Specific Inhibitors of Tyrosine-specific Protein Kinases: Properties of 4-Hydroxycinnamamide Derivatives in Vitro

Tadayoshi Shiraishi,1 M. Koji Owada,2 Masaaki Tatsuka,3 Takashi Yamashita, Kiyoshi Watanabe, and Takeo Kakunaga2

Departments of Oncogene Research [T. S., M. T., T. K.] and Tumor Virolology [M. K. O.J, Research Institute of Microbial Diseases, Osaka University, Osaka 565, Japan, and Bioclinical Research Laboratories, Kanegafuchi Chemical Industry Co., Ltd., Takasago, Hyogo 676, Japan [T. S., T. Y., K. W.]

ABSTRACT

Inhibition by seven synthetic 4-hydroxycinnamamide derivatives, ST 271, ST 280, ST 458, ST 494, ST 633, ST 638, and ST 642, of tyrosine-specific protein kinases (tyrosine kinase) of oncogene or proto-oncogene products (p130gag-v-fps, p70gag-actin-v-fgr, pp60-src, pp60c-src) and epidermal growth factor (EGF) receptor kinase was investigated. ST 638 (α-cyano-3-ethoxy-4-hydroxy-5-phenylisothiocinnamamide) strongly inhibited more of the tyrosine kinases than any of the other compounds. The susceptibilities of these tyrosine kinases to ST 638 increased in the following order: EGF receptor > p70gag-actin-v-fgr > pp60c-src > p130gag-v-fps, pp60-src, with 50% inhibitory concentration values of 1.1, 4.2, 18, 70, and 87 μM, respectively. The phosphorylation of the tyrosine residues in particulate fractions from RR1022 cells expressing pp60-src was inhibited by ST 638 in a dose-dependent way, while it had a negligible effect on the phosphorylations of threonine and serine residues. Kinetic analysis showed that ST 638 competitively inhibited the phosphorylation of an exogenous substrate by the EGF receptor kinase with a Ki of 2.1 μM. ST 638 noncompetitively inhibited autophosphorylation by EGF receptor kinase. These results indicate that ST 638 is a potent and specific inhibitor of tyrosine kinases in vitro, and that its inhibitory activity is caused by competing with the substrate protein for the tyrosine kinase binding site.

INTRODUCTION

The activity of tyrosine-specific protein kinase (tyrosine kinase) is associated with several oncogene products of retroviruses (1-5) and their cellular counterparts (6). Also, some growth factor receptors have tyrosine kinase activity that is stimulated by ligand binding (7-9). Many reports suggest that tyrosine kinase activity contributes to the growth of some human cancers such as squamous carcinoma (10) and brain tumors (11), although nonproliferating systems and differentiated systems (12) also have tyrosine kinase activity. Thus, tyrosine kinase seems to be involved in the control of cell proliferation, carcinogenesis, and cell differentiation and more information is needed to understand its role. Specific inhibitors of tyrosine kinases are useful for studying the properties and functions of tyrosine kinase both in vitro and in vivo. Several synthetic compounds containing 4-hydroxycinnamic acid as a common skeleton are potent inhibitors of the tyrosine kinase activity associated with the epidermal growth factor EGF receptor. These 4-HC derivatives have 50% inhibitory concentrations of 10^-6 to 10^-7 M. These compounds have a minimal effect on such serine/threonine-protein kinases as cAMP-dependent protein kinase (A kinase), Ca^2+/phospholipid-de-
SPECIFIC INHIBITORS OF TYROSINE KINASE

Phosphorylation Assay. Protein kinases were mixed with 23 µl of kinase buffer (20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂ for pp60v-src and pp60c-src; 20 mM Tris-HCl, pH 7.5, 10 mM MnCl₂ for p130gag-v-fps and p70gag-actin-v-fgr; 20 mM Tris-HCl, pH 7.5, 10 mM MnCl₂, and 100 ng EGF for EGF receptor) and 2 µl of [γ-32P]ATP (3000 Ci/mmol), and incubated for 15 min at 30°C. The reaction was stopped by heating in Laemmli's sample buffer (22). After separation of the reaction products by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the gel was dried and autoradiographed. Appropriate bands were cut from the gel and their radioactivities were measured by Cerenkov counting.

