Inhibition of Lymphocyte Kinase Lck and Phosphatidylinositol 3-kinase by a Novel Immunosuppressant, Lymphostin

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Lck is a Src-family tyrosine kinase that is expressed predominantly in T cells, where it plays important roles in T-cell activation. Lymphostin was isolated from Streptomyces sp. as an inhibitor of Lck. As previously reported, lymphostin inhibited Lck (IC50 0.05 μM) and the mixed lymphocyte reaction (IC50 0.009 μM). We have now examined the mechanism of inhibition by lymphostin. Lymphostin inhibited protein-tyrosine kinase activity in Jurkat T cells, demonstrating the effectiveness of the compound at the cellular level. Furthermore, lymphostin suppressed delayed-type hypersensitivity in mice. However, the inhibitory activity against Lck at the cellular level was weaker than that against the mixed lymphocyte reaction. Thus, we examined the effects of lymphostin on other kinases. Interestingly, lymphostin also inhibited phosphatidylinositol 3-kinase (IC50 0.001 μM). Consequently, we conclude that lymphostin inhibits the mixed lymphocyte reaction and delayed-type hypersensitivity not only through the blockade of Lck, but through the blockade of phosphatidylinositol 3-kinase as well.

Key words: Lck; phosphatidylinositol 3-kinase; immunosuppressant; T lymphocyte; protein tyrosine kinase

Lyophilocyte kinase, Lck (p56\(^{Lck}\)), is a Src-family tyrosine kinase that is expressed exclusively in lymphoid cells, predominantly in thymocytes and peripheral T cells, where it plays important roles in T-cell development and activation.\(^{11}\) Lck binds to the cytoplasmic domains of CD4 and CD8 T-cell co-receptors,\(^{21}\) and following T-cell receptor stimulation, phosphorylates T-cell receptor zeta chains, which then recruit ZAP-70 kinase to promote T-cell activation.\(^{31}\) A mutant human Jurkat T cell line lacking functional Lck fails to respond to anti-T cell receptor antibodies,\(^{4}\) and Lck knock-out mice are defective in thymocyte development and do not have T cell-mediated effects including allo-skin graft rejection.\(^{5,6}\)

Cyclosporin A (CsA) and FK506 have been used as clinically effective immunosuppressants. CsA and FK506 form complexes with immunophilins such as cyclophilin and FK506 binding protein (FKBP), respectively. The drug-immunophilin complexes suppress various T cell-related immune responses, such as the production of interleukin 2 (IL-2), through the inhibition of the Ca\(^{2+}\)- and calmodulin-dependent phosphatase, calcineurin.\(^{8}\) This mechanism of action, however, leads to severe side effects, i.e. nephrotoxicity, neurotoxicity, and diabetogenicity, which limit their clinical use. Accordingly it is considered that Lck inhibitors could be novel potential immunosuppressants with different mechanisms of action from these drugs.

Recently, we reported that in the course of screening for Lck inhibitors from microorganisms, we found a novel microbial alkaloid, lymphostin (Fig. 1), possessing a pyrrolo[4,3,2-de]quinoline skeleton, which is rare among natural products.\(^{9,10}\) The IC50 for enzyme inhibition was 0.05 μM. In previous studies, the immunosuppressive activity of lymphostin was assessed using the mixed lymphocyte reaction (MLR), which is a measure of T-cell activation followed by IL-2 dependent T-cell proliferation. This is considered to be an in vitro model of allograft rejection. Lymphostin was a potent inhibitor of the MLR, with an IC50 of 0.009 μM, while the IC50 of CsA was 0.05 μM. The IC50 for the antiproliferative activity of lymphostin against NFS-60, a murine myeloid leukemia cell line, was 0.16 μM.

