Selective inhibitor of Janus tyrosine kinase 3, PNU156804, prolongs allograft survival and acts synergistically with cyclosporine but additively with rapamycin

Stanislaw M. Stepkowski, Rebecca A. Erwin-Cohen, Fariba Behbod, Mou-Er Wang, Xienui Qu, Neelam Tejpal, Zsuzsanna S. Nagy, Barry D. Kahan, and Robert A. Kirken

Janus kinase 3 (Jak3) is a cytoplasmic tyrosine (Tyr) kinase associated with the interleukin-2 (IL-2) receptor common gamma chain (γc) that is activated by multiple T-cell growth factors (TCGFs) such as IL-2, -4, and -7. Using human T cells, it was found that a recently discovered variant of the undecylprodigiosin family of antibiotics, PNU156804, previously shown to inhibit IL-2–induced cell proliferation, also blocks IL-2–mediated Jak3 auto-tyrosine phosphorylation, activation of Jak3 substrates signal transducers and activators of transcription (Stat) 5α and Stat5b, and extracellular regulated kinase 1 (Erk1) and Erk2 (p44/p42). Although PNU156804 displayed similar efficacy in blocking Jak3-dependent T-cell proliferation by IL-2, -4, -7, or -15, it was more than 2-fold less effective in blocking Jak2-mediated cell growth, its most homologous Jak family member. A 14-day alternate-day oral gavage with 40 to 120 mg/kg PNU156804 extended the survival of heart allografts in a dose-dependent fashion. In vivo, PNU156804 acted synergistically with the signal 1 inhibitor cyclosporine A (CsA) and additively with the signal 3 inhibitor rapamycin to block allograft rejection. It is concluded that inhibition of signal 3 alone by targeting Jak3 in combination with a signal 1 inhibitor provides a unique strategy to achieve potent immunosuppression. (Blood. 2002;99: 680-689) © 2002 by The American Society of Hematology

Introduction

Complete activation of T cells requires 3 threshold-limited sequential signals. Signal 1, delivered by antigens that engage a specific T-cell receptor (TCR), is followed by signal 2, delivered by a B7-CD28 interaction. Within seconds to minutes of TCR engagement, the CD3ζ chain is tyrosine (Tyr) phosphorylated during the autoactivation of Zap70, Lck, and Fyn protein Tyr kinases. Concomitantly, calcium (Ca++) mobilization triggers the catalytic activation of calcineurin (CaN) phosphatase to dephosphorylate nuclear factor of activated T cell (NFAT)—a necessary step for NFAT to translocate to the nucleus and bind discrete DNA-binding elements within the promoter of the interleukin-2 (IL-2) gene. Signals 1 and 2 are critical for the synthesis and secretion of IL-2, which, in concert with other T-cell growth factors (TCGFs) such as IL-4, -7, -9, -13, and -15, deliver signal 3 through cytokine receptors, a necessary step for driving the clonal expansion of T cells. These cytokine receptors share a common gamma chain (γc) that, when combined with a private α chain for each cytokine, delivers intracellular signals by Janus Tyr kinase 1 (Jak1) and Jak3 and that activates signal transducers and activators of transcription 1 (Stat1), Stat3, Stat5α/b, and Stat6. Current clinical immunosuppressive regimens are dominated by CaN inhibitors (cyclosporine [CsA] or FK506) that block T-cell progression through the early G1 stages of the cell cycle. However, ubiquitous expression of CaN in many different tissues contributes to several adverse side effects, including nephrotoxicity and neurotoxicity. One therapeutic approach aimed at improving immunosuppression while simultaneously reducing CsA-induced toxicity is to develop combination therapy. Rapamycin (RAPA), an agent recently approved by the United States Food and Drug Administration for use in clinical transplantation, disrupts late G1 and early S cell-cycle progression by inhibition of the serine (Ser)–threonine (Thr) kinase, the mammalian target of rapamycin (mTOR). Our extensive preclinical and recent clinical studies have documented that a combination of CsA and RAPA produces potent synergistic interactions allowing for a 50% reduction of CsA trough blood levels. Although RAPA is not nephrotoxic alone, however, the ubiquitous tissue distribution of mTOR leads to other toxicities such as myelosuppression and hyperlipidemia. The current study explores the hypothesis that the inhibition of molecular targets unique to lymphocytes, particularly those activated by TCGFs, would provide a novel and selective means to block T-cell function and allograft rejection when used alone or in combination with CaN inhibitors. In particular, we seek to identify antagonists of Jak3, thereby inhibiting an entire family of TCGF-dependent pathways. Indeed, Jak3 (primarily expressed in T, B, and natural killer [NK] cells) is activated through the γc and plays a critical role in T-cell development and function. Humans or mice genetically deficient in this enzyme or unable to activate Jak3 because of genetic disruption of the γc manifest severe combined immunodeficiency disease. Recent evidence suggests that disruption of...
Jak3 results in the inactivation of 2 critical Jak3 substrates, Stat5a and Stat5b. Although double Stat5a/b gene-deficient mice are not as severely immunosuppressed as Jak3 or γc knockouts, their T cells fail to proliferate in the presence of TCGFs such as IL-2,25 presumably because of greatly reduced levels of several cell-cycle proteins, including the cyclin-dependent kinase-6 and cyclins A, D2, D3, and E.27

