Expedited Articles

3,5-Bis(trifluoromethyl)pyrazoles: A Novel Class of NFAT Transcription Factor Regulator

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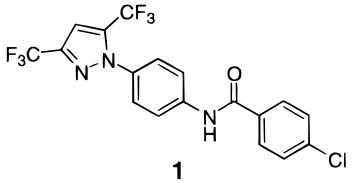
A series of bis(trifluoromethyl)pyrazoles (BTPs) has been found to be a novel inhibitor of cytokine production. Identified initially as inhibitors of IL-2 synthesis, the BTPs have been optimized in this regard and even inhibit IL-2 production with a 10-fold enhancement over cyclosporine in an ex vivo assay. Additionally, the BTPs show inhibition of IL-4, IL-5, IL-8, and eotaxin production. Unlike the IL-2 inhibitors, cyclosporine and FK506, the BTPs do not directly inhibit the dephosphorylation of NFAT by calcineurin.

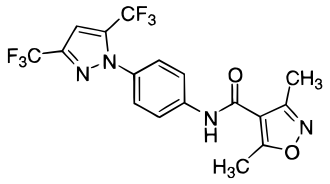
Introduction

The interaction of T-lymphocytes with antigens triggers a complex signaling cascade that switches on the gene program leading to T-cell activation. During this process, T-cells express the autocrine growth factor interleukin-2 (IL-2), which promotes cell proliferation by interacting with its receptor, also expressed by activated T-cells. The transcriptional regulation of the IL-2 gene has been extensively analyzed with the IL-2 promotor, a 275-bp region located upstream of the transcriptional start site of the gene. Cis-acting elements for several transcription factors have been identified within this regulatory region. The factors which bind to these motifs include AP-1, NF-κB, and nuclear factor of activated T-cells (NFAT).[1] The transcription factor NFAT plays an essential role in IL-2 expression. Binding sites for NFAT have also been found within the promotor regions of several cytokines, including IL-3, IL-4, and IL-5. NFAT is composed of a complex whose binding specificity is mediated by a cytosolic subunit and an inducible nuclear component comprised of AP-1 family members. The cytoplasmic subunit is encoded by a family of genes constituted by at least four structurally related NFAT members: NFATp/NFAT1, NFATc, NFAT3, and NFATX/NFAT4/NFATc3. These members translocate to the nucleus upon calcium mobilization during T-cell activation by a mechanism involving the calcium dependent dephosphorylation of the transcription factor by calcineurin. The immunosuppressive drugs cyclosporin A (CsA) and FK506 block the phosphatase activity of calcineurin, thus preventing the subsequent dephosphorylation and translocation of NFAT to the nucleus. Hence, NFAT can be considered a secondary target of the action of the immunosuppressive drugs, whose inhibition accounts, at least in part, for the transcriptional inhibitory activity of the immunosuppressants.[2] Both CsA and FK506 have been shown to be effective in preventing organ graft rejection in the clinic.[3] 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.[4] These side effects are likely to be caused by the pleotropic metabolic effects these agents exert through binding to immunophilins.[5]

Chart 1. High-Throughput Screening Leads

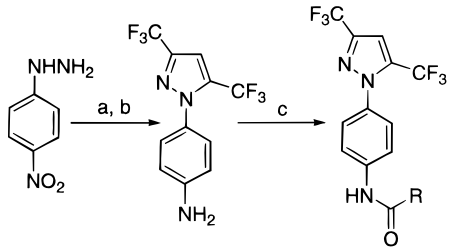


1



2

Scheme 1[a]



[a] (a) $CF_3C(O)CH_2C(O)CF_3$, HCl, EtOH, heat; (b) H_2, Pd−C, EtOAc; (c) RC(O)Cl, base, CH_2Cl_2 or RCO_2H, EDC, DMAP.

