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Natural flavonoids are potent inhibitors of glycogen phosphorylase

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There is little known about effects to be expected from high intake of flavonoids with respect to regulation of glucose/glycogen homeostasis. Glucose/glycogen homeostasis is mainly regulated by glycogen synthase (GS) and glycogen phosphorylase (GP). We investigated effects of naturally occurring flavonoids with varying substitution pattern on the activity of isolated muscle GP. Almost all flavonoids tested inhibited phosphorylated, active GPa, as well as unphosphorylated, adenosine monophosphate-activated GPb. However, inhibition of GPa was two-to-fourfold stronger than that of GPb. The flavonol quercetin and the anthocyanidins cyanidin and delphinidin turned out to be the most potent inhibitors of GPa, with concentration values where enzymatic activity is 50% of the respective control in the low micromolar range (<5 μM). Furthermore, by comparing GPa inhibitory activity of typical representatives from all known flavonoid classes, structural elements of flavonoids required for effective GP inhibition could be identified.

Keywords: Flavonoids / Glucose/glycogen homeostasis / Glycogen phosphorylase / Inhibition / Structure activity relationship

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1 Introduction

Flavonoids are dietary polyphenols ubiquitously found in foods of plant origin like fruits and vegetables [1]. Basic structures are given in Fig. 1, individual structures, reflecting varying substituent patterns, in Table 1.

Various biological activities, such as antibacterial, antithrombotic, vasodilatory, anti-inflammatory and anticarcinogenic effects, are attributed to flavonoids [2]. Several cohort studies in humans with flavones, flavonols and flavanones argue for an inverse relation between dietary intake of these flavonoids and the incidence of various chronic diseases, including coronary heart disease, stroke, cancer and type 2 diabetes [3]. Information on effects of flavonoids on glucose/glycogen homeostasis is scarce and in part contradictory [4–7]. In the last decades scientific and public interest in health effects of flavonoids has increased dramatically. To gain deeper knowledge on potential effects of these compounds concerning glucose/glycogen homeostasis, in the present study the effect of several naturally occurring flavonoids on the activity of isolated muscle glycogen phosphorylase was investigated.

Glucose/glycogen homeostasis is mainly regulated by two enzymes: glycogen phosphorylase (GP) and glycogen synthase (GS). Glycogen phosphorylase (EC 2.4.1.1), the key enzyme of glycogen breakdown, catalyzes the degradative phosphorolysis of glycogen to glucose-1-phosphate (G-1-P). The enzyme consists of two identical 97-kDa subunits and exists in two interconvertible forms: GPa, the Ser^{14}-phosphorylated form with high activity and high substrate affinity, and GPb, the unphosphorylated form with low activity and low substrate affinity. GPa has been found to play a major role in the regulation of glycogen degradation in proliferating cells [8]. Furthermore, both forms can exist in two different conformational states, depending on the presence or absence of allosteric inhibitors or activators. The less active tensed conformation (T-state) is present in the absence of glycogen and of allosteric inhibitors like caf-

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Abbreviations: AMP, adenosine monophosphate; **DH**, glucose-6-phosphate dehydrogenase; **DHF**, 3′,4′-dihydroxyflavone; **GPa/b**, glycogen phosphorylase a/b; **G-1-P**, glucose-1-phosphate; **G-6-P**, glucose-6-phosphate; **IC_{50}**, concentration where enzymatic activity is 50% of the respective control; **NADP**, nicotinamide adenine dinucleotide phosphate; **PGM**, phosphoglucomutase