A recently published report28 revealed that PNU156804, an analogue of the toxic parent compound undecylprodigiosin, blocks IL-2–induced T-cell proliferation in the late G1 phase of the cell cycle. On the molecular level, these authors showed that PNU156804 inhibits the activation of NF-κB and AP-1 transcription factors, the expression of cyclins D2 and E and cyclin-dependent kinases 2 and 4, and the hyperphosphorylation of the retinoblastoma protein. In contrast, IL-2 receptor (IL-2R) messenger RNA (mRNA) expression of α and γ chains was not affected by PNU156804.28 However, the exact molecular target for PNU156804 was not identified in this earlier study. The current experiments investigate whether PNU156804 inhibits TCGF-induced T-cell proliferation through blockade of the Jak3, Stat5a/b, and mitogen-activated protein kinase (MAPK) pathways. We also examined whether PNU156804, alone or in combination with CsA or RAPA, can effectively block allograft rejection.

Materials and methods

Cell culture and treatment

The rat T-cell line Nb2-11c, originally developed by Dr Peter Gout (Vancouver, BC, Canada), was grown in RPMI 1640 with 10% fetal calf serum (FCS; catalog no. 1020-90; Intergen, Purchase, NY), 2 mM L-glutamine, 5 mM HEPES, pH 7.3, and penicillin-streptomycin (50 U/mL and 50 μg/mL, respectively), at 37°C–5% CO2. Freshly explanted normal human T lymphocytes purified by isocentrifugation (Ficoll; EM Science, Gibbstown, NJ) were phytohemagglutinin (PHA) activated for 72 hours as previously described.29 T lymphocytes were made quiescent by washing and incubating for 24 hours in RPMI 1640 medium containing 1% FCS before exposure to cytokines. Next, cells were treated with varying concentrations of PNU156804 (generously provided by Dr Roberto D’Alessio, Department of Pharmacology, Pharmaccia and Upjohn Research Center, Nerviano, Italy) as described in the figure legends. All cells were then stimulated with 100 nM recombinant human IL-2 (Hoffman-LaRoche, Basel, Switzerland), anti-CD3 (PharMingen, San Diego, CA), or ovine prolactin (PRL) supplied by the National Hormone and Pituitary Program (National Institute of Diabetes, Digestive, and Kidney Disease, Bethesda, MD) at 37°C. Cell pellets were frozen at −70°C.

Proliferation assays

Quiescent human primary T cells, human Jurkat cells, or rat T cells (5.0 × 105/well) were plated in flat-bottom, 96-well microtiter plates in 200 μL quiescent media in the absence or presence of 1 nM IL-2, −4, −7, or −15 (PeproTech, Rock Hill, NJ) or of PRL. Next, cells were treated for 16 hours with PNU156804 or the inactive control, PNU159744, and were pulsed for 4 hours with [3H]-thymidine (0.5 μCi [0.0185 MBq]/200 μL) and harvested onto fiberglass filters. [3H]-thymidine incorporation was analyzed by liquid scintillation counting as previously described.29

Solubilization of membrane proteins, immunoprecipitation, and Western blot analysis

Frozen cell pellets were thawed on ice and solubilized in lysis buffer (104 cells/mL) as previously described.29 For human T cells, supernates were incubated, rotating end-over-end for 2 hours at 4°C with either 5 μL/mL polyclonal rabbit antiserum raised against peptides derived from the unique carboxyl group (COOH) termini of Jak3 (amino acid [aa] 1104-1124) or carboxyl termini of human Stat5a (aa 775-794) or Stat5b (aa 777-787), and phosphoserine Stat5 rabbit polyclonal antibodies (pAbs) were generated against a phosphopeptide surrounding S725 of human Stat5a.30 For rat Nb2-11c and T cells, Jak3, Jak2, p56Lck, and antiphosphotyrosine antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Proteins bound to antibodies were captured by incubation for 30 minutes with Protein A–Sepharose beads (Pharmacia, Piscataway, NJ), sedimented for purification, and eluted by boiling in 2× sodium dodecyl sulfate (SDS) sample buffer (20% glycerol, 10% 2-mercaptoethanol, 4.6% SDS, 0.0084% bromophenol blue in 0.125 M Tris, pH 6.8) for 4 minutes. For phospho-MAPK assays, approximately 25 μg total cell lysate was dissociated in SDS sample buffer and was separated on 10% (all others on 7.5%–polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Proteins were transferred to polyvinylidene difluoride (PVDF) (Immobilon; catalog no. IPVH 0010; Millipore, Bedford, MA) as previously described.11 Western blot analysis was performed with either pAbs, marine antiphosphotyrosine monoclonal antibodies (mAbs) (4G10; catalog no. 05-321; Upstate Biotechnology, Lake Placid, NY), or phospho-p44/42 MAPK (catalog no. 9101; New England Biolabs, Beverly, MA). Western blots with the above antibodies, rabbit antiphospho-ERK1/2 (extracellular regulated kinase 1/2), and monoclonal pan-ERK (catalog no. E17120; PharMingen, San Diego, CA) were diluted 1:1000 in blocking buffer and were used as previously described.11

