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In vitro effects of selected flavonoids on the 5′-nucleotidase activity

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A series of structurally related flavonoids and related compounds were evaluated whether they have inhibitory properties on the 5′-nucleotidase (5′-ribonucleotide phosphohydrolase; EC 3.1.3.5, 5′-NT) activity. Some of the flavonoids tested inhibit the enzyme such as quercetin, morin, apigenin, chrysin, myricetin, luteolin, diosmetin, (±)naringenin and diosmin. Rutin, naringin, hyperosid, (±)catechin, caffeic acid and rosmarinic acid had no inhibitory effect on the 5′-NT activity. Myricetin and quercetin were the most potent inhibitors for 5′-NT with IC_{50} values of 1.1 and 1.4 μM, respectively. Kinetic analysis showed a mixed type of inhibitor for both myricetin ($K_i = 1.5$ μM at pH 7.45), and quercetin ($K_i = 0.6$ μM at pH 7.45). The K_m value for 5′-adenosine monophosphate (5-AMP) was determined with 77 μM at pH 7.45. The differential inhibitory potencies of flavonoids seem to be structurally related (hydroxylation pattern). The results demonstrate that some flavonoids are strong inhibitors of 5′-NT activity which can be correlated to their pharmacological effects.

1. Introduction

Flavonoids are phenolic compounds found in several fruits, plants and vegetables, which have variable effects on the living system [1, 2]. They are universally present as pigments in aerial parts of plants and integral part of the human diet. It has been known for a long time that they have low mammalian toxicity and remarkable antiinflammatory, antiallergic, antianginal and spasmolytic activities [3]. In previous studies, it has been established that some flavonoids were able to reduce ischaemic myocardial size [4]; others have antineoplastic properties [5]. They have also been shown to have cytotoxic [6], antimicrobial [7, 8], antiviral [9], but also mutagenic [8, 10] and carcinogenic [6, 8] activity.

Important biologic activities displayed by flavonoids are inhibitory effects on the enzyme systems critically involved in the initiation and maintenance of the inflammatory and immune response including several kinases, phospholipases, lipoxygenases and cyclooxygenases [5, 11, 12]. Biochemical investigations of the mechanisms of the whole-organism toxicity of the flavonoids have shown that they inhibit a wide variety of enzyme systems, including ATPase [7, 8, 10, 13, 14], xanthine oxidase [15] and adenosine deaminase [16, 17].

DNA turn-over enzymes are included in the purine and pyrimidine metabolism. Of them, Adenosine deaminase (ADA), 5′-Nucleotidase (5′-NT), Purine nucleotid phosphorylase (PNP), Guanase (GUA), Xanthine oxidase (XO) and Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) have key functions in catabolic and salvage pathway of nucleotides. Therefore, the activities of these enzymes in cancer processes are changed in significant pattern due to specific metabolic conditions.

As far as we know, no attempt has been made until now to investigate effects of flavonoids on the 5′-NT activity. In the present study we investigated the effect of several flavonoids and related compounds on this enzyme as one of the key enzyme in the DNA turn-over. Additionally, it was the aim of this study to find structures connected with inhibitory properties.

2. Investigations and results

The result of a screening regarding the effect of flavonoids and related compounds on 5′-NT activity is shown in the Table, listing the substances in their rank order of potency as inhibitors of 5′-NT activity. Fig. 1 demonstrates the comparison of 4 selected flavonoids in the inhibition assay indicating that all IC_{50} values are in the range between 1 and 10 μM. Myricetin and quercetin, with IC_{50} of 1.1 and 1.4 μM, respectively, were the most potent inhibitors among the flavonoids tested, while diosmin at concentrations up to 100 μM was the weakest compound. The glycosidic flavonoids did not inhibit the enzyme or only very weak. Our studies also demonstrated that myricetin and quercetin showed mixed type inhibition (changes in both K_m and V_{max}, Fig. 2). Myricetin had a $K_i = 1.5$ μM at pH 7.45, and for quercetin a K_i of 0.6 μM at pH 7.45 was determined. The K_m value for 5′-adenosine monophosphate (5′-AMP) was calculated with 77 μM at pH 7.45.

