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DIFFERENTIAL EFFECTS OF FLAVONOIDS AS INHIBITORS OF TYROSINE PROTEIN KINASES AND SERINE/THREONINE PROTEIN KINASES

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Abstract—The inhibitory potencies of bioflavonoids on various tyrosine protein kinases and serine/threonine protein kinases were investigated. The phosphotransferase activity of an oncogene product, $pp130^{fps}$, and a growth factor receptor, insulin receptor, were inhibited by myricetin, a derivative of quercetin, However, tyrosine kinase activity in the particulate fraction from human platelets (PM-TPK) was resistant to myricetin. Apparent K_i values of myricetin for tyrosine protein kinases of $pp130^{fps}$ and insulin receptor were 1.8 and 2.6 μM, respectively. The K_i values for serine/threonine kinase activities of myosin light chain kinase (MLC-kinase), casein kinase I, casein kinase II, cAMP-dependent protein kinase, and protein kinase C were 1.7 μM, 9.0 μM, 0.6 μM, 27.5 μM, and 12.1 μM, respectively. Lineweaver–Burk plots revealed that myricetin competitively inhibits $pp130^{fps}$ tyrosine kinase, myosin light chain kinase, casein kinase I and II with ATP, but does not inhibit other protein kinases. Since myricetin is a hydroxylated derivative of quercetin, the inhibitory effects of a series of seven flavonoids with various numbers of hydroxy residues were examined. Structure activity studies exhibited that the inhibitory potencies of the flavonoids for tyrosine kinases of $pp130^{fps}$ and insulin receptor correlated with the number of hydroxy residues on the flavone rings (γ = 0.974 and 0.926, respectively), whereas the hydroxylation influenced to a lesser extent the inhibitory potencies for serine/threonine protein kinase. The hydroxy residues at position 3′ and 5′ did not affect the activities of cAMP-dependent protein kinase, and protein kinase C, and the hydroxylation at position 5′ is detrimental for the inhibition of MLC-kinase, and casein kinase I and II. Thus, flavonoids may be useful tools to elucidate the active site of tyrosine and serine/threonine protein kinases.

Bioflavonoids, like quercetin; 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-1-benzopyran-4-one, inhibits growth and proliferation of certain malignant cells *in vitro* [1] and suppress the effect of tumor promotors on skin papilloma formation in mice, as initiated with 7,12-dimethylbenz[*a*]-anthracene [21]. Recent studies indicated that quercetin also inhibits several protein kinases, including tyrosine kinases associated with DMBA-induced rat mammary tumor [3] and $pp60^{src}$ [4], casein kinase II [5], phosphorylase kinase [6] and Ca^{2+}-phospholipid-dependent protein kinase (protein kinase C) [7]. The inhibitions of casein kinase II and $pp60^{src}$ tyrosine kinase by quercetin apparently compete with ATP [4, 5]. We reported that isoquinolinesulfonamides such as H-7 and H-8, which interact with the ATP-binding site of enzymes, potently inhibited protein kinase C and cyclic nucleotide-dependent protein kinases but had a comparatively weak inhibition for casein kinase I and II and myosin light chain kinase [8], thereby suggesting that isoquinolinesulfonamides can be used as molecular probes for mapping of active sites of protein kinases. We have now investigated the molecular mechanism of inhibitory effects of myricetin, a more potent derivative of quercetin, on tyrosine protein kinases and serine/threonine protein kinases, the objective being to determine (a) whether the effects of bioflavonoids are restricted to tyrosine protein kinases associated with malignant cells or all the tyrosine protein kinases, and (b) whether bioflavonoids exert inhibitory effects on tyrosine and serine/threonine protein kinases, via a common molecular mechanism.

MATERIALS AND METHODS

Histones H1 and H2B were purchased from Boehringer-Mannheim Biochemicals. Phosphatidylserine (pig liver) was purchased from Serdary Research Laboratories, Inc. A synthetic peptide Glu 4:Tyr 1 was from Sigma. Chloroform was removed from this phospholipid by a stream of nitrogen, and the phospholipid was sonicated in water for 1 min to produce a suspension of 0.5 mg/ml. [γ-^{32}P]ATP and [γ-^{32}P]GTP was obtained from Amersham, U.K. All other chemicals of the highest grade were obtained from commercial sources. The structures of the compounds tested are illustrated in Fig. 1. Quercetin and apigenin were obtained from Sigma Chemical Co., and myricetin and other flavonoids were from Aldrich Chemical Co.

