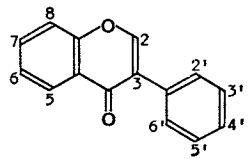
INHIBITION OF TYROSINE PROTEIN KINASE ACTIVITY BY SYNTHETIC ISOFLAVONES AND FLAVONES

Sir:

Tyrosine protein kinase is known to be associated with oncogene products of the retroviral *src* gene family[1]. This kinase activity is closely correlated with the ability of retroviruses to transform cells, since mutants of viruses which lack tyrosine kinase activity are defective in malignant transformation[2]. Similar kinase activities are also associated with the cellular receptors for growth factors such as epidermal growth factor (EGF)[3], PDGF[4], insulin[5], insulin-like growth factor I[6] and CSF-1[7]. It is supposed therefore that tyrosine-phosphorylation plays an important role for diverse cell functions,

Table 1. Effects of isoflavone derivatives on tyrosine protein kinase activity of EGF receptor and growth of RSV-transformed 3Y1 (RSV3Y1) cells.



Compound	Position				IC_{50} (μg/ml)	
	2	5	7	4′	PKI[a]	RSV3Y1
PKI-1 (Genistein)	—	OH	OH	OH	0.7	7.0
PKI-2	CH_3	OH	OH	OH	2.0	25.0
PKI-3	CH_3	OH	OH	OCH_3	11.0	15.0
PKI-4	CH_3	OAc	OAc	OCH_3	>100	40.0
PKI-5	COOH	OH	OH	OCH_3	10.0	>100
PKI-6	$COOC_2H_5$	OH	OH	$OCOCOOC_2H_5$	1.0	>100
PKI-7	COOH	OH	OH	OH	2.0	>100
PKI-8	$CH_2N(CH_3)_2$	OH	OH	OCH_3	>100	18.0
PKI-9	CH_2NH–C$_6$H$_5$	OH	OH	OCH_3	>100	16.0
PKI-10	$CH_3NHCH_2CH_2OH$	OH	OH	OCH_3	>100	>100
PKI-11	CH_2N–C$_5$H$_{10}$	OH	OH	OH	10.0	9.5
PKI-12	$COOC_2H_5$	OH	OH	OH	2.0	50.0
PKI-13	$CONH_2$	OH	OH	OH	10.0	>100
PKI-14	$CONHCH_3$	OH	OH	OH	15.0	>100
PKI-15	$CONHCH_2CH_2OH$	OH	OH	OH	15.0	>100
PKI-16	CH_2SCH_3	OH	OH	OH	5.0	20.0
PKI-17	CH_2S–C$_3$H$_3$NS	OH	OH	OH	5.0	20.0
PKI-18	$CH_2SCH_2CH_2COOH$	OH	OH	OH	10.0	90.0
PKI-19	$COOCH_3$	OH	OH	OH	2.0	60.0
PKI-20	$COOCH(CH_3)_2$	OH	OH	OH	10.0	25.0
PKI-21	$COOCH_2CH_2OCH_3$	OH	OH	OH	6.0	60.0
PKI-22	$CH_2SCH_2COOC_2H_5$	OH	OH	OH	4.0	>100
PKI-23	$CH_2SCH_2CHCH_2OH$ (OH)	OH	OH	OH	4.0	>100
PKI-24	CH_2N–C$_3$H$_3$NS	OH	OH	OH	15.0	>100

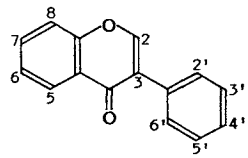
[a] Protein kinase inhibition.

cellular differentiation and malignant transformation. From this point of view, a specific inhibitor for tyrosine protein kinase is expected to be a good tool for understanding the physiological role of tyrosine-phosphorylation of the various functional cellular proteins. In this line of screening, we isolated genistein, an isoflavone, from fermentation broth of a *Pseudomonas* species[8]. We report here the inhibitory effect of isoflavones and flavones on tyrosine protein kinase activity and growth of Rous sarcoma virus(RSV)-transformed cells.

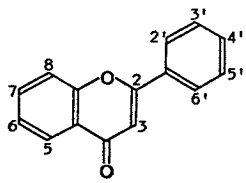
Isoflavone derivatives (PKI-1 to PKI-24) were synthesized in our laboratories and listed in Table 1. Acacetin, prunetin and daidzein were purchased from Laboratories Sarget. Biochanin A was obtained from Aldrich Chemical Co.; flavone, kaempferol and apigenin from Sigma Chemical Company and quercetin from Nakarai Chemicals, Ltd.

