Potent Inhibition of NFAT Activation and T Cell Cytokine Production by Novel Low Molecular Weight Pyrazole Compounds*

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NFAT (nuclear factor of activated T cell) proteins are expressed in most immune system cells and regulate the transcription of cytokine genes critical for the immune response. The activity of NFAT proteins is tightly regulated by the Ca\(^{2+}\)/calmodulin-dependent protein phosphatase 2B/calcineurin (CaN). Dephosphorylation of NFAT by CaN is required for NFAT nuclear localization. Current immunosuppressive drugs such as cyclosporin A and FK506 block CaN activity thus inhibiting nuclear translocation of NFAT and consequent cytokine gene transcription. The inhibition of CaN in cells outside of the immune system may contribute to the toxicities associated with cyclosporin A therapy. In a search for safer immunosuppressive drugs, we identified a series of 3,5-bistrifluoromethyl pyrazole (BTP) derivatives that block Th1 and Th2 cytokine gene transcription. The BTP compounds block the activation-dependent nuclear localization of NFAT as determined by electrophoretic mobility shift assays. Confocal microscopy of cells expressing fluorescent-tagged NFAT confirmed that the BTP compounds block calcium-induced movement of NFAT from the cytosol to the nucleus. Inhibition of NFAT was selective because the BTP compounds did not affect the activation of NF-κB and AP-1 transcription factors. Treatment of intact T cells with the BTP compounds prior to calcium ionophore-induced activation of CaN caused NFAT to remain in a highly phosphorylated state. However, the BTP compounds did not directly inhibit the dephosphorylation of NFAT by CaN in vitro, nor did the drugs block the dephosphorylation of other CaN substrates including the type II regulatory subunit of protein kinase A and the transcription factor Elk-1. The data suggest that the BTP compounds cause NFAT to be maintained in the cytosol in a phosphorylated state and block the nuclear import of NFAT and, hence, NFAT-dependent cytokine gene transcription by a mechanism other than direct inhibition of CaN phosphatase activity. The novel inhibitors described herein will be useful in better defining the cellular regulation of NFAT activation and may lead to identification of new therapeutic targets for the treatment of autoimmune disease and transplant rejection.

Engagement of the T cell antigen receptor (TcR)\(^{1}\) with the antigen-major histocompatibility complex on antigen-presenting cells triggers a complex TcR signaling cascade that leads to T cell activation and cytokine secretion (1). During this process, T cells express the autocrine growth factor interleukin 2 (IL-2), which promotes T cell proliferation by interacting with the IL-2 receptor, which is also up-regulated on activated T cells. The transcriptional regulation of the IL-2 gene has been extensively analyzed at the IL-2 promoter, a 275-bp region located upstream of the transcriptional start site of the gene (2, 3). Several transcription factors have been identified to bind elements within this regulatory region, including AP-1, NF-κB, and the nuclear factor of activated T cells (NFAT) (2).

The transcription factor NFAT plays an essential role in IL-2 expression. Binding sites for NFATs have also been found within the promoter regions of several other cytokine genes, including IL-3, IL-4, IL-5, IL-8, IL-13, tumor necrosis factor α, granulocyte-macrophage colony-stimulating factor, and γ-IFN (4, 5). NFAT is a complex composed of a cytoplasmic subunit and an inducible nuclear component comprised of AP-1 (Fos/Jun) family members. At least four structurally related NFAT cytoplasmic subunit members, NFATp/NFAT1, NFATc/NFAT2, NFAT3, and NFATX/NFATc3/NFAT4, have been identified (5). NFAT proteins share a conserved domain located toward the C terminus (6) that binds DNA and also participates in cooperative protein-protein interactions with AP-1 transcription factors (7, 8). Immediately N-terminal to the DNA-binding domain is a second conserved module of amino acids in the form of an NFAT-h domain (9). The N terminus of NFAT, including the NFAT-h region, regulates nuclear/cyttoplasmic trafficking in response to changes in intracellular Ca\(^{2+}\) concentrations. In resting T cells, the protein is retained in the cytoplasm and its NFAT-h domain is heavily phosphorylated. Engagement of the TcR or treatment of cells with the Ca\(^{2+}\) ionophore activates the Ca\(^{2+}\)/calmodulin-dependent Ser/Thr phosphatase, calcineurin. CaN dephosphorylates the NFAT-h domain, resulting in translocation of NFAT to the nucleus (9).

The clinically important immunosuppressive drugs, cyclosporin A and FK506, act by binding to their respective immunophilins, cyclophilin and FKBP12 (9). The immunophilin-drug

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complex binds to CaN and inhibits CaN phosphatase activity, thus preventing the dephosphorylation and translocation of NFAT to the nucleus (10–12). As a substrate of CaN, NFAT is a secondary target of the action of these immunosuppressive drugs. NFAT inhibition is believed to account, at least in part, for the transcriptional inhibitory activity of these immunosuppressants.