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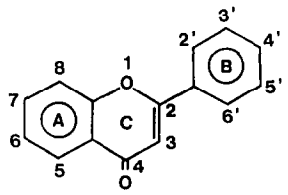

Compounds	-OH
Flavone	—
7-hydroxyflavone	7
Chrysin	5,7
Apigenin	4',5,7
Fisetin	4',5',3,7
Quercetin	3',4',3,5,7
Myricetin	3',4',5',3,5,7

Fig. 1. Chemical structures of flavonoids.

Tyrosine protein kinase preparation and assay. Fujinami virus-infected cells and anti pp130^{fps} mouse monoclonal antibody used in the experiments described here were prepared by Greiser-Wilke *et al.* [9]. pp130^{fps} was purified from these cells by immunoaffinity chromatography. The phosphotransferase activity of pp130^{fps} was measured in a 50 μl reaction mixture containing 20 mM Tris–HCl (pH 7.5), 10 mM $MnCl_2$, 0.1 mM EDTA, 0.2 mg/ml myosin light chain, and various concentrations of [γ-^{32}P]ATP in the presence of approximately 1.0×10^7 cpm radio activity labelled nucleotide (5 μCi/nmol). The reactions were incubated at 30° for 15 min and terminated by addition of 20 μl of five times concentrated electrophoresis sample buffer and by heating at 95° for 2 min (electrophoresis sample buffer: 70 mM Tris–HCl (pH 6.8), 10% glycerol, 2% SDS, 0.01% bromophenol blue, 2% 2-mercaptoethanol). The products of the reaction were resolved by polyacrylamide gel electrophoresis (1 mm thick; 11.5 × 14 cm; 10% acrylamide, 0.26% bisacrylamide) using the buffer systems described by Laemmli [10]. The gels were fixed in 10% acetic acid, 5% methanol, dried onto Whatman 3 MM paper and the labeled proteins were visualized by autoradiography using Kodak X-Omat R film. The radioactive bands were cut out of the dried gels and the radioactivity was quantified by liquid scintillation spectrometry.

Insulin receptor tyrosine kinase was prepared from rat liver and measured by the method of Freidenberg *et al.* [11]. Lectin-purified extracts of liver membranes (20 μl, 0.2 μg protein) were used as the enzyme fractions. After an overnight incubation at 4° in the absence or presence of insulin (1 μM), the phosphorylating reaction was initiated by the addition of 30 μl of a reaction mixture calculated to give final concentrations of 50 mM Tris–HCl (pH 7.4), 5 mM $MnCl_2$, 2 mM $MgCl_2$, 1 mg/ml synthetic polypeptide (Glu:Tyr = 4:1) and various concentrations of [γ-^{32}P]ATP (1 μCi/nmol). After incubating this solution at 30° for 15 min, a 35 μl of the supernatant was spotted on a piece of phosphocellulose paper (Whatman, P81). The paper was extensively washed in 75 mM phosphoric acid and placed in a vial containing Aquasol (New England Nuclear). Duplicates of papers were prepared from each reaction mixture and ^{32}P incorporated into the peptide was quantitated in a liquid scintillation counter. The kinase activity of insulin receptor was calculated, excluding that without insulin. Platelet membrane tyrosine kinase was prepared and assayed by the method of Nakamura *et al.* [12]. Tyrosine protein kinase activity of the platelets particular fraction (30 μg/tube) was measured by stimulating the phosphorylation of angiotensin II using 50 mM $MgCl_2$, 0.05% of Nonidet P-40 and various concentrations of [γ-^{32}P]ATP (1 μCi/nmol). The phosphorylation reaction was initiated by adding 5 μl of [γ-^{32}P]ATP, incubated at 30° for 10 min, and halted by adding of 50 μl of 5% trichloroacetic acid and 20 μl of bovine serum albumin (10 mg/ml). The proteins were precipitated by centrifugation for 5 min. A 70 μl aliquot of the supernatant was spotted on a piece of phosphocellulose paper (Whatman, P81) and washed in 75 mM phosphoric acid.