In this study, we examined the mechanism of inhibition of Lck by lymphostin. Lymphostin inhibited
autophosphorylation of Lck, as well as Lck-mediated phosphorylation of exogenous substrate, and was effective at the cellular level. However, the inhibitory activity against the protein-tyrosine kinase activity of Lck at the cellular level occurred at drug concentrations that were much higher than those required for inhibition of MLR. These results suggest that lymphostin may inhibit MLR through a mechanism(s) other than the blockade of Lck. We, therefore, examined the effects of lymphostin on other protein kinases and phosphatidylinositol 3-kinase (PI3-kinase). Interestingly, lymphostin also potently inhibited the kinase activity of PI3-kinase, with an IC₅₀ of 0.001 μM. PI3-kinase is coupled to multiple T-cell molecules (reviewed by Ward et al.¹¹). The PI3-kinase inhibition by lymphostin may be involved in the MLR inhibition. Thus, we also examined the mechanism of inhibition of PI3-kinase activity by lymphostin.

In addition, we report the immunosuppressive activity of lymphostin in a mouse experimental model. Our data indicate that lymphostin is a potent immunosuppressant with inhibitory activities against Lck and PI3-kinase.

Materials and Methods

Reagents and materials. Lymphostin was isolated from the fermentation broth of Streptomyces sp. KY11783 in our laboratories, as described previously.⁹ The peptide substrate (Tyr-Ala-Glu)₇ used in the assay of Lck activity was synthesized in our laboratories, as described previously.⁹ Lck and PI3-kinase was partially purified from bovine thymus according to the method of Cushman et al.¹² PI3-kinase was isolated from the fermentation broth of Streptomyces sp. KY11783 in our laboratories, as described previously.⁹ Lck was partially purified from bovine thymus tissue according to the method of Cushman et al.¹₂ PI3-kinase was partially purified from bovine thymus tissue as described by Shibasaki et al.¹₃ [γ³²P]ATP and anti-phosphotyrosine antibody PY20 conjugated to horseradish peroxidase were purchased from Amersham Corp. (Tokyo, Japan); anti-Lck (N-terminal) antiserum and anti-PI3 kinase p85 antiseraum complexed to protein G-Sepharose beads were from Upstate Biotechnology, Inc. (Lake Placid, NY); OKT3 was from Becton Dickinson (Franklin Lakes, NJ). All other reagents were of analytical grade.

Enzyme assay. The activity of Lck was assayed using a peptide substrate (Tyr-Ala-Glu)₇⁴ and [γ³²P]ATP in the presence of various concentrations of the test compounds, as described previously.⁹ The peptide (0.5 mg/ml) was phosphorylated in a reaction mixture comprising 12 mM MOPS (pH 7.5), 12 mM MgCl₂, 60 μM Na₃VO₄, partially purified Lck (156 μg/ml), 50 μM [γ³²P] ATP (1000–2000 cpm/pm), and 1% DMSO as a carrier for the compounds in a final volume of 25 μl. After a 60-minute incubation at 30°C without ATP, the reaction was started by the addition of [γ³²P] ATP at 30°C and stopped by the addition of 25 μl of 10% trichloroacetic acid, after 15 minutes. The acid-precipitable materials were collected on a nitrocellulose membrane filter and washed with 10% trichloroacetic acid. The radioactivity on the filter was measured in toluene scintillation fluid using a scintillation counter.

The activity of autophosphorylation of Lck was measured using an immunocomplex assay. Jurkat T cells were lysed in lysis buffer containing 1% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, and 2 mM EDTA by rotating for 30 min, and the lysate was centrifuged at 12000 rpm for 20 min at 4°C. The supernatants were incubated with rabbit anti-mouse IgG (DAKO; Kyoto, Japan), followed by Protein G-Sepharose beads prepared as a 50% (w/v) suspension. The cleared lysates were incubated with the anti-Lck (N-terminal) antiserum, followed by protein G-Sepharose beads prepared as a 50% (w/v) suspension. After washing, the beads were suspended in 20 μl of a reaction buffer containing 40 mM PIPES, pH 7.1, and 10 mM MnCl₂. The mixture was incubated at 30°C for 10 min in the presence of various concentrations of the test compounds. [γ³²P] ATP (10 μCi/assay) was added to start the reaction, and the mixture was incubated at 30°C for 10 min. The reaction was stopped by the addition of 40 μl of 2× SDS sample buffer. The samples were boiled for 5 min, and the supernatants were run on a 5–20% SDS polyacrylamide gel. The radioactivity on the dried gel was measured with a Fuji BAS2000 Biomaging Analyzer (FujiFilm; Tokyo, Japan).

