Selective Itk Inhibitors Block T-Cell Activation and Murine Lung Inflammation

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ABSTRACT: Nonreceptor protein tyrosine kinases including Lck, ZAP-70, and Itk play essential roles in T-cell receptor (TCR) signaling. Gene knockout studies have revealed that mice lacking these individual kinases exhibit various degrees of immunodeficiency; however, highly selective small molecule inhibitors of these kinases as potential immunosuppressive agents have not been identified. Here we discovered two novel compounds, BMS-488516 and BMS-509744, that potently and selectively inhibit Itk kinase activity. The compounds reduce TCR-induced functions including PLCγ1 tyrosine phosphorylation, calcium mobilization, IL-2 secretion, and T-cell proliferation in vitro in both human and mouse cells. The inhibitors suppress the production of IL-2 induced by anti-TCR antibody administered to mice. BMS-509744 also significantly diminishes lung inflammation in a mouse model of ovalbumin-induced allergy/asthma. Our findings represent the first description of selective inhibitors to probe human Itk function and its associated pathway, and support the hypothesis that Itk is a therapeutic target for immunosuppressive and inflammatory diseases.

Although proper immune responses rely on T-cell activation upon antigen presentation, deregulation of T-cell stimulation often results in autoimmunity and inflammatory diseases (*1*). Inhibition of T-cell activation has been one of the strategies for developing immunosuppressive agents to treat autoimmune disorders and inflammation. Suppression of host immune functions by blocking T-cell activation is also a successful modality for preventing organ transplant rejection (*2*). T-cell-specific nonreceptor tyrosine kinases including Lck, ZAP-70, and Itk play essential roles in T-cell receptor (TCR)[1] signaling by regulating T-cell activation following engagement of antigen-presenting cells (APC) (*3*). Gene knockout studies have revealed various degrees of immunodeficiency in mice lacking these different kinases (*4*−*7*). Itk$^{-/-}$ mice have reduced numbers of T-cells, particularly CD4^{+} T-cells, and mature T-cells isolated from these mice are defective in TCR-mediated responses such as calcium mobilization, IL-2 secretion, and proliferation (*6*, *7*). These mice have diminished mean survival time when infected with the intracellular pathogen *Toxoplasma gondii* (*7*). In addition, Itk-deficient mice are unable to develop functional Th2 cells, resulting in their inability to expel the nematode *Nippostrongylus brasiliensis* from the gut (*8*). These studies led us to speculate that selective, small molecule inhibitors of Itk could be useful as immunosuppressive and antiinflammatory agents and might have value for immunological and inflammatory diseases where T-cell activation contributes to the disease pathophysiology. In this report, we discovered two potent and selective Itk inhibitors which blocked T-cell activation in vitro and suppressed inflammation in a murine asthma/allergy model.

EXPERIMENTAL PROCEDURES

Preparation of the Tec Family Kinases. The cDNAs corresponding to the kinase domains of the Tec family tyrosine kinases were cloned into the baculovirus transfer vector pAcGHLT-C (BD Biosciences) for expression as glutathione *S*-transferase (GST) fusion proteins in Sf9 cells. The protein regions cloned and primers used (with cloning sites underlined) are listed as follows: Itk (accession number Q08881) amino acids 352−620, 5′-GAGAGAGAATTCAAGATACGGGAAATGGGTGATCG-3′ and 5′-GAGAGACTCGAGCTAAAGTCCTGATTCTGCAATTTCAGC-3′; Txk (accession number P42681) amino acids 264−527, 5′-GAGAGTGAATTCTGAGATAGATCCATCTGAGTTGGCTTTTATAAAGG-3′ and 5′-GAGACTGCGGCCGCTCACCAGGTTTCCGCAATCTCTGTGAC-3′; Tec (accession number P42680) amino acids 364−631, 5′-GAGAGAGAATTCAGAGATTAACCCTTCAGAACTGAC-3′ and 5′-GAGAGAGCGGCCGCTTATCTTCCAAAAGTTTCTTCACATTC-3′; Btk (accession number Q06187) amino acids 396−659, 5′-GAGAGACTCGAGAGAAATTGATCCAAAGGACCTGACC-3′ and 5′-GAGAGTGCGGCCGCTCAGGATTCTTCATCCATGACATCTAGAATATTGC-3′; Bmx (accession number P51813) amino acids 411−675, 5′-GAGAGACCATGGAGAACTGAAAAGAGAAGAGATTACC-3′ and

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[1] Abbreviations: TCR, T-cell antigen receptor; APC, antigen-presenting cells; EGF, epidermal growth factor; PLCγ1, phospholipase Cγ1; PBMC, peripheral blood mononuclear cells.

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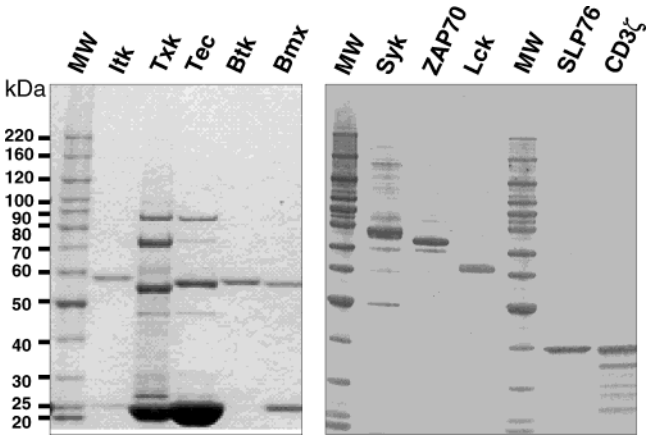

FIGURE 1: SDS−PAGE analysis of the protein preparations. One microgram of purified protein (labeled on the top of the lane) was analyzed on 4−12% NuPAGE gels (Invitrogen). The molecular masses of the Tec family kinase domains are expressed as GST fusion proteins ranging from 55 to 60 kDa. MW = molecular mass markers in kilodaltons.

