The Mannich Base NC1153 Promotes Long-Term Allograft Survival and Spares the Recipient from Multiple Toxicities

Stanislaw M. Stepkowski, Judy Kao, Mou-Er Wang, Neelam Tejpal, Hemangshu Podder, Lucrezia Furian, Jonathan Dimmock, Amitabh Jha, Umashankar Das, Barry D. Kahan and Robert A. Kirken

J Immunol 2005;175;4236-4246

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

The Mannich Base NC1153 Promotes Long-Term Allograft Survival and Spares the Recipient from Multiple Toxicities[1]

Stanislaw M. Stepkowski,* Judy Kao,† Mou-Er Wang,* Neelam Tejpal,* Hemangshu Podder,* Lucrezia Furian,* Jonathan Dimmock,‡ Amitabh Jha,‡ Umashankar Das,‡ Barry D. Kahan,* and Robert A. Kirken[2†]

JAK3 is a cytoplasmic tyrosine kinase with limited tissue expression but is readily found in activated T cells. Patients lacking JAK3 are immune compromised, suggesting that JAK3 represents a therapeutic target for immunosuppression. Herein, we show that a Mannich base, NC1153, blocked IL-2-induced activation of JAK3 and its downstream substrates STAT5a/b more effectively than activation of the closely related prolactin-induced JAK2 or TNF-α-driven NF-κB. In addition, NC1153 failed to inhibit several other enzymes, including growth factor receptor tyrosine kinases, Src family members, and serine/threonine protein kinases. Although NC1153 inhibited proliferation of normal human T cells challenged with IL-2, IL-4, or IL-7, it did not block T cells void of JAK3. In vivo, a 14-day oral therapy with NC1153 significantly extended survival of MHC/non-MHC mismatched rat kidney allografts, whereas a 90-day therapy induced transplantation tolerance (>200 days). Although NC1153 acted synergistically with cyclosporin A (CsA) to prolong allograft survival, it was not nephrotoxic, myelotoxic, or lipotoxic and did not increase CsA-induced nephrotoxicity. In contrast to CsA, NC1153 was not metabolized by cytochrome P450 3A4. Thus, NC1153 prolongs allograft survival without several toxic effects associated with current immunosuppressive drugs. ***The Journal of Immunology,*** **2005, 175: 4236–4246.**

Despite improvement in organ allograft survivals, the efficacy of modern immunosuppression is marred by a variety of side effects contributing to functional graft deterioration (1, 2). The main reason for drug-induced toxicities is the ubiquitous expression of the target molecules and the consequent widespread uncoupling of tissue homeostasis (3, 4). For example, the serine/threonine phosphatase calcineurin (CaN)[3] is expressed in a variety of cell types, including neurons, cardiac and skeletal muscles, and lymphoid tissue (5, 6). Consequently, immunosuppressants that target CaN, cyclosporin A (CsA) and tacrolimus, cause toxicities affecting renal, neural, and hepatic tissues (7, 8). Similarly, sirolimus (SRL) blocks mammalian target of rapamycin (mTOR), a molecule critical for controlling nutrient and growth factor-induced cell growth and differentiation (9, 10). Experimental models have shown a complex pharmacokinetic and pharmacodynamic interaction between SRL and CsA with potent therapeutic, but also toxic, synergism (11, 12). Detailed pharmacokinetic analysis have showed that SRL elevated CsA levels in kidneys and in other tissues more than in the bloodstream (13). Modeling in salt-depleted rats revealed that SRL worsened CsA-induced nephrotoxicity and vice versa; CsA increased two SRL-mediated toxicities: myelodepression and hypercholesterolemia (14). Double-blinded clinical trails confirmed improved therapeutic efficacy by SRL-CsA combinations to prevent allograft rejection (synergism) but also revealed their unique side effects, namely, thrombocytopenia, hypercholesterolemia, and hypertriglyceridemia (1). Indeed, targeting molecules exclusively expressed in lymphocytes should eliminate these toxicities, yet maintain therapeutic potency and synergism when used in combination with other agents.

Selective targets may be identified among molecules important for T cell activation. Full T cell activation requires the three sequential and threshold-limiting signals (15): signal 1 delivered by alloantigen engagement of a specific TCR followed by signal 2 delivered by a B7/CD28 interaction that induces production of IL-2 and other T cell growth factors (TCGF), such as IL-4, IL-7, IL-9, and IL-15 (16, 17). Both CsA and tacrolimus disrupt CaN phosphatase activity required for dephosphorylation of NFAT, leading to its translocation to the nucleus and binding to the discrete DNA binding elements of TCGFs (16, 17). Although signals 1 and 2 initiate production of TCGFs, signal 3 delivered through the TCGF-specific cytokine receptors promotes T cell clonal expansion via G_1-S transition (18). Many TCGFs share a common γ-chain (γ_c), which associates with an affinity-conferring α-chain for each cytokine and occasionally with a shared β-chain (IL-2 and IL-15) to deliver intracellular signals initiating T cell proliferation and differentiation (19). For example, IL-2 binding through the high-affinity IL-2Rα/IL-2Rβ/IL-2Rγ_c recruits and activates the IL-2Rβ-associated JAK1 and γ_c-associated JAK3 (20, 21). Autoactivation of these enzymes promotes Tyr phosphorylation of IL-2Rβ-chain, attracting other signaling elements, including STAT5a

*Division of Immunology and Organ Transplantation, Department of Surgery, University of Texas Medical School at Houston, Houston, TX 77030; †Department of Integrative Biology and Pharmacology, University of Texas Medical School at Houston, Houston, TX 77030; and ‡College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

Received for publication December 2, 2004. Accepted for publication July 13, 2005.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by grants from the National Institute of Diabetes and Digestive Kidney Diseases (38016-12), the National Institutes of Allergy and Infectious Diseases (AI39026 and AI053566), and the Canadian Institute of Health Recourses (NOP-53171).