3. Discussion

Hydrolysis of nucleoside 5′-monophosphates at different evolutionary levels and within different cellular systems and pools serves varying physiological functions. 5′-NT represents the final enzymatic step within a cascade that leads from ATP to the nucleoside which is used for the salvage of nucleotides. The role of the nucleoside which is

Table: Maximal inhibition of 5′-NT by selected flavonoids and related compounds

Test substance	Maximal inhibition in % (mean ± SD)	IC_{50} (μM)
Myricetin	92.5 ± 0.5	1.1
Quercetin	83.5 ± 2.7	1.4
Luteolin	89.7 ± 0.6	1.8
Morin	94.9 ± 1.5	3.5
Apigenin	74.0 ± 2.4	11.4
Chrysin	62.0 ± 3.9	23.2
Diosmetin	60.4 ± 2.4	38.7
(±)-Naringenin	47.2 ± 1.5	n.d.
Diosmin	43.6 ± 1.7	n.d.
Rutin	No effect	
Naringin	No effect	
Hyperosid	No effect	
(±)-Catechin	No effect	
Caffeic acid	No effect	
Rosmarinic acid	No effect	

All compounds were tested up to a maximal concentration of 100 μM, with the exception of quercetin (10 μM). n.d. = no determined

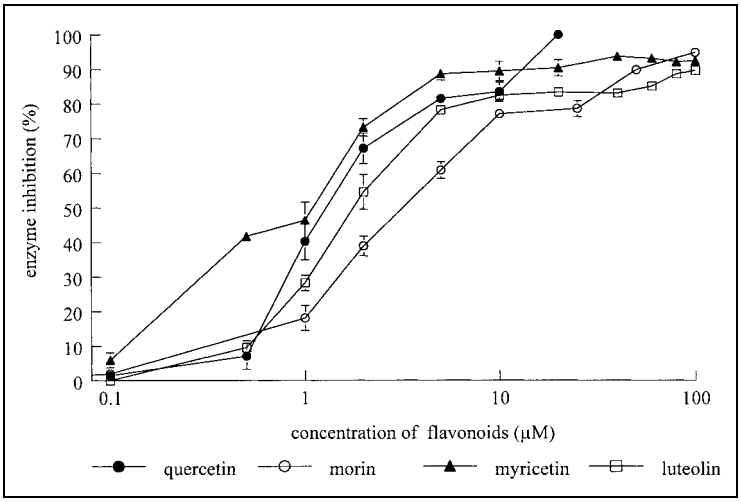

Fig. 1:
Dose-dependent inhibition of 5′-NT activity by selected flavonoids

used for the salvage of nucleotides. The role of the cytoplasmic 5′-NT is connected to intracelluar degradation of ATP via AMP to adenosine or via IMP to inosine. These enzymes are thought to be of particular functional importance in situations leading to lowered energy charge such as increased work load, anoxia or ischaemia. The AMP concentration within the cells needs to be under control to prevent free interconversion of AMP, ADP and ATP by adenylate kinase as well as excessive activation of glycogen mobilization and glycolysis [18]. Together with the significance of 5′-NT in DNA turn-over the dephosphorylation activity seems to be an interesting point of attack in the regulation of metabolic processes. The enzyme catalyzes the dephosphorylation of purine and pyrimidine ribo- and deoxyribonucleoside 5′-monophosphates. It is present as both cytoplasmic- and membrane-associated form in bacterial, plant and vertebrate cells [18]. Extracellular production of adenosine by membrane associated 5′-NT is linked to a number of adenosine receptor-mediated events, such as vasodilation [19, 20], glomerular filtration rate [21], or inflammatory response [22]. Cytoplasmic 5′-NT is involved in regulating intracellular levels of nucleotides, as well as in controlling the secretion of adenosine [18].

Flavonoids are a ubiquitous family of secondary products that serve to protect plants from environmental stress. The biologic effects of flavonoids in mammalians have been studied for over about 60 years [23]. Active flavonoids tend to inhibit cell activation at various levels. It has been shown that flavonoids are capable of protecting the lysis of human erythrocytes following photosensitized oxidation [24]. A number of flavonoids have also been found to scavenge free radicals directly and to inhibit lipid peroxidation [25, 26]. Many flavonoids, such as quercetin, inhibit protein kinase C in the μM range. Inhibition of many other mammalian enzymes by flavonoids has been studied [14, 17]. Some flavonoids are carcinogenic and turn on genes or activate cells via fatty acid mobilization. It is also clear that some of the biologic effects of flavonoids are not fully explicable by any previously identified mechanism. Recently, some synthetic and semisynthetic chemicals having inhibitory activities on enzymes included in the DNA turnover have been used as antineoplastic drugs [27–29]. Therefore, the discovery of new natural chemicals having inhibitory activities on these enzymes seems to be of importance. Some previous studies suggest that flavonoids have these kinds of functions [14, 30].

Lymphocytes show very low levels of purine de novo biosynthesis and by that they are dependent on the purine salvage pathway [31]. In this regard, 5′-NT has drawn special attention. Deficiencies in 5′-NT activity have been studied in several disease states, including hemolytic anemia, multiple myeloma and hairy cell, chronic B-lymphocytic and acute lymphoblastic leukemia and different type cancerous human tissues [32–36].