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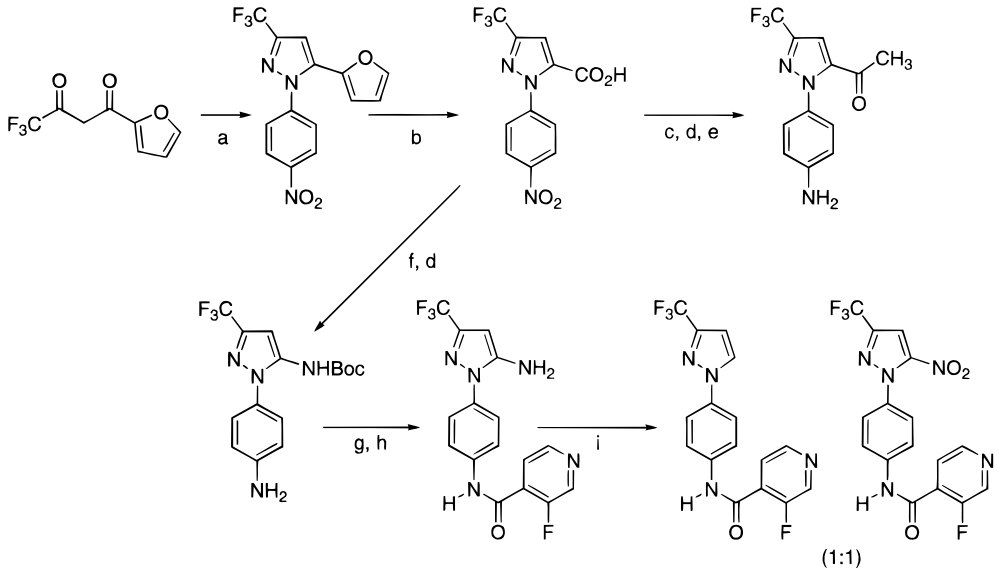
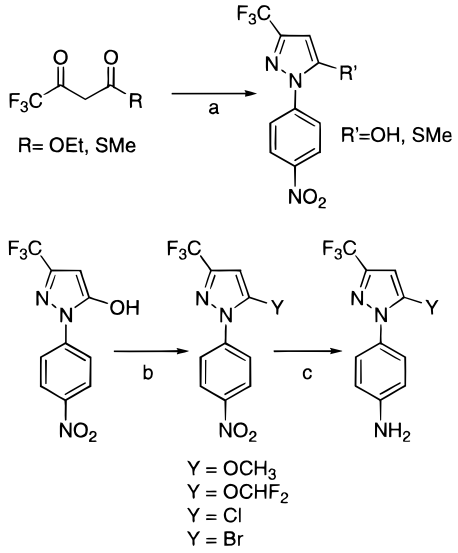
Scheme 2[a]



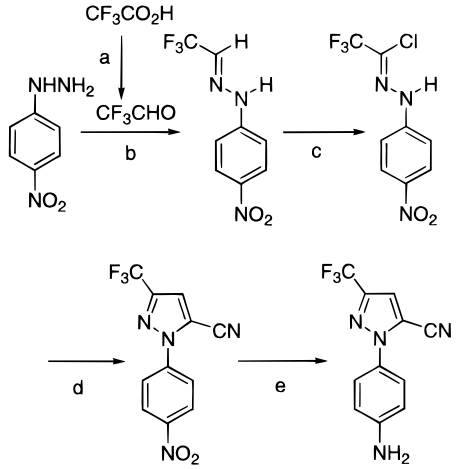
[a] (a) 4-Nitrophenylhydrazine, HCl, EtOH, heat; (b) $KMnO_4$, *t*-BuOH, 0.5 N NaOH; (c) $CH_3NH(OCH_3)$·HCl, HOBt, EDC, CH_2Cl_2; (d) Fe powder, NH_4Cl, EtOH, H_2O, heat; (e) CH_3Li, THF; (f) DPPA, Et_3N, *t*-BuOH, toluene, heat; (g) 3-fluoroisonicotinic acid, EDC, DMAP, CH_2Cl_2; (h) trifluoroacetic acid, CH_2Cl_2; (i) $NaNO_2$, $CuSO_4$, CH_3CN, H_2O.