[2] Address correspondence and reprint requests to Dr. Robert A. Kirken at the current address: Department of Biological Sciences, University of Texas at El Paso, 500 West University Avenue, El Paso, TX 79968-0001. E-mail address: rkirken@utep.edu

[3] Abbreviations used in this paper: CaN, calcineurin; CI, combination index; CsA, cyclosporin A; mTOR, mammalian target of rapamycin; GIC, graft-infiltrating cell; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MST, mean survival time; pAb, polyclonal Ab; PRL, prolactin; SRL, sirolimus; TCGF, T cell growth factor; γ_c, common γ-chain.

0022-1767/05/$02.00

and STAT5b (22). After docking through SH2 domains to selected receptor phosphotyrosines, STAT5a/b are tyrosine- and serine-phosphorylated and dissociate from their receptors (23). Formed dimers translocate to the nucleus, binding to the promoter sites on multiple genes that control cell growth and differentiation (24).

Given the aforementioned limitations of SRL, JAK3 has been explored as a unique target to block T cell signaling of TCGFs through the γ_c pathway (25–27). Indeed, JAK3 is primarily expressed in T, B, and NK cells and is critical for T cell development and function (28, 29). Humans or mice genetically deficient of JAK3 or γ_c manifest a SCID phenotype (30). Published work has already suggested (28, 29) that the JAK3-inactivating compounds derived from tyrphostin AG490 (25), prodigiosin PNU156804 (26), dimethoxyquinazoline JANEX-1 (31), and CP-690,550 (27) extend allograft survival. However, undesirable effects on JAK2, a closely related kinase that is widely used by many cell types, may impair the clinical application of JAK3 inhibitors lacking specificity. In fact, AG490 displayed similar effects on both kinases, whereas PNU156804 was slightly more effective toward JAK3 than JAK2 (25, 26). Similarly, although CP-690,550 caused no apparent metabolic abnormalities, CP-690,550 treatment of cynomolgus monkeys was associated with anemia, most likely related to JAK2 inhibition (27). Although efficacy of a new JAK3 inhibitor to prevent allograft rejection is paramount, drug selectivity to JAK3 over JAK2 may predetermine its improvement over standard immunosuppressants.

The present study examined the therapeutic efficacy and toxicity profiles of a putative immunosuppressant. A Mannich base compound, NC1153, preferentially inhibited JAK3 as opposed to several other kinases. More importantly, NC1153 alone prolonged kidney allograft survival and could induce transplantation tolerance. The combination of NC1153 with CsA displayed therapeutic synergism. However, NC1153 was neither nephrotoxic nor affected hematopoiesis and lipid metabolism. We also showed that NC1153 is not metabolized by the cytochrome P450 3A4 isoform, the primary metabolizing enzyme of CsA and SRL (32, 33). Therefore, we postulate that NC1153 may provide very unique clinical benefits for transplant patients that can be extended to other T cell-mediated diseases.

Materials and Methods

Cell culture and treatment

The rat T cell line Nb2-11c, developed by Dr. P. Gout (Department of Cancer Endocrinology, British Columbia Cancer Agency, Vancouver, Canada), was grown in RPMI 1640 with 10% FCS (catalog no. 1020-90; Serologicals), 2 mM L-glutamine, 5 mM HEPES, pH 7.3, and penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively), at 37°C-5% CO_2. Freshly explanted normal human lymphocytes purified by isocentrifugation (Ficoll; EM Science) were PHA activated for 72 h as previously described (34). Cells were rested and treated with varying concentrations of NC1153 as described in the figure legends. All cells were then stimulated, as described in the legends, with recombinant human IL-2 (Hoffman-LaRoche), TNF-α (PeproTech), or ovine prolactin (PRL) supplied by the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases. Cell pellets were frozen at −70°C. NC1153 was prepared as previously described (35).

Flow cytometry

T lymphocytes were purified via a negative T cell isolation kit (Dynal Biotech) and subsequently activated with PHA for 72 h and then treated without or with 50 μM NC1153 for 16 h; cells were then stained with BD Pharmingen reagents, including PE-labeled mouse anti-human IL-2Rα (catalog no. 555-432), PE-conjugated mouse anti-human IL2Rβ (catalog no. 554-525), and PE-labeled mouse anti-human γ_c (catalog no. 555-898) Abs. Cell staining and FACScan were performed as previously described (36).

Proliferation assays

Quiescent human T cells or Jurkat cells (5.0×10^4/well) were plated in flat-bottom, 96-well microtiter plates in 200 μl of quiescent medium containing RPMI 1640 and 1% FCS in the absence or presence of 1 nM IL-2, IL-4, or IL-7 or PRL. Next, cells were treated for 16 h with NC1153 and then pulsed for 4 h with [^{3}H]thymidine (0.5 μCi/200 μl) and harvested onto fiberglass filters and analyzed by liquid scintillation counting as previously described (34).