Both CsA and FK-506 have been shown to be effective in preventing organ graft rejection in the clinic (13). In addition, CsA has been shown to be beneficial in reducing joint erosion and disease progression in rheumatoid arthritis patients (14, 15). However, side effects observed with the clinical use of both of these compounds, notably nephrotoxicity, neurotoxicity, diabetogenicity, and gastrointestinal toxicity, have markedly reduced their impact (16). These side effects are likely to be caused by the pleiotropic metabolic effects these agents exert through binding to immunophilins and inhibiting CaN (or immunophilin peptidyl-prolyl isomerase activity) in cells outside the immune system (17–19).

The identification of NFAT as a molecular target in T cell activation suggests a more direct, molecular based approach to the development of immunosuppressive agents with the potential for improved efficacy and reduced side effects. One of the approaches toward the identification of novel immunotherapeutics is to identify low molecular weight entities that selectively target the NFAT transcription factor without inhibiting the Ca²⁺-dependent phosphatase, CaN. In this communication we describe the identification and molecular characterization of a novel series of NFAT regulators that exert their biological effects via a mechanism that does not involve inhibition of the Ca²⁺-dependent phosphatase, CaN.

**EXPERIMENTAL PROCEDURES**

**IL-2 Promoter-Luciferase Gene Construct—**The human IL-2 promoter containing the DNA sequence from −325 to +24 (20) was generated by polymerase chain reaction from human genomic DNA (Promega, Madison, WI) and subcloned into pT7-Blue (Novagen, Madison, WI). DNA was isolated from individual colonies. Clones expressing the correct DNA sequence were identified, and the IL-2 promoter fragment was purified HindIII/HindIII fragment and digested to RgI/HindIII-digested pRC/RSV (Invitrogen). This ligation replaced the RSV promoter of pRC/RSV with the IL-2 promoter to generate pRC/IL-2. The firefly luciferase gene plus an SV40 intron were isolated from pGL2-Basic (Promega) by digestion with HindIII and HpaI. The pRC/IL-2 was digested with XhoI. The cohesin ends were made blunt with fill-in with polymerase I Klenow fragment and then digested with HindIII. The purified HindIII/HpaI luciferase fragment was ligated to the blunt XhoI/HindIII pRC/IL-2 to generate pIL-2-Luc. For permanent cell lines, Jurkat E6.1 cells (American Type Culture Collection, Manassas, VA) were transfected with linearized pIL-2-Luc plus linearized pME4A, using electroporation as previously described (21). Clones were selected for resistance to 400 μg/ml hygromycin, and a stably transfected cell line, Jurkat E10,E2 (abbreviated Jurkat E2), was created.

**IL-2 Reporter Expression in Jurkat E2 Cells—**Jurkat E2 cells were routinely cultured in RPMI 1640 plus 10% fetal bovine serum containing 400 μg/ml hygromycin and 500 μg/ml geneticin. Prior to assay, Jurkat E2 cells were resuspended at 4 × 10⁵ cells/ml in RPMI 1640 plus 5% fetal bovine serum (lacking antibiotics) and stimulated with 10 ng/ml PMA and 2 μM ionomycin and placed in 96-well microtiter plates in a final volume of 200 μl/well. The cells were incubated for 18 h at 37°C (5% CO₂) in a humidified incubator and pelleted by centrifugation at 400 × g over Histopaque-1077 (Sigma). Responder cells from two individuals were washed three times in RPMI 1640 medium at 400 × g for 10 min. The remaining PBMCs (stimulator cells) were treated with 25 μg/ml mitomycin C (Sigma) for 30 min at 37°C (5% CO₂, 100% humidity) and washed three times in RPMI 1640 medium. Cultures were incubated in RPMI 1640 medium consisting of RPMI 1640 (Gibco, Grand Island, NY) supplemented with 4 μM t-glutamine (Sigma), 50 μM 2-mercaptoethanol, and 10% fetal bovine serum (Hyclone, Logan, UT). Cultures were incubated at 37°C (5% CO₂, 100% humidity) for 4 days. On day 4, 0.5 μCi of [³H]thymidine (PerkinElmer Life Sciences) was added to each well during the last 6 h of culture. Cultures were harvested onto glass-fiber filter mats using a 96-well harvester (Tomtec, Hamden, CT). [³H]Thymidine uptake was measured by direct β-counting using a Matrix 9600 β-counter (Packard Instrument Co., Downers Grove, IL).

**Concanavalin A Proliferation Assay—**Test compounds were added to appropriate wells on 96-well tissue culture plates (Corning Glass Works) in 20 μl of supplemented RPMI 1640. Human peripheral blood mononuclear cells were added to each well in 5-μl volumes (final cell concentration equal to 5 × 10⁵ cells/well). After 15 min, 100 μl of 5 μg/ml concanavalin A (Sigma) in supplemented RPMI 1640 was added to each well to a final concentration of 2.5 μg/ml. Plates were incubated for 3 days at 37°C with 5% CO₂. On day 3, plates were pulsed with 0.5 μCi/well tritiated thymidine (PerkinElmer Life Sciences). After 6 h, plates were harvested on a Tomtec 96-well harvester. Glass filter mats were counted on a Matrix 9600 direct β counter (Packard Instrument Co.).

