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INHIBITION OF PROTEIN-TYROSINE KINASE ACTIVITY BY FLAVANOIDS AND RELATED COMPOUNDS

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ABSTRACT.—A series of 22 flavanoids and related compounds were tested for their ability to inhibit the activity of a protein-tyrosine kinase purified from bovine thymocytes (p40). Flavones or flavanols with hydroxyl groups at C-5 and C-7 or with three hydroxyl groups on the phenyl ring were potent inhibitors of p40. The replacement of hydroxyl groups with methoxyl groups led to a substantial loss of inhibitory activity. The presence of methoxyl or rhamnosyl substituents at C-3 also abolished inhibitory activity. Kinetic analyses indicated that the flavone apigenin [**2**] was a competitive inhibitor of p40 with respect to ATP. Flavanones and isoflavones were relatively inactive as protein-tyrosine kinase inhibitors. The isoflavone genistein [**17**], which has been reported as a potent inhibitor of both $pp60^{v\text{-}src}$ and the epidermal growth factor receptor, was not an inhibitor of p40.

The protein-tyrosine kinases constitute a family of enzymes that are thought to play critical roles in the regulation of cell proliferation. They include the transmembrane receptors for several polypeptide growth factors, the products of at least eight distinct retroviral oncogenes and their corresponding cellular proto-oncogenes, and several additional enzymes characterized by their activities or by their sequence similarity to other known tyrosine kinases (for recent reviews see 1–4). All of these enzymes catalyze the transfer of phosphate from ATP to the hydroxyl of tyrosyl residues on protein substrates. Because this activity is essential for the transduction of mitogenic signals by the growth factor receptor kinases and for the malignant transformation of cells by the viral oncogene-encoded kinases, it is inferred that the normal role of all cellular protein-tyrosine kinases is in the regulation of some aspect of cell growth or differentiation.

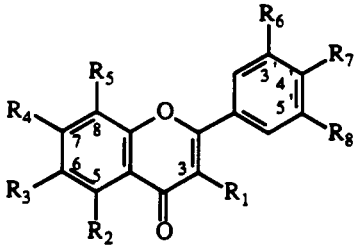
The probable significance of protein-tyrosine kinases in cell growth regulation makes them important targets for the discovery and development of specific inhibitors to serve both as potential chemotherapeutic agents and as pharmacological tools for defining the physiological roles of tyrosine kinases. Potential inhibitors reported to date include both synthetic compounds (5–11) and natural products (12–17). The latter includes flavones such as quercetin (12) and myricetin (13), and the isoflavone genistein (14). Both quercetin and genistein have been reported to inhibit the activity of the epidermal growth factor receptor and of $pp60^{v\text{-}src}$, the transforming protein of the Rous sarcoma virus. Our own interests are centered on a protein-tyrosine kinase known as p40, which is a major protein-tyrosine kinase activity in normal thymocytes (18). In this study we have examined a range of flavanoids and related compounds for their ability to inhibit the activity of the purified p40 kinase.

RESULTS AND DISCUSSION

Several naturally occurring flavanoids and related compounds were tested for their ability to inhibit the phosphorylation of a tyrosine-containing peptide catalyzed by the thymocyte protein-tyrosine kinase, p40. Compounds were tested initially at concentrations of 1000, 500, and 100 μg/ml. Compounds that exhibited half-maximal inhibition at concentrations <500 μg/ml were then reexamined at a lower range of concentrations (2.5–100 μg/ml).

Quercetin [**1**], which had previously been shown to inhibit the activity of $pp60^{v\text{-}src}$ and the EGF-receptor, was also a potent inhibitor of p40 (ID_{50} = 11 μg/ml). Several additional polyphenols of related structure (apigenin [**2**], fisetin [**3**], robinetin [**4**], and myricetin [**5**]) were also potent inhibitors, showing half-maximal inhibition at concentrations comparable to that seen for quercetin. These results (Table 1) indicated that several different polyhydroxylated flavones and flavonols were capable of interacting with p40 with high affinity. β-Naphthoflavone, which lacks any hydroxyl groups, was completely inactive as an inhibitor (not shown). A hydroxyl group at C-3 was not necessary for inhibitory activity (e.g., **2**), but the presence of a bulky substituent at this position (contrast **5** with **6**) substantially blocked inhibitory activity.