Assay of the Tyrosine Kinase Inhibitory Activity. The tyrosine kinases were incubated with various concentrations of inhibitors for 30 min at 0°C, and then phosphorylation assays were started by the addition of [γ-32P]ATP. The products were separated and the radioactivities of appropriate protein bands were counted as above.

Calculation of IC₅₀. The IC₅₀ values were measured from three or more graphs drawing five points (vertical axis, representing the inhibition percentage and the abscissa, the logarithmic concentrations of inhibitor) as inhibitor concentrations resulting in a 50% loss of tyrosine kinase activity.

Phosphoamino Acid Analysis. One-dimensional and two-dimensional analysis of phosphoamino acids were done as described (20).

RESULTS

Effects of 4-HC Derivatives on Various Tyrosine Kinases. To investigate the inhibition of various tyrosine kinases by the 4-HC derivatives ST 271, 280, 458, 494, 633, 638, and 642, we selected p130gag-v-fps, p70gag-actin-v-fgr, and pp60v-src as tyrosine kinases of activated oncogene products, and pp60c-src as that of the proto-oncogene product, and EGF receptor kinase. To confirm the specificity of these tyrosine kinase activities, the phosphoamino acid compositions of the reaction products were analyzed as shown in Fig. 2. The predominant residue phosphorylated in immunocomplexes of p130gag-v-fps, p70gag-actin-v-fgr, and pp60v-src as tyrosine kinases of activated oncogene products, and pp60c-src as that of the proto-oncogene product, and EGF receptor kinase. To confirm the specificity of these tyrosine kinase activities, the phosphoamino acid compositions of the reaction products were analyzed as shown in Fig. 2. The predominant residue phosphorylated in immunocomplexes of p130gag-v-fps, p70gag-actin-v-fgr, pp60v-src, and pp60c-src was tyrosine, while phosphorylation of the threonine or serine residues did not occur. The same results were obtained with A431 plasma-membrane fractions as previously reported (13). Therefore, these oncogene products are suitable for studying the inhibition of tyrosine kinase activity by 4-HC derivatives.

We examined the effects of the 4-HC derivatives on the tyrosine kinase activity associated with p70gag-actin-v-fgr. The 4-HC derivative’s inhibitory effects were also compared with that of quercetin, an inhibitor of pp60v-src tyrosine kinase activity (23) as well as serine-/threonine-protein kinases (13, 24). The compound ST 280 markedly decreased the incorporation of the 32P into the M₇₀,₀₀₀ proteins in a dose-dependent manner at 1.0 to 25 µM. ST 458, 633, and 638 behaved similarly. ST 271 and 494 moderately decreased the incorporation of 32P in this concentration range. Quercetin was a weak inhibitor (Fig. 3).

The effects of 4-HC derivatives and quercetin on the tyrosine kinase activities of p130gag-v-fps, pp60v-src, pp60c-src, and the EGF receptor were examined. The IC₅₀ values of 4-HC derivatives are listed in Table 1. The compounds ST 638 and quercetin moderately inhibited the p130gag-v-fps kinase activity, while the others had weak or no inhibitory activity against the same enzyme. The compounds ST 271, 280, 638, and quercetin weakly or moderately inhibited the tyrosine kinase activities associated with the RR1022 particulate fractions. The compounds ST 271, 280, 458, 633, 638, and quercetin potently

Fig. 1. Chemical structures of 4-hydroxycinnamamide derivatives.

Fig. 2. One-dimensional phosphoamino acid analysis of proteins phosphorylated with oncogene or proto-oncogene product tyrosine kinases. The immunocomplexes of tyrosine kinases were phosphorylated and phosphoamino acid compositions of the products were analyzed (20). /, immunoglobulin G heavy chain (IgG-H) phosphorylated with the pp60v-src; 2, autophosphorylated p130gag-v-fps; 3, autophosphorylated p70gag-actin-v-fgr; 4, IgG-H phosphorylated with the pp60c-src.