5′-GAGACTGCGGCCGCTCAATGCTTGTCTTTTTCCCGAAGTG-3′.

After sequence verification, the recombinant baculoviruses were then generated using the BaculoGold system according to the manufacturer's procedure (BD Biosciences). For production of the recombinant kinases, Sf9 cells growing in log phase were infected with the corresponding baculoviruses at MOI = 5 for 2 days. The cells were harvested and resuspended in ice-cold buffer A [50 mM Tris-HCl, pH 7.5, 50 mM NaF, 100 mM NaCl, 1 mM Na_3VO_4, 1 mM DTT, 10% glycerol, 1% NP-40, and Complete protease inhibitors (1 tablet/50 mL; Roche Diagnostics)]. After centrifugation (16000*g*, 20 min, 4 °C), the supernatants were incubated with glutathione−Sepharose 4B (Amersham Pharmacia Biotech; 1 mL bed volume/L of lysate) for 1 h at 4 °C and washed two times with 15 bed volumes of buffer A and two times with 15 bed volumes of buffer B (50 mM Tris-HCl, pH 7.5, 50 mM NaF, 100 mM NaCl, 1 mM Na_3VO_4, 10% glycerol, and 1 mM DTT). The Sepharose beads were then resuspended in 2 bed volumes of buffer B and poured into disposable columns. The GST fusion proteins were eluted with buffer B containing 10 mM glutathione and stored at −70 °C. The purity of these protein preparations is shown in Figure 1.

Preparation of Lck, Syk, and ZAP-70. The cDNAs corresponding to full-length Lck (accession number M36811), Syk (accession number L28824), and ZAP-70 (accession number L05148) were cloned into the baculovirus donor vector pFastBacHTa (Invitrogen) using the following primer sets: Lck, 5′-GGCGGAATTCATGGGCTGTGGCTGCAGC-3′ and 5′-GGGCCTCGAGAGGCCTCCTCTCAAGGC-3′; Syk, 5′-CCCGAATTCATGGCCAGCAGCGGCATGGCTG-3′ and 5′-TTGCTCGAGTGATCAAAGGCAGCCACCGAC-3′; ZAP-70, 5′-CCCAGAATTCATGCCAGACCCCGCG-GCGCACC-3′ and 5′-CAGCGTCGACTCAGGCACAGG-CAGCCTCAGCC-3′. The recombinant baculoviruses were generated using the Bac-to-Bac system (Invitrogen) according to the manufacturer's suggestion. Procedures for infection of Sf9 and purification of the recombinant enzymes were similar to those described above for the Tec family kinases except 1 mM DTT and Complete protease inhibitor tablet in buffers A and B were replaced with 5 mM β-mercaptoethanol and EDTA-free Complete protease inhibitor tablet (Roche Diagnostics). The recombinant enzymes were captured on TALON metal affinity resin (BD Biosciences/Clontech), eluted by 100 mM imidazole, and dialyzed in buffer B. The purity of the enzymes is shown in Figure 1.

Production of the Substrate Proteins GST-SLP-76 and GST-CD3ζ. The cDNAs corresponding to the amino acids from 95 to 175 of human SLP-76 (accession number U20158) and the amino acids from 52 to 163 of the human CD3ζ chain (accession number J04132) were cloned into the pGEX4T-1 vector (Amersham Pharmacia Biotech) for expression in *Escherichia coli*. The primers used for cloning are as follows: SLP-76, 5′-GCGGCCGGATCCACAGAAA-GCCACGAAGAGGAC-3′ and 5′-CTCGGTCGACGTC-GATGTACATGGAGTTGGAGTTGG-3′; CD3ζ chain, 5′-CCTGGAATTCAGAGTGAAGTTCAGCAGGAGC-3′ and 5′-TCCCGGAATTCTTAGCGAGGGGGCAGGGC-3′. To express the recombinant proteins, the *E. coli* BL21(DE3) with pLysS was transformed with the pGEX expression vectors, grown to OD_{600} = 0.8, and induced by adding 1 mM IPTG for 4 h at 37 °C. The bacteria were harvested and suspended in PBS containing 1% Triton X-100 and Complete protease inhibitors (1 tablet/50 mL). The bacteria were lysed by freezing and thawing, digested with DNase I (Sigma-Aldrich), and centrifuged (16000*g*, 20 min, 4 °C). GST-SLP-76 and GST-CD3ζ were purified from the supernatants using glutathione−Sepharose 4B and the procedure described above for the Tec family kinases except that the washing buffer was PBS. The purified protein was dialyzed in buffer C (50 mM HEPES, 100 mM NaCl, and 1 mM DTT) and stored at −70 °C. The purity of both proteins is shown in Figure 1.