Solubilization of membrane proteins, immunoprecipitation, and Western blot analysis

Frozen cell pellets were thawed on ice and solubilized in a lysis buffer (10^8 cells/ml) as previously described (34). For human lymphocytes, supernatants were incubated rotating end over end for 2 h at 4°C with either 5 μl/ml polyclonal rabbit antisera raised against peptides derived from the unique COOH termini of JAK3 (aa 1104–1124), as well as JAK2 (catalog no. 06-255), phosphotyrosine (catalog no. 16-101), or Shc Ab (catalog no. 06-203; Upstate Biotechnology) and C termini of human STAT5a (aa 775–794) or STAT5b (aa 777–787) Ab (37). Proteins bound to Abs were captured by incubation for 30 min with protein A-Sepharose beads (Pharmacia Biotech), sedimented for purification, and eluted by boiling in SDS sample buffer (38). For phosphor-MAPK assays, 25 μg of total cell lysate were dissociated in SDS sample buffer and separated on 10% (all others on 7.5%) SDS-PAGE under reducing conditions. Proteins were transferred to polyvinylidene difluoride (catalog no. 1PVH 00010, Immobilon; Millipore) as reported (38). Western blot analysis was performed with polyclonal Ab (pAb) to phospho-p44/42 MAPK (catalog no. 9101; Cell Signaling Technology), and blots were reprobed with *pan*-ERK mAb (catalog no. E17120; BD Pharmingen) after diluting 1/1000 in blocking buffer (26).

EMSA

Nuclear extracts from cytokine-treated human T cells were isolated as reported earlier (37). For EMSA analyses (39), 1 μg of ^{32}P-labeled oligonucleotide corresponding to the β-casein promoter (5′-agatttctaggaat tcaatcc-3′) or NF-κB DNA binding element (5′-agttgaggggggactttccag-3′) was labeled with [^{32}P]dATP (fill-in reaction) and then incubated with nuclear extracted protein. In some experiments, a supershift was performed by preincubating 5 μg of nuclear extracted protein with 1 μl of either normal rabbit serum or specific rabbit antisera raised against peptides derived from the unique C termini of STAT5a or N terminus for STAT5a and STAT5b (Advantex Bioreagents) (26). Complexes were separated on polyacrylamide gels (5%), dried, and exposed to x-ray film (Kodak) as previously described (34).

Tyrosine kinase assay

JAK3 autokinase assays were performed by immunopurifying human JAK3 from T cells that were washed three times with lysis buffer followed by a single wash with kinase buffer containing 25 mM HEPES, pH 7.3, 0.1% Triton X-100, 100 mM NaCl, 10 mM $MgCl_2$, 3 mM $MnCl_2$, and 200 μM sodium orthovanadate. Isotope-free tyrosine kinase reactions were initiated by the addition of 100 μM unlabeled ATP and allowed to incubate at 37°C for 15 min in the presence of NC1153 (40). The reactions were quenched by washing the protein A-Sepharose beads with lysis buffer and eluting bound material by boiling in SDS-sample buffer for 4 min and resolved by 7.5% SDS-PAGE. Anti-phosphotyrosine immunoblotting was performed as described above. Quantitations of tyrosine-phosphorylated JAK3 and total JAK3 reblots were assessed using an Expression 633 scanner (Epson; Hewlett-Packard), and densitometry of bands was standardized against background. Normalized phosphorylation was calculated by dividing the absorbance ratio of phosphorylated-JAK3/total-JAK3 from vehicle or NC1153-treated samples. Analysis of NC1153 effects on other growth factor receptors, *Src* family members, and protein kinases A and C were performed according to the Upstate Biotechnology Cell Signaling Solutions and Kinase Profiler. All NC1153 inhibitory kinase reactions were initiated in the presence of 100 μM ATP and normalized to vehicle. Values are presented as the percentage of mean ($n = 2$) of control (without drug) and SD, which is reported as the range/$\sqrt{2}$.

Measurement of P450 activity

Effect of NC1153 on different P450 isoforms (3A4, 2D6, 2C19, 1A2, 2C8, and 2C9) were tested using the previously described method (41). Positive controls with selective substrates were used to monitor P450 3A4 (ketoconazole), P450 2D6 (quinidine), P450 2C19 (tranylcypromine), P450 1A2 (puratyline), P450 2C8 (quercetin), and P450 2C9 (sulfaphenazole) conversion to substrate. Pooled human microsomes (BD Biosciences) were

assayed with known P450-selective substrates and competition by escalating concentrations (−8 to −4 log M concentration) of NC1153. Data are plotted as percent inhibition of P450 isoform activity with a competitive substrate without or with NC1153 × 100.

Rat kidney and spleen transplants

ACI (WF; RT1^a) and Lewis (RT1^l) rats (160–200 g) obtained from Harlan Sprague Dawley were cared for according to the guidelines of the University of Texas Animal Welfare Committee. Rats were housed in light- and temperature-controlled quarters and given chow and water ad libitum. Orthotopic kidney transplantation was performed using a standard microsurgical technique of end-to-side anastomoses to recipient aorta and vena cava (39). The cold ischemia times were <30 min. Graft survival time was defined as the last survival day of bilaterally nephrectomized recipients. Recipients remained untreated or were treated with NC1153 alone by daily i.v. injections (2.5–20 mg/kg) for 7 days or by daily oral gavage for 7 days (20–160 mg/kg) or 14 days (40–240 mg/kg); combined therapy comprised 7-day oral gavage with NC1153 (20–160 mg/kg) and/or 3-day oral gavage with CsA (2.5–20 mg/kg). Some recipients were treated with 160 mg/kg NC1153 for 14 days and thereafter three times a week for up to 90 days with 160 or 240 mg/kg NC1153. The results, presented as mean survival time (MST) ± SD, were assessed for statistical significance by Gehan's survival test. In addition, the interaction between NC1153 and CsA was evaluated by the median effect analysis (42, 43). Combination index (CI) value of <1 suggests synergistic, >1 antagonistic, or =1 additive interactions (43). Spleen transplantation was performed by a previously described method (25). Irradiated (750 rad) Lewis rat spleen allografts were transplanted to ACI recipients that remained untreated or were treated with 160 mg/kg NC1153 for 7 days.

