Humboldt-Universität zu Berlin, Institut für Pharmazie, Germany

Inhibition of metallopeptidases by flavonoids and related compounds

H. BORMANN and M. F. MELZIG

To elucidate possible mechanisms of activity in medicinal plants containing flavonoids, the inhibitory potency of twenty flavones, flavonols, flavanones, phenylacrylic acids and various hydroxylated phenylacetic acids on the activity of neutral endopeptidase (NEP; EC 3.4.24.11), angiotensin-converting enzyme (ACE; EC 3.4.15.1) and aminopeptidase N (APN; EC 3.4.11.2) was investigated *in vitro*. The screening generally resulted that inhibition of these enzymes requires free hydroxyl groups at the flavone molecule. Flavone and methoxylated compounds (sinensetin) were without effects. Flavonoids with free hydroxyl functions in position 3′, 4′ and 5,7 inhibited the activity of NEP (quercetin, luteolin, fisetin), with myricetin (IC_{50} = 42 μM) as strongest inhibitor. Inhibition of ACE and APN did not depend on this class of compounds and substitution pattern. E.g. 3,4-dihydroxyphenylacetic acid and 4-methylcatechol (urinary metabolites of flavonoids) also inhibited both APN and ACE activity, but not NEP activity. The results demonstrate that some of the pharmacological activities of flavonoids might be related to the inhibition of metallopeptidases responsible for the splitting of regulatory neuropeptides.

1. Introduction

Flavonoids are phenolic compounds occurring ubiquitously in vascular plants. They show a broad spectrum of pharmacological effects. A variety of medicinal plants are therapeutically used because of their anti-inflammatory, anticarcinogenic, spasmolytic, vessel stabilizing or diuretic actions. Certain flavonoids are known to interfere with several enzymes, such as adenosine deaminase, cAMP phosphodiesterase, trypsin or cytosolic aminopeptidase (EC 3.4.11.1) [1]. We carried out a screening of flavonoids, phenylacrylic acids and various hydroxylated phenylacetic acids, urinary metabolites of flavonoids [2], against three metallopeptidases, to elucidate the mechanism of activity *in vitro*. Neutral endopeptidase (NEP; EC 3.4.24.11), angiotensin-converting enzyme (ACE; EC 3.4.15.1) and aminopeptidase N (APN; EC 3.4.11.2) are located at the outer membrane of different cells (ectoenzymes) and contain zinc as a cofactor. NEP inactivates a variety of renal- and CNS-active peptides (substance P, bradykinin, enkephalins, atrial natriuretic factor). Inhibitors of NEP should be useful in the treatment of pain with a spectrum of activities similar to that of opioid analgesics [3]. On the other hand, NEP inhibitors protect the endogenous atrial natriuretic factor from degradation and thereby enhance the typical renal effects of ANF on diuresis and natriuretic response [4]. Inhibitors of ACE are used as established antihypertensive drugs [5]. Recently dual ACE/NEP inhibitors were developed and investigated for the treatment of severe hypertension and chronic heart failure [6]. In contrast to NEP and ACE, rather specifically acting enzymes, APN is a α-aminoacylpeptide-hydrolase with a low substrate specificity. APN is important be included into this investigation, because this enzyme is involved in metastasis and immuno-modulating activities [7, 8].

2. Investigations and results

2.1. NEP

Tables 1–3 demonstrate that flavonoids have a different inhibitory potency on NEP depending on their chemical structure. An IC_{50} could only be calculated for luteolin (127 μM), quercetin (192 μM), fisetin (220 μM) and myricetin (42 μM) (Fig.). Common characteristics are free hydroxyl functions in position 3′, 4′ and 7. Neighboring hydroxyl functions in 4′, 5′ instead of 3′, 4′ decrease the inhibitory potency (morin). This finding was supported by the properties of the substances with only one OH-function in the benzene ring (apigenin, kaempferol and naringenin with a maximum of inhibition in the range of 20–30%). Flavonoids without hydroxyl functions in position 3′ or 4′ (chrysin, hesperetin) did not show any effect in the highest available concentration of 300 μM. Inhibition of NEP requires a free OH-function in 7 position, because 7-O-glycosides (diosmin, naringin, hesperidin, eriocitrin) were without influence on NEP activity. 3-O-glycosides (hyperosid, quercitrin and rutin) were only weak inhibitors of NEP, although their aglykone quercetin is an effective inhibitor. The unsubstituted flavone molecule and the methoxylated sinensetin did not show any effect. Catechin, rosmarinic acid and other substances with two hydroxyl substituents on an aromatic ring (oleuropein, resveratrol), act as weak inhibitors. Catechin has the same hydroxylation pattern like quercetin or luteolin, but the absence of a double bond at position C-2 causes a different conformation. The various hydroxylated phenylacetic acids, metabolites of flavonoids, did not influenced NEP activity.