Flavonoids were dissolved in 100% DMSO and added to the reaction mixture at concentrations between 0.1 μM and 500 μM. The final concentration of DMSO in the reaction mixture was 5%. This concentration of DMSO had no effect on the protein kinase activities tested herein.

Serine/threonine protein kinase preparation and assay. Calmodulin (CaM)* isolated from bovine brain was prepared by the procedure described by Yazawa *et al.* [13]. Myosin light chain was prepared from chicken gizzard by the method of Hathaway and Haeberle [14]. The partially purified holoenzyme of cAMP-dependent protein kinase I (second DE-52 step) and its purified catalytic subunits were prepared from rabbit skeletal muscle, by the method of Beavo *et al.* [15]. Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C) was prepared from rabbit brain, as described by Inagaki *et al.* [16]. Ca^{2+}-CaM-dependent myosin light chain kinase was purified from chicken gizzard by the method of Walsh *et al.* [17]. Casein kinase I from rat liver was prepared by the method of Meggio *et al.* [18]. Casein kinase II from rabbit skeletal muscle was prepared according to Huang *et al.* [19]. The phosphotransferase activities of cAMP-dependent protein kinase, protein kinase C, myosin light chain kinase and casein kinase I and II were assayed under the conditions described by Inagaki *et al.* [20].

cAMP-dependent protein kinase activity was assayed in a reaction mixture containing, in a final volume of 0.2 ml, 50 mM Tris–HCl (pH 7.0), 10 mM magnesium acetate, 2 mM EGTA, 1 μM cAMP or no cAMP, 3.3–20 μM [γ-^{32}P]ATP (0.2 μCi/nmol), 0.5 μg of the enzyme, 100 μg of histone H2B, and each compound, as indicated.

Protein kinase C activity was assayed in a reaction mixture containing, in a final volume of 0.2 ml, 50 mM Tris–HCl (pH 7.0), 10 mM magnesium acetate, 0.5 mM calcium chloride or 1 mM EGTA, 10 μg of PS, 3.3–20 μM [γ-^{32}P]ATP (0.2 μCi/nmol), 100 μg of histone H1, and 0.3 μg of the enzyme. MLC-kinase activity was assayed in a reaction mixture

* Abbreviations used: CaM, calmodulin; PS, phosphatidyl serine; PM-TPK, platelet membrane-related tyrosine protein kinase; MLC-kinase, myosin light chain kinase.

containing, in a final volume of 0.2 ml, 50 mM Tris–HCl (pH 7.0), 10 mM magnesium acetate, 0.1 mM calcium chloride or 1 mM EGTA, 100 ng of CaM, 5–100 μM [γ-^{32}P]ATP (0.2 μCi/nmol), 20 μM smooth muscle 20,000-Da myosin light chain, and 0.6 μg of MLC-kinase. Casein kinase I activity was assayed in a reaction mixture containing, in a final volume of 0.2 ml, 50 mM Tris–HCl (pH 7.0), 10 mM magnesium acetate, 0.5 mM EGTA, 1 mM dithiothreitol, 3.3–20 μM [γ-^{32}P]ATP (0.2 μCi/nmol), 3.0 μg of the enzyme, and 800 μg of casein (Hammerstein quality). Casein kinase II activity was assayed in a reaction mixture containing, in a final volume of 0.2 ml, 50 mM Tris–HCl (pH 7.0), 5 mM magnesium acetate, 0.5 mM EGTA, 1 mM dithiothreitol, 3.3–20 μM [γ-^{32}P]ATP (0.2 μCi/nmol or 3.3–20 μM [γ-^{32}P]GTP (0.35 μCi/nmol), 2.8 μg of the enzyme, and 800 μg of casein (Hammerstein quality).

Assays were performed for 1, 3 and 5 min at 30° and in all cases demonstrated a linear incorporation of γ-^{32}P-phosphate into the substrate, over the 5-min assay. The reaction was terminated by adding 1 ml of ice-cold 20% trichloroacetic acid, following the addition of 500 μg of bovine serum albumin as a carrier protein. The sample was centrifuged at 3000 rpm for 15 min, the pellet was resuspended in ice-cold 10% trichloroacetic acid solution and the centrifugation–resuspension cycle was repeated three times. The final pellet was dissolved in 1 ml of 1 N NaOH, and radioactivity was measured in a liquid scintillation counter.