**Electrophoretic Mobility Shift Assay—**Jurkat E2 cells were used for nuclear extract preparation per experimental point. The cells were resuspended for 5 min at 37°C in 20 μg/ml of proteinase K, then treated with 100 μg/ml of RNase A and 10 μg/ml of proteinase K. Cells were treated with 0.5% Triton X-100, then re-suspended in 50 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, 2 μg/ml of proteinase K, 20 μg/ml of cyclic AMP, 50 μM ATP, and washed with the cell lysis, and chemiluminescence was measured with a luminometer.

**Determination of IL-2 Secretion from Normal Human Peripheral Blood Mononuclear Cells—**Normal human peripheral blood mononuclear cells (PBMC) were isolated by standard procedure. Briefly, 50 ml of peripheral blood was obtained from normal donors on the morning of each assay. The heparinized blood was mixed with an equal volume of phosphate-buffered saline, and peripheral blood mononuclear cells were isolated by density centrifugation at 400 × g over Histopaque-1077 (Sigma). PBMCs (1 × 10⁶ cells/ml) were treated with 25 μg/ml mitomycin C (Sigma) at 37°C with anti-CD3 monoclonal antibody containing 0.05% Tween 20 (wash buffer) and blocked with Dulbecco’s phosphate-buffered saline containing 0.95% Tween 20 (wash buffer) and blocked with Dulbecco’s phosphate-buffered saline containing 1% bovine serum albumin and 10 mM NaN₃ (diluent-blocking buffer) for 1–3 h at room temperature or overnight. Plates were washed four times with Dulbecco’s phosphate-buffered saline containing 0.95% Tween 20 (wash buffer) and blocked with Dulbecco’s phosphate-buffered saline containing 1% bovine serum albumin and 10 mM NaN₃ (diluent-blocking buffer). Tissue culture supernatant at various dilutions was added at 100 μl/well in triplicate. Plates were incubated for 2 h at room temperature and washed four times with wash buffer. 100 μl of rabbit anti-human IL-2 (10 μg/ml, Genzyme, Cambridge, MA) were added and incubated at 4 °C for 1 h at room temperature, followed by four washes and the subsequent addition of 100 μl of 1:2000 dilution of alkaline phosphatase-conjugated goat anti-rabbit Fab(α)₂ (Biosource International). After 1 h the plates were washed four times and 100 μl of para nitrophenyl phosphate (Southern Biotech or Sigma) at 1 mg/ml in buffer was added. Color development was allowed to proceed at room temperature for 20 min before the addition of 50 μl of 2N NaOH. Absorbance at 405 nm was determined using a plate reader (Molecular Devices, Sunnyvale, CA). IL-2 concentrations were calculated using SoftMax (Molecular Devices) based on the IL-2 standard solutions.