Rat heart transplantsations

Wistar Furth (WF; RT1u) and Buffalo (BUF; RT1b) rats (each weighing 160-200 g) obtained from Harlan Sprague-Dawley (Indianapolis, IN) were cared for according to the guidelines of the Animal Welfare Committee. Rats were housed in light- and temperature-controlled quarters and given chow and water ad libitum. Heterotopic heart transplantation was performed using a standard microsurgical technique of end-to-side anastomoses to recipient aorta and vena cava.31 Cold ischemia times were less than 30 minutes. Graft survival time was defined as the last day of transabdominally palpable cardiac contractions. Recipients remained untreated or were treated by oral gavage every other day for 14 days (6 treatments) with 40, 80, or 120 mg/kg PNU156804 alone or in combination with daily oral gavage of 1.25, 2.5, or 5 mg/kg CsA or 0.25, 0.5, or 1 mg/kg RAPA. Some recipients were treated with CsA or RAPA alone. The results, presented as mean survival time ± standard deviation, were assessed for statistical significance by the Gehan survival test. In addition, the interaction between PNU156804 and CsA or RAPA was evaluated by the median effect analysis.12,33 Computer software was used to calculate combination index (CI) values: CI < 1 showed synergistic interactions, CI > 1 showed antagonistic interactions, and CI = 1 showed additive interactions.33

Histopathologic evaluation

WF recipients of BUF heart allografts were treated as described above with vehicle (n = 3) or vehicle containing 80 mg/kg PNU156804 (n = 3). At day 7 after transplantation, heart allografts were placed in Bouin fixative (Poly Scientific R&D, Bay Shore, NY). Each heart was sectioned in an identical fashion consisting of single horizontal cut followed by 3 consecutive incisions used to generate slides. Another dissection was made, and 3 more consecutive slices and slides were generated. Twelve slides per heart were stained with hematoxylin and eosin, as described previously.34 Rejection was graded in accordance with the standards established by the International Society of Heart and Lung Transplantation35: grade 0, no evidence of rejection; grade 1A, focal perivascular infiltration; grade 1B, diffused interstitial infiltration; grade 2 for moderate infiltration with uni-focal myocyte damage; grade 3A, moderate infiltration with severe focal myocyte damage; grade 3B, moderate infiltration with severe diffused myocyte damage; grade 4, severe infiltration with ongoing severe myocyte damage.

Results

PNU156804 blocks IL-2–mediated growth and Jak3 autophosphorylation

To investigate the effects of PNU156804 on T-cell proliferation, T cells that had been activated for 72 hours with PHA were then made
quiescent and admixed with ascending PNU156804 concentrations (1-100 μM) for 16 hours in the absence or presence of 1 nM IL-2. As shown in Figure 1, PNU156804 completely abolished IL-2–induced [3H]-thymidine incorporation at 25 μM (IC50 of approximately 7.5 μM). In contrast, the inactive analog PNU159744 showed minimal effect on T-cell proliferation at similar concentrations of drug, with only 20% inhibition observed at 100 μM (Figure 1). This inhibitory effect was not caused by cell death from toxicity because cell viability was typically greater than 85% based on a trypan blue dye exclusion test at this time point (16 hours), which was measured at the completion of each experiment (data not shown). Moreover, the effect of the drug appeared to be confined to activated T cells because PNU156804 did not inhibit actively growing non-Jak3–expressing Jurkat cells, a model for unactivated T cells (Figure 1).

PNU156804 fails to inhibit anti-CD3 activation of p56Lck in primary human T cells

The above experiments suggest PNU156804 inhibits activated but not unactivated T cells by disrupting the signal 3 pathway. Earlier work by Mortellaro et al28 supports this model because the expression of IL-2 and its affinity-confering α-chain, both dependent on signal 1 and 2, were not affected. To test this notion further, effects of PNU156804 on the TCR-activated pathways were investigated by stimulating T cells by anti-CD3 activation and measuring changes in p56Lck and other Tyr-phosphorylated proteins. For this assay, primary human T cells were treated with 20 mM active drug (PNU156804) for 16 hours and were stimulated with or without 5 μg/mL (μM) anti-CD3 antibody for 5 minutes at 37°C (Figure 2A). Cell lysates were clarified, and 30 μg total cell lysate was separated on SDS-PAGE from vehicle (lanes a-b) or from PNU156804-treated samples (lanes c-d). Alternatively, lysates were immunoprecipitated with antibodies to p56Lck (lanes e-h). Proteins were Western blotted with antiphosphotyrosine antibodies. Overall, no changes were observed in total Tyr-phosphorylated protein or TCR activation of the p56Lck Tyr kinase. Stripping and reprobing of the blot confirmed an equivalent loading of p56Lck.