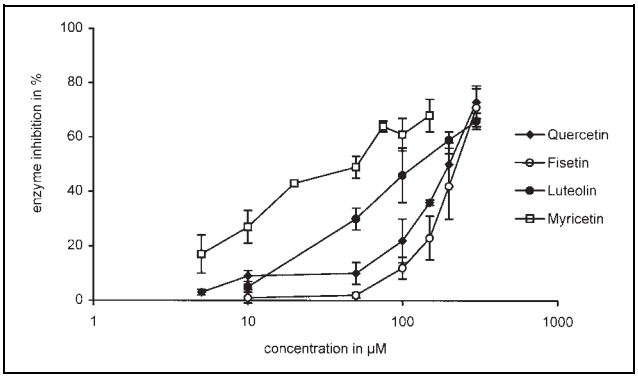
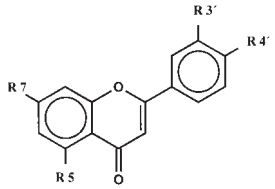


Fig. Inhibition of NEP by selected flavonoids

2.2. ACE

Tables 1–3 demonstrates the influence of the different flavonoids on ACE. No substance could be found, for which an IC_{50} value could be calculated within the maximal concentration of 300 μM. The inhibitors of NEP showed only a weak effect on this enzyme. Luteolin, diosmin, querci-

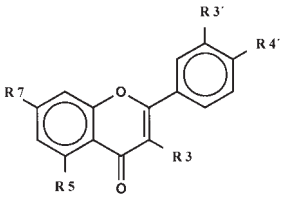
Table 1: Enzyme inhibition by flavones (at maximal concentration of 300 μM)



	Max. inhibition of			R5	R6	R7	R3′	R4′
	NEP	ACE	APN					
Apigenin	31% R = 6	18% R = 2	42% R = 21	OH		OH		OH
Acacetin	14% R = 2	16% R = 4	21% R = 2	OH		OH		OCH_3
Luteolin	66% R = 7	55% R = 28	10% R = 2	OH		OH	OH	OH
Homoorientin	n.i.	15% R = 3	n.i.	OH		OH	OH	OH 6-C-gluc
Baicalein	36% R = 11	10% R = 3	57% R = 6	OH	OH	OH		
Chrysin	n.i.	n.i.	49% R = 6	OH		OH		
Sinensetin	n.i.	n.i.	n.i.	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3
Diosmin	n.i.	44% R = 20	n.i.	OH		O-rut	OH	OCH_3
Vitexin	n.i.	n.i.	n.i.	OH		OH		OH 8-C-gluc
Diosmetin	n.i.	n.i.	45% R = 2	OH		OH	OH	OCH_3
Flavone	n.i.	n.i.	n.i.					

n.i.: no inhibition; R: range of min. and max. value in %

Table 2: Enzyme inhibition by flavonols (at maximal concentration of 300 μM)



	Max. inhibition of			R3	R5	R7	R3′	R4′	R5′
	NEP	ACE	APN						
Quercetin	73% R = 13	23% R = 5	14% R = 4	OH	OH	OH	OH	OH	
Hyperosid	24% R = 2	n.i.	n.i.	O-galac	OH	OH	OH	OH	
Quercitrin	22% R = 5	51% R = 17	n.i.	O-rham	OH	OH	OH	OH	
Rutin	25% R = 2	n.i.	n.i.	O-rut	OH	OH	OH	OH	
Fisetin	71% R = 15	23% R = 5	n.i.	OH		OH	OH	OH	
Kaempferol	21% R = 4	n.i.	12% R = 3	OH	OH	OH		OH	
Myricetin	68% R = 12	26% R = 11	48% R = 9	OH	OH	OH	OH	OH	OH
Rhamnetin	n.i.	23% R = 2	54% R = 5	OH	OH	OCH_3	OH	OH	
Isorhamnetin	n.i.	n.i.	49% R = 2	OH	OH	OH	OCH_3	OH	
Morin	33% R = 5	14% R = 2	18% R = 13	OH	OH	OH		OH	OH 2′OH

n.i.: no inhibition; R: range of min. and max. value in %

trin and naringin (inhibition approx. 45%) turned out as the most effective compounds. It is remarkable, that, except luteolin, the glycosides are more active than their aglykones (diosmetin, quercetin and naringenin). The urinary metabolites 3,4-dihydroxyphenylacetic acid and 4-methylcatechol were as effective as the flavonoids (inhibition 60 and 40%; 500 μM). Rosmarinic acid and caffeic acid showed a remarkable inhibitory potency on ACE (inhibition 54 and 60%; 500 μM).