Histopathological evaluation

At day 7 posttransplant, kidney allografts derived from recipients receiving drug treatment were diced, and pieces were placed in Bouin's fixative (Poly Scientific R&D), sectioned, and stained with H&E as described earlier (44).

Toxicity study

After a 7-day conditioning period on low-salt chow, rats ($n = 5$ or 6) were randomly assigned to treatment for 28 days with p.o. 160 or 240 mg/kg NC1153 alone, 10 mg/kg CsA alone, 1.6 mg/kg SRL alone, or NC1153-CsA or CsA-SRL combinations. In addition, there was an untreated control group (six rats) fed a low-salt diet. At day 28, the animals were placed in metabolic cages for 24-h urine collections. Blood samples were used for serum hematocrit and hemoglobin, as well as creatinine, total cholesterol, and high-density lipoprotein (HDL)- and low-density lipoprotein (LDL)-cholesterol determinations. Creatinine clearance values were calculated based on urinary (milligrams per milliliter) and plasma creatinine concentrations (milligrams per milliliter). Results are presented as mean values ± SD, and statistical significance was compared; $p < 0.05$ was considered as significant.

The kidney sections, stained with progressive H&E, were evaluated using a semiquantitative five-grade scale for tubular and glomerular changes: 0, none; 1+, <5%; 2+, 6–25%; 3+, 26–50%; and 4+, >50%. A similar scale was used for vascular changes: 0, none; 1+, minimal; 2+, mild; 3+, moderate; and 4+, severe. The right femurs sectioned and stained with H&E were estimated as the percentage of the marrow space occupied by cellular as opposed to adipose tissue elements. The average number of megakaryocytes in four high-power fields was used to estimate platelet formation.

Results

Selection of NC1153 based on inhibition of TCGF-induced proliferation

Based on its role in T cell function and its limited tissue distribution, JAK3 represents a unique therapeutic target. Although we had identified two agents with JAK3 inhibitory activity (AG490 and PNU156804), we sought to identify inhibitors with greater selectivity (25, 26). Therefore, we expanded our search through the repository of the National Cancer Institute (NCI) Drug Discovery Database Program, which comprises thousands of small molecules (45). Agents that displayed a high correlation coefficient (>0.6) to our "seed" compound, tyrphostin AG490, were assessed for their ability to block proliferation of quiescent PHA-activated human lymphocytes in response to IL-2. Of nine selected compounds, NC1153 potently inhibited IL-2-induced T cell proliferation (Fig. 1*A*). To exclude any direct effects on the IL-2R, we found that NC1153 had no impact on the expression of all three IL-2 receptor chains (α, β, and γ_c; Fig. 1*B*) by FACS analysis. To determine whether NC1153 may preferentially target JAK3-containing lymphocytes, JAK3-deficient Jurkat T cells or JAK3-containing PHA-primed human PBLs made quiescent were pretreated with ascending concentrations of NC1153 in the presence of IL-2 and then assayed for [^{3}H]thymidine incorporation. NC1153 almost completely abolished uptake of radiolabeled thymidine at 10 μM (IC_{50} of ~2.5 μM) in contrast to its effect on Jurkat T cells void of JAK3 (Fig. 1*C*). To further support the notion that NC1153 events are not limited to IL-2, but may preferentially inhibit JAK3, the aforementioned human lymphocytes were similarly assayed in the presence of increasing concentration of NC1153 and stimulated with IL-4 or IL-7 γ_c cytokines. Indeed, [^{3}H]thymidine incorporation was reduced in a dose-dependent manner by NC1153 (0.01–25 μM) regardless of the JAK3-activating cytokine used (Fig. 1*D*). IL-2 stimulation of other responsive cell lines (e.g., YT, Nb2, and CTLL-2) was similarly inhibited compared with negatively isolated human T cells (data not shown).

NC1153 disrupts IL-2-induced autophosphorylation of JAK3 and its substrates

Previous work has documented that IL-2, IL-4, or IL-13 stimulated the catalytic activity of JAK3 with unlabeled ATP and anti-phosphotyrosine immunoblotting (38, 46, 47), with these sites having been mapped to Tyr^{980} and Tyr^{981} (48). To validate that JAK3 is a viable target for NC1153, immunopurified JAK3 enzyme was isolated from PHA-activated PBLs that had been challenged with IL-2 (100 nM) for 1 min. Next, JAK3 was exposed to ascending concentrations of NC1153 (0–50 μM) and 100 μM unlabeled ATP. When reactions were stopped by addition of SDS-PAGE sample buffer, the experimental samples were probed for Tyr phosphorylation (*top blot*, Fig. 2*A*) and then reprobed for total JAK3 protein (*bottom blot*, Fig. 2*A*). Following densitometric analysis of JAK3 Tyr phosphorylation normalized to the total protein, a 50% reduction in IL-2-mediated kinase activity was observed at ≈2.5–5.0 μM (Fig. 2*A*) (49, 50). These results suggest that blockade of TCGF-driven proliferation in the presence of NC1153 (Fig. 1) could be mediated in part via JAK3.