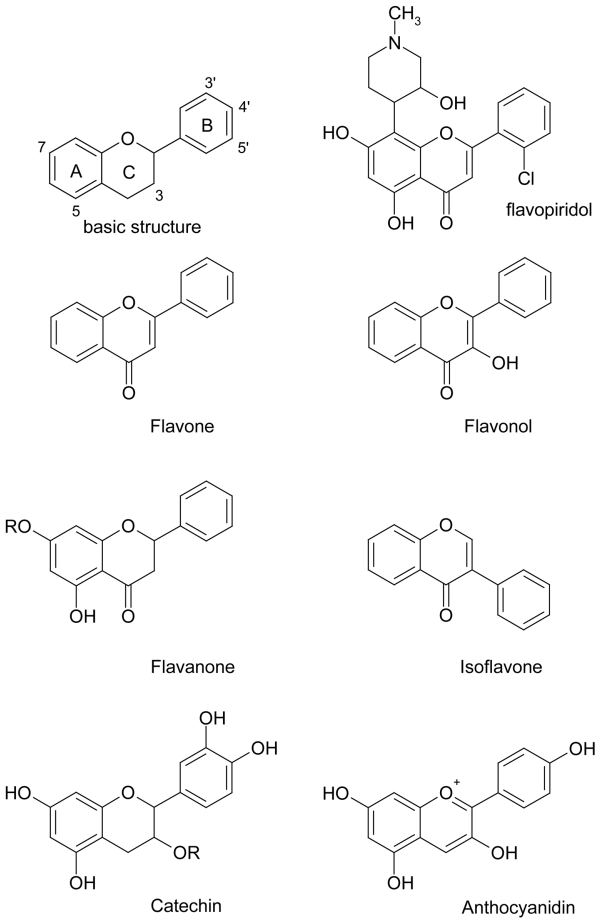

Figure 1. Structures of flavonoid classes and the flavonoid derivative flavopiridol.

feine, glucose-6-phosphate (G-6-P), glucose and adenosine triphosphate (ATP). The more active relaxed conformation (R-state) is promoted by substrate as well as allosteric effectors, including adenosine monophosphate (AMP) and phosphorylation [9]. At least six potential regulatory sites have been identified for GP. These include the catalytic site that binds glycogen, G-1-P, glucose and glucose-analogues, the inhibitor site where caffeine and related compounds are bound, the allosteric site binding AMP, ATP and G-6-P, the Ser^{14}-phosphate recognition site, the glycogen storage site and a new allosteric inhibitor site, located at the dimer interface where the potential antidiabetic drug CP320626 is bound [10].

The synthetic flavonoid derivative, flavopiridol, (Fig. 1) has been shown to potently inhibit isolated and cellular GP activity [11]. Co-crystallization experiments with GPb clearly demonstrated that flavopiridol binds at the inhibitor site of the enzyme [12]. Information concerning effects of natural flavonoids on GP activity is still missing, however.

Table 1. Substitution pattern of tested flavonoids. Numbering of substituents according to the basic structure given in Fig. 1

	3	5	7	3′	4′	+ OH	+ OCH_3
Flavones							
DHF				OH	OH		
Chrysin		OH	OH				
Apigenin		OH	OH		OH		
Luteolin		OH	OH	OH	OH		
Tricin		OH	OH	OCH_3	OH		5′
Baicalein		OH	OH			6	
Flavonols							
Quercetin	OH	OH	OH	OH	OH		
Flavanones							
Naringenin		OH	OH		OH		
Hesperitin		OH	OH	OH	OCH_3		
Eriodictyol		OH	OH	OH	OH		
Isoflavones							
Daidzein			OH		OH		
Genistein		OH	OH		OH		
Catechins							
(+)-Catechin	OH	OH	OH	OH	OH		
(−)-Epicatechin	OH	OH	OH	OH	OH		
Epigallocatechin	OH	OH	OH	OH	OH	5′	
ECG[a)]	Galloyl	OH	OH	OH	OH		
EGCG[b)]	Galloyl	OH	OH	OH	OH	5′	
Anthocyanidins							
Pelargonidin	OH	OH	OH		OH		
Cyanidin	OH	OH	OH	OH	OH		
Delphinidin	OH	OH	OH	OH	OH	5′	
Peonidin	OH	OH	OH	OCH_3	OH		
Malvidin	OH	OH	OH	OCH_3	OH		5′

a) Epicatechin gallate.
b) Epigallocatechin-3-gallate.

