The Degree of Inhibition of Protein Tyrosine Kinase Activity by Tyrphostin 23 and 25 Is Related to Their Instability

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Abstract

Tyrphostins, a series of compounds with hydroxy cis-cinnamonicitrile backbone structures, are used as protein tyrosine kinase inhibitors to study signal transduction. While studying the inhibition of pp60^c-src protein tyrosine kinase activity with tyrphostins 23 and 25 (3,4-di- and 3,4,5-trihydroxy cis-cinnamonicitrile), we found the inhibitors to be quite unstable. The inhibition of pp60^c-src activity corresponded to the formation of products derived from the parent tyrphostin compound. One of these isolated products was at least 10-fold more inhibitory to both pp60^c-src and epidermal growth factor receptor kinase activity than the parent tyrphostin. The generation of compounds more inhibitory than the parent tyrphostin may explain the delayed inhibition reported with epidermal growth factor receptor kinase activity. Since these tyrphostins are unstable and form compounds more inhibitory towards protein tyrosine kinase activity, any results obtained with these compounds must be interpreted with caution.

Introduction

The association of PTKs with cellular transformation present them as potential targets for drug design (1, 2). Inhibitors would uncouple the PTKs from their signal transduction pathway and thus would also be valuable reagents for studying the role of tyrosine kinases in signal transduction. Several inhibitors have been developed (1–5) that are competitive against ATP and/or the peptide substrate. Most of these have been found to either lack specificity or chemical stability and sometimes both. Hence, they are of limited use. Early reports on tyrphostins (4–6), however, have suggested that they may have the stability and specificity required of a PTK inhibitor. In the studies reported here, we observed an inhibition of pp60^c-src tyrosine kinase activity by tyrphostins 23 and 25 which increased with time. Our studies indicate that these two tyrphostins are unstable and form products that are more potent inhibitors of PTK activity.

Materials and Methods

Tyrphostin 23 and 25 were obtained from Biomol Research Labs, Inc. Recombinant pp60^c-src was purified from baculovirus-infected insect cells as described previously (7). Synthetic poly Glu:Tyr, 4:1 (poly E4Y; range, Mr 20,000–50,000) was obtained from Sigma Chemical Co. EGFR PTK activity was assayed as an immunoprecipitate from A431 cells using monoclonal antibody clone Alo8 (8).

The PTK activity of pp60^c-src and EGFR were assayed in a reaction medium consisting of 0.15 M EPPS-NaOH (pH 8.0), with 6 mM MgCl2, 0.2 mM [γ-32P]ATP (0.2–0.4 mCi/μmol), 10% glycerol, 0.01% Triton X-100, and poly E4Y (2 mg/ml) as the substrate. MnCl2 was not included in the assay unless specifically stated. The final reaction volume was 50 μl. Assays were performed at 30°C. Reactions were initiated by the addition of [γ-32P]ATP and MgCl2. After 30 min, 35 μl of the reaction mixture was spotted onto 3-mm (Whatman) filter paper and immediately dropped into warm (70°C) 5% trichloroacetic acid. The paper was then washed and counted for the incorporation of 32P into the substrate. For the determination of IC50, 0–1000 μg/ml of tyrphostin was used.

For the pH studies, 5 μM tyrphostin was solubilized in a sonication bath (30 min; 22°C) with 10 mM citrate-NaOH (pH 5.0) and 10 mM 4-morpholinepropanesulfonic acid-HCl (pH 7.2) or 10 mM 2-(N-cyclohexylamino)ethanesulfonic acid-NaOH (pH 8.6). The tyrphostin preparations were then analyzed within 30 min as pp60^c-src inhibitors as described above.

HPLC was performed on a Hewlett-Packard 1090 A liquid chromatograph equipped with a diode array detector using an RP-300 C8 column (Brownlee Labs). The effluent was monitored at multiple wavelengths (245, 260, and 304 nm). Solvent A was water with 0.1% trifluoroacetic acid; solvent B was acetonitrile with 0.1% trifluoroacetic acid. A gradient of 0–33% B was used for 26 min at a flow rate of 1.5 ml/min at ambient temperature. The peak fractions (P1, P2, and P3) were collected, retoevaporated to approximately 1 ml, lyophi-

ized, and weighed. Of the products recovered, 50% was P1, 25% was P2, and 4% was P3, by weight. Both P2 and P3 were very hygroscopic. The isolated compounds (P1, P2, and P3) were dissolved by sonication in 0.15 M EPPS-NaOH (pH 8.0) for determination of their IC50.