To monitor identical events in intact cells, IL-2-responsive YT cells were cultured with ascending concentrations of NC1153 (0–100 μM) for 3 h and challenged with 100 nM IL-2. Next, cell lysates were immunoprecipitated with Abs for JAK3, STAT5a, or STAT5b and Western blotted with anti-phosphotyrosine mAb (Fig. 2*B*). NC1153 caused a concentration-dependent loss of Tyr phosphorylation of JAK3 with similar IC_{50} of 2.5 μM (Fig. 2*B*, *top panel*). Because catalytically active JAK3 is required for IL-2-driven Tyr phosphorylation of STAT5a/b (38), both substrates STAT5a (Fig. 2*B*, *middle panel*) and STAT5b (Fig. 2*B*, *bottom panel*) also showed reduced Tyr phosphorylation in IL-2-stimulated YT cells cultured with NC1153. The same blots were reblotted with Abs for total JAK3, STAT5a, or STAT5b protein to verify equal loading (Fig. 2*B*). In addition, IL-2-engaged T cells also initiate activation of the Shc/Ras/Raf/MAPK pathway via the adapter protein Shc, which binds to phosphorylated Tyr^{338} of the IL-2R β-chain (51, 52). To evaluate whether NC1153 disrupts this event, YT cells were treated with vehicle alone or with ascending concentrations of NC1153 and examined for Shc activation. Total cell lysates immunoprecipitated with anti-Shc pAb were

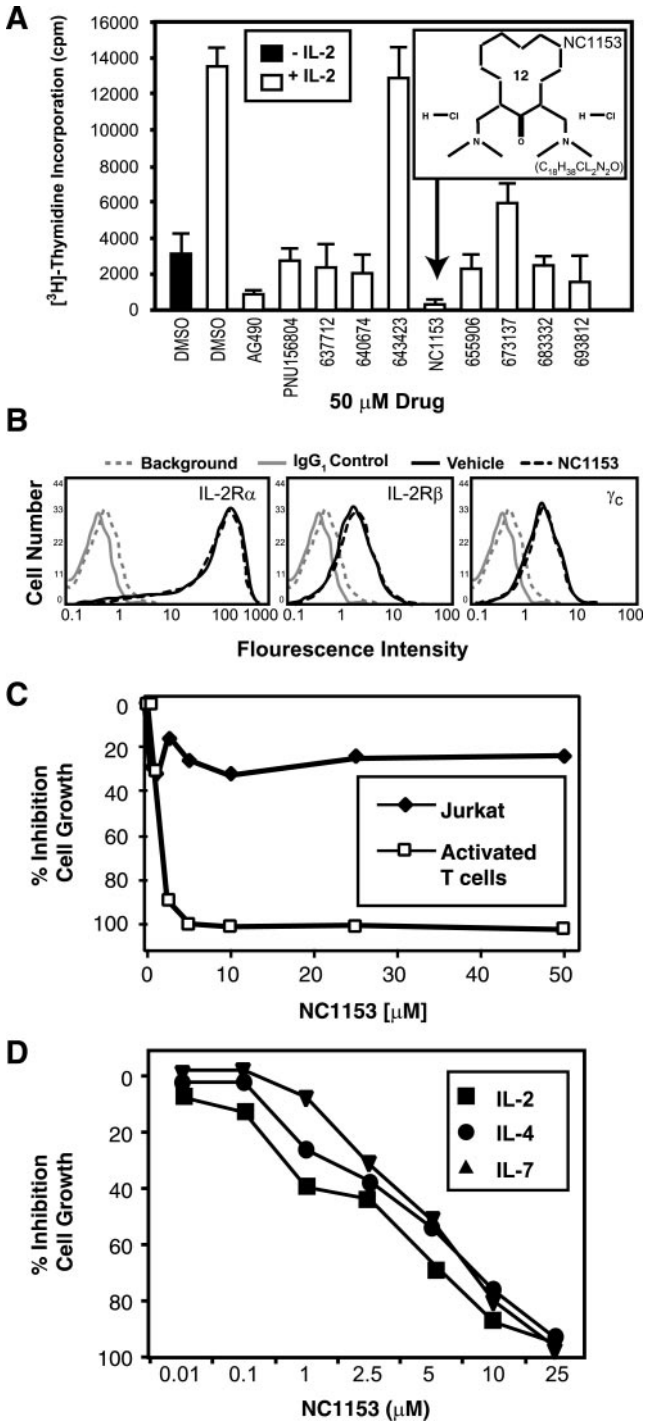


FIGURE 1. NC1153 inhibits γ_c-induced proliferation of T cells. *A*, Human PHA-activated PBLs (5.0×10^4 cells/well) were rested overnight and rechallenged with IL-2 (1 nM) without or with 50 μM NCI compounds (ordinate) or vehicle (DMSO) for 16 h at 37°C. Cells were then pulsed with [^{3}H]thymidine (0.5 μCi/200 μl) for 4 h, with incorporated radiolabeled probe plotted on the abscissa. *Inset*, Structure and chemical composition of NC1153. *B*, PHA-activated PBL were treated with 50 μM NC1153 as in *A*, and expressions of IL-2Rα, IL-2Rβ, and γ_c were measured using FACS. *C*, Jurkat T cells or PHA-activated human PBLs were challenged in the presence of 1 nM IL-2 with ascending concentrations of NC1153. *D*, PHA-activated human PBLs were stimulated in the presence of 1 nM IL-2, IL4, or IL7 with ascending concentrations of NC1153. DNA synthesis in *C* and *D* was measured as described in *A*. Uptake of radiolabeled probe is plotted on the abscissa and expressed as "% inhibition" of vehicle alone-treated cells in the presence of corresponding cytokine. Each point was run in triplicate.