2.3. APN

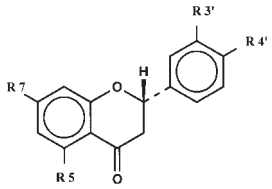
Quercetin, fisetin and luteolin, the inhibitors of NEP, were without effect on APN. More lipophilic compounds, however with less hydroxyl functions (apigenin, chrysin, baicalein) or methoxlyated substances (diosmetin, rhamnetin, isorhamnetin) showed APN inhibition (Tables 1–3). Myricetin with five hydroxyl functions (5, 7, 3′, 4′, 5′) is the only one among the flavonoids which inhibited all three peptidases. A direct relationship between flavonoid structure and inhibitory potency could not be found.

The higher solubility of the urinary metabolites allowed to test them in concentrations up to 1 mM. In this highest achievable concentration, 3,4-dihydroxyphenylacetic acid and 4-methyl-catechol inhibited more than 40% of the total enzyme activity.

3. Discussion

The effects of quercetin, fisetin, luteolin and myricetin on NEP seems to have pharmacological significance, especially when enriched in a tissue. The effects of flavonoids

Table 3: Enzyme inhibition by flavanones (at maximal concentration of 300 µM)



	Max. inhibition of			R5	R7	R3′	R4′
	NEP	ACE	APN				
Naringenin	20% R = 3	22% R = 2	16% R = 2	OH	OH		OH
Naringin	n.i.	37% R = 23	n.i.	OH	O-(2O-rham)-gluc		OH
Hesperetin	n.i.	18% R = 1	10% R = 3	OH	OH	OH	OCH_3
Hesperidin	n.i.	34% R = 2	n.i.	OH	O-rut	OH	OCH_3
Eriocitrin	n.i.	28% R = 7	n.i.	OH	O-rut	OH	OH

n.i.: no inhibition; R: range of min. and max. value in %

could not to be compared with the competetive NEP inhibitors phosphoramidon or thiorphan with IC_{50} values in the nanomolar range. Despite of that weak pharmacological effects of single plant constituents, the efficiency of various medicinal plant extracts could be caused by a synergism of flavonoids with other compounds in the plant. This opinion is emphasized by our results that two of the urinary metabolites showed an influence on ACE and APN. Phenylpropane derivatives occur together with flavonoids in plants extracts, so it can be suggested that e.g. inhibition of ACE by rosmarinic and caffeic acid contributes to the overall pharmacological or therapeutic effects. Results of a systematic screening of flavonoids and related compounds for the inhibition of ACE and NEP has not been published so far. Studies investigated ACE inhibition by single flavonoids e.g. astragalin and isoquercetin [9] or morin and amentoflavon [10] isolated from traditional medicinal drugs. Kameda et al. [9] showed that the effects of flavonol glucosides and their gallates on ACE activity might be due to the flavonol moiety. As a maximum, they found 67% inhibition by astragalin at 300 µg/ml (= 670 µM). Wagner et al. [10] found ACE inhibition by

Table 4: Enzyme inhibition by related compounds of flavonoids

	Enzyme inhibition		
	NEP	ACE	APN
Urinary metabolites			
3,4-Dihydroxyphenylacetic acid	n.i.	max. 60%; 500 µM R = 8	max. 42%; 1 mM R = 4
4-Hydroxyphenylacetic acid	n.i.	n.i.	n.i.
3-Methoxy-4-hydroxyphenyl acetic acid	n.i.	n.i.	n.i.
3-Hydroxyphenylacetic acid	n.i.	n.i.	n.i.
4-Methylcatechol	n.i.	max. 40%; 500 µM R = 3	max. 47%; 1 mM R = 2
Phenylpropane-derivatives			
Rosmarinic acid	max. 27%; 300 µM R = 3	max. 54%; 500 µM R = 2	n.i.
Caffeic acid	max. 14%; 500 µM R = 4	max. 65%; 500 µM R = 8	n.i.
Related compounds of flavonoid structure			
Ononin (isoflavone)	n.i.	n.i.	n.i.
Catechin	max. 21%; 300 µM R = 6	max. 40%; 300 µM R = 9	max. 16%; 300 µM R = 3
Other compounds with orthohydroxyfunctions			
Alizarin	max. 21%; 300 µM R = 3	max. 16%; 300 µM R = 2	max. 25%; 300 µM R = 5
Aesculetin	n.i.	n.i.	n.i.
Ellagic acid	max. 36%; 300 µM R = 5	max. 34%; 300 µM R = 2	n.i.
Oleuropein	max. 15%; 300 µM R = 2	max. 38%; 300 µM R = 9	n.i.
Resveratrol	max. 24%; 250 µM R = 4	n.i.	n.i.

