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SPECIFIC INHIBITORS OF TYROSINE-SPECIFIC PROTEIN KINASE, SYNTHETIC 4-HYDROXYCINNAMAMIDE DERIVATIVES

Tadayoshi Shiraishi, Takeshi Domoto, Naohiro Imai,
Yoshio Shimada, and Kiyoshi Watanabe

Biochemical Research Laboratories, Kanegafuchi Chemical Industry Co., Ltd., Takasago-cho, Takasago-shi, Hyogo-ken 676, Japan

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SUMMARY: Several newly synthesized 4-hydroxycinnamamide derivatives such as 3-(3',5'-di-isopropyl-4'-hydroxybenzylidene)-2-oxindol (ST 280), 3-(3',5'-dimethylthiomethyl-4'-hydroxybenzylidene)-2-oxindole (ST 458), α-cyano-3-ethoxy-4-hydroxy-5-phenylthiomethylcinnamamide (ST 638) and 3-(3'-ethoxy-4'-hydroxy-5'-phenylthiomethylbenzylidene)-2-pyrolidinone (ST 642) were found to inhibit tyrosine-specific protein kinase activity of the epidermal growth factor (EGF) receptor with IC_{50} values of 0.44 μM, 0.44 μM, 0.37 μM and 0.85 μM, respectively. None of them showed inhibitory effect on the enzyme activities of serine- and/or threonine-specific protein kinases such as cAMP-dependent protein kinase, Ca^{2+}/phospholipid-dependent protein kinase C, casein kinase I and casein kinase II. In addition, none of them had effect on Na^+/K^+-ATPase or 5'-nucleotidase. The results suggest that the compound ST 280, ST 458, ST 638 and ST 642 are potent and specific inhibitors of tyrosine-specific protein kinase.

Tyrosine-specific protein kinase (tyrosine kinase) is a group of enzymes which catalyze the transfer of phosphate from ATP to tyrosine residues in protein substrate. This enzyme activity was initially detected in several oncogene products of RNA tumor viruses such as pp60v-src, p70gag-yes, p130gag-fps and p70gag-fgr, and some growth factor receptors such as EGF receptor and insulin (1). These receptors were phosphorylated on tyrosine residues in a short periods after the binding of EGF and insulin (2,3). The phosphorylation of these receptors was thought to be an important initial event of signal transduction mechanisms in cells, which was triggered by growth factors. Recently, another type of tyrosine kinases which was named as cellular tyrosine kinase was detected in the nonproliferating or differentiated systems such as rat spleen (4). The significant increase in the number of the EGF receptor and their associated tyrosine kinase activity was observed in the cultured epidermoid carcinomas and biopsyspecimens from epidermoid tumors or brain tumors (5-7).

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These results and many other reports suggest that the tyrosine kinase contributes to important mechanisms of cell proliferation, carcinogenesis and cell differentiation, although the exact nature of such mechanisms is unknown. Therefore, a specific inhibitor of tyrosine kinases should be useful in investigating the mechanisms of carcinogenesis, cell proliferation and differentiation and it may be effective in prevention and chemoterapy of cancer.

In the course of search for specific inhibitors of tyrosine kinases, we found that several newly synthesized 4-hydroxycinnamamide derivatives such as 3-(3',5'-di-isopropyl-4'-hydroxybenzylidene)-2-oxindol (ST 280), 3-(3',5'-dimethylthiomethyl-4'-hydroxybenzylidene)-2-oxindole (ST 458), α-cyano-3-ethoxy-4-hydroxy-5-phenylthiomethylcinnamamide (ST 638) and 3-(3'-ethoxy-4'-hydroxy-5'-phenylthiomethylbenzylidene)-2-pyrolidinone (ST 642) showed potent inhibitory activities against the phosphorylation of plasma membrane fractions from human epidermoid carcinoma cell line A-431. Furthermore, we studied some properties of these inhibitors in vitro.