separated on 10% SDS-PAGE and Western blotted with anti-phosphotyrosine Abs that likely recognize activated Tyr^{239}, Tyr^{240}, and Tyr^{317} sites on this adapter molecule (53, 54). As shown in Fig. 2*C*, Tyr phosphorylation of Shc was inhibited in a dose-dependent manner (*lanes f–i*). To examine ERK1/2 that are downstream counterparts of Shc, the same cell lysates were separated on 10% SDS-PAGE and blotted with phospho-ERK1/2 pAbs that recognize phosphoactivated The^{202} and Tyr^{204} sites on both enzymes. Activation of ERK1 and ERK2 was inhibited at 5–10 μM NC1153 (*lanes f–i*). Because two additional experiments confirmed the loss of active Shc and ERK1/2 proteins observed at similar NC1153 concentrations and reblotting with total Shc and ERK1/2 protein verified equivalent loading, NC1153 blocks IL-2-driven Shc and ERK1/2 (p44/42) activity. Thus, NC1153 subsequently disrupts MAPK activation in intact IL-2-activated T cells.

NC1153 fails to block activation of other effector molecules

To further demonstrate selectivity, we assessed NC1153 for its ability to selectively inhibit JAK3/STAT5 activation compared with multiple non-JAK3 signaling molecules. Because JAK-regulated STAT5a/b Tyr/Ser phosphorylation is required for dimerization, nuclear translocation, and gene transcription of STAT5a/b (55), we tested whether NC1153 ablates IL-2-induced STAT5/DNA binding activity (Fig. 3*A*, *top panel*). Protein nuclear extracts (5 μg/well) from 10 to 100 μM NC1153-treated PHA-activated T cells were mixed with a ^{32}P-labeled β-casein probe corresponding to the STAT5a/b DNA binding element. In contrast to untreated cells, equivalent amounts of protein (5 μg/well) obtained from NC1153-treated T cells displayed greatly reduced STAT5a/b DNA-binding efficiency (Fig. 3*A*, *lanes c–f*). Furthermore, the STAT5-DNA binding complexes were verified by super shifting with STAT5a/b pAb (not shown). Because STAT5a/b is critical for IL-2-mediated cell cycle progression (18), we conclude that the loss of IL-2-inducible T cell proliferation is caused, at least in part, by the disruption of STAT5a/b activation. The same cells were stimulated with TNF-α (50 nM) for 10 min to monitor NF-κB activation (Fig. 3*A*, *bottom panel*). TNF-α induced binding of p50/p65 NF-κB components to a ^{32}P-labeled probe, which was not affected even at 100 μM NC1153 (*lanes c–f*) compared with untreated controls (*lanes a* and *b*). These experiments confirmed that NC1153 selectively blocks IL-2-mediated DNA binding of STAT5a/b without affecting DNA binding of NF-κB activated by TNF-α. Thus, from the NCI screen, NC1153 inhibited γ_c-cytokine signaling, whereas the remaining NCI compounds failed to display this high degree of selectivity.

Next, we examined NC1153 effects on JAK2, the closest homologue of JAK3 (50% identity), which is critical for hemopoietic development. Indeed, JAK2 is recruited by receptors for erythropoietin, CSF, G-CSF, and thrombopoietin (56–58). A recent study revealed that a JAK3 inhibitor (CP-690,550) blocked in vitro JAK2 activity at low concentrations (20–325 nM) but in vivo caused anemia in cynomolgus monkeys, as documented by decreased blood hematocrit and hemoglobin levels (27). To exclude similar effects of NC1153, we used the rat T cell line (Nb2-11c), which responds to PRL that activates JAK2 (59). Nb2-11c cells were treated with ascending concentrations of NC1153 (1–100 μM) and then challenged with PRL for 10 min. As shown from representative data in Fig. 3*B*, 100 μM NC1153 failed to significantly affect Tyr phosphorylation of JAK2. A similar study using the IL-3-dependent pro-B cell line Ba/F3 revealed similar results with NC1153, failing to affect IL-3 activation of JAK2 and STAT5 (data not shown). Nonetheless, NC1153-mediated blockade of JAK3 vs JAK2 normalized data suggests NC1153 preferentially

