A-770041, a Novel and Selective Small-Molecule Inhibitor of Lck, Prevents Heart Allograft Rejection


Abbreviations: IL, interleukin; A-770041, 1-methyl-1H-indole-2-carboxylic acid (4-[1-[4-(4-acetyl-piperazin-1-yl)-cyclohexyl]-4-aminopyrazolo[3,4-d]pyrimidin-3-yl]-2-methoxy-phenyl)-amide; A-420983, 1-methyl-1H-pyrazolo[3,4-d]pyrimidin-3-yl}-2-methoxy-phenyl)-amide; A-770041, 1-methyl-1H-indole-2-carboxylic acid (4-[1-[4-(4-acetyl-piperazin-1-yl)-cyclohexyl]-4-aminopyrazolo[3,4-d]pyrimidin-3-yl]-2-methoxy-phenyl)-amide.

Abstract

Lck, one of eight members of the Src family of tyrosine kinases, is activated after T cell stimulation and is required for T-cell proliferation and interleukin (IL)-2 production. Inhibition of Lck has been a target to prevent lymphocyte activation and acute rejection. Here, we report the pharmacologic characterization of 1-methyl-1H-indole-2-carboxylic acid (4-[1-[4-(4-acetyl-piperazin-1-yl)-cyclohexyl]-4-aminopyrazolo[3,4-d]pyrimidin-3-yl]-2-methoxy-phenyl)-amide (A-770041), an orally bioavailable pyrazolo[3,4-d]pyrimidine with increased selectivity for Lck compared with previously reported compounds. A-770041 is a 147 nM inhibitor of Lck (1 mM ATP) and is 300-fold selective against Fyn, the other Src family kinase involved in T-cell signaling. Concanavalin A-stimulated IL-2 production in whole blood is inhibited by A-770041 with an EC50 of approximately 80 nM. A-770041 is orally bioavailable (F = 34.1 ± 7.2% at 10 mg/kg) and has a t1/2 of 4.1 ± 0.1 h. Concanavalin A-induced IL-2 production in vivo is inhibited by oral administration of A-770041 (in vivo EC50 = 78 ± 28 nM). Doses of A-770041 at or above 10 mg/kg/day prevent rejection of hearts transplanted heterotopically in rats from Brown Norway donors to Lewis recipients across a major histocompatibility barrier for least 65 days. Grafs from animals treated with 20 mg/kg/day A-770041 or 10 mg/day Cyclosporin A had minimal microvascular changes or multifocal mononuclear infiltrates. However, mineralization in myocytes from the grafts from A-770041-treated animals was less than animals treated with Cyclosporin A. Lck inhibition is an attractive target to prevent acute rejection.

One of the most proximal signaling proteins downstream of the T cell receptor is the Src family tyrosine kinase Lck (Straus and Weiss, 1992). T cells are activated when they contact antigen associated with MHC proteins inducing multimerization of CD4 or CD8 coreceptors present on the T cell. Autophosphorylation of Lck associated with these receptors occurs, resulting in increased kinase activity. CD4/CD8 coreceptors associate with the T cell receptor ζ chain resulting in the phosphorylation of its immunoreceptor tyrosine-based activation motifs (Barber et al., 1989). ZAP-70 binds to phosphorylated ζ chain through its tandem SH-2 domains, where it is phosphorylated by Lck and subsequently undergoes autophosphorylation (Duplay et al., 1994). These events ultimately lead to phospholipase Cγ activation and the release of calcium from intracellular stores, generating the calcium signal required for calcineurin activation and production of cytokines such as IL-2 and interferon-γ (Weiss and Littman, 1994).

Lck plays a crucial role in the maturation of lymphocytes in the thymus and in mature T cell activation and proliferation (Molina et al., 1992; Straus and Weiss, 1992). Recruitment of Lck to the cell membrane by CD4 has been postulated to enhance T cell sensitivity and may regulate the amount of antigen required to activate the T cell (Li et al., 2004). Lck−/− mice have very few circulating mature T cells because Lck is required for thymocytes to progress to the double negative stage (Molina et al., 1992). Mice with an inducible Lck transgene on an Lck−/− background are reported to have normal numbers of circulating T cells, but the cells do not produce IL-2 or proliferate in response to CD3/CD28 stimulation (Legname et al., 2000; Seddon et al., 2000).