Scheme 3[a]



[a] (a) 4-Nitrophenylhydrazine, HCl, EtOH, reflux; (b) for Y = OCH_3: $(CH_3O)_2SO_2$, K_2CO_3, CH_3CN, for Y = $OCHF_2$: CHF_2Cl, K_2CO_3, DMF, heat, for Y = Cl: $PhP(O)Cl_2$, heat, for Y = Br: $POBr_3$, heat; (c) Fe powder, NH_4Cl, EtOH, H_2O.

Scheme 4[a]



[a] (a) $LiAlH_4$, Et_2O; (b) HCl, EtOH, heat; (c) NCS, DMF; (d) 2-chloroacrylonitrile, Et_3N, toluene, heat; (e) Fe powder, NH_4Cl, EtOH, H_2O, heat.

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. In this article, we describe the identification and characterization of a novel series of NFAT regulators that exert their biological effects via a mechanism that does not involve inhibition of the Ca^{2+}-dependent phosphatase calcineurin.[6]

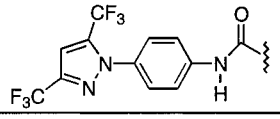
We initially identified the bis(trifluoromethyl)pyrazole (BTP) derivatives **1** and **2** (Chart 1) as leads from a high-throughput reporter gene-based screen for IL-2 synthesis inhibitors which featured PMA plus ionomycin stimulation of the Jurkat T-cell line transfected with the luciferase gene under the transcriptional control of a full-length IL-2 promotor.[7,8] Subsequent in vitro testing was done in a concanavalin A (Con A)-induced proliferation assay using human and rat peripheral blood mononuclear cells (PBMC proliferation, rat or human) followed by an assay measuring IL-2 synthesis inhibition in human whole blood stimulated by PMA/ionomycin (IL-2 synthesis in human blood). These data are shown in Tables 1 and 2.

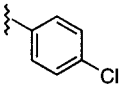
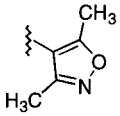
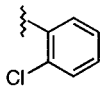
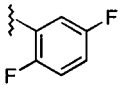
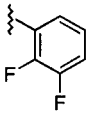
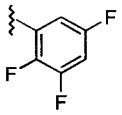
Chemistry

A 350-member focused amide library was prepared by solution-phase parallel synthesis (Scheme 1) rapidly providing analogues with optimized potency in the Con A-stimulated human PBMC proliferation assay (Table 1). Subsequent SAR studies allowed for optimization of the physicochemical and pharmacokinetic properties of further analogues. The profiles of these compounds are summarized in the biology section.

The general synthetic plan for this class of compounds centered upon preparation of pyrazolyl anilines which

Table 1. SAR for Terminal Amide Modifications



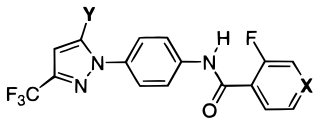
compound	substructure	IC$_{50}$ (nM)[a] PBMC proliferation[b] human	PBMC proliferation[b] rat	IL-2 synthesis in human blood[c]	human plasma protein binding (%)[d]
1		445 ± 44 (141)	9603 ± 6589 (8)	6032 ± 2412 (26)	99.9
2		340 ± 23 (8)	1266 ± 338 (7)	1978 ± 665 (6)	99.6
3		190 ± 32 (8)	687 ± 104 (9)	1735 ± 547 (6)	99.8
4		138 ±61 (5)		914 ± 3 (2)	99.9
5		154 ± 29 (8)	1279 ± 329 (5)	701 ± 180 (6)	99.7
6		84 ± 20 (4)		2269 ± 434 (4)	99.9
7		39 ± 8 (4)		372 ± 76 (4)	99.9
8		33 ± 2 (6)	152 ± 22 (17)	405 ± 195 (4)	99.9
9		25 ± 4 (10)	122 ± 21 (4)	1467 ± 392 (4)	99.9
10		42 ± 2 (4)	141 ± 27 (6)	496 ± 82 (5)	99.7

[a] IC$_{50}$ values are expressed as the mean ± SEM of *n* assays. [b] Peripheral blood mononuclear cell (PBMC) proliferation stimulated by Con A. [c] PMA/ionomycin-induced IL-2 synthesis in human whole blood. [d] See ref 19 for a description of this assay.

were subsequently coupled with appropriate carboxylic acids or acid chlorides. Two synthetic routes were used to access the pyrazolyl anilines. The first consisted of condensation between 4-nitrophenylhydrazine and either a 1,3-diketone or a β-ketoester. These condensation products were often further modified to provide the requisitely functionalized pyrazole. The second method invoked a 1,3-dipolar cycloaddition to construct the pyrazole nucleus.