Results

The inhibitory effect of tyrphostins 23 and 25 on pp60^c-src kinase activity was studied by assaying the enzyme in the presence of variable concentrations of the inhibitors. The IC50 for tyrphostins 23 and 25 were 440 and 150 μM, respectively.

When the tyrphostins were dissolved in 0.15 M EPPS-NaOH (pH 8.0), the solutions changed color with time. Both tyrphostins 23 and 25 changed from light yellow to dark brown within 24 h and then back to a lighter brown color. Although it has been reported (4) that these tyrphostins are chemically stable, this observation led us to question their stability.

Consequently, we followed the UV-VIS spectra (220–500 nm) of tyrphostin in 0.15 M EPPS-NaOH (pH 8.0) for 24 h and observed that the main peak at 340 nm shifted to 440 nm. This indicated that the tyrphostins were unstable under these conditions. Manganese (Mn2+) and iron (Fe2+ and Fe3+) have been shown to catalyze the oxidation of compounds like caffeic acid (9) and erbstatin (2), respectively. Hence, we examined the effect of pH and manganese ions on the stability of tyrphostins by determining if the pH used to solubilize them and/or the presence of MnCl2 in the reaction medium influenced their inhibition of pp60^c-src PTK activity (Table 1). Inhibition was greatest if the compounds were dissolved in an acidic buffer. This correlated with the relatively rapid destruction of tyrphostins at acidic pH versus basic pH, which was observed by HPLC (data not shown). In addition, the presence of MnCl2 in the PTK reaction medium increased the amount of inhibition. Iron (5 μM FeSO4 or 5 μM FeCl3) also enhanced the inhibition of pp60^c-src by tyrphostin 23. The inhibition by 1 μM tyrphostin 23 increased from 40 to 80% in the presence of iron.

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2 To whom requests for reprints should be addressed, at Department of Neuro-Oncology, Box 316, The University of Texas, M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.

3 The abbreviations used are: PTK, protein tyrosine kinase; EGFR, epidermal growth factor receptor; IC50, 50% inhibitory concentration; HPLC, high performance liquid chromatography; EPPS, N-(2-hydroxyethyl)piperazine-N'-3-propanesulfonic acid.

Corrections to page 867

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The instability of tyrphostins 23 and 25 was studied as a function of time. The compounds were dissolved by sonication in 0.15 M EPPS-NaOH buffer (pH 8.0) at a concentration of 5 mM. At different time points, samples were analyzed by reverse-phase chromatography. Within 24 h, the parent compound formed several products with different retention times (Fig. 1). With tyrphostin 23, the parent peak (P₁) had a retention time of 19 min and diminished in size while several new peaks appeared, the major ones occurring at retention times of 8 min (P₂) and 23 min (P₃). The P₁ parent peak decreased from 100% at 1 h to nearly 5% by 96 h. The loss of peak P₁ correlated with the formation of P₂ and P₃, which increased from 0% at 1 h to 95% at 96 h (Fig. 2). Similar results were obtained with tyrphostin 25.

We also examined the inhibitory effect of the isolated tyrphostin-derived products on the PTK activity of pp60<sup>src</sup> and EGFR. Tyrphostin 23 was solubilized in the 0.15 M EPPS-NaOH buffer (pH 8.0) by sonication, and after 24 h, peaks P₁, P₂, and P₃ (see Fig. 1) were isolated by reverse-phase HPLC. The peak fractions were pooled, lyophilized, and tested for their effect on pp60<sup>src</sup> and EGFR kinase activity (Table 2). With respect to pp60<sup>src</sup> activity, P₂ and P₃ were on average 5- and 13-fold more inhibitory than the parent compound. For the EGFR kinase activity, P₂ was not more inhibitory, whereas P₃ was 10-fold more inhibitory. The compounds isolated as P₂ and P₃ were stable as judged by their chromatographic behavior after an additional 24 h of incubation at pH 8.0 (data not shown).