MATERIALS and METHODS

Materials. The synthetic 4-hydroxycinnamamide derivatives, ST 280, ST 458, ST 638 and ST 642, were newly prepared in our laboratories. The chemical structures of these compounds are shown in Fig. 1. The methods for synthesis of these compounds will be reported elsewhere. A-431 cells were kindly provided by Dr. K. Onodera from University of Tokyo. [r ^{32}P]ATP was purchased from Amasham Japan. Quercetin was from Wako Pure Chemical Industries. EGF, bovine serum alubmin, cAMP-dependent protein kinase (A-kinase), histone type II-AS, histone III-S, α-casein, phospho-L-serine, phospho-L-threonine and phospho-L-tyrosine were from Sigma.

Growth of A-431 cells and preparation of their plasma membrane fractions. A-431 cells were grown in Dulbeco's modified Eagle's medium (Nissui) supplemented with 10% fetal calf serum (Flow Laboratories), penicillin G (50 IU/ml), streptomycin (50 μg/ml) and kanamycin (25 μg/ml). Intact plasma membrane fractions from A-431 cells were prepared by the method of Carpenter et al.(8). Protein was quantitated by the method of Bradford (9) using bovine serum albumin as a standard.

Preparation and assay of protein kinases. Phosphorylation of intact plasma membrane fractions of A-431 cells was performed as described by Carpenter et al.(8). The activity of A-kinase was measured by the method of Corbin and Reimann (10). Ca^{2+}/phospholipid-dependent protein kinase C (C-kinase) in the supernatant of homogenate of cerebra of Wistar male rats was purified 250-

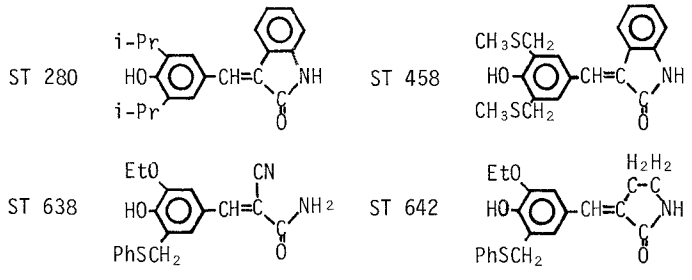


Fig. 1. The chemical structures of 4-hydroxycinnamamide derivatives.

fold by DEAE-cellulose column chromatography and Sephadex G-150 gel filtration. The activity of C-kinase was measured by the method of Kikkawa et al. (11). Casein kinase I and II in the supernatant fraction of lysate of rabbit red blood cells were purified 372-fold and 22800-fold, respectively, by column chromatography of DEAE-cellulose and phosphocellulose. The activities of casein kinase I and II were measured by the methods of Hathaway et al.(12), and Hathaway and Traugh (13). The activities of Na+/k+-ATPase and 5'-nucleotidase in plasma membrane fractions from A-431 cells were measured by methods of Wallach and Kamat (14), and Widnell (15), respectively, and released phosphates were measured by method of Chen et al.(16).

RESULTS and DISCUSSION

Inhibition of tyrosine kinase activity of EGF receptor by 4-hydroxycinnamamide derivatives. The novel synthetic compound ST 280, ST 458, ST 638 and ST 642 were found to show a marked inhibitory activity against the phosphorylation of plasma membrane fractions from A-431 cells. The phosphorylated proteins in the reaction mixture were analyzed by 7.5 % SDS-polyacrylamide gel electrophoresis as shown in Fig.2. The EGF-dependent phosphorylation of 170 K protein (EGF receptor) was observed under the control phosphorylation condition. The compound ST 280 and ST 638 caused a marked decrease in the phosphorylation of 170 K protein in the dose-dependent manner. Almost complete inhibition was observed with 10 μM of ST 280 and ST 638. Similar results were obtained with ST 458 and ST 642 (data not shown). These results suggeste that ST 280, ST 458, ST 638 and ST 642 are potent inhibitors of EGF receptor kinase. In order to confirm that these derivatives really inhibit the phosphorylation of tyrosine residues in protein substrate, the phosphoamino acid analysis of the phosphorylated proteins was carried out as