**Human Mixed Leukocyte Response Assay—**50 ml of peripheral blood was obtained from four normal, unrelated donors on the morning of each assay. The heparinized blood was mixed with an equal volume of phosphate-buffered saline, and PBMCs were isolated by density centrifugation at 400 × g over Histopaque-1077 (Sigma). Responder cells from two individuals were washed three times in RPMI 1640 medium at 400 × g for 10 min. The remaining PBMCs (stimulator cells) were treated with 25 μg/ml mitomycin C (Sigma) for 30 min at 37°C (5% CO₂, 100% humidity) and washed three times in RPMI 1640 medium. Cultures were incubated in RPMI 1640 medium consisting of RPMI 1640 (Gibco, Grand Island, NY) supplemented with 4 μM t-glutamine (Sigma), 50 μM 2-mercaptoethanol, and 10% fetal bovine serum (Hyclone, Logan, UT). Cultures were incubated at 37°C (5% CO₂, 100% humidity) for 4 days. On day 4, 0.5 μCi of [³H]thymidine (PerkinElmer Life Sciences) was added to each well during the last 6 h of culture. Cultures were harvested onto glass-fiber filter mats using a 96-well harvester (Tomtec, Hamden, CT). [³H]Thymidine uptake was measured by direct β-counting using a Matrix 9600 β-counter (Packard Instrument Co.).
ttin A) and resuspended in 1 ml of buffer B (buffer A plus 0.05% Nonidet P-40). The nuclei pellet was resuspended in 315 μl of buffer C (50 mM HEPES pH 7.8, 50 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 10% glycerol), 35 μl of 3 M ammonium sulfate was added and mixed for 30 min in the cold room. The precipitated nuclei were centrifuged in the assay, dissolved in 1 M ammonium sulfate with of the supernatant was transferred to a new tube, and an equal volume of 3 M ammonium sulfate was added, mixed, and incubated on ice for 30 min. The precipitated nuclei extract was pelleted at 100,000 × g for 10 min and resuspended in 50 μl of buffer C. The nuclear proteins were desalted by passage over a Bio-Rad PDSG column. Protein concentrations were determined by Bradford assay (22). The N-terminal domain of GFP was carried out with 10 μg of nuclear extract in a solution consisting of 10 mM Tris-HCl (pH 7.5), 0.5 mM EDTA, 50 mM NaCl, 5% glycerol, and 2 mg of poly(dI-dC). The protein solutions were incubated at room temperature for 45 min with 0.2-0.5 μg of end-labeled double-stranded oligonucleotides. Oligonucleotides used in these assays included IL-2 oligonucleotides. Oligonucleotides used in these assays include IL-2 NFAT1-(1-415), TTCCAAAGAGTCATCAG; NFAT1-(1-328) fusion peptide in CHO cells was monitored by time-lapse microscopy using a Bio-Rad MRC 1000 UV laser scanning confocal microscope and a ×10 objective lens. The cells, grown in coverslip chambers, were placed onto a temperature-controlled stage and maintained at 37 °C throughout the experiment. GFP fluorescence was excited using the 488 nm line of a krypton/argon laser and visualized using a 522 nm bandpass filter. The microscope software was set to automatically acquire images at intervals of 30 s to 3 min. We examined the ability of drug compounds to inhibit ionomycin-stimulated translocation of GFP-NFAT1-(1-415) into the nucleus. The cells were incubated with drug for 20-45 min prior to adding ionomycin. Time-lapse imaging was begun immediately after adding ionomycin, and images were acquired every 30 s for 20 min. We also investigated whether the drugs could reverse ionomycin-induced nuclear translocation of GFP-NFAT1-(1-415). Ionomycin was added first, and cells were imaged every 2 min for 20 min to follow nuclear export of GFP-NFAT1-(1-415). BTPs, CsA, or vehicle was then added, and images were taken every 3 min for 90 min to follow the rate of nuclear export of GFP-NFAT1-(1-415). BTP Compounds Block the Nuclear Localization of NFAT—In CHO Cells—NFAT1-(1-415) was cloned into the pEH6 vector, and the construct was used to stably transfect CHO cells. 5 × 10⁶ test cells were washed and treated with either drug or vehicle control for 30 min at 37 °C. The cells were stimulated for 30 min at 37 °C with 1 μg/ml ionomycin. After stimulation the cells were washed one time with cold PBS and resuspended in 2 ml of radioimmune precipitation buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 5 mM MgCl₂, 0.5% SDS, 1% Triton X-100, 1% deoxycholate, 5 μM AEBSF, 100 mM aprotinin, 25 mM leupeptin, 10 mM iodoacetamide, 50 mM NaF, 1 mM Na₃VO₄, and 30 mM sodium pyrophosphate). The cell suspension was thawed once and the lysate detergent four times in sample buffer. Proteins were resolved by 10% SDS-PAGE and NFAT1-(1-415) was detected by Western blotting with monoclonal anti-NFATc1 antibody (Affinity BioReagents, Golden, CO).

Elk-1 Dephosphorylation Assay—Starved COS cells were washed twice with ice-cold phosphate-buffered saline and lysed by scraping in buffer A (50 mM HEPES pH 7.5, 150 mM NaCl, 8 mM β-mercaptoethanol, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfon fluoride) supplemented with 1% Triton X-100. The cell lysates were clarified by centrifugation at 12,000 × g for 20 min at 4 °C, and the supernatants were used for in vitro dephosphorylation assays. In vitro phosphatase assays were performed using phosphorylated GST-Elk-1c as a substrate. “32P-Labeled GST-Elk-1c was prepared by incubating with active MEK1 and ERK1 in kinase buffer (18 μM HEPES, pH 7.5, 10 mM magnesium acetate, and 50 μM ATP) for 30 min at 30 °C in the presence of “32P]ATP. Phosphorylated GST-Elk-1c was purified with glutathione-agarose (Sigma) and eluted in buffer (10 mM Tris-Cl, pH 7.5) containing 1 M ammonium sulfate. Phosphorylated GST-Elk-1c (1 μg) was incubated with the indicated amount of cell lysate in a total volume of 30 μl of buffer A containing various phosphatase inhibitors as indicated. The reactions were allowed to proceed for 30 min at 30 °C and then stopped by adding SDS-polyacrylamide gel electrophoresis sample buffer and separated by SDS-PAGE. Phosphorylated Elk-1c was detected by autoradiography.
shift assay. The NFAT inhibitory effect of BTP1 (1 μM) was similar to the inhibition observed with CsA (1 μM) or FK506 (1 nM) (Fig. 5). The BTP compounds did not affect the ability of NFAT to bind directly to its IL-2 enhancer element when added directly to the NFAT/oligonucleotide binding reaction in vitro (data not shown). The BTP compounds showed selective inhibition of NFAT. For example, they did not affect the activation, nuclear localization, or binding of NF-κB or AP-1 to their respective IL-2 enhancer elements (Fig. 5). Binding sites for NFATs have also been found within the promoter regions of several other cytokine genes, including IL-4, IL-5, and γ-IFN (4, 5). Consistent with their ability to inhibit NFAT activation, the BTP compounds inhibited IL-4 and IL-5 secretion from activated Hut 78 cells and γ-IFN from stimulated peripheral blood T cells with ED50 values of 100–300 nM.2