The activity of PI3-kinase was measured as described previously.¹⁵ In an ordinary solution assay, partially purified PI3-kinase from calf thymus tissue was used at a final concentration of 20 ng/ml. In the case of the immunocomplex assay, PI3-kinase activity was measured in immunoprecipitates with anti-PI3-kinase p85 antiseraum complexed with protein G-Sepharose beads. Jurkat T cells were harvested, washed in serum-free RPMI 1640 medium and lysed in 1% Nonidet P-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM sodium orthovanadate, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride for 20 min. The lysates were clarified by centrifugation at 12000 rpm for 20 min. The clarified lysates were
cleared by incubation with normal rabbit serum and protein G-Sepharose beads. The cleared lysates were incubated with the anti-P13-kinase p85 antisemur at 4 µg/ml, followed by protein G-Sepharose beads prepared as a 50% (w/v) suspension. The beads were washed sequentially with Nonident P-40 lysis buffer, LiCl solution (0.5 M LiCl, 0.1 M Tris-HCl, pH 7.5), distilled water, and washing buffer (0.1 M NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 7.5). The partially purified enzyme or the immunoprecipitates were suspended in 50 µl of a buffer containing 20 mM Tris-HCl, pH 7.5, 20 mM MgCl$_2$, 100 mM NaCl, and 0.5 mM EGTA. Then, 1 µl of 10 mg/ml PI dissolved in dimethyl sulfoxide was added to the reaction mixture to make micelles of PI. The mixture was incubated at 30°C for 10 min in the presence of various concentrations of the test compounds. [$\gamma$-$^3$P] ATP (1.0 µCi/assay; final concentration, 10 µM) was added to start the reaction, and the mixture was incubated at 30°C for 60 min. The reaction was stopped by the addition of 100 µl of chloroform, methanol and 11.6 N HCl (100:200:2). Following centrifugation, the lower organic phase was collected for thin-layer chromatography on silica gel plates to be developed in chloroform, methanol, 25% ammonium hydroxide and water (43:38:5:7). The radioactivity in Jurkat T cells was measured as follows. Cells (4 × 10$^6$ in 2 ml of medium) were incubated for 30 min with test compounds before a 6-min incubation with 10 µg/ml of OKT3. Reactions were stopped by the addition of cold phosphate-buffered saline. Cells were harvested, washed in cold PBS, and lysed in 1% Triton X-100, 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1 mM sodium orthovanadate, 2 mM EDTA, 1 mM (p-amidinophenyl)methanesulfonil fluoride, 20 µM leupeptin, and 0.15 U/ml aprotinin for 20 min. Insoluble materials were removed by centrifugation at 14000 rpm for 10 min. The lysates were added to 2 × sample buffer and boiled at 100°C for 5 min. For immunoblots, the samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The blots were blocked with 1% bovine serum albumin in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl and 0.1% Tween-20. Blots were incubated with anti-phosphotyrosine antibody, PY20, conjugated with horseradish peroxidase followed by washing and then detected with ECL system (Amersham Corp.; Tokyo, Japan). The densities of p20 band were measured using a Pdi scanner (Howtek Inc.; Hudson, NH).

Delayed-type hypersensitivity (DTH) reaction. To induce DTH$^{16}$ to trinitrophenyl (TNP), 0.1 ml of 10 mM trinitrobenzene sulfonic acid (TNBS) solution (pH 7.4) was subcutaneously (s.c.) injected into two separate sites on the dorsal flanks of the Balb/c mice. The compound dissolved in 10% DMSO was intraperitoneally injected (i.p.) once a day from day 0 to day 4. Ten percent DMSO was used as the solvent control. Challenge was done 5 days later by injecting 0.05 ml of 10 mM TNBS solution (pH 7.4) into the right footpad. Twenty-four hours after the footpad challenge, DTH reactivity was assessed by measuring the swelling of the footpad. The magnitude of the DTH was expressed as the increment of the thickness of the challenged right footpad, compared with the untreated left footpad. Each group consisted of five mice. Lymphostin was dissolved in DMSO and administered by i.p. injection for 5 consecutive days, beginning on the day of sensitization.