2 Materials and methods

2.1 Chemicals

Flavonoids (Table 1) were obtained from Extrasynthèse (Genay, France), quercetin from Sigma (Taufkirchen, Germany). For the GP- and phosphoglucomutase (PGM)-assay, substances were freshly dissolved in DMSO (1% final DMSO concentration). Enzymes (GPa, GPb, PGM and glucose-6-phosphate-dehydrogenase, DH), glycogen and β-nicotinamide adenine dinucleotide phosphate (β-NADP) were purchased from Sigma.

2.2 GP-assay

Enzyme activity was measured according to a method described by Kaiser *et al.* [11], with slight modifications. In principle, GP activity is determined in the direction of glycogen breakdown, generating G-1-P. The latter is converted by PGM to G-6-P, which in turn is dehydrogenated by DH, generating NADPH from NADP, which can be followed spectrophotometrically at 340 nm. Briefly, highly purified rabbit muscle GPa or GPb were dissolved in buffer A (40 mM β-glycerophosphate pH 6.8, 8 mM cysteine) and stored at 4°C. GP solutions were further diluted in 760 µL buffer B (20 mM sodium phosphate pH 7.2, 2 mM $MgSO_4$) in a cuvette to a final concentration of 1.5 µg/reaction. Afterwards, 1 mM NADP, 1.4 U/mL DH, 3 U/mL PGM

and, in the case of GPb-assay, 0.2 mM AMP were added and carefully stirred at 25°C. Reaction was started by adding 50 µL glycogen (20 mg/mL), yielding a final glycogen concentration of 0.1%. Kinetics of all assays was studied at 25°C over a 9-min interval with a Cary 1 Bio UV-Visible Spectrophotometer (Fa. Varian, Germany). Calculations were done with CaryWIN-UV-Software (Fa. Varian).

2.3 PGM-assay

Potential interaction of flavonoids with downstream enzymes of the GP-assay (PGM, DH) was excluded by following the reaction using G-1-P as starting substrate. NADP (1 mM), 1.4 U/mL DH, same amount of buffer A as used in the GP-assay and 5 mM G-1-P were added to 850 µL buffer B and treated identically. After adding 3 U/mL PGM the reaction was followed over a 5-min interval at 340 nm and kinetics calculated as described.

3 Results

3.1 Inhibition of GPa by flavonoids

Mammalian glycogen phosphorylase is expressed as three isoforms, liver-, muscle- and brain-type. The isoforms are to be distinguished by structural and functional properties, as well as by different expression profiles in tissues [13]. Most adult tissues express all three isoforms at varying levels, depending on the species. In some human tumor cells, however, normal GP expression and activity is deregulated and brain-type GP has been shown to be the major isoenzyme expressed in gastric cancer and renal cell carcinoma [14, 15]. However, the inhibitor site is identically conserved in all mammalian GP [16], indicating that a compound inhibiting muscle GP by binding at this site is likely to inhibit also liver and brain GP as well.

To answer the question, whether naturally occurring flavonoids can modulate GP activity, we used highly purified rabbit muscle GP as an easily accessible model. GP activity was measured by assaying effects on glycogen breakdown. Compounds from all flavonoid classes were tested in concentrations up to 50 µM. As can be seen (Table 2), activity of Ser^{14}-phosphorylated, active GPa is potently inhibited by the flavonol quercetin (concentration where enzymatic activity is 50% of the respective control, IC_{50}, 4.8 µM), the flavones luteolin (IC_{50} 15.6 µM) and baicalein (IC_{50} 11.2 µM) and the catechins epicatechin gallate (IC_{50} 12.5 µM) and epigallocatechin-3-gallate (IC_{50} 7.7 µM). The anthocyanidins cyanidin and delphinidin inhibit GPa activity in the same low micromolar range (IC_{50} 3 µM). The other flavonoids tested showed only marginal effects on GPa activity (IC_{50} > 50 µM). Catechins without a galloyl group in position 3 as well as the isoflavone daidzein and the flavanones hesperetin and eriodictyol were inactive (Table 2). As a control, potential effects on PGM and DH were studied at comparable concentrations with the PGM-assay (Section 2.3). No effects on PGM or on DH in the assay system were observed under the described conditions, indicating a direct interaction of active flavonoids with GP (data not shown).