Because of their central role in maintaining intracellular levels of nucleoside 5′-monophosphates and extracellular levels of adenosine, cytoplasmic and membrane-associated

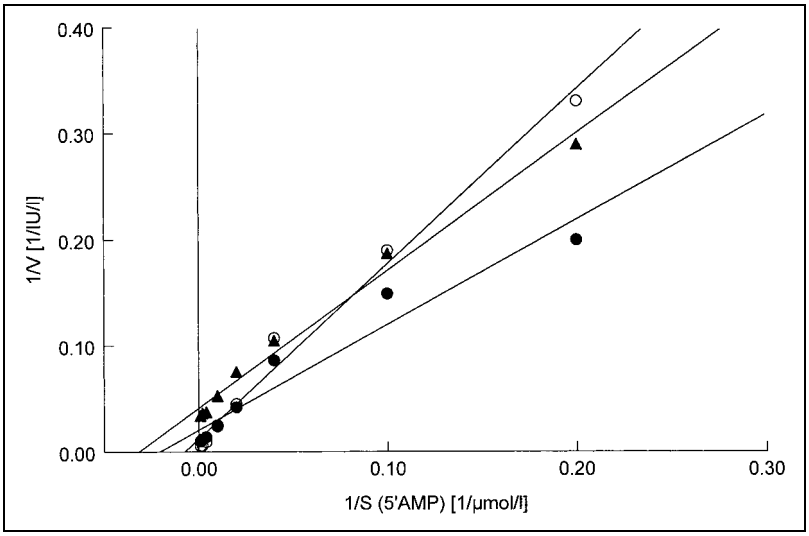

Fig. 2:
Effect of myricetin and quercetin on substrate-dependent determination of 5′-NT activity (Lineweaver-Burk-Plot, linear regression of the single points) ○ without inhibitor; ● plus 1 μM myricetin; ▲ plus 1.5 μM quercetin

5′-NT have been important targets of pharmaceutical research. Flavonoids like quercetin, myricetin and luteolin which inhibit efficiently the 5′-NT with IC_{50} values in the range of 1 to 2 μM influence the immunological system as well as cancer induction and progression. One possible mechanism included in a network of beneficial effects of flavonoids might be the inhibition of 5′-NT activity.

4. Experimental

4.1. Materials

The flavonoids and related compounds were purchased from Roth (Karlsruhe, FRG), all other chemicals were supplied by Sigma (Deisenhofen, FRG). All reagents were of analytical reagent grade; throughout the experiments bidestilled water was used.

4.2. 5′-Nucleotidase activity assay procedure

5′-NT was purchased from Sigma (from *Crotalus atrox* venom, partially purified lyophilized powder, 330 IU/mg protein). Determination of 5′-NT activity and the inhibitor studies were carried out using a method for the determination of inorganic phosphate described by Kyaw et al. [37]. The assay medium was a Tris-HCl buffer containing 50 mM pH 7.45, 10 mM $MgSO_4$, 60 mM NaCl, 5 mM KCl and 10 mM NaN_3. Briefly, 500 μl assay buffer containing the appropriate concentration of AMP (2 mM) and the inhibitor were incubated for 30 min at 37 °C. After that, 500 μl Triton X-100 (7.5%) and 500 μl ammonium molybdate (3.75% in 10 M H_2SO_4) were added and vortexed. In this assay, the liberated inorganic phosphorous from 5′-AMP was measured spectrophotometrically at 375 nm after the color reaction between inorganic phosphate and acidified ammonium molybdate in the presence of Triton X-100. 5′-NT activity was expressed in international units per liter.

4.3. Preparation of flavonoid solutions

The flavonoids were dissolved in dimethylsulfoxide (DMSO) making a stock solution, which was diluted approx. 100fold in the assay. At this concentration DMSO had no effect on 5′-NT activity as tested in control experiments.

4.4. Statistics

All experiments were done in duplicate or triplicate with 3 parallel samples. The IC_{50}, K_m and K_i values were estimated by the linear regression analyses of 5′-NT activity versus 5′-AMP or flavonoid concentration using Slide Write^R program.