Results

Mechanism of inhibition of Lck by lymphostin

We have already reported that lymphostin inhibits phosphorylation of peptide substrate (Tyr-Ala-Glu)$_7$ by Lck from bovine thymus, in a concentration-dependent manner. To identify the mechanism of the inhibition of Lck by lymphostin, we examined the time dependency of the inhibitory action. Lymphostin was incubated with the reaction mixture for different times before the addition of ATP. The reaction was started by the addition of ATP and incubated for 15 min. Incubation in the absence of the drug for up to 60 min had little effect on the enzyme activity of Lck (data not shown). The inhibitory activity of lymphostin was dependent on the first incubation times (Fig. 2). The IC$_{50}$ in the first incubation times of 0, 5, 10, 30, and 60 min were 0.6, 0.4, 0.2, 0.1, and 0.05 µM, respectively. The results suggested that lymphostin inhibits Lck in a slow-binding manner.

T-cell receptor stimulation induces Lck autophosphorylation, which increases the enzyme activity of Lck. Thus, we examined the inhibitory activity of lymphostin against Lck autophosphorylation in vitro. Lck immunocomplexes from human Jurkat T cells were incubated with lymphostin for 10 min, and the reaction was started by the addition of ATP. Lymphostin inhibited Lck autophosphorylation in a dose-dependent manner. A representative autoradiogram is shown in Fig. 3. The IC$_{50}$ of autophosphorylation was 0.33 µM, which was similar to the IC$_{50}$ for the phosphorylation of peptide substrate in a 10-min incubation (0.2 µM).
Fig. 2. Effects of Incubation Time on Susceptibility of Lck to Inhibition by Lymphostin.

1 µM (○), 0.3 µM (●), 0.1 µM (▲), 0.03 µM (▲) or 0.01 µM (▼) lymphostin was incubated with a reaction mixture lacking ATP for the indicated time. Then the reaction was started by addition of ATP, and, after 10 min, phosphorylation of the peptide substrate was measured as described in "Materials and Methods". Data are the mean from an experiment done in duplicate.

Fig. 3. Effects of Lymphostin on Autophosphorylation of Lck.
Lck immunocomplex prepared from Jurkat cells was incubated with [γ-32P]ATP for 10 min. The indicated concentrations of lymphostin were added to the reaction mixture 10 min before starting the reaction. The Lck autophosphorylation was analyzed using SDS-PAGE as described in "Materials and Methods". An autoradiogram is shown with the position of bands corresponding to Lck indicated by arrow. Data shown are from an experiment that is representative of three independent experiments with the same result.

Effects of lymphostin on T-cell tyrosine phosphorylation

Signaling through the T-cell receptor has been shown to cause rapid increases in tyrosine phosphorylation of T-cell receptor zeta chain and other substrates. A mutant of the human Jurkat T-cell line lacking Lck kinase function was defective in the induction of tyrosine phosphorylation, indicating that Lck kinase activity is essential for tyrosine phosphorylation through the T-cell receptor. Tyrosine kinase inhibitors such as genistein, herbimycin, and PP1 were reported to inhibit T-cell-receptor-induced T-cell activation. We examined the effects of lymphostin on anti-CD3-stimulated tyrosine phosphorylation in human Jurkat T-cells. Human Jurkat T-cells were treated with 10 µg/ml of antibody OKT3 alone and with OKT3 after a 30-min incubation with lymphostin or staurosporine. After lysis in 1% Triton X-100, T-cell tyrosine phosphorylation was detected using Western blotting with anti-phosphotyrosine antibody. A representative phosphotyrosine blot is shown in Fig. 4. OKT3 induced tyrosine phosphorylation of an approximately 23-kDa protein. Lymphostin inhibited tyrosine phosphorylation in a dose-dependent manner. The IC50 was 0.2 µM, as found by measuring the p23 bands. Lymphostin also reduced constitutive tyrosine phosphorylations. Lymphostin proved to be effective at the cellular level, at least in the decreased tyrosine phosphorylation of T-cell proteins. However, the concentrations required for inhibition of tyrosine phosphorylation were greater than those required to inhibit MLR. This result suggested that there may be additional targets of lymphostin.