Table 2. IC_{50} values [µM] for inhibition of GPa and GPb of flavonoids tested in concentrations up to 50 µM[a)]

Compound	IC_{50} GPa	IC_{50} GPb
Quercetin	4.8 ± 0.4 µM	20.9 ± 1.9 µM
Chrysin	> 27.5 µM[b)]	15.3 ± 1.0 µM
Apigenin	> 30 µM[b)]	no effect up to 30 µM[b)]
Luteolin	15.6 ± 1.8 µM	28.8 ± 2.8 µM
Tricin	> 50 µM	no effect
Baicalein	11.2 ± 1.5 µM	10.2 ± 1.2 µM
Naringenin	> 50 µM	no effect
Hesperetin	no effect	no effect
Eriodictyol	no effect	no effect
Catechin	no effect	no effect
Epicatechin	no effect	no effect
Epigallocatechin	no effect	no effect
ECG[c)]	12.5 ± 1.3 µM	50. ± 7.8 µM
EGCG[d)]	7.7 ± 0.7 µM	33.9 ± 2.9 µM
Pelargonidin	43.6 ± 3.0 µM	6.2 ± 0.4 µM
Cyanidin	3.0 ± 0.2 µM	9.0 ± 0.5 µM
Delphinidin	3.1 ± 0.4 µM	10.7 ± 0.4 µM
Malvidin	> 50 µM	no effect
Peonidin	25.1 ± 1.7 µM	17.6 ± 1.4 µM
Genistein	> 50 µM	no effect
Daidzein	no effect	no effect
Gallic acid	no effect	no effect
Ellagic acid	3.2 ± 0.5 µM	12.1 ± 1.4 µM
DHF	> 50 µM	> 50 µM

a) All assays were performed as described in Section 2. In GPb-assays, the enzyme was activated using 0.2 mM AMP. The data presented are the mean ± SD of at least four independent experiments. Inhibition of GP activity was calculated as percent test over solvent (1% DMSO) control T/C [%]. The IC_{50} value was calculated by linear regression.
b) Highest soluble concentration in assay buffer.
c) Epicatechin gallate.
d) Epigallocatechin-3-gallate.

3.2 Inhibition of GPb by flavonoids

Furthermore, it has been investigated, whether unphosphorylated GPb is affected by natural flavonoids differentially to GPa. To measure effects on GPb activity, prior activation by an allosteric activator, such as AMP, is required. Binding of AMP results in a conformational change from the inactive T-state to the active R-state. It was established that 0.2 mM AMP was sufficient to activate the enzyme (data not shown). Validation of the assay was achieved by using the established allosteric inhibitor caffeine (Fig. 2).

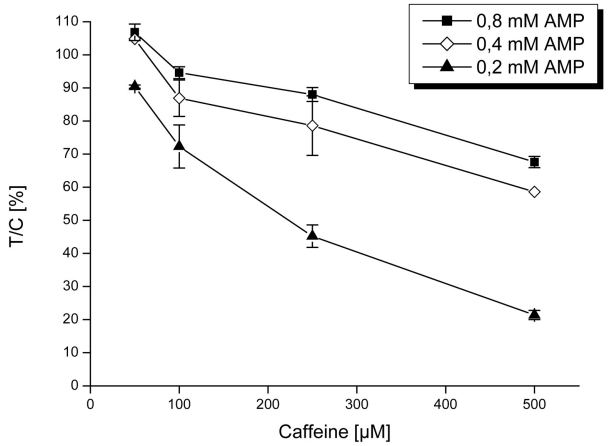

Figure 2. Inhibition of GPb by its allosteric inhibitor caffeine. All assays were performed as described in Section 2. Three different concentrations of AMP were used to activate GPb. Optimal activation of GPb was achieved by an AMP concentration of 0.2 mM.

