from the Flemish Community (Belgium).

References

Barret, R., Droppelman, D. A., Wright, K. F. (1992) N-0861 selectively antagonizes adenosine-A_1 receptors in-vivo. Eur. J. Pharm. 216: 9–16

Bruns, R. F., Lu, G. H., Pugsley, T. A. (1986) Characterization of the A_2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membranes. Mol. Pharmacol. 29: 331–346

Cheng, Y. C., Prusoff, W. H. (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 22: 3099–3108

Deray, G., Martinez, F., Cacoub, P., Baumelou, B., Jacobs, C. (1990) A role for calcium and ischemia in radiocontrast-induced intrarenal vasoconstriction. Am. J. Nephrol. 10: 316–322

Edlund, A., Sollevi, A. (1993) Renal effects of i.v. adenosine infusion in humans. Clin. Phys. 13: 361–371

Formica, J. V., Regelson, W. (1995) Review of the biology of quercetin and related bioflavonoids. Food Chem. Toxicol. 33: 1061–1080

Fuhr, U., Kummert, A. L. (1995) The fate of naringin in humans: a key to grapefruit juice-drug interactions? Clin. Pharm. Ther. 58: 365–373

Hasrat, J. A., Pieters, L., Claeys, M., Vlietinck, A. J., De Backer, J.-P., Vauquelin, G. (1997a) Adenosine-1 active ligands: cirsimarin, a flavone glycoside from *Microtea debilis*. J. Nat. Prod., 60: 638–641

Hasrat, J. A., Vlietinck, A. J., De Backer, J.-P., Vauquelin, G. (1997b) Medicinal plants in Surinam: screening of plant extracts for receptor binding activity, part 1. Phytomedicine, 4: 59–65

Haysleft, J. P., Macala, L. J., Smallwood, J. I., Kalghatgi, L., Gasalla-Herraiz, J., Isales, C. (1995) Adenosine stimulation of Na^+ transport is mediated by an A_1 receptor and a $[Ca^{2+}]_i$-dependent mechanism. Kidney Int. 47: 1576–1584

Ji, X.-D., Melman, N., Jacobson, K. A. (1996) Interactions of flavonoids and other phytochemicals with adenosine receptors. J. Med. Chem. 39: 781–788

Karton, Y., Jiang, J.-L., Ji, X.-D., Melman, N., Olah, M. E., Stiles, G., Jacobson, K. A. (1996) Synthesis and biological activities of flavonoid derivatives as A_3 adenosine receptor antagonists. J. Med. Chem. 39: 2293–2301

Knight, R. J., Collis, M. G., Yates, M. S., Bowmer, C. J. (1991) Amelioration of cisplatin-induced acute renal failure with 8-cyclopentyl-1,3-dipropylxanthine. Br. J. Pharmacol. 104: 1062–1068

Koch, H. P., Jäger, W., Groh, U., Plank, G. (1992) In-vitro inhibition of adenosine deaminase by flavonoids and related compounds. New insights into the mechanism of action of plant phenolics. Methods Find. Exp. Clin. Pharmacol. 14: 413–417

McCoy, D. E., Bhattacharya, S., Olson, B. A., Levier, D. G., Arend, L. J., Spielman, W. S. (1993) The renal adenosine system: structure, function, and regulation. Semin. Nephrol. 13: 31–40

Middleton, E. J. R, Kandaswami, C. (1994) The impact of plant flavonoids on mammalian biology: implications for immunity, inflammation and cancer. In: Harborne, J. B. (ed.) The Flavonoids, Advances in Research since 1986. Chapman & Hall, London, Chapter 15

Mizumoto, H., Karasawa, A., Kubo, K. (1993) Diuretic and renal protective effects of 8-(noradamantan-3-yl)-1,3-dipropylxanthine (KW-3902), a novel adenosine-A_1 receptor antagonist, via pertussis toxin insensitive mechanism. J. Pharmacol. Exp. Ther. 266: 200–206

Mullane, K. M., Williams, M. (1990) Adenosine and cardiovascular function. In: Williams, M. (ed.) Adenosine and Adenosine Receptors. Humana Press, Chapter 8

Okajima, F., Akbar, M., Majid, M. A., Sho, K., Tomura, H., Kondo, Y. (1994) Genistein, an inhibitor of protein tyrosine kinase, is also a competitive antagonist for P_1-purinergic (adenosine) receptor in FTRL-5 thyroid cells. Biochem. Biophys. Res. Commun. 203: 1488–1495

Osswald, H., Gleiter, Ch., Mühlbauer, B. (1995) Therapeutic use of theophylline to antagonize renal effects of adenosine. Clin. Nephrol. 43 (Suppl.): S33–S37

Schnermann, J., Briggs, J. P. (1993) The role of adenosine in cell-to-cell signaling in the juxtaglomerular apparatus. Semin. Nephrol. 13: 236–245

Schnermann, J., Weihprecht, H., Briggs, J. P. (1990) Inhibition of tubuloglomerular feedback during adenosine$_1$ receptor blockade. Am. J. Physiol. 258: F553–F561