Catalytically active Jak3 is required for IL-2–driven Tyr phosphorylation of Jak1 and Stat5a/b.26,27,36,37 To determine whether the blockade of T-cell proliferation was caused by a loss of Jak3 activity, PHA-activated T cells (72 hours) were treated with ascending concentrations of PNU156804 (0-10 μM) for 16 hours, followed by a 10-minute stimulation with 100 nM IL-2. Lysed cells were immunoprecipitated with Jak3 pAb and then Western blotted with antiphosphotyrosine mAb (Figure 2B). Tyr phosphorylation of Jak3 was notably reduced at 5 μM (lane i) and almost completely inhibited at 10 μM (lane j) concentrations. Five separate experiments confirmed the loss of Jak3 Tyr phosphorylation between 5 and 20 μM PNU156804. Inactive PNU159744 control was ineffective in blocking Jak3 activation (Figure 2B, lanes k-n). The same samples blotted with anti-Jak3 pAb to measure total Jak3 confirmed equivalent protein levels (Figure 2B, lower panel). Thus, PNU156804 disrupts Tyr phosphorylation of Jak3.

Because an earlier study demonstrated that the lack of cell proliferation in the presence of PNU156804 was not attributed to the loss of IL-2Rα or β chain expression,28 we examined whether the inhibition of Jak3 resulted from a direct effect of the drug on the Jak3 enzyme. In particular, several studies have shown a correlation between cytokine-induced Tyr phosphorylation and catalytic activation of Jak kinases.38-40 We have previously demonstrated that IL-2, α, or -γ markedly increased the catalytic activity of Jak3 using an in vitro kinase assay based on antiphosphotyrosine immunoblotting and the addition of unlabeled adenosine triphosphate (ATP).41,42 Similar autokinase assays were performed herein on Jak3 immunoprecipitates obtained from T cells exposed to medium alone or with 100 nM IL-2 (3 minutes at 37°C). Affinity-purified Jak3 protein incubated with kinase buffer in the presence or absence of cold ATP (15 μM), either dimethyl sulfoxide (DMSO) or 10 μM PNU156804, and the auto-Tyr phosphorylation samples were separated on SDS-PAGE and subsequently blotted with antiphosphotyrosine mAbs. Representative data from 2 separate experiments measured Jak3 autophosphorylation normalized to nonphosphorylated protein (phosphorylated–nonphosphorylated) to calculate a stimulatory index (SI) value for in vitro treatment with either vehicle alone (Figure 2C, lanes a-d) or with 20 μM PNU156804 (Figure 2C, lanes e-h). Immunopurified Jak3 Tyr autophosphorylation was potently inhibited by PNU156804 because the addition of cold ATP failed to stimulate Jak3 Tyr autophosphorylation (lane h) in comparison with non–ATP-treated samples (lanes g). Densitometric analysis of these sample points (SI, 1.8-2.0) showed that PNU156804 reduced Jak3 autophosphorylation by 90% in comparison with DMSO-treated control sets (SI, 3.9-7.0; Figure 1B, lanes c-d). Thus, these findings suggest that PNU156804 inhibits IL-2–mediated T-cell growth by ablating crucial Jak3-dependent signaling pathways.

PNU156804 disrupts IL-2–mediated Stat5a/b Tyr/Ser phosphorylation

Given that both Stat5a and Stat5b are downstream of Jak3 and that T cells from Stat5a/b gene-deficient mice failed to proliferate in response to IL-2 stimulation,8 we examined whether PNU156804 inhibits IL-2–induced activation of Stat5a/b. For this assay, PHA-activated quiescent human T cells were treated with 20 μM PNU156804 or inactive control PNU159744 for 16 hours, followed by IL-2 stimulation. PNU156804 blocked Stat5a (Figure 3) and Stat5b (Figure 3B) Tyr phosphorylation. In addition to Tyr phosphorylation, Stat5a/b transcription factors are (like other Stats) Ser phosphorylated.43 Indeed, interferon α/β (IFN-α/β) or IFN–γ–mediated Ser phosphorylation of Stat1α or IL-6 of Stat3 is believed necessary for its function, including maximal nuclear translocation, DNA binding, transcriptional activation, and cell cycle progression.44,45 We previously mapped a Ser phosphorylation site in Stat5a (Ser726) that is conserved in Stat5b (Ser731).46,47 As shown