The latter was found to display inhibitory activity under the selected conditions similar to that described in the literature [11]. Therefore, inhibitory effects of naturally occurring flavonoids on activated GPb were measured in the presence of 0.2 mM AMP. In concentrations up to 50 µM, flavonoids were found to be considerably less effective on GPb than on GPa activity or were not effective. Exceptions were chrysin, pelargonidin and peonidin, showing stronger inhibition of GPb than GPa and baicalein with equal inhibitory activity on both enzyme forms. PGM and DH were not affected (data not shown).

3.3 Structural requirements for GP inhibition

Typical representatives of all flavonoid classes, including also several anthocyanidins and catechins with varying substitution pattern, were compared in their inhibitory efficacy to allow conclusions on structural elements required for effective inhibition of GPa.

Quercetin (IC_{50} 4.8 µM) and cyanidin (IC_{50} 3 µM) inhibit GPa activity with similar IC_{50} values in the low micromolar range (Fig. 3A). Absence of a hydroxyl group such as in luteolin in position 3 (ring C), leads to decreased inhibitory efficacy (IC_{50} 15.6 µM). Further absence of the two hydroxyl groups in C5 and C7 (ring A), such as in 3′,4′-dihydroxyflavone (DHF), leads to complete loss of inhibitory activity towards GPa (IC_{50} > 50 µM). Eriodictyol, a compound lacking the double bond between C2 and C3, likewise was found inactive (Fig. 3A). Anthocyanidins in addition to showing hydroxyl groups at positions 3, 5 and 7 encompass varying substitution patterns on ring B (Fig. 3B). Cyanidin and delphinidin, both having vicinal hydroxyl groups in ring B were found to potently inhibit GPa activity (IC_{50} ~ 3.0 µM). Absence of one of the vicinal hydroxyl groups leads to markedly reduced inhibitory activity, as exemplified by pelargonidin (IC_{50} 43.6 µM). Replacement of one or two hydroxyl groups in these positions by a methoxy group (peonidin and malvidin) dramatically decreases inhibitory activity on GPa (IC_{50} 25 µM and ~60 µM, respectively).

Catechins do not inhibit GPa activity in the tested concentration range (Fig. 3C). However, a galloyl substituent in position 3 (ring C) provides strong inhibitory activity, comparable to quercetin and luteolin. Whereas gallic acid itself showed no effect on GPa activity, the dimer ellagic acid was found to potently inhibit the enzyme with an IC_{50} of 3.2 µM (Fig. 3C).

4 Discussion

Because of their presumed association with favorable health effects, products rich in flavonoids become more and more popular. Consumption of dietary supplements rich in flavonoids very likely result in intake levels far above those ingested with a normal diet. It is a largely unresolved question at present, whether a significantly enhanced intake as to be expected from dietary food supplements or certain functional foods, is consistent with beneficial effects on human health or whether adverse effects are also to be taken into account. Results of *in vivo* studies in diabetic and in non-diabetic rats have suggested a hypoglycemic/insulin-like effect of certain individual flavonoids [4] and of flavonoid-containing seed extracts [17, 18]. Controversial results have been reported concerning hypoglycemic or insulin-like activity of (-)-epicatechin [19–21]. Obviously, more information about effects of naturally occurring flavonoids concerning their potential influence on regulation of blood glucose level therefore is required.

In the present study, we used highly purified rabbit muscle GPa and GPb as an easily accessible model system to measure potential effects of flavonoids. Comparing effects on GPa activity of typical representatives of the different flavonoid classes allowed concluding on structural elements necessary for effective GP inhibition. In contrast to luteolin, quercetin and cyanidin, compounds with a saturated C-ring (eriodictyol and catechin) did not inhibit GPa activity. Therefore, planarity of the molecule seems to be a mandatory element for potential inhibition of this enzyme. By comparing inhibitory efficacy of flavonoids with vicinal hydroxyl groups in 3′ and 4′ positions, the flavonol quercetin and the anthocyanidin cyanidin were found to be the most potent inhibitors of GPa activity. Absence of the hydroxyl group in C3 (luteolin) however, reduces the inhibitory effect about threefold. Additional absence of two hydroxyl groups in C5 and C7 on ring A as exemplified by DHF, abrogates inhibitory activity towards GPa (IC_{50} >