Inhibition of PI3-kinase by lymphostin

In order to identify additional targets of lymphostin, we examined the effects of lymphostin on various kinases. We found that lymphostin inhibited PI3-kinase at an approximately 50-fold lower concentration than that required for Lck inhibition. PI3-kinase activity was measured using the [γ-32P] ATP radioisotopic method. Phosphorylated products of PI were extracted and separated by TLC. The effects of lymphostin on PI3-kinase activity are shown in
Lymphostin, a Lck and Phosphatidylinositol 3-kinase Inhibitor

Fig. 5. Inhibition of PI3-Kinase by Lymphostin.

Anti-PI3-kinase p85 immunoprecipitates prepared from Jurkat cells was incubated with \([p-32P]ATP\) and phosphatidylinositol for 60 min. The indicated concentrations of lymphostin were added to the reaction mixture 10 min before start of the reaction. The lipid products were extracted and separated by TLC as described under "Materials and Methods". An autoradiogram of the TLC is shown with the position of spots corresponding to PI-3P and origin (Ori) indicated by arrows. Data shown are from an experiment that is representative of two independent experiments with the same result.

Fig. 6. Effects of Treatment Time on Susceptibility of PI3-Kinase to Inhibition by Lymphostin.

Anti-PI3-kinase p85 immunoprecipitates prepared from Jurkat cells were treated with vehicle (lane 1) or with 0.01 \(\mu\)M of lymphostin (lane 2–4) for 1 min (lane 2), 10 min (lane 3), or 30 min (lane 1 and lane 4). After the immunoprecipitates were washed 5 times with washing buffer used in their preparation, PI3-kinase activity in the washed immunoprecipitates was assayed. The lipid products were extracted and separated by TLC as described under "Materials and Methods". An autoradiogram of the TLC is shown with the position of spots corresponding to PI-3P and origin (Ori) indicated by arrows. Data shown are from an experiment that is representative of two independent experiments with the same result.

Table 1. Effects of Lymphostin on Delayed-type Hypersensitivity (DTH) in mice by lymphostin

B6D2F1 mice (n = 5) were s.c. immunized with trinitrobenzene sulfonic acid (TNBS) on day 0 and compounds were i.p. injected once a day from day 0 to day 4. TNBS was challenged on day 5 and footpad swelling was measured 24 hours later as described in "Materials and Methods".

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>Inhibition (%)</th>
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<tbody>
<tr>
<td>Lymphostin</td>
<td>1 × 5i.p.</td>
<td>65</td>
</tr>
<tr>
<td>Cs A</td>
<td>0.3 × 5i.p.</td>
<td>27</td>
</tr>
<tr>
<td>Cs A</td>
<td>30 × 5i.p.</td>
<td>91</td>
</tr>
</tbody>
</table>

1 mg/kg × 5 of lymphostin suppressed DTH responses by 65%. In comparison, cyclosporin A showed 91% inhibition by i.p. injection of 30 mg/kg × 5.