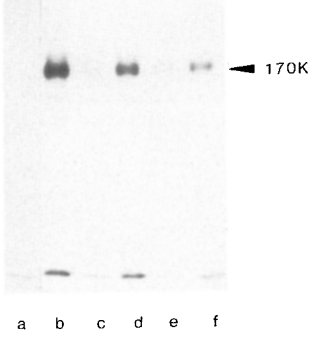


Fig. 2. Inhibition of the phosphorylation of 170 K protein by ST 280 and ST 638. The phosphorylation reaction was carried out in the presence or absence of ST 280 or ST 638. After the reaction, the 20 ul aliquots of reaction mixture were added equal volume of 2-fold concentration Lammli's SDS-sample buffer, and boiled at 100 °C for 3 min. The boiled sample were subjected to 7.5 % SDS-polyacrylamide gel electrophoresis as reported by Lammli (25). The autoradiography was performed by using Fuji X-ray film AIF new RX with two intensifier screens at -80 °C. a, no EGF; b, EGF alone; c, EGF + ST 280 (10 μM); d, EGF + ST 280(1 μM); e, EGF + ST 638(10 μM); f, EGF + ST 638(1 μM).

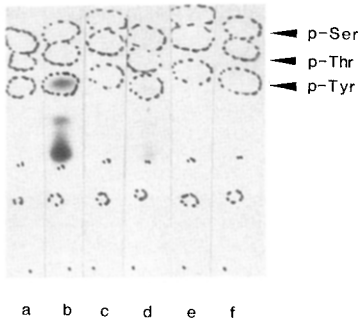


Fig. 3. Inhibition of EGF dependent phosphorylation on tyrosine residues by ST 280 and ST 638. The phosphorylation reactions were carried out with or without EGF in the presence or absence of ST 280 or ST 638. The 50 µl aliquots of reaction mixtures were added 10 volumes of ice cold 10 % TCA/10 mM pyrophosphate. After standing for 120 min at 4 °C, the insoluble materials were collected by centrifugation and washed three times with 500 µl of the same TCA solution, once with ethanol and ethanol/ethylether(1:1), and dried in air. The pellets were dissolved in 40 µl of 6N-HCl, saturated with N_2-gas and hydrolysed at 100 °C for 60 min. The hydrolysates were dried up in vacuo, added water and again dried up. After three times repeating of this treatment, dried materials were dissolved in 10 µl of water containing 50 µg each of p-tyrosine, p-threonine and p-serine. The 4µl aliquots were spotted on cellulose TLC (Eastman Chromatogram Sheet 6065) and subjected to electrophoresis at 200 V for 120 min with pH 3.5 buffer (acetic acid/pyridine/water = 50:5:945). Autoradiography was carried out as in Fig 2. The dotted circles indicate the ninhydrin positive areas. a, no EGF; b, EGF alone; c, EGF + ST 280(10 µM); d, EGF + ST 280(1 µM); e, EGF + ST 638(10 µM); f, EGF + ST 638(1 µM)

shown in Fig. 3. The EGF-dependent incorporation of the $^{32}PO_4$ into tyrosine residues was markedly decreased by the addition of 1 µM of ST 280, and was completely inhibited by 10 µM of ST 280. Similar results were obtained with ST 638. These results indicate that ST 280 and ST 638 are potent inhibitors of the EGF receptor kinase.

Effect of 4-hydroxycinnamamide derivatives on various protein kinases, Na+/K+-ATPase and 5'-nucleotidase activities. To confirm the specificities of enzyme inhibition, the inhibitory effects of ST 280, ST 458, ST 638 and ST 642 on the EGF receptor kinase, serine- and/or threonine-specific protein kinases such as A-kinase, C-kinase, casein kinaseI and II were examined in comparison with quercetin, since quercetin is known to inhibit not only tyrosine kinase but other types of protein kinases (17-20). The result is shown in Table I. The compound ST 280, ST 458, ST 638 and ST 642 were found to potently inhibit the EGF receptor kinase activity with IC_{50} values of 0.44 µM, 0.44 µM, 0.37 µM and 0.85 uM, respectively, as demonstrated in Fig 2 and 3. However, none of them had inhibitory effect on A-kinase, C-kinase, casein kinase I or casein kinase II by the concentration up to 100 uM. These protein kinases were known to phosphorylate the serine and/or threonine residues in protein substrate (10-14). On the other hand, quercetin was active in inhibiting all of protein kinases except for A-kinase, and its inhibitory effect on EGF receptor kinase