Condensation between 4-nitrophenylhydrazine and a 1,3-diketone was used to construct the majority of the pyrazoles. Illustrative of this approach was the synthesis of the BTP analogues as shown in Scheme 1. 4-Nitrophenylhydrazine was condensed with 1,1,1,5,5,5-hexafluoro-2,4-pentanedione under acidic conditions to provide the pyrazole.[9] Reduction of the aromatic nitro group, most frequently with either hydrogenation or iron powder, to the corresponding aniline provided the amine partner required for amide bond formation. Coupling with acid chlorides in the presence of a base

Table 2. Evaluation of Pyrazole Substituents



			IC_{50} (nM)[a]			rat pharmacokinetics (4 mpk, po)[b]			
compound	X	Y	PBMC proliferation[c] human	PBMC proliferation[c] rat	IL-2 synthesis in human blood[d]	$T_{1/2}$ (h)	*F*%	C_{max} (μg/mL)	AUC (μg·h/mL)
11	C	CN	79 ± 10 (4)	613 (1)	304 ± 68 (4)	6.2	31	0.28	3.24
12	N	CN	266 ± 14 (16)	1524 ± 418 (3)	240 ± 31 (20)	4.3	78	1.09	10.90
13	C	$C(O)CH_3$	170 ± 14 (4)	869 ± 76 (2)	1575 ± 241 (8)		5	0.12	1.11
14	N	NO_2	410 ± 86 (4)		440 ± 112 (4)	1.7	18	0.22	0.31
15	N	OH	−23%[c] ± 6 (2)		16%[e] ± 6 (3)				
16	C	OCH_3	66 ± 16 (10)	284 ± 11 (2)	570 ± 146 (6)		26	0.07	0.97
17	N	OCH_3	185 ± 77 (4)	501 (1)	261 ± 59 (4)	1.0	68	1.48	3.05
18	N	SCH_3	43 ± 8 (4)	158 ± 35 (3)	944 ± 263 (10)	5.2	63	0.87	7.79
19	N	$OCHF_2$	82 ± 18 (6)	146 (1)	182 ± 19 (40)	6.8	74	0.61	7.75
20	N	Cl	75 ± 8 (12)	149 ± 38 (2)	158 ± 20 (23)	4.8	79	0.78	11.05
21	N	Br	44 ± 2 (4)	224 (1)	288 ± 92 (10)	7.2	94	1.04	16.42

[a] IC_{50} values are expressed as the mean ± SEM of *n* assays. [b] Pharmacokinetic data were normalized to 4 mpk, po unless otherwise specified. [c] Peripheral blood mononuclear cell (PBMC) proliferation stimulated by Con A. [d] PMA/ionomycin-induced IL-2 synthesis in human whole blood. [e] Response at maximum concentration tested (10 μM).

scavenger or with carboxylic acids activated with carbodiimides completed the synthesis. On some occasions, solid-phase reagents or scavengers were used to simplify reaction workup.[10] For example, in the coupling with acid chlorides, poly(4-vinylpyridine) was used as the acid scavenger and a polymer-bound benzylamine was used to remove excess acid chloride.