Knockout studies support the notion that inhibition of Lck should prevent acute rejection of transplanted organs. Skin grafts transplanted from wild-type mice to Lck−/− mice across a major histocompatibility barrier survive indefinitely (Wen et al., 1995). Knockout mice are useful in the study of
cell development but are less valuable in understanding the kinase inhibitory effects and function in mature T cells; hence, inhibitors of Lck are being investigated to prevent rejection of transplanted solid organs. A nonselective Lck inhibitor discovered by Abbott Laboratories, A-420983, has been shown to limit rejection in a nonvascularized neonatal heart transplantation model and prevent acute rejection of pancreatic β cells transplanted across major histocompatibility barriers (Waegell et al., 2002; Borhani et al., 2004).

Although A-420983 is a 37 nM Lck inhibitor, it lacks selectivity within the Src family. In particular, it exhibits only 8-fold selectivity against Fyn, a kinase that also plays a role in T cell signaling (Borhani et al., 2004). Besides the role of Fyn in T cell maturation and activation, it plays a role in myelination of neurons (Umemori et al., 1994, 1999), Sertoli cell maintenance of germ line stem cells in the testicle (Maekawa et al., 2002), and degranulation of mast cells (Parravini et al., 2002). A-420983 is also equipotent against Src and only 10-fold selective against Fgr. Src plays a role in bone formation and osteoclast function (Lowe et al., 1993), and Src −/− mice develop osteopetrosis as a result of the decreased resorption of bone by osteoclasts (Soriano et al., 1991). Hck/Fgr double knockout mice show defects in neutrophil adhesion and activation leading to defective respiratory burst and degranulation that could have consequences innate immunity (Lowell et al., 1996; Lowell and Berton, 1998). We continued our chemistry effort to find a molecule with greater selectivity for Lck to minimize the possibility that efficacy was driven by coinhibition of both Fyn and Lck. We also wanted to find a molecule with greater selectively against Src and Fgr to avoid the potential detrimental effects that have been implied from studies from knockout mice for these Src family members.

Subsequent synthetic chemistry efforts resulted in the discovery of A-770041 (Fig. 1), a compound with increased selectivity for Lck over other Src family members compared with A-420983. The purpose of these studies was to determine whether A-770041, a more selective Lck inhibitor that does not inhibit Fyn, could prevent T cell activation, IL-2 production in vivo, and rejection of vascularized transplanted heart in a rodent model. In the present studies, it is demonstrated that A-770041 abolishes the production of IL-2 in vitro and in vivo. Furthermore, A770041 prevents the rejection of fully mismatched vascularized heart allografts in rats and results in prolonged survival of grafts, similar to treatment with Cyclosporin A.

Materials and Methods

Animals. Lewis and Brown Norway rats (male, 200–300 g) were obtained from Charles River Laboratories, Inc. (Wilmington, MA), maintained on a 12-h light/dark cycle, and provided food and water ad libitum. All animal studies were reviewed by the Abbott Bioresearch Center Institutional Animal Care and Use Committee and complied with Association for Assessment and Accreditation of Laboratory Animal Care guidelines and the Guide for the Care and Use of Laboratory Animals.

Compounds. A-770041 was synthesized by Abbott Bioresearch Center (Worcester, MA). Compound was dissolved in a 5% ethanol in sterile water for all in vivo studies. Cyclosporin A (Neoral) was purchased from Novartis (Basel, Switzerland). Cyclosporin A was diluted in 5% ethanol in sterile water for all in vivo studies.