*Figure 1. PNU156804 disrupts cell growth of activated, but not of unactivated, human T cells in a dose-dependent manner. Proliferation of quiescent PHA-activated human T cells (5.0 × 10^6 cells/well) in the presence of 1 nM IL-2 was examined after treatment with increasing concentrations of PNU156804 (○) or inactive control PNU159744 (●) for 16 hours at 37°C. Conversely, Jurkat cells were treated in an identical fashion with PNU156804 (▲). All cells were then pulsed with [3H]-thymidine (0.5 μCi [0.0185 MBq/200 μL] for 4 hours, incorporated into a radiolabeled probe, and plotted on the abscissa as expressed as total cpm (n = 6).
in Figure 3A-B, PNU156804 inhibited IL-2–induced Stat5a/b Ser kinase activity. In particular, neither site was inducibly Ser phosphorylated in the presence of cytokine and PNU156804, as measured by phosphoserine-specific Stat5a/b pAb (middle panels). The same samples reblotted with anti-Stat5a/b mAb confirmed equivalent protein levels (panels A and B). These results suggest that PNU156804 inhibits Jak3 from mediating Tyr and Ser phosphorylation of Stat5. In contrast, the inactive PNU159744 analogue showed no effect on either Tyr or Ser kinase activity. Because Jak-regulated Stat Tyr/Ser phosphorylation is required for dimerization, nuclear translocation, and gene transcription,25 we conclude that IL-2–Stat5a/b–mediated gene transcription critical for IL-2–mediated cell cycle progression is one explanation for the loss of IL-2–inducible T-cell proliferation observed in Figure 1.

PNU156804 inhibits Erk1/2 Tyr/Thr phosphorylation

IL-2 potently activates the Shc/Ras/Raf/MAPK pathway by the adapter protein SHC, which binds to Tyr 338 of the IL-2Rβ chain ultimately to drive T-cell proliferation.48,49 To investigate whether PNU156804 disrupts this signaling pathway, PHA-activated T cells were treated with vehicle alone (Figure 4, lanes a-b) or with ascending PNU156804 concentrations. Total cell lysates separated on 10% SDS-PAGE were blotted with phospho-Erk1/2 pAb that recognize activated Thr202 and Tyr 204 sites on both enzymes. As shown in the representative experiment, Erk1 and Erk2 were completely inhibited at 10 μM PNU156804 (lane i). Two additional experiments confirmed the loss of active Erk1/2 proteins observed at 10 to 20 μM PNU156804 concentrations. Immunoblotting with a pan-Erk1/2 (indicated beneath phosphorylation blots)
verified equivalent loading, though with only weak p44 for Erk1.
These data support the conclusion that the inhibition of Jak3 by
PNU156804 disrupts Erk1/2 (p44/42) activity in IL-2–mediated
signaling pathways.

**PNU156804 preferentially inhibits Jak3- rather than
Jak2-dependent cell proliferation**

Because Jak3 is recruited by IL-2 and other TCGFs,6 we tested the
effect of PNU156804 on PHA-activated human T cells stimulated
with either 1 nM IL-2, -4, -7, or -15 (Figure 5). [3H]-thymidine
incorporation, plotted as percentage inhibition of total incorporated
radiolabel versus increasing concentrations of PNU156804, dis-
played similar efficacy in disrupting T-cell growth stimulated by
various TCGFs (Figure 5A). Thus, γc–Jak-dependent T-cell prolifera-
tion is equally inhibited in response to IL-2, -4, -7, or -15.

To test the selectivity of the drug, PNU156804 was added to T
cells undergoing Jak3-versus Jak2-dependent proliferation. For this
assay, the rat Nb2-11c cell line was chosen because it responds to
either PRL (Jak2) or IL-2 (Jak3) stimulation.50 As depicted in
Figure 5B, Nb2-11c cells treated with ascending concentrations of
PNU156804 showed nearly 2-fold greater inhibition for the
IL-2–Jak3-dependent pathway than for the PRL-Jak2-dependent
pathway. Moreover, though 20 μM PNU156804 reduced cell
proliferation to basal levels (~20% of total [3H]-thymidine incor-
poration), PRL-mediated growth was retained by 2-fold or inhibited
by less than 50% (Figure 5B). Using the same Nb2-11c cells, we
compared the inhibitory effect of PNU156804 on Jak3- versus
Jak2-mediated Stat5α/b activation. Nb2-11c cells treated with
ascending concentrations of PNU156804 (as described in Figure 1)
were stimulated with either IL-2 to activate Jak3 (lanes a-f) or PRL
to activate Jak2 (lanes g-l). As shown by Western blot, the Tyr
phosphorylation patterns of signaling proteins closely paralleled
their proliferative response (Figure 5B; insert). Indeed, PNU156804
inhibited IL-2–mediated Tyr phosphorylation of Jak3, Stat5α, and
Stat5b (maximum at 10 μM), whereas PRL activation of Jak2,
Stat5α, and Stat5b showed minimal inhibition even at 25 μM (lane
l). These experiments document that PNU156804 disrupts Jak3-
dependent signaling molecules and proliferation driven by TCGFs
in a similar fashion that is at least twice as efficacious as its effect
on the Jak2-dependent signaling pathway(s).