In Vitro Kinase Assays. The assays used to routinely determine the compound activity (IC_{50} values) against the Tec family kinases were performed using GST-SLP-76 as peptide substrate in the presence of 1 μM ATP in a 96-well plate. The kinase reactions (60 μL) containing 25 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mg/mL BSA, 1 μM ATP (0.4 μCi of [γ-^{33}P]ATP), 83 μg/mL GST-SLP-76, 10 ng of recombinant enzyme, and various concentrations of tested compound were carried out at room temperature for 10 min. After the reactions were terminated by addition of 100 μL of 20% TCA and 100 mM $NaPP_i$, the TCA-precipitated proteins were harvested onto GF/C unifilter plates (Perkin-Elmer) and washed. The radioactivity incorporated was then determined using a TopCount (Packard Instrument) after addition of 35 μL of Microscint scintillation fluid. For the Itk enzyme kinetics, the concentrations of ATP and GST-SLP-76 were specified in the corresponding figures and legends.

The same procedure was used for measuring compound activity (IC_{50}) against Syk and ZAP-70 using 5 ng of enzyme. For Lck (2 ng) and Fyn (40 ng; from Upstate Cell Signaling Solutions) assays, the peptide substrate was replaced with GST-CD3ζ (83 μg/mL) and poly(Glu-Tyr) (100 μg/mL), respectively.

The procedures for measuring compound activity against Cdk2, PKC, IKK2, insulin receptor (IR), and EGF receptor (EGFR) have been described previously (*9*). All other kinases including Erk1, calcium/calmodulin-dependent protein kinase II (CaMKII), glycogen synthase kinase-3β (GSK-3β), and

the catalytic subunit of protein kinase A (PKA) were purchased commercially (Upstate Cell Signaling Solutions), and the assays were performed using manufacturer's suggested procedures in the presence of 1 μM ATP.

Itk High-Throughput Assay. For the high-throughput screen, a homogeneous time-resolved fluorescence (HTRF) assay was employed. The kinase reaction (60 μL) containing 25 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 0.1 mg/mL BSA, 2.5 μM ATP, 9 μg/mL XL665-conjugated GST-SLP-76, 4 ng of GST-Itk kinase domain, and 10 μM tested compound was carried out at room temperature for 30 min. Conjugation of XL-665 to GST-SLP-76 was performed according to the manufacturer's procedure (Packard Instruments). The kinase reaction was terminated by addition of 20 μL of 120 mM EDTA followed by addition of 100 μL of europium cryptate-conjugated anti-phosphotyrosine antibody (Packard Instruments). The HTRF signal was measured using TopCount (Packard Instruments) with an excitation wavelength set at 337 nm and an emission wavelength set at 665 nm.

Analysis of Cellular Tyrosine Phosphorylation. Jurkat T-cells (1×10^7 cells in 1 mL) were treated with compounds for 20 min and stimulated with the anti-CD3 antibody G19-4 (1 μg/mL) or PBS for 2 min at 37 °C before lysates were prepared. The buffer used to prepare cell lysates was 25 mM HEPES, pH 7.4, 50 mM β-glycerophosphate, 80 mM KF, 1 mM DTT, 3 mM EGTA, 1 mM EDTA, 0.1 mM Na_3VO_4, 10% glycerol, 1% NP-40, and protease inhibitor cocktail tablet (1 tablet/50 mL, Complete; Roche Diagnostics). Cell lysates corresponding to 20 μg of cellular protein were analyzed by Western blot using the phosphotyrosine antibody 4G10 (Upstate Cell Signaling Solutions). To analyze the compound effect on PLCγ1, cell lysates corresponding to 100 μg of protein were subjected to immunoprecipitation using antibody specific to PLCγ1 (*10*). The immunoprecipitates were immunoblotted with the phosphotyrosine antibody 4G10, stripped, and reblotted with anti-PLCγ1 antibody.

A549 cells grown on 10 cm plates were serum starved for 24 h before incubation with the indicated compound (10 μM) (or 0.1% DMSO) for 20 min at 37 °C. The cells were then treated with EGF (50 ng/mL) for 5 min at 37 °C and lysed. The cell lysates corresponding to 30 μg of protein were resolved on a 4−12% NuPAGE gel (Invitrogen), transferred to a PVDF membrane, and immunoblotted with the anti-phosphotyrosine antibody 4G10. To analyze compound effects on PLCγ1, cell lysates corresponding to 400 μg of protein were subjected to immunoprecipitation using antibody specific to PLCγ1 (*10*). The immunoprecipitates were immunoblotted with the 4G10 antibody; the blots were then stripped and probed with anti-PLCγ1 antibody.

Measurement of Intracellular Calcium. Jurkat cells (5×10^5 cells/mL) were loaded with 4 μM fluo-3 AM (Molecular Probes) for 45 min at 37 °C. The cells were washed and seeded onto poly(D-lysine)-coated 96-well plates at 2×10^5 cells per well. The cells were treated with various concentrations of compounds (or 0.1% DMSO as control) for 30 min at 25 °C and placed in the fluorometric imaging plate reader (FLIPR; Molecular Devices) with an excitation wavelength set at 488 nm and an emission wavelength set at 530 nm. The fluorescence change was determined by subtracting the fluorescence before and after the addition of the anti-CD3 antibody (G19-4, 4 μg/mL).