The ability of BTP1 to block the nuclear localization of NFAT was further investigated in CHO cells stably transfected with green fluorescent protein fused with the N-terminal regulatory domain (residues 1–415) of NFAT1 (GFP-NFAT1(1–415)). As shown in Fig. 6 (top left panel), GFP-NFAT-transfected CHO cells exhibit diffuse cytoplasmic fluorescence and the absence of nuclear fluorescence. Following 20 min of stimulation with 1 μM ionomycin, the fluorescent GFP-NFAT translocates to the nucleus (Fig. 6, top right panel). However, in the presence of 5 μM BTP1, the ionomycin-induced movement of GFP-NFAT from the cytoplasm to the nucleus is completely blocked (Fig. 6, lower right panel). A similar inhibition of GFP-NFAT nuclear localization is observed with 1 μM CsA (Fig. 6, middle right panel). The nuclear localization of NFAT requires the sustained activation of CaN. As Ca2+ levels drop or CaN is inhibited with the immunosuppressive drugs FK506 or CsA, NFAT is rapidly rephosphorylated and exported from the nucleus (26). In CHO cells pretreated with ionomycin to cause nuclear accumulation of GFP-NFAT, addition of BTP compounds produced a more rapid export of GFP-NFAT from the nucleus (data not shown) compared with untreated cells.

BTP Compounds Inhibit the Calcium-induced Dephosphorylation of NFAT in Cells—The movement of NFAT from the cytosol to the nucleus is dependent upon the dephosphorylation

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2 Y.-W. Chen, S. W. Djuric, and J. M. Trevillyan, manuscript in preparation.
of the N-terminal regulatory domain of NFAT by calcium- and CaN-dependent mechanisms as NFAT activation is elicited by ionomycin and blocked by the immunosuppressive drugs CsA and FK506 (27, 28). Consequently, we determined whether the BTP-dependent block in NFAT nuclear translocation was accompanied by inhibition of NFAT dephosphorylation.

**FIG. 3.** BTPs inhibit CD3/CD28-induced IL-2 secretion from peripheral blood mononuclear cells. Peripheral blood mononuclear cells were stimulated with CD3 and CD28 monoclonal antibodies for 24 h as described under “Experimental Procedures.” Some cultures were stimulated in the presence of increasing concentrations of FK506 or BTPs 1, 2, and 3. 24-h culture supernatants were collected and IL-2 levels determined by ELISA as described under “Experimental Procedures.” Values are mean ± S.D. (n = 3).

**FIG. 4.** BTPs inhibit mitogen- and alloantigen-stimulated T cell proliferation. Human peripheral blood mononuclear cells were isolated and stimulated with concanavalin A (A) or by mixed lymphocyte reaction (B) as described under “Experimental Procedures.” Some cultures were stimulated in the presence of increasing concentrations of CsA, BTP1, BTP2, or BTP3. Cell proliferation was measured by [3H]thymidine uptake as described under “Experimental Procedures.” Values are mean ± S.D. (n = 8).

Novel Inhibitor of Cytokine Gene Transcription
ination of NFAT1 as determined by retention of the more slowly migrating species of NFAT1 (Fig. 7). The effect of BTPs on the NFAT phosphorylation state was similar to that observed with CsA, a known inhibitor of the NFAT phosphatase, CaN (Fig. 7).

BTP Compounds Fail to Inhibit CaN Activity in Vitro

The ability of BTPs to block NFAT dephosphorylation in cells caused us to investigate whether BTPs are capable of directly inhibiting the catalytic activity of CaN. Calcium/calmodulin-dependent CaN activity was determined by measuring the dephosphorylation of a $^{32}$P-radiolabeled protein kinase RII subunit peptide (DLDVPIPGRFDRRVSVAAE; Asp-X$_{17}$-Glu) by purified bovine brain CaN, as described under Experimental Procedures. For assay validation, the immunophilin, FKBP-12, was included in the assay to allow inhibition of CaN by FK506. As shown in Fig. 8, FK506 inhibited the dephosphorylation of Asp-X$_{17}$-Glu in a dose-dependent manner with an IC$_{50}$ of 10 nM. BTP1 and BTP2 failed to inhibit CaN activity in the same assay at concentrations as high as 100 $\mu$M, 100–300-fold above the concentration needed for BTP-dependent inhibition of cellular NFAT activation and IL-2 secretion (see Figs. 3 and 5). These data support the notion that BTPs do not directly inhibit CaN activity.