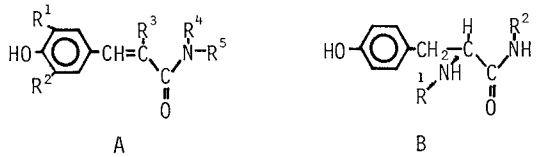
Table 1 Effects of 4-hydroxycinnamamide derivatives and quercetin on various protein kinases, Na+/K+-ATPase and 5'-nucleotidase

Compounds	50 % Inhibitory concentration (μM)						
	1) Tyr-kinase	2) A-kinase	Casein kinase-I	3) C-kinase	Casein kinase-II	Na+/K+ ATPase	5'-Nucleotidase
ST 280	0.44	>100	>100	>100	>100	74	>100
ST 458	0.44	>100	>100	>100	>100	>100	>100
ST 638	0.37	>100	>100	>100	>100	>100	>100
ST 642	0.85	>100	>100	>100	>100	>100	>100
quercetin	0.41	>100	45	22	0.10	5.5	>100

1) EGF receptor associated tyrosine kinase (plasma membrane fractions from A-431 cells)
2) cAMP-dependent protein kinase
3) Ca^{2+}/phospholipid dependent protein kinase C

was similar to those of the 4-hydroxycinnamamide derivatives. This result indicates that these derivatives are specific inhibitors of tyrosine kinase whereas quercetin is a nonspecific inhibitor. Furthermore, we examined the effect of these derivatives and quercetin on Na+/k+-ATPase and 5'-nucleotidase associated with the plasma membrane fractions from A-431 cells. As shown in Table 1, none of ST 458, ST 638 and ST 642 affected both enzyme activities at 100 μM, while ST 280 showed weak inhibitory effect on the Na+/K+-ATPase with IC_{50} value of 74 μM, but had no effect on 5'-nucleotidase activity. In contrast, quercetin was shown to potently inhibit Na+/K+-ATPase with IC_{50} value of 5.5 μM, but had no effect on 5'-nucleotidase activity. This result excludes the possibility that 4-hydroxycinnamamide derivatives act as a nonspecific inhibitor against the enzymes associaited with plasma membrane.

It is interesting why these derivatives show potent specific inhibitory activities against tyrosine kinase. We observed in the course of screening test that all compounds which showed potent inhibitory activities necessarily contained a 4-hydroxycinnamamide skeleton. It is noteworthy that the structure of tyrosine residue are similar to that of 4-hydroxycinnamamide skeleton as shownin Fig. 4. The specific inhibitory activities of these derivatives may be due to the structural similarity between tyrosine residue and the common



A B

Fig. 4. Structural similarity between 4-hydroxycinnamamide skeleton and tyrosine residue. A: common structure of 4-hydroxycinnamamide derivatives, B: tyrosine residue in protein substrate.

structure of these derivatives, although exact inhibitory mechanisms of these derivatives remains to be elucidated.

Several inhibitors of tyrosine kinase have been reported, although most of them are not so specific for tyrosine kinase. A bioflavone quercetin was reported to inhibit the tyrosine kinase activity of pp60v-src (18) and activities of C-kinase (19), phosphorylase kinase (20) and Ca^{2+}/Mg^{2+}-ATPase (21). Chloropromazine, imipramine and dibucain which are well known as Ca^{2+} antagonists also inhibit the tyrosine kinase activity of pp60v-src (22). Amiloride, an inhibitor of Na+/H+-antiporter was shown to inhibit the EGF receptor kinase activity (23). More recently, erbstatin (24) and genistain (25) which were isolated from the culture broth of microbial origin were reported to be specific inhibitors of tyrosine kinase.

The 4-hydroxycinnamamide derivatives will be valuable as a tool for elucidating the role of tyrosine kinase in cells, because of their high specific inhibitory activities. In conclusion, the present results indicate that the 4-hydroxycinnamamide derivatives, ST 280, ST 458, ST 638 and ST 642 are potent specific inhibitors of the tyrosine kinase activity of EGF receptor. Whether ST 280 and ST 638 also inhibit other types of tyrosine kinases such as pp60v-src, p130gag-fps etc. and whether these derivatives can act as a specific inhibitor of tyrosine kinase *in vivo* are now being investigated. The results will be reported elsewhere.

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