Detection of IL-2 Secretion. Experiments were performed in a 96-well plate. The cells (1×10^5 per well) were stimulated with plate-bound anti-CD3 antibodies (G19-4 for the human cells and 2C11 for the mouse cells) for 16 h in the presence of various concentrations of compounds (or 0.1% DMSO for control). The concentration of IL-2 in the cell-free media was measured by enzyme-linked immunosorbent assays (ELISA; BD Biosciences) according to manufacturer's suggestion.

Mouse Serum IL-2 Model. Balb/c mice were injected subcutaneously with the indicated dose of compound or vehicle (H_2O:ethanol:Tween 80 = 90:5:5) 15 min before intravenous administration of anti-CD3 antibody (2C11, 10 μg per mouse or as indicated in the figure). Serum was collected for the analysis of IL-2 and compound levels at 90 min after anti-CD3 antibody administration. IL-2 was measured by ELISA, and compound levels were measured by mass spectrometry.

Murine Asthma/Allergy Model. The animal model employed was essentially as described previously (*11*) with minor modifications. Balb/c mice were primed and then boosted with 100 μg of ovalbumin in aluminum hydroxide adjuvant at days 0 and 14, respectively, by intraperitoneal injection. On day 28, the animals received an ovalbumin inhalation challenge. Shortly after the ovalbumin inhalation challenge, BMS-509744 or vehicle (H_2O:ethanol:Tween 80 = 90:5:5) was administered either subcutaneously or intraperitoneally twice daily for 3 days. On day 31, bronchoalveolar lavage was performed to collect leukocytes from the airways, and percentages of various leukocyte subsets were determined by differential cell counts using microscopy.

RESULTS AND DISCUSSION

Discovery and Characterization of Small Molecule Inhibitors of Itk. In an effort to discover Itk inhibitors, we established Itk kinase assays using a recombinant Itk kinase domain as the source of enzyme and a GST fusion protein containing the amino acids from 95 to 175 of SLP-76 as the peptide substrate (Figure 1). Enzyme kinetic studies of the Itk kinase domain revealed that the apparent K_M for ATP and GST-SLP-76 were 4.9 and 16.1 μM, respectively (Figure 2). Screening of our internal compound libraries using an HTRF assay identified an aminothiazole compound, BMS-285047 (Figure 3A; ref *12*), with an IC_{50} of 1 μM against Itk. The compound exhibited good selectivity for Itk among all of the kinases tested (Table 1). Subsequent medicinal chemistry efforts by following compound structure and activity relationships led us to identify two compounds, BMS-488516 and BMS-509744 (Figure 3B,C), that potently inhibited Itk in vitro with IC_{50} values of 96 and 19 nM, respectively. Both compounds exhibited competitive kinetics with respect to ATP (Figure 4), suggesting that they bind to the ATP binding site of the Itk kinase domain. Selectivity studies in vitro further revealed that both compounds showed >200-fold selectivity versus other Tec family tyrosine kinases and at least a 30-fold potency ratio versus all of the other protein kinases tested (Table 1).

Itk Inhibitors Block TCR-Induced Responses in Human T-Cells. To further analyze compound selectivity in cells, their effects on cellular tyrosine phosphorylation were examined in Jurkat T-cells following stimulation with anti-

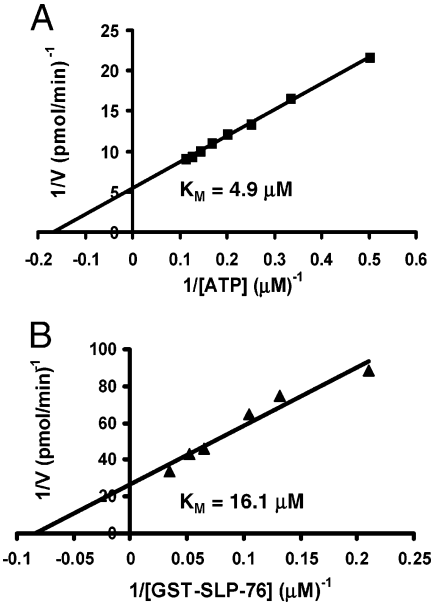

FIGURE 2: Enzyme kinetics of the Itk kinase domain for substrates ATP (A) and GST-SLP-76 (B). (A) Reactions to determine K_M for ATP were done in the presence of 20 μM GST-SLP-76 using 10 ng of enzyme at room temperature for 10 min. The data were converted to Lineweaver−Burk plots for calculating kinetics parameters. The k_{cat} value was calculated to be 106 s^{-1} in this experiment. (B) Reactions to determine K_M for GST-SLP-76 were performed in the presence of 10 μM ATP. The k_{cat} value was calculated to be 55 s^{-1} in this experiment.

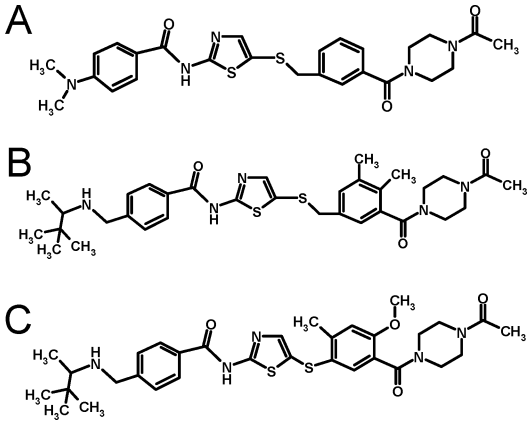

FIGURE 3: Structures of BMS-285047, BMS-488516, and BMS-509744. The chemical structures of BMS-285047 (A), BMS-488516 (B), and BMS-509744 (C) are illustrated. Their preparation procedures are detailed in the Supporting Information.