BTP Compounds Fail to Inhibit CaN Activity in Cell Lysates—CsA and FK506 inhibit CaN activity via association with their respective immunophilins (9). Consequently, although the BTPs were incapable of directly inhibiting the catalytic activity of CaN, CaN/calmodulin-dependent CaN activity was determined by measuring the dephosphorylation of a $^{32}$P-radiolabeled protein kinase RII subunit peptide (DLDVPIPGRFDRRVSVAAE; Asp-X$_{17}$-Glu) by purified bovine brain CaN, as described under Experimental Procedures. For assay validation, the immunophilin, FKBP-12, was included in the assay to allow inhibition of CaN by FK506. As shown in Fig. 8, FK506 inhibited the dephosphorylation of Asp-X$_{17}$-Glu in a dose-dependent manner with an IC$_{50}$ of 10 nM. BTP1 and BTP2 failed to inhibit CaN activity in the same assay at concentrations as high as 100 $\mu$M, 100–300-fold above the concentration needed for BTP-dependent inhibition of cellular NFAT activation and IL-2 secretion (see Figs. 3 and 5). These data support the notion that BTPs do not directly inhibit CaN activity.

BTP Compounds Fail to Inhibit CaN Activity in Cell Lysates—CaA and FK506 inhibit CaN activity via association with their respective immunophilins (9). Consequently, although the BTPs were incapable of directly inhibiting CaN activity (Fig. 8), we further tested the ability of the BTPs to inhibit CaN activity in cell lysates to determine whether additional cellular factors might facilitate BTP-dependent inhibition of CaN. For these studies an Elk-1 C-terminal peptide (Elk-1c; amino acid residues 307–428) was used as substrate. The transcription factor Elk-1 is a component of ternary complex factor and regulates gene expression in response to a variety of extracellular stimuli. Phosphorylation of the C-terminal domain of Elk-1 is mediated by extracellular signal-regulated and stress-activated protein kinases. Phosphorylated Elk-1 has been demonstrated to be a physiologic substrate of

![Fig. 5. BTP compounds block the nuclear localization of NFAT.](image)

Jurkat cells were stimulated with PMA (20 ng/ml) and ionomycin (1 $\mu$g/ml) for 3 h at 37°C. Some cultures were stimulated in the presence of 1 $\mu$m CsA, 1 $\mu$m FK506, 1 $\mu$m BTP1, or 1 $\mu$m BTP2. Following the 3-h incubation, nuclei were isolated and nuclear proteins extracted as described under “Experimental Procedures.” The nuclear protein extracts were incubated with $^{32}$P-radiolabeled oligonucleotides corresponding to binding elements for NFAT, AP-1, and NF-kB transcription factors (see “Experimental Procedures”). The protein-oligonucleotide complexes were separated from free oligonucleotide by gel electrophoresis and detected by autoradiography.

![Fig. 6. BTP compounds block ionomycin-induced nuclear translocation of NFAT1 in CHO cells.](image)

CHO cells were stably transfected with GFP-NFAT1-(1–415). Cells were grown in coverslip chambers, placed onto a temperature-controlled stage, and maintained at 37°C. GFP fluorescence was excited using the 488 nm line of a krypton/argon laser and visualized using a 522 nm bandpass filter. The cells were incubated with 1 $\mu$m CsA or 5 $\mu$m BTP1 for 20 min prior to addition of 1 $\mu$m ionomycin. Time-lapse imaging was begun immediately after adding ionomycin, and images were acquired every 30 s for 20 min. Images shown represent the 20-min time point following ionomycin addition.
CaN, which opposes the activation of Elk-1 (30). As shown in Fig. 9, A, phosphorylated Elk-1 (pElk-1) is dephosphorylated (as measured by a decrease in $^{32}$P content and an increase in electrophoretic mobility) when incubated with COS-7 cell lysate. The dephosphorylation of pElk-1 is inhibited in a dose-dependent fashion by the addition of CsA or EGTA to the cell lysate. The dephosphorylation of a $^{32}$P-radiolabeled peptide corresponding to a sequence in the R II subunit of cAMP-dependent kinase (DLD-H9262) was initiated by the addition of 10 nM purified bovine brain CaN in the presence of 100 nM purified bovine brain calmodulin, 100 $\mu$M CaCl$_2$, and 1 $\mu$M recombinant human FKBP12 as described under "Experimental Procedures." The reaction was initiated by the addition of 10 $\mu$M of 25 $^{32}$P-labeled Asp-X$_2$-Glu and proceeded for 45 min at 30 °C. The reaction was stopped using 0.5 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 5% trichloroacetic acid. Free radiolabeled inorganic phosphate was isolated by Dowex cation-exchange chromatography and quantified by liquid scintillation counting. Data represent the average of duplicates that varied by <10%.

**DISCUSSION**

The 3,5-bistrifluoromethyl pyrazole compounds were identified based on their ability to block IL-2 gene transcription. IL-2 gene transcription is dependent upon the activation of several transcription factors, including NF-$\kappa$B, AP-1 complex (composed of Fos/Jun heterodimers), and NFAT. We established that the BTP compounds selectively block the activation-dependent nuclear localization of NFAT. The activity of NFAT proteins is tightly regulated by the calcium/calmodulin-dependent phosphatase, CaN. A conserved sequence motif (PX-LXXF) located at the N terminus of the NFAT regulatory domain contributes to CaN binding to NFAT, facilitating NFAT dephosphorylation (31). Dephosphorylation of NFAT by CaN is required for NFAT activation and nuclear localization. In this regard, treatment of intact T cells with the BTP compounds, followed by CaN activation, caused NFAT to remain in a highly phosphorylated state localized to the cytosol. However, the BTP compounds did not directly inhibit CaN phosphatase activity. These data, in total, demonstrate that the BTP compounds block NFAT dephosphorylation by a mechanism other than direct inhibition of CaN activity and thus represent novel immunosuppressive agents targeting NFAT.