**In vivo effect of PNU156804 alone or in combination with CsA
or RAPA on heart allograft survival**

We also examined whether the Jak3 inhibitor PNU156804
displays immunosuppressive activity in vivo. As shown in
Figure 6, WF (RT1u) recipients of BUF (RT1b) heart allografts
oral gavaged on alternate days for 14 days with DMSO alone
showed a mean survival time of 6.3 ± 0.5 days (Figure 6). Alternate-
day oral gavage of ascending doses of PNU156804 (40, 80, or 120 mg/kg) significantly extended heart allograft survival in a dose-dependent fashion (all P < .01). The signal 1 inhibitor CsA, delivered daily alone by oral gavage for 14 days
(1.25, 2.5, or 5.0 mg/kg), produced in vivo effects similar to
those produced by PNU156804. However, combinations of
PNU156804 and CsA administered at ratios ranging from 64:1
to 1:1 exhibited much better effects than either drug alone, with
many transplanted hearts surviving more than 100 days (Figure
6). To determine the quality of interaction between PNU156804
and CsA, results were evaluated by the median effect analysis to
calculate CI values. Although all 2-drug ratios tested were
synergistic (CI, 0.2-0.8), optimal results were produced by the
PNU156804-CsA ratio of 4:1 (CI, 0.22).

Next, we examined the interaction of PNU156804 with another
signal 3 inhibitor, RAPA, which targets the 256-kd Ser/Thr kinase
and regulator of protein translation, mTOR. RAPA given alone
daily by oral gavage for 7 days (0.25, 0.5, or 1.0 mg/kg) resulted in
modest, albeit significant, prolongation of heart allograft survival.
Combinations of PNU156804 and RAPA produced only additive
effects (CI, 0.9-1.0). These results showed that selective inhibition
of the Jak3-dependent signal 3 pathway blocks allograft rejection.
This effect is synergistic with a signal 1 inhibitor but only additive
with another signal 3 inhibitor.

**Inhibition of Jak3 blocks graft damage and reduces
leukocyte cell infiltration**

For histologic examination, heart allografts were obtained from
recipients that had received alternate-day oral gavage of DMSO
alone or DMSO combined with 80 mg/kg PNU156804. Each heart was uniformly cut horizontally, and 12 hematoxylin-eosin–stained sections per heart were scored as described in "Materials and methods." Untreated heart allografts examined on day 7 after grafting displayed maximum grade 4 damage with extensive myocardial necrosis involving more than 50% of the complete cross-section. In the areas of necrosis, the myocardial fibers were totally destroyed up to the epicardium. The remaining areas of the samples without necrosis showed intense infiltration with polynuclear and mononuclear cells (Figure 7A). In contrast, heart allografts from recipients treated with PNU156804 showed only grade 1B changes with mild infiltration of epicardium, myocardium, and endocardium, but without any evidence of myocyte damage (Figure 7B). Given that similar changes were observed on all sections in each group, we conclude that selective inhibition of Jak3 blocks graft damage and reduces infiltration of leukocytes.

Discussion
The current results demonstrate that PNU156804 inhibits TCGF-induced T-cell growth (IC50, approximately 7.5 μM; Figure 1A) by the disruption of Jak3 autokinase activity (Figure 2B-C). Consequently, PNU156804 (but not the inactive control compound PNU159744) blocks the activation of Jak3 substrates, namely, Stat5a and Stat5b, as assessed by phosphotyrosine and phosphoserine Western blots (Figure 3). In fact, PNU156804 completely disrupts not only Jak3-dependent downstream activation of Stat5a and Stat5b Ser kinases (Figure 3), it also disrupts the downstream Ser/Thr kinases, p44/Erk1 and p42/Erk2 (Figure 4). Although the inhibitory activity of PNU156804 was equally effective in blocking Jak3-driven T-cell proliferation by either IL-2, -4, -7, or -15 (Figure 5A), the drug was 2-fold less efficient in inhibiting growth by PRL through a closely homologous Tyr kinase, Jak2 (Figure 5B). Last,
PNU156804 alone significantly extends cardiac allograft survival and acts synergistically in combination with CsA (CI, 0.2-0.8) and additively in combination with RAPA (CI, 1.0; Figure 6). Thus, PNU156804 represents a selective Jak3 inhibitor with sufficient potency to block allograft rejection.