**Discussion**

The increased inhibition of pp60<sup>src</sup> and EGFR kinase activity by the formation of products from the parent tyrphostin is similar to that reported for caffeic acid (10). Caffeic acid and its first oxidation product (a quinone) lack antigonadotropic activity. However, the products (oligomers) arising from the caffeoquinone are inhibitors of gonadotropin. A similar oxidation mechanism may be involved in the instability of tyrphostins. In addition, metal ions, such as manganese and iron, have been shown to catalyze the oxidation of caffeic acid (9) and erbstatin (2), respectively. We have observed a similar effect with the tyrphostins. All of these compounds (caffeic acid, erbstatin, and tyrphostins) are structurally very similar in having a polyhydroxy benzene ring. Since manganese is often used in PTK assays and it stimulates the formation of compounds that are more inhibitory than the precursor tyrphostin, we would expect that inhibition by tyrphostins would be greater with in vitro assays where MnCl₂ is used. In addition, the serum used in cell culturing contains iron (2-5 μM) which has an effect similar to that of manganese. Therefore, the in vitro and in situ experiments used to test tyrphostins will generally contain metal ions that promote the destruction of tyrphostin and thus may enhance the inhibition observed with these compounds.

Previous studies have shown that tyrphostins, including tyrphostin 23, at concentrations of 50–100 μM can inhibit the PTK activity of the EGFR in vitro and in situ (11). Delayed inhibition (16–24 h) after treatment of the cells with the tyrphostins was attributed to the slow action of these compounds, and the authors suggested that the rate of tyrphostin entry into cells may be slow. However, in another study, Faaland et al. (12) demonstrated that rapid uptake of the tyrphostins at concentrations of 50–100 μM occurred with peak intracellular concentration at 1 h. However, no inhibition of tyrosine kinase activity was observed during that time. Faaland et al. (12) hypothesized that

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**Table 1** Inhibition of pp60<sup>src</sup> activity by tyrphostin 23 solubilized at different pH values<sup>a</sup>

<table>
<thead>
<tr>
<th>pH</th>
<th>-MnCl₂</th>
<th>+MnCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>98</td>
<td>96</td>
</tr>
<tr>
<td>7.2</td>
<td>47</td>
<td>83</td>
</tr>
<tr>
<td>8.6</td>
<td>15</td>
<td>78</td>
</tr>
</tbody>
</table>

<sup>a</sup> Tyrphostin (5 mM) was solubilized by sonication at pH 5.0, 7.2, or 8.6 and then analyzed as an inhibitor of pp60<sup>src</sup> under standard assay conditions (pH 8.0). MnCl₂ was either omitted or included in the [γ<sup>32</sup>P]ATP mixture used to initiate the phosphorylation of poly E<sub>4</sub>Y. When MnCl₂ was included, it was also added to the control samples (0 mM tyrphostin and 2 mg/ml poly E<sub>4</sub>Y set equivalent to 100% activity).

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**Table 2** Inhibition of PTK activity by tyrphostin 23 (P₁) and the tyrphostin 23-derived products (P₂ and P₃) isolated by reverse phase HPLC<sup>b</sup>

<table>
<thead>
<tr>
<th>Sample</th>
<th>IC₅₀ (μg/ml)</th>
<th>EGFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak 1 (P₁)</td>
<td>81, 75</td>
<td>170, 153</td>
</tr>
<tr>
<td>Peak 2 (P₂)</td>
<td>15, 18</td>
<td>160, 165</td>
</tr>
<tr>
<td>Peak 3 (P₃)</td>
<td>6, 6</td>
<td>11, 22</td>
</tr>
</tbody>
</table>

<sup>b</sup> See legend of Figure 1 for conditions.

<sup>b</sup> Results from two determinations are shown.
Tyrphostin did not directly inhibit the EGFR kinase activity but functioned indirectly. The data reported in this paper indicate that tyrphostin 23 is unstable and forms inhibitory compounds more potent than the parent compound. The delayed inhibition of the EGFR kinase activity with tyrphostin (11, 12) may thus be due to the formation of such products.

Recently, it has been reported that tyrphostins and other PTK inhibitors also inhibit presumably PTK-independent functions such as fatty acid synthesis and mitochondrial oxidative phosphorylation (13). These data indicate that a great deal of caution must be taken in using these compounds to understand the role of specific PTKs in signal transduction since the effect of their products on other PTKs and PTK-independent events cannot be predicted.

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References

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