RESULTS

Inhibitory effect of myricetin on tyrosine protein kinases

Figure 2 shows the effect of the presence of increasing concentrations of myricetin, a derivative of quercetin, on the activity of three different types of tyrosine protein kinases, namely pp130^{fps}, insulin receptor and platelet membrane-associated (PM-TPK) tyrosine protein kinases. About 50% of pp130^{fps} and insulin receptor tyrosine kinase activities were inhibited by 3 or 5 μM of myricetin, respectively; however, PM-TPK was not significantly affected by myricetin concentrations, in the micromolar range.

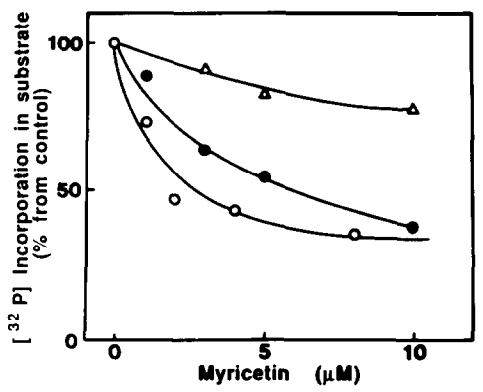

Fig. 2. Effect of myricetin on the activities of tyrosine protein kinases. Tyrosine kinases of pp130^{fps} (○), insulin receptor (●) and platelet particulate fraction (△) were assayed as described under Materials and Methods, with various concentrations of myricetin added as indicated. The presented data are the mean of two independent experiments.

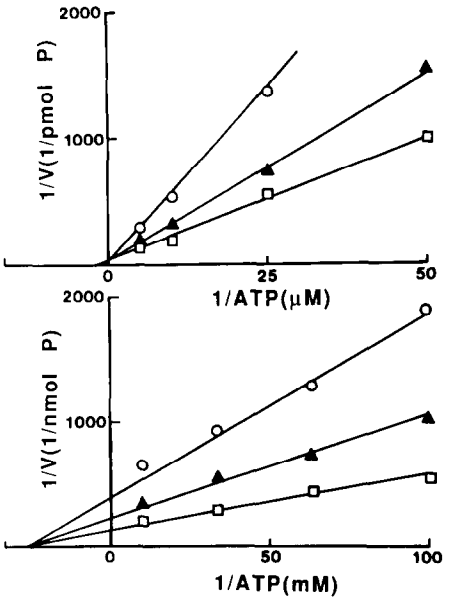

Fig. 3. Kinetic analysis of inhibitory effect of myricetin on tyrosine protein kinases. (A) Reciprocal velocity of pp130^{fps} tyrosine kinase versus 1/[ATP] with 0 (□), 2.0 (▲) and 5.0 (○) μM of myricetin. (B) Reciprocal velocity of insulin receptor tyrosine kinase versus 1/[ATP] with 0 (□), 5.0 (▲) and 10 (○) μM of myricetin. All other conditions are as described under Materials and Methods.

Mechanism of tyrosine protein kinase inhibition by myricetin

To characterize further the mechanism of tyrosine protein kinase inhibition by myricetin, the enzyme activities were assayed at various ATP concentrations and in the presence of increasing concentrations of myricetin. The corresponding data were evaluated using Lineweaver–Burk plots, as shown in Fig. 3. Myricetin apparently behaves as a competitive inhibitor toward ATP in the phosphorylation reaction of pp130^{fps} but revealed a non-competitive inhibition of insulin receptor tyrosine kinase. Using these data, the K_i values of pp130^{fps} and insulin receptor tyrosine protein kinase were calculated to be 1.8 and 2.6 μM, respectively.