Because presently used clinical immunosuppressants act in a ubiquitous fashion, they produce potent side effects. For example, an active metabolite of 6-mercaptopurine, 6-thioinosinic acid incorporates into nucleic acids causing DNA and RNA breakage in many cells, thereby causing severe bone marrow depression.51,52 Although glucocorticoids inhibit the production of many cytokines within different cell types, they produce multiorgan side effects, such as Cushingoid features, growth retardation, poor wound healing, and many others deleterious effects.52 CsA or FK506 can disrupt the Ser-Thr phosphatase activity of CaN in several non–T-cell types, thereby contributing to nephrotoxicity and neurotoxicity.53 Moreover, mTOR inhibition of RAPA ablates not only cytokine-mediated growth of T and B cells but many other cells, which can result in myelosuppression and hyperlipidemias.18,19,21,22 Given that the fundamental problem for all available immunosuppressants is the ubiquitous distribution of their targets, we must seek highly specific agents that inactivate molecules uniquely expressed in resting T cells (eg, Zap70) or in activated T and B cells (eg, Jak3).

Several recent studies have revealed that the Jak3 Tyr kinase enzyme is an essential signaling intermediate for the development and function of T and B cells and of NK cells.23,24 Indeed, the retroviral introduction of Jak3 enzyme into Jak3-deficient mice restores normal T-cell development.54 Although understanding of the signaling pathways activated by Jak3 (directly or indirectly) is incomplete, Jak3 signaling by Stat5a/b is necessary to regulate genes required for cellular proliferation.26 As shown here, PNU156804 abolishes IL-2-dependent T-cell proliferation by the inhibition of Jak3-mediated autokinase activity and Stat5a/b Tyr phosphorylation. Consequently, PNU156804 prevents Stat5a/b dimerization by their SH2 domains and by Stat5a/b translocation to the nucleus. Given the limited pattern of Jak3 expression, the g-Jak3-Stat5 pathway is likely to represent a convergence point by which TCGFs drive T-cell clonal expansion, thereby making it a preferred pathway for novel and selective immunosuppression.

We have recently reported that AG-490 blocks T-cell proliferation by the inhibition of Jak3 autokinase activity and Stat5a/b Tyr phosphorylation.55,56,57 However, AG-490 is equally potent in inhibiting Jak2 activation, including eosinophils stimulated by granulocyte-macrophage colony-stimulating factor and in vascular smooth muscle cells and cardiac myocytes activated by angiotensin 2.56-58
PNU156804 inhibits IL-2–induced proliferation of human T cells without affecting the expression of IL-2Rα and γc chains. As shown herein, PNU156804 disrupts not only IL-2–dependent T-cell proliferation by selective inhibition of the γc-Jak3-Stat5a/b–dependent signaling pathway, it also disrupts IL-4, -7, and -15–dependent T-cell proliferation. Although PNU156804 diminished B-cell proliferation in response to killed *Staphylococcus aureus* antigens, however, it also inhibited CD40-triggered activation of NF-κB. These latter results suggest a role for Jak3 Tyr kinase activity in the CD40-initiated signaling pathway. Indeed, it is readily established that CD40 signaling is critical for B-cell growth, survival, differentiation, and immunoglobulin class switching, yet the CD40-CD40 ligand complex is not thought to recruit γc. Jak3 and Stat3 can be directly activated by the CD40 receptor, activation events necessary for the subsequent expression of CD23, intercellular adhesion molecule-1, and lymphotixin-α genes and for the production of immunoglobulin E. Another study reported that CD40 receptor activation by the CD40 ligand could also activate Jak3 and Stat5a in monocytes but not in B cells. It seems plausible to expect that PNU156804 may prove useful in determining whether Jak3 is an active participant in the CD40 signaling pathway.

Our recent study showed that AG-490 inhibits IL-2–dependent proliferation of PHA-stimulated human T cells with an IC50 of approximately 20 μM. In contrast, PNU156804 is at least twice as effective in inhibiting human T cells when performed under identical experimental conditions by displaying an IC50 of approximately 7.5 μM for IL-2 and other T-cell growth factors that activate the Jak3 cascade. Furthermore, PNU156804 shows 2-fold greater specificity to block Jak3-dependent T-cell proliferation in response to IL-2 compared to Jak2-dependent cell proliferation (Figure 5). Whether in vivo comparison with both drugs will support the hypothesis that PNU156804 is more efficacious remains to be determined.

In addition to Tyr phosphorylation, Stats undergo Ser phosphorylation on a conserved Ser residue that plays a critical role in cytokine-induced nuclear translocalization, maximal gene transcription, and cell cycle progression. Earlier evidence has been presented that shows minichromosome maintenance-5 protein is required for full DNA replication by binding to phosphorylated Ser727 on Stat1α located within the transactivation domain. Interestingly, blockade of Jak3 by PNU156804 inhibits the phosphorylation of a similarly conserved proline-Ser-proline (Pro-Ser-Pro) motif in Stat5a and Stat5b. In contrast to the conserved Ser in Stat1, Stat3, and Stat4, which is located within a Pro-X-Ser-Pro motif and MAPK consensus phosphorylation site, the Ser phosphorylation site of Stat5a/b lies within a Pro-Ser-Pro motif lacking the invariant amino acid. This evidence suggests that Stat5a/b Ser kinase represents a unique molecular target for regulating T-cell activity to produce selective immunosuppressive effects.