Homogeneous Time-Resolved Fluorescence-Kinase Assay. The purified recombinant tyrosine kinase to be tested was mixed with a biotinylated peptide substrate and varying inhibitor concentrations with 1 mM ATP, 10 mM Mg2+, and 2 mM Mn2+. After incubation for 60 min, a europium cryptate-labeled anti-phosphotyrosine and streptavidin-labeled allophycocyanin were added to the well. The ratio of the signal of 620 and 665 nm was used to calculate IC50.

Anti-CD3-Induced IL-2 in Whole Blood. Heparinized human whole blood was stimulated with αCD3 monoclonal antibody and phorbol 12-myristate 13-acetate in the presence of A-770041 (0–30 μM). IL-2 release into plasma was determined 2 h after stimulation by an enzyme-linked immunosorbent assay.

Concanavalin A-Induced Cytokine Production. Male Lewis rats were dosed with 2.5 mg/kg A-770041 or vehicle (1 ml/kg). At 2, 6, 10, and 22 h after dosing of the test compound, rats were administered 5 mg/kg concanavalin A (0.5 ml in saline, no. 2000-06; GE Healthcare, Little Chalfont, Buckinghamshire, UK) i.v. via the tail vein. Two hours after concanavalin A administration (i.e., 4, 8, 12, and 24 h after test compound dosing), rats were euthanized with CO2 blood, collected by cardiac puncture into heparinized tubes, and plasma was separated. The plasma samples were divided into two aliquots, one for the assay of A-770041 concentration (200 μl) and one for the detection of IL-2 by enzyme-linked immunosorbent assay (100 μl).

Heterotopic Heart Transplantation. Hearts were transplanted between Brown Norway (donor) and Lewis (recipient) rats essentially as described by Korecky and Masika (1990) with modifications. Recipient animals were treated with compounds beginning on day −1, and the day of surgery was denoted as day 0. The donor and recipient were anesthetized by inhalation of isoflurane, and the dorsal fur was shaved. The graft harvest in the donor and preparation of the recipient for implantation occurred simultaneously, using one surgeon for each procedure to minimize ischemic time. In the donor, the superior and inferior vena cava, the pulmonary veins, and the aorta were isolated and transected just proximal to the first branch of the pulmonary artery. The organ was immediately flushed with 5 ml of ice-cold, sterile, lactated Ringer’s solution and submerged in the solution. The aorta and pulmonary artery were dissected, and surrounding adipose tissue was removed. The recipient was prepared by making an incision along the midline. The abdominal contents were wrapped in gauze soaked with warm (37°C) Ringer’s solution and moved to the left. The inferior vena cava and
abdominal aorta were dissected and separated. The heart was implanted by end-to-side anastomoses of the graft aorta to the recipient abdominal aorta and the graft pulmonary artery to the vena cava with 7-0 synthetic suture. The clamp was released, and the anastomoses were compressed with a collagen pad to stop bleeding. Once the heart began to beat and the bleeding was stopped, the abdomen was closed, and anesthesia was withdrawn. The abdomen was palpated daily to determine whether the graft was beating. Blood was collected either via the tail vein (200 μl) during survival studies or from the vena cava upon scheduled sacrifice, allowed to clot, and serum was separated and kept at −70°C until analysis.

**Detection of A-770041.** A-770041 concentrations in plasma or serum were analyzed by protein precipitation and liquid chromatography-mass spectrometry/mass spectrometry analysis. The drug precipitation protocol was performed using robotic sample preparation on a Tecan Genesis workstation. To 50 μl of plasma or serum was added 200 μl of internal standard solution (100 nM A-420983 in 100% acetonitrile); the resultant precipitate was filtered using a 96-well format 3M filter plate. The filtrate was diluted 1:5 ml with 35% acetonitrile in water, and 10 μl was injected onto a 3.5 μM Xterra C18 high-performance liquid chromatography column (Waters, Milford, MA), eluted at 0.85 ml/min using a gradient mobile phase consisting of acetonitrile and 0.2% formic acid. The concentrations were determined by liquid chromatography-mass spectrometry/mass spectrometry using an API 4000 instrument (Applied Biosystems, Foster City, CA) in positive electrospray mode. The calibration curve ranged from 1 to 3 μM.