These data suggested that multiple hydroxyl groups are important for the interaction of flavanoids with p40. A similar observation has been made for the interaction of flavanoids with the $pp130^{fps}$ tyrosine kinase and the insulin receptor (13). The results from testing a series of polymethoxyflavones supported this hypothesis. In general, the replacement of hydroxyl groups with methoxyl substituents yielded much weaker inhibitors. For example, replacement of two of the three hydroxyl groups on **2**, a potent inhibitor, with methoxyl groups (compound **10**), completely blocked its inhibitory ac-



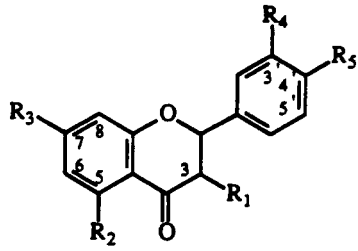
1 R_1=R_2=R_4=R_6=R_7=OH, R_3=R_5=R_8=H (quercetin)
2 R_2=R_4=R_7=OH, R_1=R_3=R_5=R_6=R_8=H (apigenin)
3 R_1=R_4=R_6=R_7=OH, R_2=R_3=R_5=R_8=H (fisetin)
4 R_1=R_4=R_6=R_7=R_8=OH, R_2=R_3=R_5=H (robinetin)
5 R_1=R_2=R_4=R_6=R_7=R_8=OH, R_3=R_5=H (myricetin)
6 R_2=R_4=R_6=R_7=R_8=OH, R_3=R_5=H, R_1=rhamnosyl (myricitrin)
7 R_2=R_4=OH, R_3=R_5=R_7=OMe, R_1=R_6=R_8=H
8 R_2=R_4=OH, R_1=R_7=OMe, R_3=R_5=R_6=R_8=H
9 R_2=OH, R_1=R_4=R_7=OMe, R_3=R_5=R_6=R_8=H
10 R_2=OH, R_4=R_7=OMe, R_1=R_3=R_5=R_6=R_8=H

TABLE 1. Effect of Flavanoids on the Protein-Tyrosine Kinase Activity of p40.

Compound	ID_{50} (μg/ml)	Compound	ID_{50} (μg/ml)
1	11	**12**	270
2	90	**13**	>500
3	35	**14**	80
4	7	**15**	300
5	11	**16**	>500
6	>500	**17**	>500
7	48	**18**	>500
8	>500	**19**	>500
9	>500	**20**	>500
10	>500	**21**	350
11	>500	**22**	450

tivity. The one methoxyderivative that exhibited reasonable inhibitory activity (compound **7**) had two hydroxyl groups at C-5 and C-7 and was not modified at C-3.

Flavanones were considerably less active than the corresponding flavones or flavanols. For example, **1**, **2**, and **3** were all considerably more active as inhibitors than were the flavanones **12**, **11**, and **14**, which bear hydroxyl substituents at the same relative positions. The three isoflavones that were tested (**17**, **18**, and **19**) all lacked signifi-

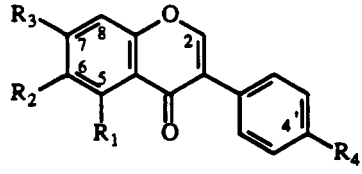


11 $R_1=R_3=R_4=OH$, $R_2=H$ (fustin)
12 $R_1=R_2=R_3=R_4=R_5=OH$ (taxifolin)
13 $R_2=R_3=OH$, $R_1=R_4=R_5=H$ (pinocembrin)
14 $R_2=R_3=R_5=OH$, $R_1=R_4=H$
15 $R_2=R_3=OH$, $R_5=OMe$, $R_1=R_4=H$
16 $R_3=OH$, $R_1=R_2=R_4=R_5=H$

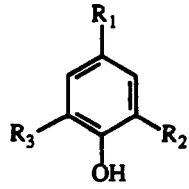
cant inhibitory activity, which is consistent with the observation that substituents other than hydroxyl groups present at the 3 position block inhibitory activity. Small-molecular-weight biosynthetic precursors to the flavanoids (syringic acid [**20**], ferulic acid [**21**], and coumaric acid [**22**]) were largely inactive as inhibitors.

Based on these studies, some general structural features required for the interaction of flavanoids with the p40 tyrosine kinase can be determined. High affinity interactions with p40 required a flavone or flavanol ring structure; the corresponding flavanones or isoflavones were relatively inactive as inhibitors. The most potent inhibitors contained hydroxyl groups at ring positions 5 and 7 (compounds **1**, **2**, **5**, and **7**) or, alternatively, had 3 hydroxyl groups on the phenyl ring (compound **4**). Methoxyl substituents could not substitute for the hydroxyl groups, suggesting the importance of hydrogen-bonding interactions between the flavanoid and the kinase. A hydroxyl group at position 3 did not decrease inhibitory activity, but the presence of other substituents [e.g., rhamnosyl (compound **6**) or methoxyl (compounds **8** and **9**)] at this position completely blocked inhibitory activity.

The inhibition of $pp60^{v\text{-}src}$ by quercetin was previously shown to be competitive with respect to ATP and noncompetitive with respect to a protein substrate (12). To determine if p40 was inhibited by flavones via a related mechanism, the effects of varying fixed concentrations of apigenin on the phosphorylation of the peptide substrate an-



17 $R_1=R_3=R_4=OH$, $R_2=H$ (genistein)
18 $R_1=R_3=OH$, $R_4=OMe$, $R_2=H$ (biochanin A)
19 $R_2=R_3=R_4=OH$, $R_1=H$



20 R_1=COOH, R_2=R^3=OCH^3 (syringic acid)
21 R^1=C=CHCOOH, R^2=OCH^3, R^3=H (ferulic acid)
22 R^1=C=CHCOOH, R^2=R^3=H (coumaric acid)

giotensin I were examined. As shown in Figure 1, apigenin was also a competitive inhibitor of p40 with respect to ATP (K_i = 10 μM) and noncompetitive with respect to the peptide substrate. This latter observation has been used to suggest that flavanoids might interact with protein-tyrosine kinases at multiple sites (14). Similar kinetics have been obtained for the inhibition of the EGF-receptor by genistein (14).