Effect of myricetin on serine/threonine protein kinases

From the data of myricetin inhibition, tyrosine protein kinases were classified in three types. To examine the specificity of myricetin, we investigated the effect of myricetin on various serine/threonine protein kinases as cAMP-dependent protein kinase, protein kinase C, myosin light chain kinase, and casein kinase I and II. In the micromolar range, myricetin potently inhibited only myosin light chain kinase and casein kinase II (Fig. 4), but at higher

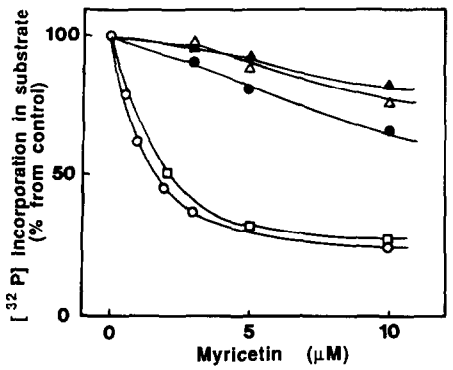

Fig. 4. Effect of myricetin on the activities of serine/threonine protein kinases. Phosphotransferase activities of protein kinase C (▲), cAMP-dependent protein kinase (△), casein kinase I (●), casein kinase II (○) and myosin light chain kinase (□) were assayed as described under Materials and Methods, with various concentrations of myricetin added as indicated. The presented data are means of two independent experiments.

concentrations, myricetin also inhibited the other protein kinases tested.

Kinetic analysis using Lineweaver–Burk plots showed that myricetin competitively inhibited myosin light chain kinase and casein kinase I and II with ATP (Fig. 5). The K_i values of each protein kinase are summarized in Table 1.

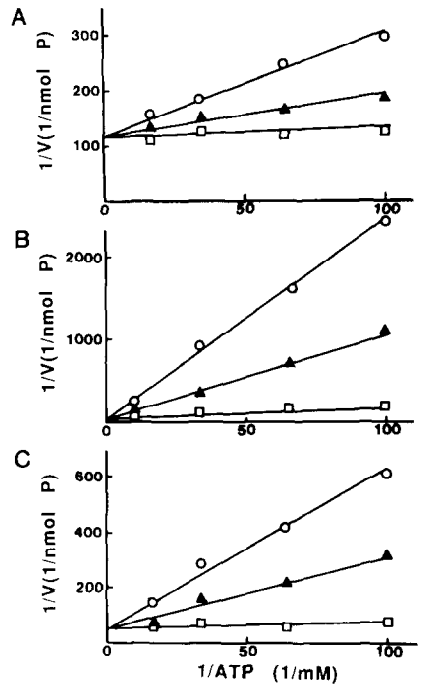

Fig. 5. Kinetic analysis of inhibitory effect of myricetin on serine/threonine protein kinases. (A) Reciprocal velocity of casein kinase II versus 1/[ATP] with 0 (□), 1.0 (▲) and 3.0 (○) μM of myricetin. (B) Reciprocal velocity of myosin light chain kinase versus 1/[ATP] with 0 (□), 5 (▲) and 10 (○) μM of myricetin. (C) Reciprocal velocity of casein kinase I versus 1/[ATP] with 0 (□), 100 (▲) and 200 (○) μM of myricetin. All other conditions are as described under Materials and Methods.

Table 1. Comparison of inhibitory potency of myricetin on serine/threonine protein kinases

Enzymes	K_i values (μM)
Casein kinase II	0.6
Myosin light chain kinase	1.7
Casein kinase I	9.0
Protein kinase C	12.1
cAMP-dependent protein kinase	27.5

Each K_i values are mean of two independent experiments.

Structure–activity study of seven flavonoids on protein kinases

To elucidate the difference of flavonoids' inhibition on tyrosine and serine/threonine protein kinases, a structure–activity relationship concerning polar substituents (hydroxy groups) was examined