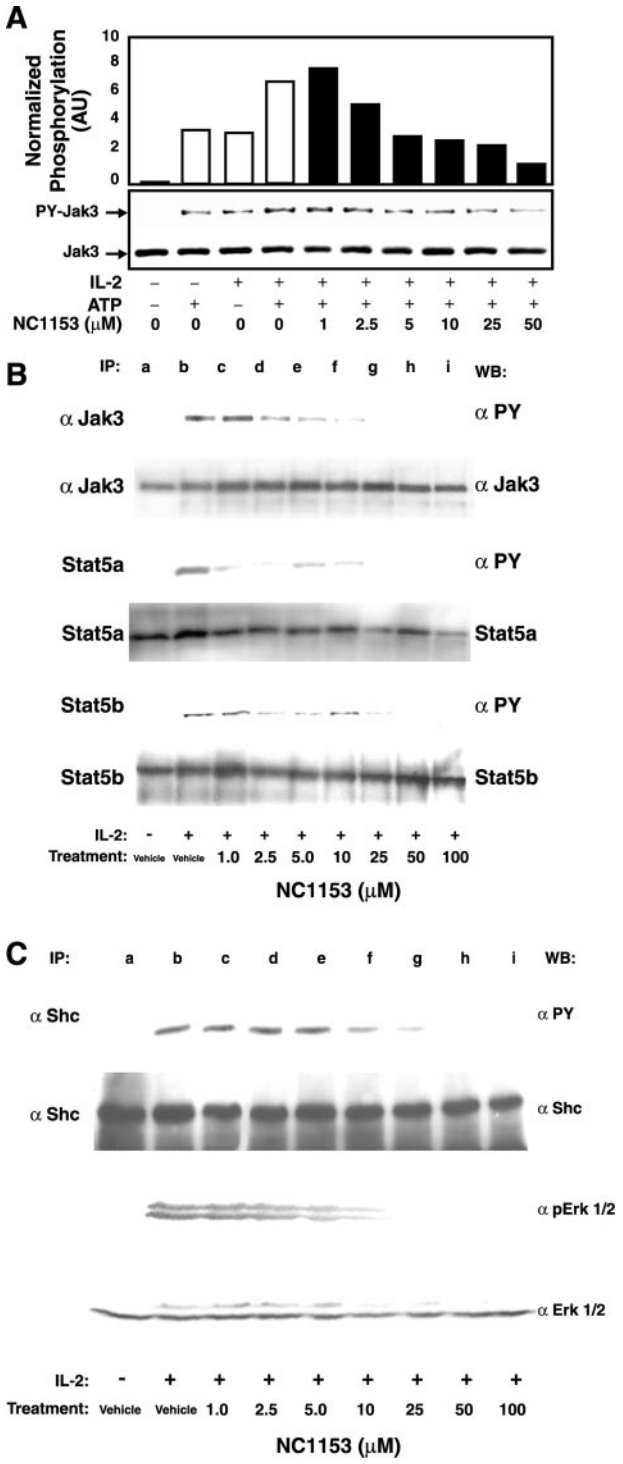


FIGURE 2. NC1153 inhibits IL-2-induced activation of JAK3 and its substrates. *A*, Anti-phosphotyrosine (PY) immunoblot of PHA-activated T cells stimulated for 10 min with (+) or without (−) 100 nM IL2 for 1 min and then immunoprecipitated with anti-JAK3 Abs. Next, immunopurified JAK3 was directly treated for 15 min on ice with ascending concentrations of NC1153 (0–50 μM). The mixture was then incubated for 20 min at 37°C in the absence (−) or presence (+) of 100 μM unlabeled ATP in kinase buffer, and reaction was allowed to proceed. JAK3 was separated on 7.5% SDS-PAGE, transferred to membrane, and blotted with anti-PY Ab (αPY). Anti-JAK3 immunoblot (αJAK3; *top panel*) verified equivalent loading of enzyme. Densitometric analysis of Tyr phosphorylated JAK3 was normalized against total JAK3 obtained from the blots is shown in the *top panel* as normalized Tyr phosphorylation in arbitrary units (AU) plotted on the abscissa, whereas treatments are indicated on the ordinate. *B*, YT cells challenged without (−) or with (+) IL-2 (10 min) in the presence of different concentrations (0–100 μM) of NC1153; lysates were then immunoprecipitated (IP) with Abs to JAK3, STAT5a, or STAT5b and Western blotted (WB) with anti-phosphotyrosine (αPY) mAb. All blots were reprobed with corresponding Ab to verify equal protein loading. *C*, YT cells challenged without (−) or with (+) IL-2 (10 min) in the presence of ascending concentrations (0–100 μM) of NC1153 were immunoprecipitated with anti-Shc Abs and Western blotted with anti-phosphotyrosine Ab, whereas total cell lysates (25 μg/lane) were blotted with phospho-ERK1/2 Ab. Both blots were reprobed for total protein (indicated beneath respective phosphoblots) to verify equivalent protein loading.

inhibits JAK3 (Fig. 3*C*). Finally, selectivity of NC1153 was further documented by using assays for multiple kinases (Fig. 3*D*). Indeed, 50 μM NC1153 failed to affect several kinases, including growth factor receptor Tyr kinases (fibroblast growth factor receptor 3 and platelet-derived growth factor receptor α), *src* family members (Src, Fyn, Lck, Yes, and Zap70), and serine/threonine protein kinases (protein kinase Cα and protein kinase A). These results suggest that NC1153 selectively inhibits γ_c-cytokines driven by activation of JAK3, without affecting several related signaling molecules.

In vivo NC1153 prolongs kidney allograft survival and is synergistic with CsA

Untreated ACI (RT1^a) recipients rejected Lewis (RT1^l) kidney allografts at a MST of 8.8 ± 0.5 days (Fig. 4*A*). In contrast, a dose-dependent extension in survival of the MHC/non-MHC-mismatched allografts was observed after a 7-day therapy. In particular, orally delivered NC1153 doses (20–160 mg/kg) produced similar results as i.v. delivered 8-fold lower NC1153 doses (2.5–20 mg/kg). For example, because an oral dose of 80 mg/kg or an i.v. dose of 10 mg/kg extended the survival to 18.8 ± 1.1 days and 18.6 ± 5.3 days, respectively, oral bioavailability was calculated at 12.5% (Fig. 4*A*). Similarly, a 14-day oral course with 40–240 mg/kg NC1153 produced dose-dependent effects with a 240 mg/kg dose producing a MST of 50.6 ± 14.3 days (Fig. 4*B*). When a 14-day daily course of 160 mg/kg NC1153 was extended with three times per week treatments for up to 90 days, most of recipients (75%) displayed graft survivals beyond 200 days. An additional extended 90-day therapy with 240 mg/kg produced long-term acceptance of kidney allografts in all recipients (Fig. 4*C*). Because long-surviving recipients accepted donor-type *LEW* (>100 days; $n = 3$) and rejected third-party Buffalo (RT1^b) heart allografts, these results suggest development of transplantation tolerance.