Fig. 3. Effects of 4-hydroxycinnamamide derivatives on the tyrosine kinase activity of p70gag-actin-v-fgr. The immunocomplexes prepared from NIH/3T3 cell lysates by specific p70gag-actin-v-fgr antisera were incubated in the presence of various concentrations of ST 271, 280, 458, 633, 638, 642, and quercetin. X-ray film was exposed at -80°C for 10 h. In A: /, buffer alone; 2, 5 µM ST 271; 3, 1 µM ST 271. In B: /, 5 µM ST 280; 2, 5 µM ST 280; 3, 5 µM ST 280. In C: /, 5 µM ST 458; 2, 1 µM ST 458. In D: /, 5 µM ST 633; 2, 1 µM ST 633; 3, 0.25 µM ST 633. In E: /, 5 µM ST 638; 2, 1 µM ST 638. In F: /, 5 µM ST 642; 2, 5 µM ST 642. In G: /, 25 µM quercetin; 2, 5 µM quercetin; 3, 1 µM quercetin.
Specified Inhibitors of Tyrosine Kinase

Table 1 Effects of 4-hydroxycinnamamides on various tyrosine kinases

<table>
<thead>
<tr>
<th>Compounds</th>
<th>p130gag-v-fps</th>
<th>pp60-src</th>
<th>p70gag-actin-v-fgr</th>
<th>pp60c-src</th>
<th>EGF receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSV9</td>
<td>ST271</td>
<td>RR1022</td>
<td>NIH/fg</td>
<td>NIH/fg</td>
<td>NIH/3T3</td>
</tr>
<tr>
<td>i</td>
<td>p</td>
<td>i</td>
<td>i</td>
<td>i</td>
<td>m</td>
</tr>
<tr>
<td>ST271</td>
<td>&gt;100</td>
<td>97</td>
<td>52</td>
<td>23</td>
<td>4.4</td>
</tr>
<tr>
<td>ST280</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>38</td>
<td>8.0</td>
<td>6.8</td>
</tr>
<tr>
<td>ST458</td>
<td>85</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>1.2</td>
<td>1.0</td>
</tr>
<tr>
<td>ST633</td>
<td>47</td>
<td>105</td>
<td>&gt;100</td>
<td>3.2</td>
<td>5.5</td>
</tr>
<tr>
<td>ST638</td>
<td>70</td>
<td>87</td>
<td>75</td>
<td>4.2</td>
<td>18</td>
</tr>
<tr>
<td>ST642</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>ST494</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>Quercetin</td>
<td>28</td>
<td>63</td>
<td>24</td>
<td>90</td>
<td>1.2</td>
</tr>
</tbody>
</table>

| Quercetin (MM) | 0 | 0.25 | 0.85 | 1.2 | 4.4 | 6.5 | 8.5 | 1.1 | 1.1 | 1.5 | 0.25 | 1.1 | 0.85 | 1.1 |

Table 2 Specific inhibition of tyrosine phosphorylation in particulate fractions from RR1022 cells

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (µM)</th>
<th>P-Tyr</th>
<th>P-Thr</th>
<th>P-Ser</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6.0 (100)</td>
<td>17.5 (100)</td>
<td>76.5 (100)</td>
<td></td>
</tr>
<tr>
<td>ST271</td>
<td>3.1 (52)</td>
<td>18.4 (105)</td>
<td>78.5 (103)</td>
<td></td>
</tr>
<tr>
<td>ST638</td>
<td>2.0 (33)</td>
<td>13.7 (78)</td>
<td>84.3 (110)</td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>3.9 (65)</td>
<td>18.1 (103)</td>
<td>78.0 (103)</td>
<td></td>
</tr>
</tbody>
</table>

Indicate that ST271 and ST638 are specific inhibitors of tyrosine kinases, and that quercetin is a nonspecific inhibitor of protein kinases.

Kinetics of Inhibition by 4-Hydroxycinnamamid Derivatives

To investigate the manner of inhibition of 4-HC derivatives, competition experiments between ST638 and either ATP, a phosphatase donor, or α-casein, a phosphatase acceptor, were done.
As indicated in Fig. 5, ST 638 was a noncompetitive inhibitor of ATP for EGF receptor autophosphorylation and for \( \alpha \)-casein phosphorylation by the EGF receptor kinase. Similar results were obtained with ST 633 or with \( \text{p130gag-v-fps} \) as a tyrosine kinase (data not shown). ST 638 competitively inhibited the phosphorylation of \( \alpha \)-casein by EGF receptor kinase, because increasing the concentration of \( \alpha \)-casein in the reaction affected the \( K_m \) of the reaction (Fig. 6B). The \( K_m \) of \( \alpha \)-casein for this reaction was 10 \( \mu \)M and the \( K_i \) was 2.1 \( \mu \)M. By contrast, ST 638 was unable to compete for the EGF receptor autophosphorylation with \( \alpha \)-casein (Fig. 6A). Similar results were obtained with ST 633 or with \( \text{p130gag-v-fps} \) (data not shown). These results indicate that ST 633 and ST 638 can competitively inhibit the phosphorylation of an exogenous substrate by the EGF receptor kinase and \( \text{p130gag-v-fps} \) kinase. To exclude the possibility that nonspecific binding of 4-HC derivatives to \( \alpha \)-casein decreased the amount of the derivatives available in the reaction mixture, the concentration of the available ST 638 was analyzed by high performance liquid chromatography. The results indicated that \( \alpha \)-casein did not affect the concentration of the available ST 638 (data not shown). It was unlikely, therefore, that \( \alpha \)-casein's ability to compete with ST 638 arose from nonspecific binding.