n.i.: no inhibition; R: range of min. and max.. value in %

flavonoids and proanthocyanidins at concentrations of 0.33 mg/ml (ca. 1 mM). They investigated morin, as we did, in a other *in vitro* system and in a 3-fold concentration. So it is possible to explain the difference between our low value of 14% and their value of 64% inhibition of ACE activity. Meunier et al. [11] reported about ACE inhibition by proanthocyanidin oligomers. In their *in vitro* and *in vivo* tests only catechin as monomer produced enzyme inhibition, but in a range comparable to our findings. Parellada et al. [12] carried out studies of the effects of flavonoids on cytosolic aminopeptidase (EC 4.4.11.1), carboxypeptidase A (EC 3.4.17.1) and also on APN. They tested various flavonoids in a concentration of 100 µM and found a weak inhibition of APN, not higher than 35%. Their kinetic studies and experiments with addition of Zn^{2+} did not support interactions of flavonoids with the active site zinc of metalloenzymes.

In general, all inhibitory activities described are related to polyhydroxylated flavonoides. These interactions might be explained by the generationen of chelate complexes with the zinc atom within the active site or more possible by a formation of hydrogen bridges between the inhibitor molecule and aminoacids near or at the active site, especially of their ortho-dihydroxyl groups in the case of myricetin, quercetin, fisetin and luteolin.

The results demonstrate that some of the weak pharmacological activities of flavonoids combined with their urinary metabolites or associated phenylpropanes might be related to an inhibition of metallopeptidases responsible for splitting of regulatory neuropeptides, like angiotensins, ANP or bradykinin.

4. Experimental

4.1. Materials

Flavonoids were purchased from Carl Roth GmbH or Sigma Chemical Co. Phenylacetic acid derivatives were provided by Prof. P. G. Pietta, Milan (Italy). L-leucine-p-nitroanilide, Suc-L-Ala-L-Ala-L-Phe-7-amido-3-methylcoumarin (SAAP-AMC) and phosphoramidon were obtained from Sigma. Hip-L-His-L-Leu was purchased by Bachem. Lisinopril was a gift of Schering & Plough (USA).

Test compounds were dissolved in 0.01 N NaOH or in DMSO and then diluted with assay buffer. The influence of DMSO on enzyme activity was considered in controls.

NEP and ACE: Stock solutions of 2.5 mM in 0.01 N NaOH were prepared, except for acacetin, sinensetin, diosmetin, flavone, oleuropein, ononin (10 mM DMSO).

APN: All flavonoids were dissolved in DMSO (10 mM), related compounds in 0.01 N NaOH (2.5 mM) except for oleuropein and ononin (DMSO 10 mM).

4.2. Enzymes

Source of NEP and ACE was boar sperm obtained from the Institute of Reproduction of Farm Animals in Schönow. By complete inhibition of the ACE with lisinopril only the NEP activity remained to cleave the substrate SAAP-AMC. For the assay of ACE no enzyme inhibitor was necessary because of the specificity of Hip-L-His-L-Leu for ACE. Aminopeptidase N (leucine aminopeptidase; EC 3.4.11.2 type IV-S from porcine kidney microsomes) was obtained from Sigma Chemical Co.

4.3. Determination of NEP activity

For determination of the NEP activity we used a two-step assay according to Melzig et al. [13]. Briefly, 50 µl lisinopril (8 µM), 50 µl SAAP-AMC (400 µM) and 200 µl HEPES-buffer (50 mM + 154 mM NaCl, pH 7.4) with and without test compounds were added and mixed. The first enzymatic reaction was started by addition of 150 µl of a boar sperm preparation and incubated for 60 min (37 °C). The reaction was stopped by addition of 50 µl phosphoramidon solution (50 µM).

20 µl of APN-solution (1 : 235) were added and the reaction mixture was incubated again for 60 min (56 °C). The reaction was terminated by addition of 800 µl acetone. The fluorescence of the released AMC was measured (λ_{exit} = 367 nm; λ_{emiss} = 440 nm).