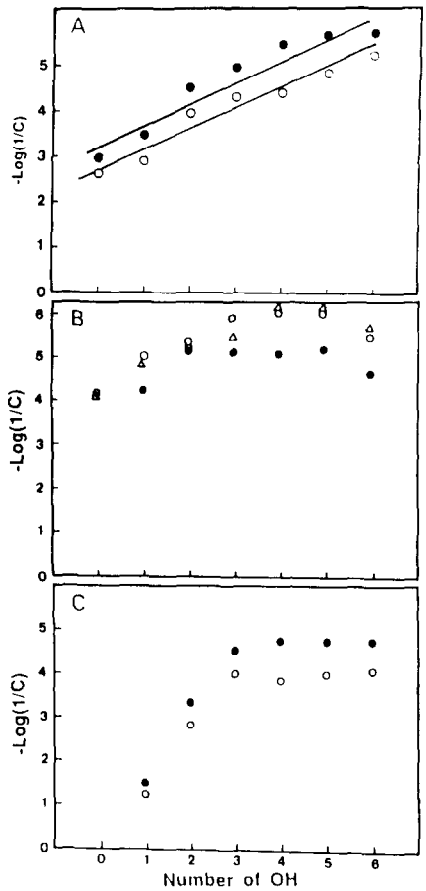

Fig. 6. Activity–structure relationships of flavonoids. The ordinate expresses the log ($1/IC_{50}$) of each flavonoid. The IC_{50} value is defined as the concentration of the compound required to produce a 50% inhibition of each enzyme activity. The abscissa exhibits the number of hydroxy residues of each flavonoid, as shown in Fig. 1. (A) Activity structure for $pp130^{fps}$ (●) and insulin receptor (○) tyrosine kinase. (B) Activity structure for casein kinase I (●) casein kinase II (△), and myosin light chain kinase (○). (C) Activity structure for cAMP-dependent protein kinase (○) and protein kinase C (○).

using the aforementioned assay. The corresponding data are illustrated in Fig. 6A. Hydroxylation of the flavone rings leads to substantial enhancement of the inhibitory potencies to pp130^{fps} and insulin receptor tyrosine protein kinases. Although the hydroxylation patterns influenced the potencies of flavonoids as inhibitors of serine/threonine kinase, it is likely that factors beyond the number of hydroxyl substituents take part in determining the potency of the inhibitors (Fig. 6B, C).

DISCUSSION

The inhibitory effect of myricetin, a derivative of quercetin, on tyrosine protein kinases was investigated using an oncogene product (130^{fps}), a growth factor receptor (insulin receptor), and a tyrosine kinase from non-proliferative cells (PM-TPK). The characteristic property of each tyrosine protein kinase was thus revealed: (1) susceptibility to flavonoids and ATP competes with the flavonoids (pp130^{fps}); (2) susceptibility to flavonoids and ATP does not compete with flavonoids (insulin receptor); (3) resistant to flavonoids (PM-TPK). Whether or not these classifications reveal a general property of oncogene-encoded, growth factor receptor associated or non-proliferative cell associated tyrosine protein kinases remains an open question. Graziani *et al.* reported that quercetin also inhibited pp60^{src} tyrosine kinase in competition with ATP and the K_i value was in the range of 6–11 μM [4]. From these data pp60^{src} may have characteristics in common with pp^{130}fps. The data suggest that the flavonoid binding site is placed at or near the ATP binding site of the oncogene-encoded protein kinases, whereas the flavonoid binding site of the insulin receptor does not share the ATP binding site. In contrast to the accumulative data on oncogene-encoded or growth factor receptor associated tyrosine protein kinases, tyrosine kinase activity in the particulate fraction from human platelets (PM-TPK) was less well understood. Nakamura *et al.* reported that the apparent M_r value of PM-TPK was 52,000, as determined by sucrose density gradient sedimentation [21] and that PM-TPK may be identical with the 60 kDa protein reported by Tuy *et al.* [22]. Golden *et al.* showed that the levels of pp60$^{c\text{-}src}$ kinase activity are elevated in blood platelets [23]. Whether or not the flavonoids-resistant tyrosine protein kinase is identical with pp60$^{c\text{-}src}$ or other unidentified tyrosine protein kinase will have to be given attention.

We also examined the inhibitory effects of flavonoids on five kinds of serine/threonine protein kinases. The quercetin-induced inhibition of casein kinase II, phosphorylase kinase and protein kinase C has been reported [5–7]. In addition, we obtained evidence that: (1) bioflavonoids at micromolar concentration ranges potently inhibit myosin light chain kinase; (2) at higher concentrations of bioflavonoid, including quercetin, they inhibit even cAMP-dependent protein kinase (purified catalytic subunit) and casein kinase I; (3) the inhibition of casein kinase I and II and myosin light chain kinase by a flavonoid myricetin but not that of cAMP-dependent protein kinase and protein kinase C was competitive with ATP. These findings correspond to the results reported by Gschwendt *et al.* [7] that protein kinase C was inhibited by quercetin interacting with phospholipids [7].