Unsymmetric diketones provided access to functionalization at either C3 and/or C5 of the pyrazole dependent on the nature of each diketone as depicted in Scheme 2. For example, when 4,4,4-trifluoro-1-(2-furyl)-1,3-butanedione was reacted with 4-nitrophenylhydrazine, the furan regiochemistry was nearly exclusively at C5.[11] This compound proved to be a useful intermediate to supply the C5 carboxylic acid which was obtained by oxidation with potassium permanganate.[12] Conversion to the acetyl derivative was achieved by addition of methyllithium to the corresponding Weinreb's amide after the nitro group had been reduced. The same carboxylic acid underwent a Curtius rearrangement to introduce a nitrogen functionality at C5. The nitro group was obtained on the pyrazole by treatment with sodium nitrite and copper sulfate following aniline production, amide bond formation, and deprotection.[13]

One of the more versatile intermediates for introducing functionality at C5 on the pyrazole was the condensation product of 4-nitrophenylhydrazine and the β-ketoester, ethyl 4,4,4-trifluoroacetoacetate, which is illustrated in Scheme 3. Although the C5 hydroxy analogues lacked activity, alkylation[14] or transformation to a halogen[15] produced some of the more biologically interesting analogues. The key intermediate was also accessed with *S*-methyl 4,4,4-trifluoro-3-oxothiobutyrate which additionally gave a limited amount of the C5 *S*-methyl ether.

Dipolar cycloaddition provided a second alternative path to the pyrazolyl anilines.[16] This route gave complete regiochemical control over the position of substituents on the pyrazole ring and is shown in Scheme 4. The hydrazone formed from reaction of trifluoroacetaldehyde and 4-nitrophenylhydrazine was chlorinated to provide the dipolar precursor.[17] Upon exposure to base and a dipolarophile (2-chloroacrylonitrile is illustrated here) the C5 nitrile was obtained in good yield.[18] Once again, the aniline and subsequent amide were formed in the usual manner.

Biological Data and Conclusions

The profiling of the compounds began with the Con A-stimulated human PBMC proliferation assay. The leads **1** and **2** indicated that the terminal amide was amenable to modification. Certainly the majority of compounds made were synthesized in parallel by formation of the amide bond depicted in Scheme 1. Although this terminal position proved accepting of a great deal of modification, the most potent compounds were found in the halogenated aromatic series. In particular, fluorine in the ortho position boosted potency as is noted in a comparison of **1**, **4**, and **6** (Table 1). Multiple fluorines on the phenyl ring maximized potency in this assay as shown with **7**−**9**. Fluorinated aromatic heterocyles such as **10** maintained this potency. Potency of compounds observed in the Con A-stimulated human PBMC proliferation assay was, in general, not maintained in moving to the human whole blood paradigm using IL-2 synthesis as the readout, presumably due to extensive plasma protein binding.[19] The compounds shown in Table 1 all exhibited a great deal of protein binding (>99%) despite a clog *P* range of 3.2−5.6. As expected, the heterocycles were at the lower end of the range. Apparently, the BTP portion of these compounds was overriding any benefit gained by heterocyclic substituents, and at least one of the lipophilic trifluoromethyl groups on the pyrazole ring had to be replaced to reduce protein binding. Further SAR studies focused on substituent modification of the pyrazole ring did produce analogues with reduced protein binding affinity and substantially greater potency in the assay measuring inhibition of IL-2 in human whole blood (Table 2). As a case in point, compounds **10** and **12** were compared. As with all the BTPs, **10** was highly protein bound (99.7%) despite a modest clog *P* (3.47). Substitution of a trifluoromethyl group with a nitrile supplied **12** with reduced protein binding (96.2%) and clog *P* (2.05). Modifications

Table 3. Efficacy in Primate Model of Acute Asthma[a]

			respiratory system resistance[c]		bronchoalveolar lavages[d]			
compound	dose (mg/kg)[b]	*n*	10 min	6 h	48 h eos	6 h IL-5	6 h IL-8	6 h eotaxin
prednisolone	1.0	8	24*	57*	106	94	9	67**
cyclosporin A	25	6	13	90**	137	92	68	62*
19	30	6	61**	69**	185*	73*	69*	56***

[a] Data represent mean percentage inhibition versus crossover vehicle control. [b] Compounds were administered by oral gavage at −26, −2, and +22 h relative to antigen inhalation (QD). [c] Bronchoconstriction was assessed by measuring respiratory system resistance at baseline, +10 min (immediate response), and +6 h (late-phase response). [d] Bronchoalveolar lavages were collected at −5 day (baseline), +6 h (for IL-5, IL-8, and eotaxin), and +48 h (for eosinophil influx, eos). Paired *t*-test vs vehicle controls: $*p < 0.05$, $**p < 0.01$, $***p = 0.054$.

are required on both the pyrazole and terminal amide portions of these molecules to reduce protein binding. Although the protein binding remains high, there was a sufficient free fraction of compound **12** to give comparable IC_{50} values between the proliferation assay (low-protein environment) and the IL-2 synthesis inhibition assay (whole blood environment). The other potent compounds in Table 2 have a similar profile.