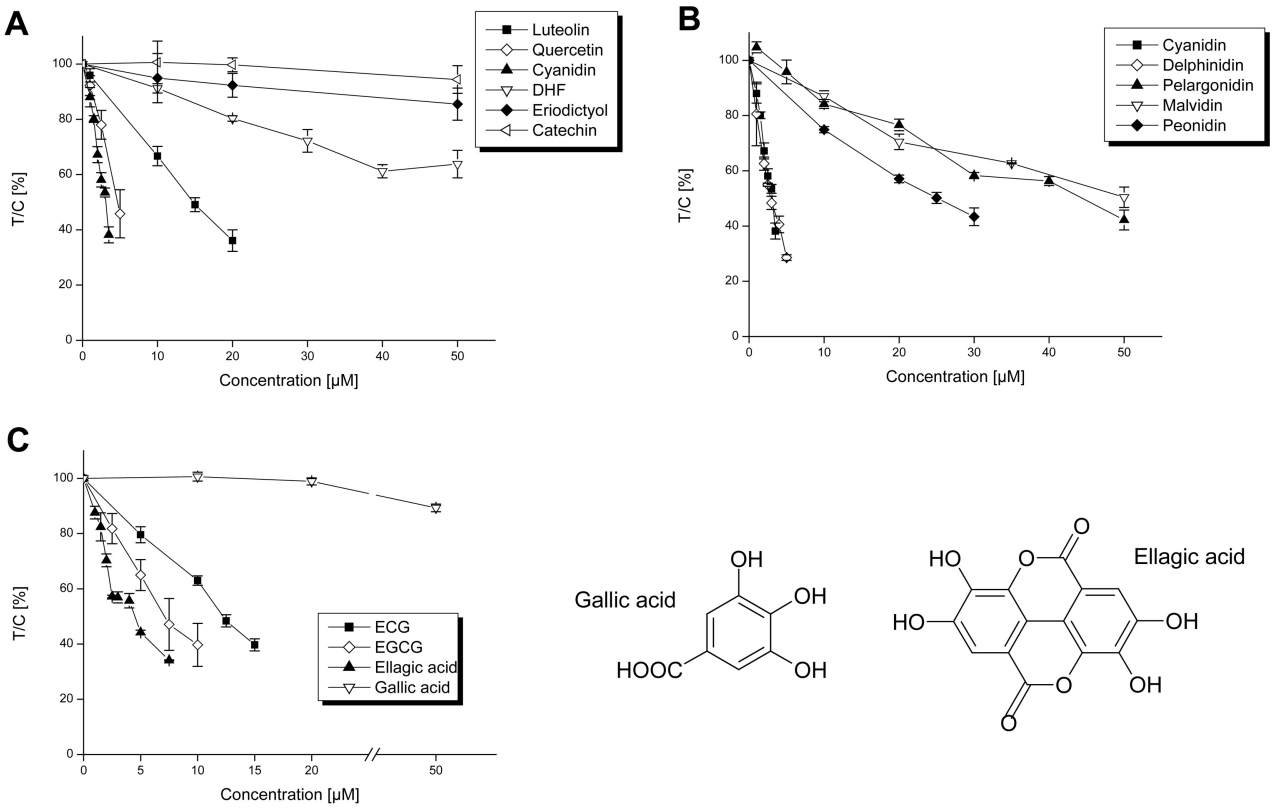

Figure 3. Inhibition of GPa by different flavonoids. All assays were performed as described in Section 2. The data presented are the mean ± SD of at least four independent experiments. Inhibition of GP activity was calculated as percent test over solvent (1% DMSO) control T/C [%]. (A) Comparison of typical representatives of flavonoid classes. (B) Influence of the substitution pattern of ring B of anthocyanidins. (C) Inhibition by epicatechingallate (ECG), epigallocatechingallate (EGCG), gallic acid and ellagic acid.