Structure–activity studies using seven flavonoids were performed to investigate characteristics of the active site of these protein kinases. Our studies indicated that the inhibitory effects of flavonoids on tyrosine kinases closely correlated with the number of hydroxy residues on the flavon rings (correlation coefficient (r) = 0.974). The inhibition of pp130^{fps} tyrosine kinase was also in accord with the number of hydroxy groups (r = 0.926). The introduction of hydroxy groups into the flavone rings results in a decrease in retention time on the reversed phase column [24], hence, there may be a decrease in flavonoid hydrophobicity and an increase in polarity. These results suggest that the hydrophobicity of flavonoids is detrimental for inhibition of the tyrosine kinases, although the nucleotide binding sites of several enzymes with dinucleotide folds are in a hydrophobic pocket. The structural requirements for inhibition of nucleotide-binding enzymes or human platelets functions have been investigated. Beretz *et al.* [25] showed that OH groups are detrimental for the inhibition of platelets. Verma *et al.* [26] indicated that increasing the number of OH groups in ring B from one to two enhances the inhibitory activity on aldose reductase, but additional OH groups are slightly depotentiating. Ferrell *et al.* [27] reported that changing the planarity of the heterocyclic ring greatly decreases the inhibitory potency to cyclic AMP phosphodiesterase or bovine pancreatic ribonuclease and that the lowest empty molecular orbitals (LEMO) for both cAMP and flavonoids, calculated by CNDO/2, are π-type orbitals with significant contributions from pyrimidine ring atoms of cAMP or the C-ring atoms of flavonoids. According to their calculations, % C ring in LEMO was myricetin > quercetin > fisetin > flavone. This order is consistent with the inhibitory potencies of tyrosine protein kinases.

To test whether these findings hold true for other protein kinases, we investigated the effects of OH groups on serine/threonine kinase. The hydroxylation pattern also influenced the inhibition of casein kinase I and II, and myosin light chain kinase, albeit less markedly.

The fact that quercetin and fisetin are more potent inhibitors than myricetin is a common characteristic of these protein kinases. Steric factors of three OH groups of the B ring may be involved in the decrease of inhibitory potency of myricetin. As for protein kinase C and cAMP-dependent protein kinase, similar structural requirements were observed. The inhibitory potencies of compounds substituted with fewer OH groups (flavone, 7-hydroxyflavone, and chrysin) were more markedly dependent on the hydroxylation, but hydroxylation did not increase the activities of flavonoids with a higher number of hydroxyl substituents (apigenin, fisetin, quercetin and myricetin).

We reported that isoquinolinesulfonamides such as H-7 and H-8, which interact with the ATP-binding site of enzymes, potently inhibited protein kinase C and cyclic nucleotide-dependent protein kinases but revealed comparatively weak inhibition for casein

kinase I and II and myosin light chain kinase [8]. All the accumulated data on isoquinoline-sulfonamides and flavonoids suggest the structural resemblance of the ATP binding sites of casein kinase I, casein kinase II and myosin light chain kinase.

Parker *et al.* [28] and our groups [29] reported the amino acid sequence of protein kinase C and suggested that the catalytic part of protein kinase C is highly homologous to the catalytic subunit of cAMP-dependent protein kinase. In addition, the domain organization of myosin light chain kinase from chicken gizzard and rabbit skeletal muscle was reported by Guerriero *et al.* [30] and Takio *et al.* [31], respectively. Our pharmacological observations are of interest as flavonoids are expected to be useful molecular probes for mapping of the active site of protein kinases.