CD3 antibody (G19-4). As reported previously (*13*), anti-CD3 antibody stimulated the tyrosine phosphorylation of several cellular proteins within 2 min in Jurkat T-cells (Figure 5A). Both BMS-509744 and BMS-488516, at concentrations from 0.1 to 10 μM, only inhibited the tyrosine phosphorylation of a single protein with a molecular mass of 145 kDa (Figure 5A). The 145 kDa tyrosine-phosphorylated protein that was selectively inhibited by Itk inhibitors was likely to be phospholipase Cγ1 (PLCγ1) as previously identified (*13*). To confirm this assumption, we analyzed tyrosine phosphorylation of PLCγ1 in Jurkat T-cells after immunoprecipitation using anti-PLCγ1 antibody. As expected, BMS-509744 and BMS-488516 dose-dependently inhibited tyrosine phosphorylation of PLCγ1 induced by anti-CD3 antibody in Jurkat T-cells (Figure 5B). These data support

Table 1: Kinase Selectivity of BMS-285047, BMS-488516, and BMS-509744

	IC_{50} (nM)		
enzyme	BMS-285047	BMS-488516	BMS-509744
Itk	1000	96	19
Txk	>50000	>50000	11000
Tec	>50000	>50000	17000
Btk	49000	>50000	4100
Bmx	20000	>50000	>50000
Lck	>25000	27000	2400
Fyn	ND[a]	8600	1100
Syk	>50000	>50000	>50000
ZAP-70	>50000	>50000	>50000
IR	ND	4400	1100
EGFR	>50000	>50000	>50000
Cdk2	27000	3300	29000
ERK-1	>50000	>50000	>50000
PKA	>50000	>50000	>50000
PKC	>50000	21000	24000
Akt1	ND	>50000	>50000
IKKβ	ND	>50000	>50000
CaMKII	ND	>50000	33500
GSK-3β	ND	47400	36000

[a] ND, not determined.

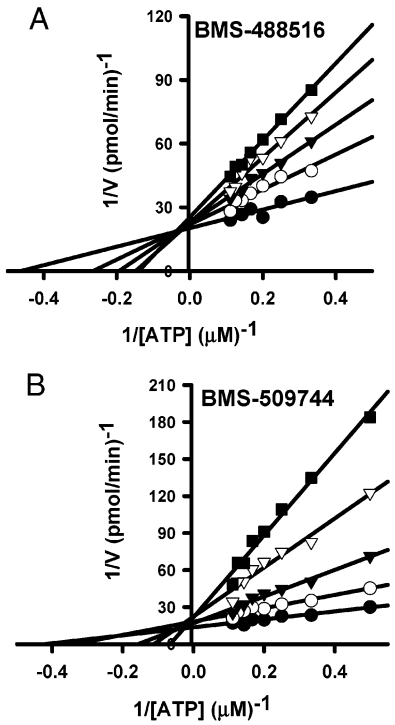

FIGURE 4: BMS-488516 (A) and BMS-509744 (B) exhibit competitive kinetics with respect to ATP. The kinase reactions were performed in the presence of 10 μM GST-SLP-76 and various concentrations of ATP as indicated for 10 min using 10 ng of enzyme. (A) Symbols for BMS-488516: solid circle, no compound; open circle, 20 nM; solid triangle, 40 nM; open triangle, 60 nM; solid square, 80 nM. (B) Symbols for BMS-509744: solid circle, no compound; open circle, 5 nM; solid triangle, 10 nM; open triangle, 20 nM; solid square, 30 nM.

that PLCγ1 is a major downstream substrate of Itk in T-cells. We also determined the effects of BMS-509744 and BMS-488516 on cellular tyrosine phosphorylation in non-T-cells where Itk expression was not evident. We found that neither compound, at concentrations up to 10 μM, significantly affected EGF-induced cellular tyrosine or PLCγ1 phosphorylation in A549 lung carcinoma cells (Figure 5C,D).