**Histology.** On day 65 after transplantation, the animal was euthanized with CO2, and the graft was removed and placed in formalin. The tissue was embedded, cut, and stained with hematoxylin and eosin. All sections were assessed by a pathologist blinded to the treatments.

**Statistics.** Data presented as the mean ± S.D. of at least n = 4 measurements. Data from the survival studies are presented as percentages with at least n = 6 per treatment group.

**Results**

**Structure, Physical and Chemical Characteristics, and Selectivity of A-770041.** The structure, molecular weight, and ClogP of A-770041, an ATP-competitive inhibitor of Lck, are shown in Fig. 1. A-770041 is a pyrazolo[3,4-d]pyrimidine with an IC50 of 147 nM against recombinant human Lck (64-509) in the presence of 1 mM ATP (Km = 12 μM). Inhibition of the kinase activity of other Src family members by A-770041 is shown in Table 1. A-770041 is 60-fold selective against Src, 95-fold selective against Fgr, and 300-fold selective against Fyn. A-770041 was also greater than 200-fold selective against a secondary battery of approximately 20 serine/threonine and tyrosine kinases outside of the Src family and IC50 values greater than 10 μM in a CEREP panel of approximately 70 molecular targets (data not shown).

**Effect of A-770041 on T Cell Activation In Vitro.** The effect of A-770041 on anti-CD3-stimulated IL-2 production in whole blood was determined (Fig. 2). IL-2 production was decreased in a dose-dependent manner. The EC50 for inhibition of anti-CD3 induced IL-2 was 80 nM, and IL-2 was suppressed greater than 90% at all concentrations above 1 μM.

**Single-Dose Pharmacokinetics of A-770041.** Lewis rats were dosed intragastrically with A-770041 at 10 mg/kg or i.v. at 5 mg/kg. Blood was collected from the tail vein at several different time points throughout a 36-h period. The plasma concentration of A-770041 was determined and is shown in Fig. 3. A-770041 has a t1/2 of approximately 4.2 h and a bioavailability of 34% with a Cmax of 2186 ± 410 nM at 5.3 h at a dose of 10 mg/kg. Single-dose pharmacokinetic studies showed that the area under the curve scaled linearly with dose up to 30 mg/kg when the compound was given orally (data not shown). Pharmacokinetic modeling of the exposures from single oral dose studies was completed to determine an acceptable dosing regime of A-770041 that would be expected to maintain Cmin, blood concentrations above 1 μM with repeated dosing. This concentration is expected to significantly inhibit Lck throughout the dosing period. It was determined that twice daily oral dosing in rats is required to maintain Cmin above 1 μM.

**Effect of A-770041 on Concanavalin A-Induced IL-2 Release in Vivo.** Compounds that inhibit Lck in vivo should prevent the activation of T cells by concanavalin A and the subsequent release of IL-2 in a dose-dependent manner. Animals were dosed intragastrically with 2.5 mg/kg A-770041. Concanavalin A was administered i.v. via the tail vein at various times after drug treatment, and blood was collected 2 h after concanavalin A, as described under Materials and Methods. The correlation of concanavalin A-induced IL-2 levels to A-770041 concentrations in the serum is shown in Fig. 4. Inhibition of concanavalin A-induced IL-2 was shown to be dependent upon plasma concentration of A-770041 (●) with an in vivo EC50 of 78 ± 28 nM. The inhibition of IL-2 in vivo by A-770041 fits to a sigmoidal curve (r2 = 0.79, Fig. 4, line).