**DISCUSSION**

In this study, we found that certain 4-hydroxycinnamamide derivatives specifically inhibited tyrosine kinases in vitro. ST 638 was a very potent inhibitor of many tyrosine kinases including \( \text{p70gag-actin-v-fgr} \), \( \text{p130gag-v-fps} \), \( \text{pp60v-src} \), \( \text{pp60c-src} \) and the EGF receptor. The 4-HC derivatives inhibited the autophosphorylation reaction of EGF receptor, \( \text{p130gag-v-fps} \), and \( \text{p70gag-actin-v-fgr} \) and the phosphorylation of exogenous substrate by either \( \text{p130gag-v-fps} \) or EGF receptor kinases. The 4-HC derivatives also inhibited tyrosine phosphorylation in RR1022 particulate fractions without affecting the phosphorylation of serine and threonine residues. These results are consistent with our previous results which suggested that 4-HC compounds inhibit the tyrosine kinase activity of the EGF receptor but not serine-/threonine-protein kinases such as A- and C-kinases (13). ST 642 specifically inhibited EGF receptor kinase, and ST 458 was the most potent inhibitor against \( \text{pp60c-src} \) kinase with no inhibition of \( \text{pp60v-src} \) kinase. ST 494 had no activity against any of the tyrosine kinases tested.

The compounds ST 633 and 638 seemed to inhibit the EGF receptor kinase and \( \text{p130gag-v-fps} \) kinase by competing for the same site that the exogenous substrate binds. Kinetic analysis showed that ST 633 and 638 competitively inhibited the ability
of the EGF receptor and p130c-raf-v-fps kinases to phosphorylated exogenous substrates. All of the potent tyrosine kinase inhibitors found in this study have a skeleton of 4-hydroxycinnamic acid, and this structure is similar to that of tyrosine residues in the substrate protein (13) suggesting that the inhibitory properties of the 4-HC derivatives arise from their structural similarity to the tyrosine residues in the substrate protein. The mechanism by which the 4-HC derivatives inhibit tyrosine kinase appears to be quite different from that of quercetin (23), genistein (27), amiloride (28), and ATP analogues like diadenosine 5',5''-P'P'4 tetraphosphate (29), all of which inhibit tyrosine kinases competitively with respect to ATP (23, 27–29). The 4-HC derivatives are more similar to the phosphorylation site analogue (30) which is a competitive inhibitor of substrate binding. The kinetic analysis indicate that ST 633 and ST 638 inhibit the phosphorylation of exogenous substrate and the tyrosine kinase autophosphorylation reaction differently, suggesting that the mechanism of these two phosphorylation reactions are distinct from each other. Our results are consistent with the observations of Weber et al. (31) who reported that the phosphorylation of the exogenous substrate by the EGF receptor kinase was an intermolecular reaction but the autophosphorylation reaction was an intramolecular reaction. Rees-Jones et al. have found that the phosphorylation of the endogenous substrate p120 and α-casein by the insulin receptor kinase was competitive, although the autophosphorylation of insulin β-subunit was unaffected by α-casein (32). The 4-HC derivatives are the first reported inhibitors of tyrosine kinase inhibiting the phosphorylation of exogenous substrate and tyrosine kinase autophosphorylation differently.

The mechanisms by which the 4-HC derivatives inhibit tyrosine kinase are of special interest since crucial substrates for tyrosine kinases in intact cells have not been identified.

REFERENCES

Specific Inhibitors of Tyrosine-specific Protein Kinases:
Properties of 4-Hydroxycinnamamide Derivatives \textit{in Vitro}

Tadayoshi Shiraishi, M. Koji Owada, Masaaki Tatsuka, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/49/9/2374

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.