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REFERENCES

1. E. M. Soulinina, R. N. Buchsbaum and E. Racker, *Cancer Res.* **35**, 1865 (1975).
2. H. Nishino, A. Iwashita, H. Fujiki and R. Sugimura, *Gann* **75**, 113 (1984).
3. J. Levy, I. Teuerstein, M. Marbach, S. Radian and Y. Sharoni, *Biochem. biophys. Res. Commun.* **123**, 1227 (1894).
4. Y. Graziani, E. Erikson and R. Erikson, *Eur. J. Biochem.* **135**, 583 (1983).
5. C. Cochet, J. J. Feige, F. Pirollet, M. Keramidas and E. M. Chambaz, *Biochem. Pharmacol.* **31**, 1357 (1982).
6. A. K. Srivastava, *Biochem. biophys. Res. Commun.* **131**, 1 (1985).
7. M. Gshwendt, F. Horn, W. Kittstein and F. Marks, *Biochem. biophys. Res. Commun.* **117**, 444 (1983).
8. H. Hidaka, M. Inagaki, S. Kawamoto and Y. Sasaki, *Biochemistry* **23**, 5036 (1984).
9. I. Greiser-Wilke, K. M. Owada and K. Moelling, *Virology* **39**, 325 (1981).
10. U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
11. G. R. Freidenberg, H. H. Klein, R. Cordera and J. M. Olefsky, *J. biol. Chem.* **260**, 12444 (1985).
12. S. Nakamura, F. Takeuchi, H. Kondo and H. Yamamura, *FEBS Lett.* **170**, 139 (1984).
13. M. Yazawa, M. Sakuma and K. Yagi, *J. Biochem. (Tokyo)* **87**, 1313 (1980).
14. D. R. Hathaway and J. R. Haeberle, *Analyt. Biochem.* **135**, 37 (1983).
15. J. A. Beavo, P. J. Bechtel and E. G. Krebs, *Meth. Enzymol.* **38**, 299 (1974).
16. M. Inagaki, M. Watanabe and H. Hidaka, *J. biol. Chem.* **260**, 2922 (1985).
17. M. P. Walsh, S. Hinkins, R. Dabrowska and D. J. Hartshorne, *Meth. Enzymol.* **99**, 278 (1983).
18. F. Meggio, A. Donella-Deana and L. A. Pinna, *FEBS Lett.* **106**, 76 (1979).
19. K. P. Huang, E. Itarte, T. J. Singh and A. Akatsuka, *J. biol. Chem.* **257**, 3236 (1982).
20. M. Inagaki, S. Kawamoto, H. Itoh, M. Saitoh, M. Hagiwara, J. Takahashi and H. Hidaka, *Molec. Pharmac.* **29**, 577 (1986).
21. S. Nakamura, F. Takeuchi, T. Tomizawa, N. Takahashi, H. Kondon and H. Yamamura, *FEBS Lett.* **184**, 56 (1985).
22. F. P. D. Tuy, J. Henry, C. Rosenfeld and A. Kahn, *Nature, Lond.* **305**, 435 (1983).
23. A. Golden, S. Nemeth and J. Brugge, *Proc. natn. Acad. Sci. U.S.A.* **83**, 852 (1986).
24. V. S. Bankova, S. S. Popov and N. L. Marekov, *J. Chromatogr.* **242**, 135 (1982).
25. A. Beretz, J. P. Cazenare and R. Anton, *Agents and Actions* **12**, 382 (1982).
26. S. D. Verma, I. Mikuni and J. H. Kinoshita, *Science* **188**, 1215 (1977).
27. J. E. Ferrell, Jr., P. D. G. C. Sing, G. Loew, R. King, J. M. Mansour and T. E. Mansour, *Molec. Pharmac.* **16**, 556 (1979).
28. J. Parker, L. Coussens, N. Totty, L. Rhee, S. Young, E. Chen, S. Stabel, M. D. Waterfield and A. Ulrich, *Science* **233** 853 (1986).
29. S. Ohno, H. Kawasaki, S. Imajoh, K. Suzuki, M. Inagaki, H. Yokokura, T. Sako and H. Hidaka, *Nature, Lond.* **325**, 161 (1987).
30. V. Guerriero, Jr., M. A. Russo, N. J. Olson, J. A. Putkey and A. R. Means, *Biochemistry* **25**, 8372 (1986).
31. K. Takio, R. D. Wade, S. B. Smith, E. G. Krebs, K. A. Walsh and K. Titani, *Biochemistry* **23**, 4207 (1984).