**Fig. 7.** BTP compounds block ionomycin-induced dephosphorylation of NFAT1-(1–415) in CHO cells. CHO cells were stably transfected with pHM6/NFAT1-(1–415). Where indicated, cells were pretreated for 30 min with varying concentrations of BTP1, BTP3, or CsA. Cells were then stimulated for 30 min with 1 $\mu$g/ml ionomycin. Cell lysates were prepared, and NFAT1-(1–415) was detected by Western blotting as described under "Experimental Procedures." The more highly phosphorylated forms of NFAT have slower mobility in the gel and are indicated with an arrow as pNFAT1-(1–415). Dephosphorylated forms of NFAT have higher mobility and are indicated by an arrow as NFAT1-(1–415).

**Fig. 8.** BTPs do not directly inhibit CaN phosphatase activity. Calcium/calmodulin-dependent CaN activity was determined by measuring the dephosphorylation of a $^{32}$P-radiolabeled peptide corresponding to a sequence in the RII subunit of cAMP-dependent kinase (DLD-VPIPGFDRRVSVAEE, Asp-X$_2$-Glu). FK506, BTP1, or BTP2 was pre-incubated with 10 nM purified bovine brain CaN in the presence of 100 nM purified bovine brain calmodulin, 100 $\mu$M CaCl$_2$, and 1 $\mu$M recombinant human FKBP12 as described under "Experimental Procedures." The reaction was initiated by the addition of 10 $\mu$M of 25 $^{32}$P-labeled Asp-X$_2$-Glu and proceeded for 45 min at 30 °C. The reaction was stopped using 0.5 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 5% trichloroacetic acid. Free radiolabeled inorganic phosphate was isolated by Dowex cation-exchange chromatography and quantified by liquid scintillation counting. Data represent the average of duplicates that varied by <10%.
Novel Inhibitor of Cytokine Gene Transcription

Procedures.

Cell lysates containing various phosphatase inhibitors as indicated: 0.01, 0.1, and 10 μM BTP1, 30 μM BTP3, or CsA (1 μM, 10 μM) for 30 min. The cells were then washed and cell lysates were prepared as described under “Experimental Procedures.” GST-Elk was phosphorylated by ERK1 in the presence of [γ-32P]ATP. The extent of dephosphorylation of GST-Elk by COS lysates was determined by autoradiography. In some reactions, the CaN inhibitors CsA (5 μM) or EGTA (5 μM) were added directly to the lysates. B, GST-Elk was phosphorylated by ERK1 in the presence of [γ-32P]ATP. The phosphorylated GST-Elk was incubated with the indicated amount of cell lysates containing various phosphatase inhibitors as indicated: 0.01, 0.1, and 1 μM CsA; 5 mM EGTA, 30 μM BTP1, and 30 μM BTP3. The extent of dephosphorylation of GST-Elk by COS lysates was determined by autoradiography.

![Fig. 9. BTP compounds do not inhibit Elk-1 dephosphorylation by COS cell lysates. A, COS cells were first treated with 30 μM BTP1, 30 μM BTP3, or CsA (1 μM, 10 μM) for 30 min. The cells were then washed and cell lysates were prepared as described under “Experimental Procedures.” GST-Elk was phosphorylated by ERK1 in the presence of [γ-32P]ATP. The extent of dephosphorylation of GST-Elk by COS lysates was determined by autoradiography. In some reactions, the CaN inhibitors CsA (5 μM) or EGTA (5 μM) were added directly to the lysates. B, GST-Elk was phosphorylated by ERK1 in the presence of [γ-32P]ATP. The phosphorylated GST-Elk was incubated with the indicated amount of cell lysates containing various phosphatase inhibitors as indicated: 0.01, 0.1, and 1 μM CsA; 5 mM EGTA, 30 μM BTP1, and 30 μM BTP3. The extent of dephosphorylation of GST-Elk by COS lysates was determined by autoradiography.](image)

![Fig. 10. NFAT dephosphorylation by CaN in COS-7 cell lysates is not inhibited by BTP3. COS-7 cells were transiently transfected with pHM6/NFAT1-(1–415), and the expressed histidine-tagged NFAT1-(1–415) was purified by Ni2+ chelation chromatography. 30 μg NFAT1-(1–415) was added to 50 μg of COS-7 cell lysate in 50 μl of reaction buffer and incubated at 30 °C for 30 min in the absence (no inhibitor) or presence of CsA or BTP3 at the concentrations indicated. Reactions were stopped with 25 μl of NuPAGE sample buffer and reducing reagent, and 30 μl of each final mixture was subjected to 10% gel electrophoresis. NFAT1-(1–415) was detected by Western blotting as described under “Experimental Procedures.” The more highly phosphorylated forms of NFAT have slower mobility in the gel and are indicated with an arrow as pNFAT-(1–415). Dephosphorylated forms of NFAT have higher mobility and are indicated by an arrow as NFAT1-(1–415).](image)

inhibitors or inhibitors of NFAT/CaN interaction. The data suggest that the BTP compounds either enhance NFAT phosphorylation or inhibit NFAT dephosphorylation by a mechanism other than direct inhibition of CaN phosphatase activity.