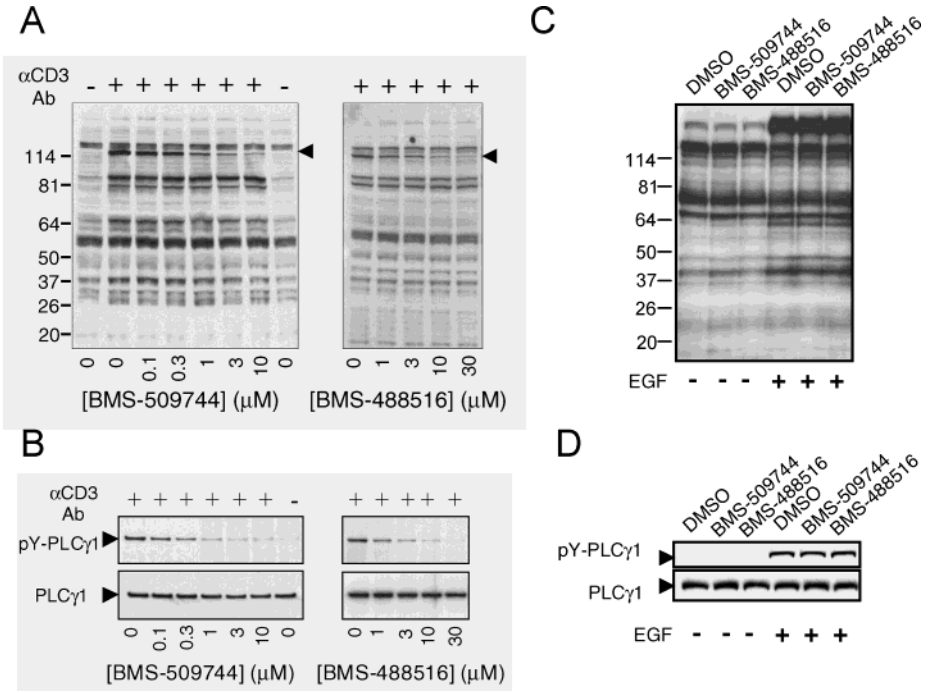

FIGURE 5: Compound effects on total cellular tyrosine and PLCγ1 phosphorylation. (A) Effects on cellular tyrosine phosphorylation induced by anti-CD3 antibody in Jurkat cells. The arrowhead symbol denotes the only protein band that is inhibited by the compounds. Molecular mass markers are in kilodaltons. (B) Inhibition of PLCγ1 tyrosine phosphorylation in Jurkat T-cells induced by anti-CD3 antibody. The arrowhead symbols denote tyrosine-phosphorylated PLCγ1 and total PLCγ1. (C) Effects on EGF-stimulated total cellular tyrosine phosphorylation in A549 cells. Lanes: 1 and 4, vehicle (0.1% DMSO); 2 and 5, 10 μM BMS-509744; 3 and 6, 10 μM BMS-488516. (D) Effects on EGF-stimulated PLCγ1 phosphorylation in A549 cells. Lanes: 1 and 4, vehicle (0.1% DMSO); 2 and 5, 10 μM BMS-509744; 3 and 6, 10 μM BMS-488516.

Upon antigen presentation in T-cells, PLCγ1 catalyzes the breakdown of phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol trisphosphate (IP_3), which stimulates Ca^{2+} mobilization. The rise in cytoplasmic free calcium concentration ($[Ca^{2+}]_i$) can trigger transcriptional activation, cytokine production, and cell proliferation. Since the Itk inhibitors selectively inhibited PLCγ1 phosphorylation in T-cells, we investigated their effects on the downstream functions of PLCγ1 including Ca^{2+} mobilization and IL-2 secretion upon anti-CD3 antibody stimulation. BMS-488516 and BMS-509744 dose-dependently inhibited anti-CD3 antibody-induced Ca^{2+} mobilization with submicromolar IC_{50}s (Figure 6A,B). The two compounds were evaluated for their potential inhibition of IL-2 secretion from Jurkat T-cells and showed potent dose-dependent suppression (Figure 6C). Similar results in inhibiting IL-2 secretion were observed in peripheral blood mononuclear cells (PBMC) isolated from healthy volunteers, although with slightly reduced potency (Figure 6C). BMS-509744 appeared to be more potent than BMS-488516 in inhibiting both calcium and IL-2, and the data are consistent with the potency of the compounds on enzymatic inhibition of Itk in vitro and blockade of PLCγ1 tyrosine phosphorylation in cells.

Effects of Itk Inhibitors on Mouse T-Cells. In addition to human cells, we evaluated both compounds in murine cells using the T lymphoma cell line EL4 and freshly isolated mouse splenocytes. Both compounds dose-dependently inhibited anti-CD3 antibody-induced IL-2 secretion in murine EL4 cells and splenocytes (Figure 6D). The EL4 cells appeared to be more sensitive to Itk inhibitors when compared to the primary splenocytes (Table 2). This observation, together with the similar trend found in human cells (Jurkat cells versus PBMCs), suggests that Itk plays a more significant role for the IL-2 secretion function in the transformed cell lines. The enhanced role of Itk in transformed cell lines may result from more active upstream signals, such as the higher level of phosphatidylinositol 3,4,5-trisphosphate (PIP_3) observed in Jurkat cells (*14*).

To evaluate whether Itk inhibitors had significant effects on non-TCR-mediated cytokine secretion in PBMCs, we evaluated their activity on lipopolysaccharide- (LPS-) induced TNFα release. BMS-488516 exhibited minor (IC_{50} = 11 μM) and BMS-509744 insignificant (IC_{50} > 25 μM) inhibitory activity against LPS-induced TNFα production from PBMCs, confirming the significant selectivity window for both compounds.

We also analyzed the compound effects on primary T-cell proliferation and found that human T-cell expansion in vitro induced by irradiated APC was suppressed by both BMS-488516 and BMS-509744 (Figure 6E). The data in Figure 6E showing nearly equal potency of both compounds against T-cell proliferation seem inconsistent with their potency against Itk enzyme in vitro and IL-2 secretion in T-cells. We suspect that BMS-488516 may have an unidentified activity that contributes to its inhibition of T-cell proliferation, as well as its minor activity against LPS-induced TNFα release in PBMC. This unknown activity is not chemical cytotoxicity since both compounds were noncytotoxic to any of the cell types used in our studies as measured by a lactate dehydrogenase release assay (data not shown).