Discussion

In this study, we show that a novel immunosuppressant, lymphostin, inhibited autophosphorylation of Lck as well as Lck phosphorylation of exogenous substrate, and anti-CD3-induced protein tyrosine phosphorylation in Jurkat T-cells. In a mouse experimental model, lymphostin inhibited DTH in vivo (1 mg/kg × 5 i.p.: 65%). However, the inhibitory activity against Lck in the cellular level was less potent (IC\(_{50}\) 0.2 \(\mu\)M) than that against MLR (IC\(_{50}\) 0.009 \(\mu\)M). These results suggest that there may be another target for lymphostin besides Lck. Thus we examined the effects of this compound on various kinases. As a
result, we found that lymphostin was a potent inhibitor of PI3-kinase. The IC$_{50}$ was 0.001 $\mu$m, which was lower than that of wortmannin, a known potent PI3-kinase inhibitor (IC$_{50}$ 0.003 $\mu$m$^{19}$).

PI3-kinase catalyzes the formation of D-3 phosphoinositide lipids by transferring the terminal phosphate of ATP to the D-3 position of the inositol head groups of phosphoinositide lipids (reviewed by Kapeller and Cantley$^{22}$; Rameh and Cantley$^{22}$). PI3-kinase is composed of a catalytic p110 subunit and a regulatory p85 subunit that binds tyrosyl-phosphorylated proteins via its src homology 2 domains. P85 also has a src homology 3 domain that can interact with proteins containing proline-rich regions. Considerable evidence indicates that PI3-kinase may function as a key component of multiple signaling pathways, including T-cell activation. The accumulation of D-3 phosphoinositide lipids occur in response to ligation of several surface molecules involved in T-cell activation and proliferation, including the T-cell receptor,$^{23}$ CD4,$^{24,25}$ CD28,$^{26}$ CD2,$^{23}$ CTLA-4,$^{27}$ and interleukin 2 receptor.$^{28}$ Lymphostin inhibited the PI3-kinase activity of a partially purified preparation from calf thymus and of an anti-PI3-kinase p85 immunoprecipitated from human Jurkat T-cells, with an IC$_{50}$ of 0.001 $\mu$m. This potency is consistent with the effects of the inhibitor on celluar response of MLR. Therefore, lymphostin is considered to inhibit the MLR and DTH not only through the blockade of Lck but also through the blockade of PI3-kinase.

Wortmannin, which is a potent and selective PI3-kinase inhibitor, has immunosuppressive activity in whole animals.$^{29}$ Wortmannin treatment of allogeneic donor lymphocytes in vitro failed to generate a significant graft-vs-host disease in irradiated mouse recipients.$^{30}$ Wortmannin inhibited the MLR with an IC$_{50}$ of 0.027 $\mu$m (data not shown), but it did not inhibit the Lck (IC$_{50}$ > 100 $\mu$m, data not shown). Together with this study, these data also suggest that PI3-kinase and Lck could be potential targets of immunosuppressants.

To investigate the involvement of Lck and PI3-kinase independently, derivatives of lymphostin with specific inhibitory activity need to be synthesized. Figure 4 indicated that lymphostin inhibited the phosphorylation of constitutively tyrosine-phosphorylated proteins as well as p23. So we cannot rule out possible involvements of other tyrosine kinase inhibitions.

The results obtained from the incubation studies indicated that lymphostin inhibited Lck and PI3-kinase through a direct interaction with the enzymes and that the inhibition was irreversible. Lymphostin has a $\beta$-methoxy enone group that would be attacked by an amino group of the protein to form a vinylous amide. Irreversible inhibition of Lck and PI3-kinase by lymphostin could be explained by such a covalent reaction of the compound with the enzyme proteins.

The biological roles of PI3-kinase have been examined by using PI3-kinase inhibitors such as wortmannin as molecular probes. However, the function of PI3-kinase has not yet been fully identified. Therefore, lymphostin and wortmannin will be good tools to study the role of this enzyme.

Many kinase inhibitors have been developed, since kinases are considered potential targets for the treatment of a variety of diseases, such as cancer, atherosclerosis, and psoriasis, and a large number of inflammatory responses, such as septic shock.$^{31}$ Potent kinase inhibitors, such as staurosporine and wortmannin, have been found in microorganisms. It is noteworthy that lymphostin has potent kinase inhibitory activity, but its structure differs from those of known kinase inhibitors. Therefore, lymphostin provides a promising lead for developing novel kinase inhibitors.

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**References**