Dephosphorylation of NFAT is necessary for subsequent import into the nucleus. In resting cells, NFAT1 is phosphorylated on at least 18 serine residues in the N-terminal regulatory domain (28). Thirteen of these are dephosphorylated upon activation with the calcium ionophore, ionomycin, resulting in the unmasking of a nuclear localization sequence within the regulatory domain and promoting nuclear import. This nuclear localization of NFAT requires the sustained activation of CaN. As Ca2+ levels drop or CaN is inhibited (e.g. with immunosuppressive drugs, CsA or FK506), NFAT is rapidly dephosphorylated (26, 33). The rephosphorylation of NFAT promotes the masking of the nuclear localization sequence and the exposure of a nuclear export sequence (28). In this regard, the addition of BTP compounds to ionomycin-treated CHO cells caused a more rapid export of GFP-NFAT from the nucleus compared with untreated cells (data not shown). The BTPs might promote the retention of NFAT in the cytosol by enhancing the activity (or blocking the inactivation) of an NFAT kinase. A number of different kinases have been implicated in the nuclear export of NFATs including glycogen synthase kinase (GSK) 3, casein kinase (CK) 1, MAP kinase kinase kinase (MEKK) 1, Jun N-terminal kinase (JNK) 2, and p38 mitogen-activated protein kinase (34–37). Our preliminary studies have failed to demonstrate an effect of BTPs on the activity of GSK3, JNK2, and p38 (data not shown). However, in cells, these kinases are themselves regulated by kinase cascades (involving kinases and phosphatases). It remains to be tested whether BTPs affect the activation pathways for these or other kinases (MEKK, CK1) implicated in NFAT phosphorylation.

The BTP compounds can be distinguished from CsA by their inability to inhibit CaN activity in vitro. However, in cells, the BTP compounds and CsA produced very similar inhibitory effects on NFAT activation and cytokine gene transcription. Under conditions of sustained increased intracellular free calcium (e.g. with ionomycin), both BTPs and CsA caused NFAT1 to localize to the cytosol in a hyperphosphorylated state and blocked a very similar profile of cytokine gene expression.2 Under these conditions it is unlikely that BTPs inhibit CaN by regulating intracellular free Ca2+ levels. The high intracellular Ca2+ levels induced by ionomycin would be predicted to bypass the cellular signaling events (i.e. TcR-induced protein kinase activation, inositol triphosphate formation, and activation of capacitative calcium channels) normally required for sustained calcium mobilization leading to cellular CaN activation (38). BTPs might indirectly affect the ability of CaN to dephosphorylate NFAT in cells. Recently, it has been reported that the peptideyl-prolyl cis-trans isomerase, Pin1, interacts specifically with the phosphorylated form of NFAT (39). The WW domain of Pin1 and the serine/proline-rich regulatory domain of NFAT mediate the NFAT-Pin1 interaction. The binding of Pin1 to
NFAT inhibits the dephosphorylation of NFAT by CaN in vitro, and overexpression of Pin1 in T cells blocks Ca\(^{2+}\)-dependent activation of NFAT in vivo (39). Consequently, BTPs might act by enhancing Pin1-NFAT binding and thus block CaN-dependent dephosphorylation of NFAT. Additional studies are needed to test this possibility.

NFAT requires extensive dephosphorylation at multiple serine residues for nuclear import and transcriptional activation (28). Thus, one might speculate that other phosphatase(s) in addition to CaN may participate in the regulation of NFAT dephosphorylation. If so, the BTP compounds might affect the NFAT phosphorylation state by regulating a non-CaN phosphatase. Further studies comparing and contrasting the sites of NFAT1 regulatory domain phosphorylation in the presence of CaS and BTPs will be useful in testing this possibility.

We have previously reported the chemical synthesis and optimization of the bistrifluoromethyl pyrazole series of cytokine inhibitors with respect to bioavailability, half-life, and efficacy to inhibit IL-2 synthesis in rodents and nonhuman primates (32). This report extends these findings by providing evidence that the BTP compounds work at the level of NFAT regulation. To our knowledge this is the first report of low molecular weight, cell-permeable molecules capable of targeting NFAT directly to inhibition of CaN in cells outside the immune system (17). Furthermore, the novel inhibitors described herein will be useful in better defining the cellular regulation of NFAT activation and may lead to identification of new therapeutic targets for the treatment of autoimmune disease and transplant rejection.

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