**TABLE 1**

<table>
<thead>
<tr>
<th>Activity against Src family kinases</th>
<th>IC50 (μM)</th>
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<tbody>
<tr>
<td>Lck</td>
<td>0.147</td>
</tr>
<tr>
<td>Src</td>
<td>9.1</td>
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<tr>
<td>Fgr</td>
<td>14.1</td>
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<td>Fyn</td>
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![Fig. 2. Effect of A-770041 on CD3-mediated IL-2 production in whole blood. IL-2 released from human whole blood was determined as described under Materials and Methods. A-770041 was added (0–30 μM) at time 0. IL-2 was determined at 24 h. Data are mean ± S.D. (n = 4).](image-url)
Effect of A-770041 on Rejection of Heterotopically Transplanted Hearts. Recipient animals were treated with vehicle control or 2.5 to 20 mg/kg/day of A-770041 in equally divided doses 12 h apart beginning the day before surgery. Hearts were transplanted heterotopically from a Brown Norway donor to a Lewis recipient, as described under Materials and Methods. Animals were treated for 14 days, and graft viability was assessed by abdominal palpation each day. As expected, hearts transplanted into animals receiving the vehicle control ceased beating between days 6 and 7 (Fig. 5). A-770041 dosed at 2.5 mg/kg/day did not lengthen graft survival time. There was a dose-dependent increase in survival with doses of 5 and 10 mg/kg/day. At doses of 10 and 20 mg/kg/day of A-770041, 100% of transplanted grafts were still beating at 14 days.

Plasma concentrations of A-770041 (Table 2) were determined 12 h after compound administration on days 3, 7, and 14 (C_{12h}) just before the administration of the next dose or termination of the study. As expected, plasma concentration of A-770041 increased almost linearly with dose and had reached steady state by day 3.

With the positive result from the 14-day study, a subsequent experiment was completed to determine the effect of A-770041 on long-term survival of heart allografts and comparison with Cyclosporin A. Recipients were treated beginning the day before heterotopic heart transplantation with 10 or 20 mg/kg/day of A-770041 in equally divided doses 12 h apart or 10 mg/kg/day of Cyclosporin A once per day for 65 days. All grafts (n = 6/group) in animals receiving 10 or 20 mg/kg/day A-770041 or 10 mg/kg/day Cyclosporin A survived to 65 days after transplantation. C_{12h}, serum concentrations of A-770041 from both groups on days 7, 34, 60, and 65 were comparable with the concentrations seen in the 14-day study (data not shown).

Animals were sacrificed at day 65 to harvest the transplanted graft for histological analysis. Representative photomicrographs of allografts are shown in Fig. 6. Allografts from rats dosed with 10 mg/kg/day A-770041 (A and B) or 10...
mg/kg/day Cyclosporin A (E and F) had minimal multifocal mononuclear infiltrates in allografts (arrows). The allografts from animals dosed with 10 mg/kg/day A-770041 had an increased incidence of minimal vascular changes characterized by tunica media hypertrophy, vacuolated myocytes, and reactive endothelial cells (vessel, Fig. 6B) compared with the Cyclosporin A or 20 mg/kg/day A-770041 groups. In addition, one graft from the 10 mg/kg/day A-770041 group had minimal vascularitis (data not shown). There was also increased incidence of minimal edema (e) within the 10 mg/kg/day A-770041 and 10 mg/kg/day Cyclosporin A groups. Mineralization was present in allografts from the Cyclosporin A group, with minimal to mild scores in three of five allografts but was not seen in grafts from animals treated with A-770041 (data not shown). In addition, allografts from the Cyclosporin A group had neovascularization extending intramurally from the pericardium.