Itk Inhibitors Reduce TCR-Induced IL-2 Production in Vivo. To determine whether the compounds had activity in vivo, we analyzed their potential inhibitory activity on IL-2 production in mice following intravenous injection of anti-

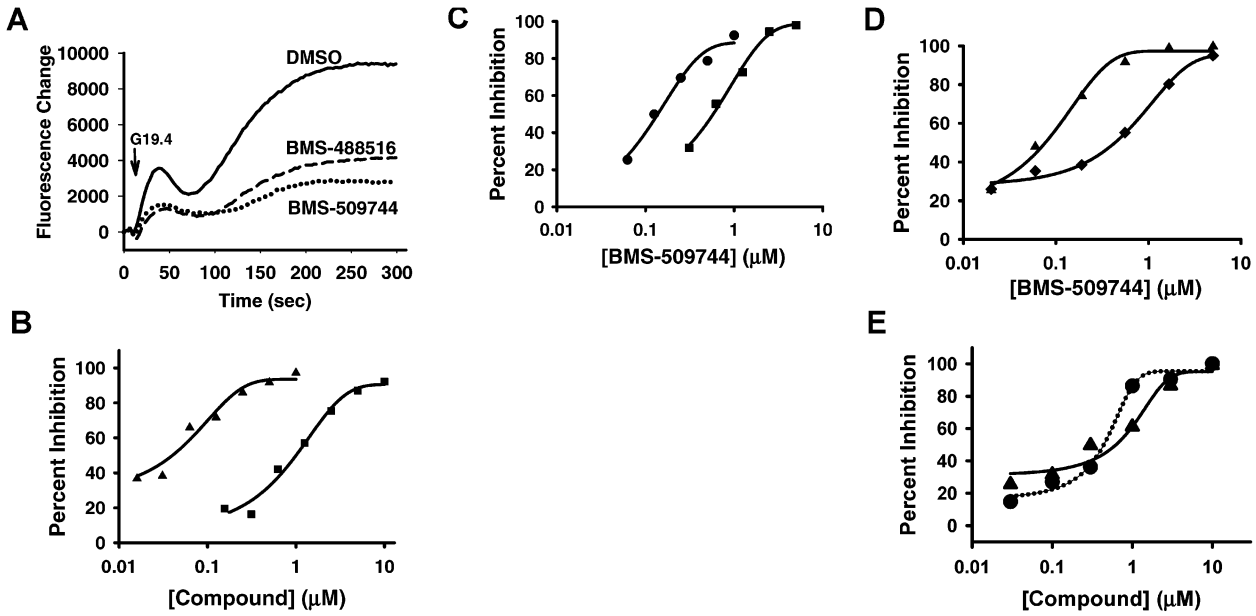

FIGURE 6: Compound effects on T-cell functions. (A) Inhibition of calcium mobilization by Itk compounds (1 μM). Anti-CD3 antibody (G19-4, 4 μg/mL) was added as indicated. Key: dotted line, BMS-488516; dashed line, BMS-509744; solid line, 0.1% DMSO. (B) Dose-dependent inhibition of calcium mobilization by BMS-509744 (▲) and BMS-488516 (■). The maximal fluorescence changes after the addition of G19-4 were used to calculate the percent inhibition of compounds. The IC_{50} values for BMS-509744 and BMS-488516 are 0.052 ± 0.018 and 1.31 ± 0.32 μM, respectively (mean $\pm$ SEM from three independent experiments). (C) Inhibition of IL-2 secretion in human T-cells. Jurkat T-cells (●) or human PBMCs (■) were stimulated with immobilized anti-CD3 antibody (G19-4) with or without various concentrations of BMS-509744 for 16 h before the IL-2 concentrations in the media were quantified. (D) Inhibition of IL-2 secretion in murine T-cells. EL4 cells (▲) or freshly dissociated mouse splenocytes (◆) were stimulated with immobilized anti-CD3 antibody (2C11) with or without various concentrations of BMS-509744 for 16 h, and the IL-2 levels in the media were analyzed by ELISA. (E) Inhibition of T-cell proliferation induced by the mixed lymphocyte reaction. The T-cells in the mixed lymphocyte reactions were exposed to BMS-488516 (●, dotted line) or BMS-509744 (▲, solid line) at the indicated concentrations for 3 days. The IC_{50} values for BMS-509744 and BMS-488516 are 0.43 ± 0.05 and 0.35 ± 0.08 μM, respectively (mean $\pm$ range from two independent experiments).

Table 2: Inhibition of IL-2 Secretion by BMS-509744 and BMS-488516[a]

	human		murine	
	Jurkat	PBMC	EL4	splenocyte
BMS-509744	0.25 ± 0.11	0.39 ± 0.16	0.072 ± 0.044	0.38 ± 0.18
BMS-488516	0.70 ± 0.18	0.65 ± 0.14	0.25 ± 0.07	1.36 ± 0.40

[a] The data are IC_{50} values in μM and are the means $\pm$ SEM from three independent experiments.