Although several of these compounds now displayed excellent potency for inhibition of IL-2 production in human PBMCs, rodent cells were much less sensitive to inhibition by this structural class. In fact, potencies were generally at least 4 times less in rodent than in human cells. This confounding property made efficacy evaluations in rodent models of autoimmune disease particularly problematic. As a consequence, we elected to evaluate the most promising compounds in a primate ex vivo IL-2 synthesis inhibition model followed by testing in a primate asthma model.[20] For these studies we chose to use the relatively optimized analogue **19** as an example. This compound displayed an excellent PK profile in rodents (as shown in Table 2) and in cynomolgus monkeys ($t_{1/2} \sim 8$ h).

To assess in vivo efficacy, the ability of orally administered **19** to block cytokine production of circulating T-cells was measured and compared to that of cyclosporin A (CsA). Blood samples obtained at 2 h after dosing, the time of peak drug concentration for both compounds, were stimulated ex vivo to produce IL-2, and these levels were compared to baseline samples from the same animals.[21] As shown in Figure 1, cyclosporine inhibited 76% at a dose of 30 mg/kg, po. This is consistent with its potency when used in cynomolgus macaques for preventing allograft rejection.[22] Compound **19** was found to have an inhibitory potency approximately 10-fold better than that of cyclosporine. Comparable inhibitory effects on T-cell IL-2 production were obtained with **19** and cyclosporine at doses of 3.0 and 30 mg/kg, po, respectively. The efficacies achieved in monkeys in vivo for blocking T-cell cytokine production suggest that **19**, and the BTP class of cytokine inhibitors, have potential similar to that of cyclosporine for use in transplantation.

As **19** was able to inhibit IL-4 and IL-5 production in human T-cell lines with similar potency to its effects on IL-2 release (data not shown), it was evaluated in an *Ascaris*-induced nonhuman primate model of asthma where, like human asthma, IL-5 is proposed to be a pathogenic mediator.[23] Difluoromethoxy ether **19** showed remarkable efficacy in this model at a dose of 30 mg/kg (oral gavage), producing a more pronounced and significant inhibition of the immediate bronchoconstriction

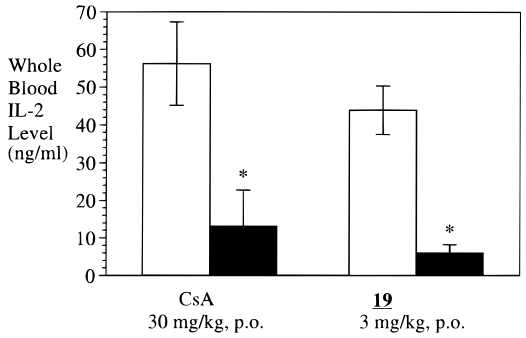


Figure 1. Inhibition of whole blood IL-2 production ex vivo following oral administration of **19** and cyclosporin A (CsA) in cynomolgus monkeys. The blood samples were drawn at 2 h after drug administration and then analyzed for IL-2 production over a 24-h incubation. This gave adequate time for drug inhibition to be maximal, regardless of whether the modes of action have differing kinetics. Preliminary pretreatment studies done in vitro with whole blood exposed to compound prior to stimulation with PMA/ionomycin showed a rapid onset of drug inhibition: bars, mean ± SEM; open bars, IL-2 production of predrug controls; filled bars, IL-2 production 2 h postdrug; $n = 3$ for **19** and $n = 6$ for CsA; $*p < 0.05$.

and IL-8 generation than cyclosporine at similar doses (data shown in Table 3).