50 µM). Evidently, for potent inhibition of GPa, vicinal hydroxyl groups on ring B are mandatory, together with the presence of hydroxyl groups at C5 and C7 on ring A. An additional hydroxyl group in C3 further enhances inhibition of GPa. In line with these observations the flavonol fisetin, distinguished from quercetin only by absence of the hydroxyl group in position 5, has been reported not to inhibit the activity of AMP-activated GPb [11]. However, the flavone baicalein, although completely lacking hydroxyl groups on ring B, was found to be a potent inhibitor of GPa and of AMP-activated GPb activity. Thus, the three adjacent hydroxyl groups in position 5, 6 and 7 on ring A might suffice to achieve effective inhibition of the enzyme, probably by allowing the compound to bind in a different manner into the respective binding site. This needs to be further explored, for example by crystal structure analysis of the enzyme-inhibitor-complex. Chrysin, displaying only two hydroxyl groups at C5 and C7, is a weaker inhibitor of GPa activity than baicalein. This finding further supports the importance of at least two adjacent hydroxyl groups in the molecule. A comparison of the efficacy of baicalein and DHF suggests that vicinal hydroxyl groups on ring B and planar structure are favorable, yet not sufficient for potent GPa inhibition. Obviously, additional hydroxyl groups at ring A are required to achieve optimum activity.

Likewise, within the anthocyanidins, number and position of hydroxyl groups also are highly influential on inhibitory activity. Cyanidin and delphinidin carry hydroxyl groups at C3, C5, C7, C3′ and C4′. They potently inhibit GPa activity in the low micromolar range. Whereas one additional hydroxyl group in 5′-position as in delphinidin does not further enhance the effect, absence of one of the vicinal hydroxyl groups at prime position (B-ring) decreases inhibitory activity on GPa markedly, as seen for pelargonidin (IC_{50} 43.6 µM). The essential role of vicinal hydroxyl groups attached to ring B is also seen by the effect of replacing it with a methoxy group, as exemplified by peonidin and malvidin, resulting in markedly reduced inhibition of GPa activity. This effect is not exclusively seen for anthocyanidins. Tricin, a flavone having hydroxyl groups in position 5, 7, 4′ together with a methoxy group in position 3′, shows a dramatically reduced inhibition of GPa activity as compared to luteolin.

Even though catechins are non-planar molecules, catechin-3-gallates inhibit GPa activity to an extent similar to quercetin and luteolin. Obviously, gallic acid attached to ring C in position 3 has a large enhancing effect on the activities of catechins and gallocatechins, inactive by themselves. Whether this effect might result, for example, from formation of a hydrogen bond between a hydroxyl group of ring B and the carbonyl function of the gallic acid ester and/or whether the three accessory hydroxyl groups in the gallic ester contribute to an enhanced inhibition of GPa activity needs to be explored further. Gallic acid itself shows no effect on GPa activity, whereas its dimer ellagic acid potently inhibits the enzyme (Fig. 3C).

Structural requirements of flavonoids for effective inhibition of GPa activity as discussed above have been described for other enzymes as well. A similar minimal flavone structure has been proposed previously for effective inhibition of DNA cleavage by topoisomerase II [22] and for inhibition of protein kinase C [23]. Furthermore, a comparable influence of the substitution pattern of ring B has also been described for inhibitory efficacy of anthocyanidins towards epidermal growth factor receptor [24] as well as human topoisomerases I and II [25].

It is not clear yet, how the active flavonoids interact with GP. Because of some structural similarity with flavopiridol, a synthetic flavonoid derivative (Fig. 1), inhibiting cyclin-dependent kinases, it might be possible, that they bind to the nucleoside inhibitor site of the enzyme, as described earlier for flavopiridol [12]. Binding to that inhibitory site of the enzyme would result in a stabilization of the inactive T-conformation..

In summary, depending on structure and substitution pattern, natural flavonoids are potent inhibitors of isolated GPa. Potent inhibitory activity is displayed by compounds having a double bond ring C together with hydroxyl groups at positions 5 and 7 in ring A and vicinal hydroxyl groups in ring B. Absence of the double bond in ring C, as exemplified by the inactive catechin structure, can be compensated for by substitution with a gallic acid ester in position 3.

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