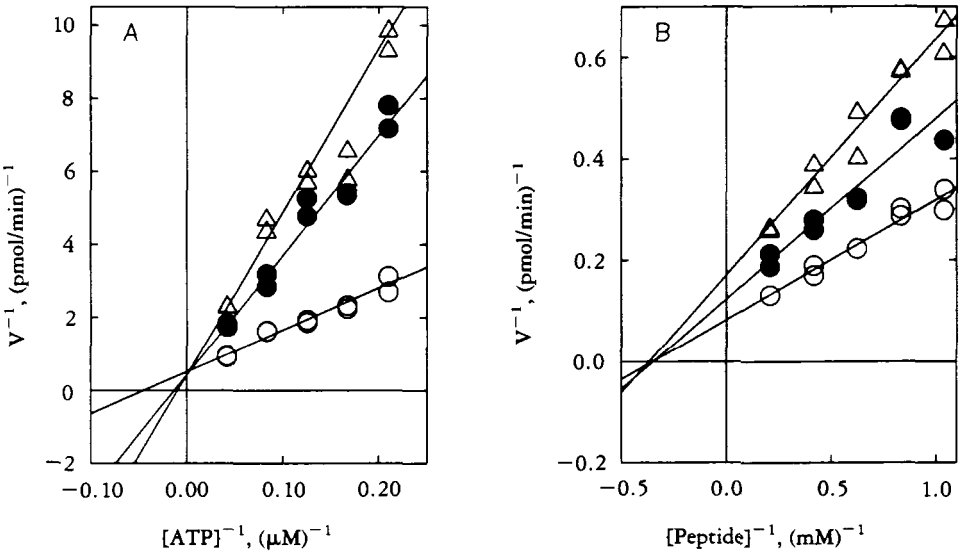


FIGURE 1. Inhibition of p40 by apigenin. A, Effect of apigenin [0 (○), 4 (●), or 8 (Δ) μg/ml] on the phosphorylation of 1.2 mM angiotensin I at varying concentrations of [γ-^{32}P]ATP. B, Effect of apigenin [0 (○), 4 (●) or 8 (Δ) μg/ml] on the phosphorylation of varying concentrations of angiotensin I (peptide) at 50 μM [γ-^{32}P]ATP.

The range of flavanoids that interacted with p40 with high affinity was more restricted than that seen for other protein-tyrosine kinases such as pp60$^{v\text{-}src}$ and the EGF-receptor. Quercetin [**1**] and related flavones and flavanols were potent inhibitors of all three enzymes (12,14). Interestingly the isoflavone, genistein [**17**], which was a potent inhibitor of both pp60$^{v\text{-}src}$ and the EGF-receptor (14), was inactive as an inhibitor of p40. Similarly, biochanin A [**18**] was a reasonably good inhibitor of the EGF-receptor kinase (14), but was inactive as a p40 inhibitor. These observations indicate a fundamental difference in the way in which different protein-tyrosine kinases interact with small molecules. Because **17** is often used as a protein-tyrosine kinase inhibitor, it is important that investigators be aware that it does not inhibit all types of protein-

tyrosine kinase. It is possible that certain isoflavones will find utility as reagents that distinguish between phosphorylation reactions catalyzed by different classes of protein-tyrosine kinases.

These results provide useful structural information on the types of compounds that interact with the p40 protein-tyrosine kinase. Studies of this nature should be helpful in the development of biological probes useful in studying the roles of specific protein-tyrosine kinases in the regulation of various cell functions and for the future discovery or design of therapeutic agents.

EXPERIMENTAL

STANDARD COMPOUNDS.—The compounds were either obtained from commercial sources or isolated and completely characterized in previous studies.

ENZYME ASSAYS.—The p40 protein-tyrosine kinase was purified to homogeneity from bovine thymus (17). The activity of p40 was measured by the phosphorylation of a synthetic tyrosine-containing peptide substrate (angiotensin I) as described previously (18), except that reactions also contained 8% DMSO (used as a solvent for all inhibitors) and varying concentrations of inhibitor. We found that the extent of inhibition of p40 by various flavanoids was not affected by the concentration of NaCl included in the assay. Values are expressed as concentrations of inhibitor that gave half-maximal inhibition of the rate of angiotensin I phosphorylation under standard conditions of 1.2 mM angiotensin I and 50 μM [γ-^{32}P]ATP.

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