Protein kinase activity of EGF receptor was determined as follows. The kinase reaction were performed in a solution containing PIPES-NaOH 20 mM, pH 7.2, $MgCl_2$ 10 mM, $MnCl_2$ 3 mM, dithiothreitol 1 mM, sodium vanadate 100 μM, [γ-^{32}P]ATP (4 mCi/μmol) 10 μM, mouse EGF (Collaborative Research) 1 μg/ml, A431 cell membrane 10 μg, which was prepared as described previously[9], and the inhibitor. The reaction was continued for 5 minutes at 0°C and terminated by addition of LAEMMLI's SDS sample buffer[10] and boiling for 2 minutes. The samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. The bands of the EGF receptor were excised from the gels and the radioactivity was counted with a liquid scintillation counter. The inhibitory activity was calculated from the re-

Table 2. Effect of flavonoids on tyrosine protein kinase activity of EGF receptor.



Compound	Position					IC_{50} (μg/ml)	
	2	5	7	3′	4′	PKI[a]	RSV3Y1
Genistein	—	OH	OH	—	OH	0.7	7.0
Prunetin	—	OH	OCH_3	—	OH	4.2	25.0
Daidzein	—	—	OH	—	OH	>100	25.0
Biochanin A	—	OH	OH	—	OCH_3	26.0	18.0
Genistin	—	OH	Glucose	—	OH	>100	>100



Compound	Position					IC_{50} (μg/ml)	
	3	5	7	3′	4′	PKI[a]	RSV3Y1
Apigenin	—	OH	OH	—	OH	25.0	11.0
Acacetin	—	OH	OH	—	OCH_3	40.0	24.0
Flavone	—	—	—	—	—	50.0	7.0
Kaempferol	OH	OH	OH	—	OH	3.2	14.0
Quercetin	OH	OH	OH	OH	OH	5.0	12.0

[a] Protein kinase inhibition.

maining protein kinase activity. Cytotoxic activity was assayed using RSV-transformed 3Y1 (RSV3Y1) cells. RSV3Y1 cells were cultured in DULBECCO's modified EAGLE's medium (DMEM) supplemented with 7% foetal bovine serum (Gibco) with or without an inhibitor. After 48 hours, the viable cells were counted by trypan blue exclusion.

In order to clarify the structure-activity relationship, the inhibitory activity of flavonoids was investigated against tyrosine kinase. Prunetin, kaempferol and quercetin exhibited high inhibitory activity (Table 2). The inhibitory activity decreased drastically either by the removal of a hydroxyl group from 5 position (flavone and daidzein) or by the addition of a methoxy group to 4′ position (biochanin A and acacetin). Addition of a methoxy group at 7 position (prunetin) also reduced the inhibitory activity. Especially a bulky group at 7 position such as *O*-glucose (genistin) completely abolished the activity. These results indicate that a hydroxyl group at 5 position is essential for inhibitory activity and that at 7 and 4′ positions is necessary for full expression of the activity. Although quercetin was highly active against tyrosine kinase, it also inhibited other enzymes such as cAMP-dependent protein kinase[11], protein kinase C[12], phosphorylase kinase[13], Na^+, K^+-ATPase[14] and 5′-nucleotidase (AKIYAMA and OGAWARA; unpublished result).

From the results described above, several isoflavones with the modification at 2 position were synthesized and tested for their activity. As shown in Table 1, the half maximum effect (IC_{50}) was observed at the range of 0.7 μg/ml of PKI-1 (genistein) to more than 100 μg/ml of PKI-4, PKI-8, PKI-9 and PKI-10. Among these compounds, PKI-2, PKI-6, PKI-7, PKI-12, PKI-19, PKI-22 and PKI-23 showed a considerably higher inhibitory activity, but genistein was the strongest. PKI-4, PKI-8, PKI-9 and PKI-10 showed IC_{50} at more than 100 μg/ml. As described previously[15], genistein scarcely inhibited serine/threonine protein kinases such as cAMP-dependent protein kinase, protein kinase C and phosphorylase kinase at 100 μg/ml. PKI-6, PKI-7 and PKI-23 exhibited similar properties. Thus, the inhibitory activity of these compounds were highly specific for tyrosine protein kinases.

Next, we examined the cytotoxic effect of isoflavones on RSV3Y1 cells (Table 1). IC_{50} values of PKI-6, PKI-7, PKI-22 and PKI-23 against the growth of RSV-transformed cells were over 100 μg/ml, although they showed a considerably high inhibitory activity against tyrosine protein kinase. Therefore, no close correlation was observed between the inhibitory activity against tyrosine kinase and the inhibition of cell proliferation. Similar results were also obtained with flavonoids (Table 2). All the flavonoids examined exhibited a fair inhibitory activity on proliferation of RSV3Y1 cells, although some compounds such as daidzein and flavone showed a poor inhibitory effect on tyrosine kinase.

Acknowledgment

This work was supported in part by the Grant-in-Aid for cancer research from the Ministry of Education, Science and Culture of Japan and Suzuken Foundation.

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(Received September 8, 1988)

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