A recently published report found that PNU156804 inhibits IL-2–induced proliferation of human T cells without affecting the expression of IL-2Rα and γc chains. As shown herein, PNU156804 disrupts not only IL-2–dependent T-cell proliferation by selective inhibition of the γc-Jak3-Stat5a/b–dependent signaling pathway, it also disrupts IL-4, -7, and -15–dependent T-cell proliferation. Although PNU156804 diminished B-cell proliferation in response to killed *Staphylococcus aureus* antigens, however, it also inhibited CD40-triggered activation of NF-κB. These latter results suggest a role for Jak3 Tyr kinase activity in the CD40-initiated signaling pathway. Indeed, it is readily established that CD40 signaling is critical for B-cell growth, survival, differentiation, and immunoglobulin class switching, yet the CD40-CD40 ligand complex is not thought to recruit γc. Jak3 and Stat3 can be directly activated by the CD40 receptor, activation events necessary for the subsequent expression of CD23, intercellular adhesion molecule-1, and lymphotixin-α genes and for the production of immunoglobulin E. Another study reported that CD40 receptor activation by the CD40 ligand could also activate Jak3 and Stat5a in monocytes but not in B cells. It seems plausible to expect that PNU156804 may prove useful in determining whether Jak3 is an active participant in the CD40 signaling pathway.

Our current findings document that the blockade of Jak3 in vivo prevents allograft rejection (Figure 6). Furthermore, histologic examination of allografts from PNU156804-treated hosts showed reduced intragraft cellular infiltration of mononuclear cells without myocyte damage (Figure 7). Lack of clonal proliferation might have allowed the generation of a sufficient number of effector T cells—cytotoxic T cells and delayed-type hypersensitivity T cells—required to mediate allograft destruction. Whether the continuous delivery of PNU156804 would inhibit the CD40-Jak3 activation pathway, reducing B-cell responsiveness required for antibody synthesis, remains to be determined. Our in vivo results do, however, support the hypothesis that selective inhibition of the Jak3-Stat5 pathway, compared with Jak2-Stat5, is sufficient to block allograft rejection.

The combination of PNU156804 and CsA results in potent synergistic interaction (CI, 0.2-0.8), extending allograft survival beyond that produced by monotherapy with each drug alone (Figure 6). Similarly, CsA and RAPA act synergistically to prolong organ allograft survival in animal models and in humans. Rats treated intravenously with combinations of RAPA (0.04-0.8 mg/kg per day) and of CsA (0.5-2.0 mg/kg per day) displayed potent synergistic interactions on heart and kidney allograft survival, as documented by CI values of 0.001 to 0.2. We have shown in this model that even subtherapeutic CsA doses reduced the expression of IL-2 mRNA at the graft site, thereby facilitating inhibition by RAPA of IL-2–dependent growth T cells and producing synergistic interaction. A similar mechanism might explain the synergistic effects of CsA in combination with PNU156804 produced at all tested PNU156804-CsA ratios (1:1-64:1), as documented by CI values of 0.2 to 0.8. Optimal synergism was produced by a 4:1 PNU156804-CsA ratio (CI, 0.22). Oral delivery of CsA and RAPA resulted in synergism with an almost identical range of CI values between 0.1 and 0.6.

In contradiction, a combination of PNU156804 with RAPA yielded an additive effect (CI, 1). These results suggest that the
sequential inhibition of signal 1 by CsA, followed by the inhibition of signal 3 by PNU156804 or RAPA, produces synergism, whereas the concomitant inhibition of signal 3 by PNU156804 and RAPA results only in an additive immunosuppressive effect. However, given that RAPA produces myelosuppressive and lipotoxic side effects because of the role of mTOR in non-cytokine-activated pathways,22 we postulate that a combination of CsA and PNU156804 would produce similarly potent immunosuppression without myelosuppressive and lipotoxic side effects. We propose that because mTOR lies downstream of Jak3 in T, B, and NK cells, TCGF signaling pathways could be selectively inhibited by PNU156804 without affecting mTOR signaling pathways in non-Jak3-expressing cell types.

In conclusion, PNU156804 preferentially disrupts Jak3 (compared with Jak2 autokinase activity), thereby selectively inhibiting γ-driven T-cell clonal expansion. Blockade of Jak3 should produce no adverse effects currently associated with RAPA. Moreover, synergy between CsA (blocking G0-G1 transition) and PNU156804 (blocking G1-S progression) proffers a novel strategy for immunosuppression by blocking sequential activation signals.

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Selective inhibitor of Janus tyrosine kinase 3, PNU156804, prolongs allograft survival and acts synergistically with cyclosporine but additively with rapamycin

Stanislaw M. Stepkowski, Rebecca A. Erwin-Cohen, Fariba Behbod, Mou-Er Wang, Xienui Qu, Neelam Tejpal, Zsuzsanna S. Nagy, Barry D. Kahan and Robert A. Kirken