Controls were necessary to consider the possibility of an influence on APN or/and fluorescence by test compounds.

4.4. Determination of ACE activity

The assay was performed according to Melzig et al. [13]. Briefly, 20 µl substrate solution (Hip-L-His-L-Leu, 24 mM in water) were added to 230 µl phosphate-buffer (83 mM $K_2HPO_4 \times 3\,H_2O$ + 326 mM NaCl, pH 8.3), including test compounds and ACE. The reaction was stopped after 30 min (37 °C) with 0.28 M NaOH (1000 µl), and a methanolic o-phthalaldehyde solution (2%, 100 µl) was added for producing the fluorescing His-Leu-o-phthalaldehyde complex. Under exclusion of light this mixture was incubated for 10 min at room temperature and terminated by addition of 2 N HCl (300 µl). The fluorescence was measured at λ_{exit} = 360 nm and λ_{emiss} = 500 nm. The inhibition rates were calculated in comparison to control without inhibitor during enzyme reaction, considering the absorbance of fluorescence light by test compounds.

4.5. Determination of APN activity

The activity of APN was determined according to Gillespie et al. [14]. The substrate L-leucine-*p*-nitroanilide was dissolved (2 mM) in HEPES-buffer (50 mM + 154 mM NaCl, pH 7.4). Aliquots of substrate (250 µl) were added to 200 µl HEPES-buffer with or without test compounds. The reaction was started by adding 50 µl enzyme solution (1 : 10 000 with HEPES-buffer, approximately 1 mU) and incubated for 60 min (37 °C). The assay was stopped by addition of 800 µl acetone. The samples were measured spectrophotometrically at 405 nm to determine the formation of *p*-nitroaniline.

4.6. Statistics

All assays were performed three times or more with at least duplicate samples. Inhibition rates were calculated in percent to controls without inhibitors. IC_{50} values were obtained from dose-effect-curves by linear regression.

References

1 Parellada, J.; Guinea, M.: J. Nat. Prod. **58**, 823 (1995)
2 Pietta, P. G.; Gardana, C.; Mauri, P. L.; Maffeii-Facino, R.; Carini, M.: J. Chromatogr. B **673**, 75 (1995)
3 Chipkin, R. E.; Ioro, L. C.; Barnett, A.; Berger, J.; Billard, W. in: Costa, E.; Trabucchi, M. (Eds.): Regulatory Peptides: From Molecular Biology to Function, p 235, Raven Press, New York, 1982
4 Bralet, J.; Mossiat, C.; Lecomte, J. M.; Charpentier, S.; Gros, C.; Schwartz, J. C.: Eur. J. Pharmacol. **179**, 57 (1990)
5 White, C. M.: Pharmacotherapy **18**, 588 (1998)
6 Menrad, A.; Speicher, D.; Wacker, J.; Herlyn, M.: Cancer Res. **15**, 1450 (1993)
7 Fujii, H.; Yosizawa, K.; Maruyama, S.; Abe, F.: Jpn. J. Antibiot. **49**, 1109 (1996)
8 Wallis, E. J.; Ramsay, L. E.; Hwettiarachi, J.: Clin. Pharmacol. Ther. **64**, 439 (1998)
9 Kameda, K.; Takaku, T.; Okuda, H.; Kimura, Y.; Okuda, T.; Hatano, T.; Agata, I.; Arichi, S.: J. Nat. Prod. **50**, 680 (1987)
10 Wagner, H.; Elbl, G.; Lotter, H.; Guinea, M.: Pharm. Pharmacol. Lett. **1**, 15 (1991)
11 Meunier, M.-T.; Villie, F.; Jonadet, M.; Bastide, J.; Bastide, P.: Planta Med. **53**, 12 (1987)
12 Parellada, J.; Suarez, G.; Guinea, M.: J. Enzyme Inhib. **13**, 347 (1998)
13 Melzig, M. F.; Pieper, S.; Siems, W.-E.; Heder, G.; Böttger, A.; Liberra, K.; Lindequist, U.: Pharmazie **51**, 501 (1996)
14 Gillespie, T. J.; Konings, P. N.; Merril, B. J.; Davis, T. P.: Life Sci. **51**, 2097 (1992)

Received May 7, 1999
Accepted August 9, 1999

Prof. Dr. Matthias F. Melzig
Institut für Pharmazie
Humboldt-Universität zu Berlin
Goethestr. 54
D-13086 Berlin
mattthias=melzig@pharma.hu.berlin.de