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References

1 Harbone, J. B.; in: Cody, V., Middleton, E., Harbone, J. (Eds): Plant flavonoids in biology and medicine: Biochemical, pharmacological and structure-activity relationship. p. 15, Alan R. Liss, New York 1986
2 Laychock, S. G.; in: Cody, V., Middleton, E., Harbone, J. (Eds): Plant flavonoids in biology and medicine: Biochemical, pharmacological and structure-activity relationship. p. 215, Alan R. Liss, New York 1986
3 Gabor, M.; in: Cody, V., Middleton, E., Harbone, J. (Eds): Plant flavonoids in biology and medicine: Biochemical, pharmacological and structure-activity relationship. p. 471, Alan R. Liss, New York 1986
4 Occhiuto, F.; Busa, G.; Ragusa, S.; De Pasquale, A.: Phyother. Res. **5**, 9 (1991)
5 Ferriola, P. C.; Cody, V.; Middleton, E.: Biochem. Pharmacol. **38**, 1617 (1989)
6 McClure, J. W.: The Flavonoids, part 2, p. 1011, Academic Press, New York 1975
7 Havsteen, B.: Biochem. Pharmacol. **32**, 1141 (1983)
8 Brown, J. P.: Mutation Res. **75**, 243 (1976)
9 Schroder, G.; Zahringer, U.; Heller, W.: Arch. Biochem. Biophys. **194**, 635 (1979)
10 Middleton, Jr. E.: Trends Pharm. Sci. **5**, 335 (1984)
11 Alcaraz, M. J.; Villar, A.; in: Boca Raton, F. L. (Ed.), CRC Handbook of free radicals and antioxidants in biomedicine. Vol. 2, p. 77, CRC Press 1989
12 Loggia, R. D.; Ragazzi, E.; Tubaro, A.; Fassina, G.; Vertura, G.: Pharmacol. Res. Commun. **20** (Suppl. 5), 91 (1988)
13 Suolinna, E. M.; Buchsbaum, R. N.; Racker, E.: Cancer Res. **35**, 1865 (1975)
14 Bohmont, C.; Aaronson, L. M.; Mann, K.; Pardini, R. S.: J. Nat. Prod. **50**, 427 (1987)
15 Costantino, L.; Rastelli, G.; Albasini, A.: Eur. J. Med. Chem. **31**, 693 (1996)
16 Melzig, M. F.: Planta Med. **62**, 20 (1996)
17 Koch, H. P.; Jager, W.; Groh, U.: Meth. Find. Exp. Clin. Pharmacol. **14**, 413 (1992)
18 Zimmermann, H.: Biochem. J. **285**, 345 (1992)
19 Duncker, D. J.; van Zon, N. S.; Pavek, T. J.: J. Clin. Invest. **95**, 285 (1995)
20 Kurtz, A.: J. Biol. Chem. **262**, 6296 (1987)
21 Weaver, D. R.; Reppert, S. M.: Am. J. Physiol. **263**, F991 (1992)
22 Resnick, M. B.; Colgan, S. P.; Patapoft, T. W.: J. Immunol. **151**, 5716 (1993)
23 Roger, C. R.: Experientia **44**, 725 (1988)
24 Sorata, Y.; Takahama, U.; Kimura, M.: Biochim. Biophys. Acta **799**, 313 (1984)
25 Baumann, J.; von Bruchhausen, F.; Wurm, G.: Prostaglandins **20**, 627 (1980)
26 Pluncket, W.: Ann. N.Y. Acad. Sci. **451**, 154 (1985)
27 Jackson, R. C.; Leopold, W. R.; Ross, D. A.: Ann. N.Y. Acad. Sci. **451**, 125 (1985)
28 Guohua, C.; Sofic, E.; Ronald, L. P.: Free Radic. Biol. & Med. **22**, 749 (1997)
29 Nosaka, K.; Takahashi, T.; Nishi, T.: Free Radic. Biol. & Med. **22**, 597 (1997)
30 Shah, T.; Simpsom, R. J.; Webster, A. D. B.; Peters, T. J.: Clin. Exp. Immunol. **66**, 158 (1986)
31 Hopkinson, D. A.; Swallow, D. M.; Marinaki, A.; Harley, E. H.: Inherit. Metab. Dis. **13**, 701 (1990)
32 Pilieri, A.; Massaia, M.; Dianzani, U.: Acta Haematol. **78** (Suppl. 1), 41 (1987)
33 Ho, A. D.; Dietz, G.; Trede, I.: Cancer **58**, 96 (1986)
34 Poplack, D. G.; Blatt, J.; Reaman, G.: Cancer Res. **41**, 4821 (1981)
35 Durak, I.; Perk, H.; Kavutcu, M.; Canbolat, O.: Free Radic. Biol. & Med. **16**, 825 (1994)
36 Durak, I.; Isik, A. Ü.; Canbolat, O.; Akyol, Ö.; Kavutcu, M.: Free Radic. Biol. & Med. **15**, 681 (1993)
37 Kyaw, A.; Maung-U, K.; Toe, T.: Anal. Biochem. **145**, 230 (1985)

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