Mechanism of action studies[24] on compounds **1** and **2** showed that the BTP compounds regulate NFAT activity by a mechanism distinct from the current immunosuppressive drugs cyclosporine and FK506. The activity of NFAT proteins is tightly regulated by the calcium/calmodulin-dependent phosphatase 2B (calcineurin). Dephosphorylation of NFAT by calcineurin is required for NFAT activation and transport from the cytosol to the nucleus. The current immunosuppressive drugs cyclosporin A and FK506 block calcineurin activity leading to inhibition of NFAT nuclear translocation and consequent cytokine gene transcription.[6] Treatment of intact T-cells with the BTP compounds, prior to calcium ionophore-induced activation of calcineurin, caused NFAT to remain in a phosphorylated state and blocked nuclear translocation. However, the BTP compounds did not directly inhibit the dephosphorylation of NFAT by calcineurin in vitro, nor did the drugs block the in vitro dephosphorylation of other calcineurin 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 calcineurin phosphatase activity. Studies to identify a molecular target are continuing.

Ongoing studies in nonhuman primate models of

autoimmune disease will allow for an assessment of the potential of this novel class of transcription factor regulator for the treatment of diseases such as asthma, rheumatoid arthritis, and transplant rejection.

Supporting Information Available: Characterization data for all compounds and elemental analyses. This information is available free of charge via the Internet at http://pubs.acs.org.

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(7) **Determination of IL-2 reporter gene expression in Jurkat E2 cells:** Jurkat E2 cells were routinely cultured in RPMI 1640 plus 10% fetal bovine serum (FBS) containing 400 μg/mL hygromycin and 500 μg/mL geneticin. Prior to assay, Jurkat E2 cells were resuspended at 4×10^5 cells/mL in RPMI 1640 plus 5% FBS (lacking antibiotics), stimulated with 10 ng/mL 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and 2 μM ionomycin (Northrop, J. P.; Crabtree, G. R.; Mattila, P. S. *J. Exp. Med.* **1992**, *175*, 1235−1245), and placed in 96-well microtiter plates in a final volume of 200 μL/well. Cells were incubated for 18 h at 37 °C, 10% CO_2 in a humidified incubator. Jurkat E2 cells were pelleted by centrifugation and the media aspirated. Cells were lysed by the addition of 20 μL of cell-lysing buffer containing 25 mM Tris-phosphate, pH 7.8/2 mM dithiothreitol/2 mM 1,2-diaminocyclohexane *N,N,N′,N′*-tetraacetic acid/10% glycerol/1% Triton X-100 and incubated at room temperature for 20 min. 100 μL of luciferase reaction buffer containing 20 mM Tricine, pH 7.8/1.07 mM $(MgCO_3)_4 \cdot Mg(OH)_2 \cdot 5H_2O$/2.67 mM $MgSO_4$/0.1 mM EDTA/33.3 mM dithiothreitol/270 μM coenzyme A/470 μM luciferin/530 μM ATP was added to the cell lysate, and chemiluminescence was measured with a luminometer. The efficacy of compounds to inhibit IL-2 gene promoter activity was determined by measuring the inhibition of TPA plus ionomycin-induced luciferase expression in Jurkat E2 cells. **Con A assay:** Serially diluted compounds were distributed to 96-well plates. Human PBMCs, isolated by ficoll/hypaque gradient centrifugation, were added to effect 2.5×10^5 cells/mL in complete RPMI 1640 medium. Con A was added to effect 2.5 μg/mL final concentration. Cells were incubated in a CO_2 incubator for 3 days and were labeled with [^{3}H]thymidine for the last 6 h of culture. [^{3}H]Thymidine incorporation, after harvesting onto glass fiber filters, was assessed by direct β-counting. **Human whole blood IL-2 inhibition assay:** Serially diluted compounds were distributed to 96-well plates. Human heparinized blood was then added to each well. PMA and ionomycin mixture was added to effect 50 ng/mL for PMA and 3 mg/mL for ionomycin. Plasma samples were collected 24 h later by centrifugation and were assayed for IL-2 by an in-house ELISA.

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