Discussion

A-770041 is a selective inhibitor of Lck that shows comparable efficacy with Cyclosporin A in preventing acute transplant rejection in solid organs. This compound has prototypical activity of an immunosuppressive agent by blocking T cell activation and IL-2 production in vitro and in vivo. Pharmacokinetic data show that A-770041 is amenable to chronic, oral, twice daily dosing in rats with compound reaching a steady-state C$_{12h}$ in blood by day 3 and maintaining it through day 65. Oral doses of A-770041 at 10 and 20 mg/kg/day prevented acute rejection for 65 days after transplantation, at which point the experiment was concluded. The 10 mg/kg/day dose limited rejection but did not completely prevent the infiltration of mononuclear cells into the allograft. These data show that a specific inhibitor of Lck is efficacious in preventing acute rejection in a rodent model. The serum concentrations of A-770041 measured in the dose-response experiments of concanavalin A-mediated IL-2 production in vivo compared with the results of 14- and 65-day transplantation studies provide some interesting predictions concerning the efficacious plasma concentrations of A-770041 needed to prevent acute rejection. One hundred percent survival of grafts at either 14 or 65 days after transplantation only occurred when the C$_{min}$ concentrations for A-770041 were maintained above the EC$_{90}$ for inhibiting IL-2 production in vivo. This point is most clearly illustrated by the lack of efficacy of A-770041 when dosed at 2.5 and 5 mg/kg/day. In both of these treatment groups, the C$_{min}$ serum concentrations are well below the EC$_{90}$ for inhibition of concanavalin-A-induced IL-2. These data suggest, similar to the data published with Cyclosporin A, that IL-2 production must be nearly maximally inhibited to prevent acute rejection of transplanted organs.

Lck is an attractive target to block T cell activation for a number of reasons (Tsutsui et al., 2003). First, Lck is crucial in the activation of T cells causing clonal expansion and generation of a cytotoxic T cell response (Weiss and Littman, 1994). Second, it is expected that inhibitors of Lck should prevent rejection of transplanted organs as evidenced by prolonged skin allograft survival in Lck$^{-/-}$ mice (Wen et al., 1995). The data presented here and in a previous publication with a first generation Lck inhibitor (A-420893) show the potential of an inhibitor of Lck to prevent acute rejection (Waegell et al., 2002; Borhani et al., 2004).

In addition, the expression of Lck is thought to be limited to T cells, natural killer cells, B1 cells, and heart, brain, and retinal neurons (Omri et al., 1998; Ping et al., 2002). Although expression of Lck in the brain and heart has been reported, Lck$^{-/-}$ mice have not been reported to have any cognitive or cardiovascular deficits. The only reported detrimental effect of knocking out Lck in nonimmune tissues is retinal dysplasia and retinal detachment (Omri et al., 1998). Whether this is a developmental or functional defect from the lack of Lck expression will need to be explored with potent, selective inhibitors of Lck such as A-770041.
Lck \(-/-\) mice show thymic atrophy from a reduction in double-positive thymocytes and profound lymphopenia with a 100-fold reduction in mature CD4 cells and a 3-fold reduction in mature CD8 cells (Molina et al., 1992). Although the lack of Lck expression through development of the knockout mouse certainly affects thymopoiesis, lack of Lck expression in peripheral mature T cells does not alter survival (Legname et al., 2000; Seddon et al., 2000). In a doxycycline-inducible Lck-transgenic mouse on an Lck \(-/-\) background, when doxycycline is fed through development, there is a relatively normal repertoire of mature T cells. Cessation of doxycycline treatment and loss of Lck expression results in decreased thymopoiesis but no effects on the numbers of circulating mature T cells for several months. Circulating T cells from the Lck \(-/-\) mice have a significantly decreased proliferative response, calcium mobilization, and IL-2 production (Straus and Weiss, 1992; Trobridge and Levin, 2001). These data suggest that the inhibition of Lck with a small molecule should have no effect on the number of mature circulating T cells or survival of these cells but should be expected to block activation and proliferation.

Inhibitors of Lck may also be efficacious in other inflammatory diseases, including rheumatoid arthritis, multiple sclerosis, inflammatory bowel diseases, type 1 diabetes, systemic lupus erythematosus, and psoriasis (Kamens et al., 2001). In conclusion, we have discovered A-770041, a selective inhibitor of Lck that is orally active, inhibits T cell activation and proliferation and prevents allograft rejection in transplanted hearts similar to Cyclosporin A for up to 65 days. These data show that selective inhibitors of Lck have the potential to be efficacious in preventing acute rejection. These inhibitors would be expected to avoid the renal effects of long-term therapy with Cyclosporin A.

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