CD3 antibody. BMS-488516 dose-dependently suppressed IL-2 production detected in serum after anti-CD3 treatment (Figure 7A). Significant inhibition was observed at doses $\geq$50 mg/kg when administered subcutaneously, and the extent of IL-2 suppression correlated with increased serum drug levels (Figure 7A). BMS-509744 also exhibited a 50% inhibitory capacity when dosed at 50 mg/kg, irrespective of the amount of induction antibody (Figure 7B).

Itk Compound Activity in a Murine Lung Inflammation Model. Allergic asthma is caused by aberrant immune responses in lung tissue mediated through T-helper type 2 (Th2) cells and their associated cytokine signaling pathways (*15*). Itk is an attractive target for modulating allergic asthma since mice lacking Itk are unable to develop Th2 cells (*8*). Attenuation of immunological symptoms of allergic asthma was also observed in mice lacking Itk (*16*). We evaluated BMS-509744 in a murine allergic asthma model (*11*). BMS-509744 dose-dependently reduced lung inflammation in these mice as a function of a reduction in both total cell and eosinophil infiltration into the lung (Figure 7C). The reduction reached statistical significance at doses of 25 mg/kg administered subcutaneously, twice daily for 3 days, starting at the time of the ovalbumin inhalation challenge.

Our discovery provides the first example of highly selective and potent Itk inhibitors identified from medicinal chemistry efforts by following a screening lead. The data reveal that Itk is a major enzyme responsible for PLCγ1 activation in human T-cells and that selective inhibition of Itk leads to blockade of PLCγ1 and its downstream signals in T-cells. Similar signaling functions in other cell types were unaffected by the inhibitors. The validation of Itk as a drug target for immune system diseases is supported through previous gene ablation studies (*6*, *7*, *16*) and is extended by the generation of specific small molecule inhibitors and their analysis in animal models of disease. The potency of the current inhibitors in vivo may in part reflect absorption, distribution, metabolism, and excretion parameters associated with these molecules. However, our studies in murine models demonstrated novel preclinical evidence that blocking T-cell activation with a selective Itk kinase inhibitor may provide a viable approach for treating immunological and inflammatory disorders. These inhibitors will have wide utility in dissecting the pathway(s) that link(s) Itk to up- and downstream components during T-cell activation. Other modes of targeting Itk, including inhibition of its protein−protein interactions or using siRNA also may have value for altering T-cell activation (*17*). Additional applications for Itk inhibitors could include treating T-cell-associated hematopoietic malignancies (*18*) and PTEN-deficient T-cell lymphoma (*14*). In PTEN-deficient T-cells, Itk is constitutively localized to the plasma membrane through its PH domain, leading to

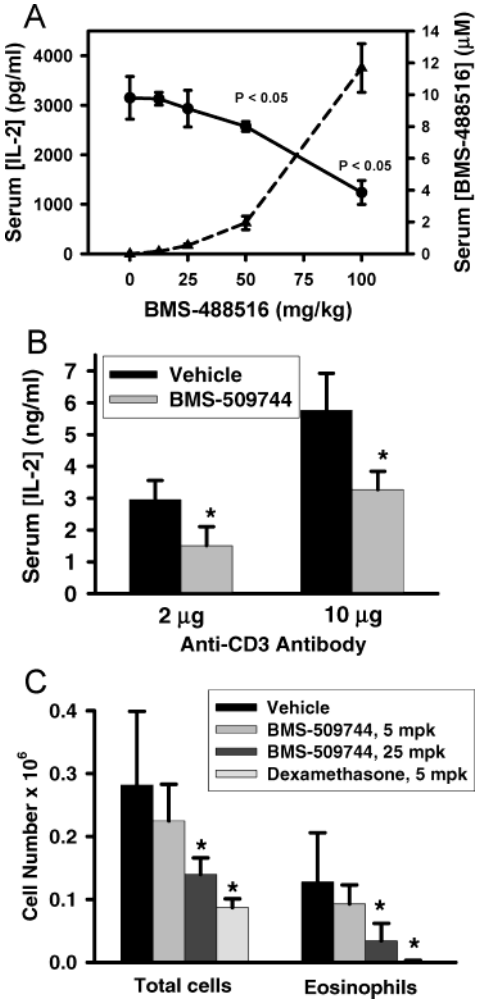

FIGURE 7: In vivo activities of BMS-488516 and BMS-509744 in murine models. (A) Dose-dependent inhibition of anti-CD3 antibody-induced serum IL-2 production (●, solid line) by BMS-488516. Data are the mean ± SD from eight animals. The right axis and the data in solid triangles (▲, dash line) represent the serum compound concentrations. (B) Suppression of anti-CD3 antibody-induced serum IL-2 production by 50 mg/kg BMS-509744. In a separate experiment, the serum BMS-509744 level was determined to be 1.89 ± 0.34 μM (n = 5). (C) Reduction of leukocyte infiltration to the lung induced by ovalbumin. The compound was dosed subcutaneously in this experiment, and similar results were obtained from a separate experiment where the compound was dosed intraperitoneally. Data are the mean ± SD from five animals. Asterisk = $p < 0.05$ (Student's t-test).

TCR-induced hyperresponsiveness (*14*). Inhibition of activated Itk in such T-cells may be a directed approach for blockade of growth and malignancy.

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SUPPORTING INFORMATION AVAILABLE

Preparation procedures for BMS-509744, BMS-488516, and BMS-285047. This material is available free of charge via the Internet at http://pubs.acs.org.

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