To examine a pharmacological interaction between NC1153 and CsA, recipients of kidney allografts were treated for 3 days with CsA (2.5–20 mg/kg) and for 7 days with NC1153 (20–160 mg/kg; Fig. 4*D*). Different ratios of CsA and NC1153 were used to determine the most effective dosing as evaluated by the median effect analysis to yield the CI values (CI <1 indicates synergy; CI = 1, additively; and CI >1, antagonism). Overall, the combination of NC1153 and CsA displayed potent synergism with CI values of 0.3–0.5. The lowest CI value occurred at NC1153-to-CsA dose ratio of 2:1 (CI = 0.3) and the highest at 16:1 (CI = 0.51). These results demonstrate that NC115 blocks allograft rejection, induces transplantation tolerance, and is synergistic with CsA to prolong allograft survival.

We also examined the mechanism of in vivo inhibition using a model of spleen allograft transplantation (25). Because untreated ACI rats acutely rejected irradiated *LEW* spleen allografts within 10 days, we could harvest a large number (≈50 × 10^6) of highly sensitized graft-infiltrating cells (GICs) on day 7 postgrafting (Fig.

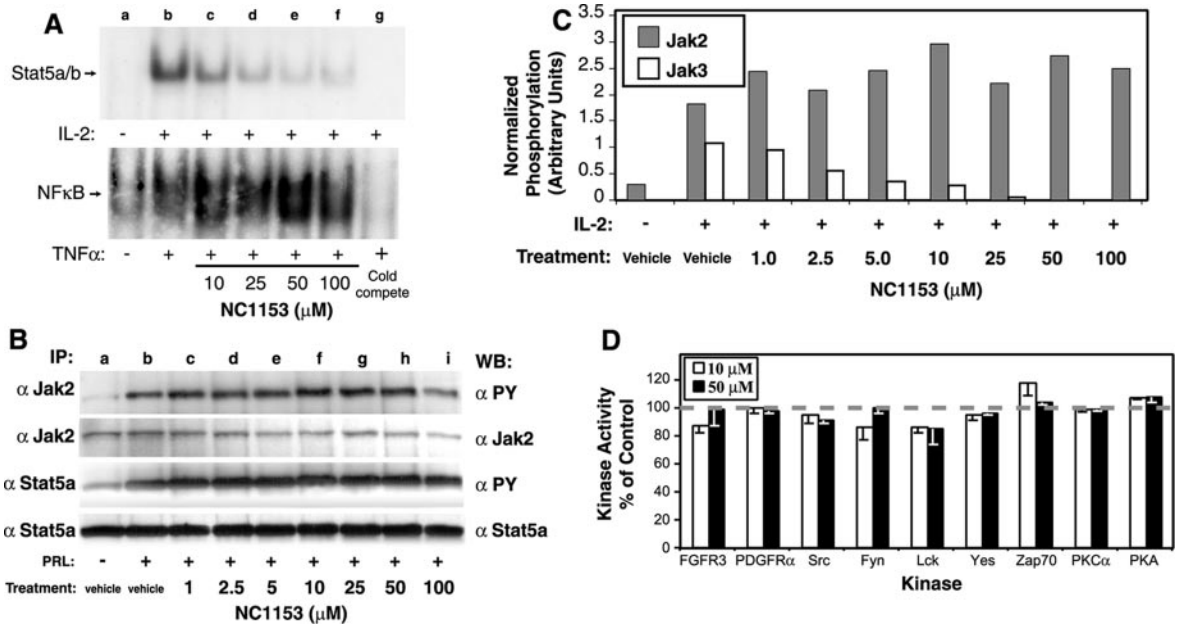
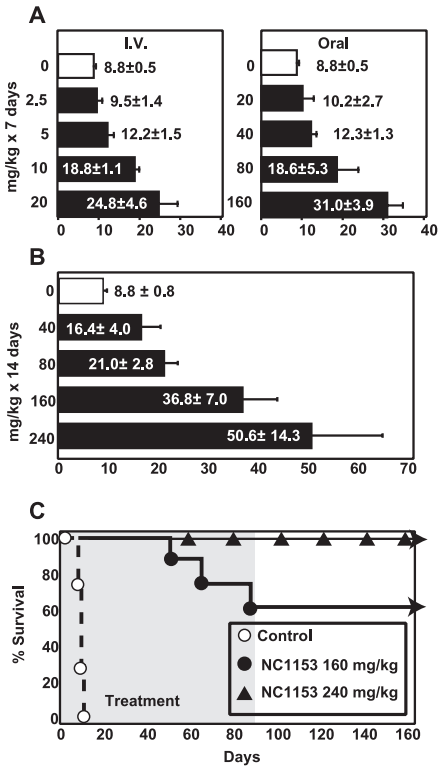
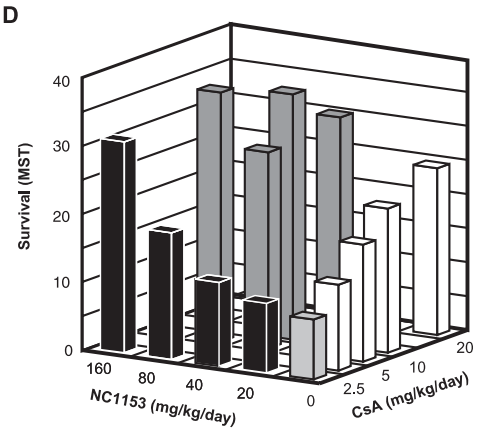


FIGURE 3. NC1153 fails to inhibit several effector molecules. *A*, Quiescent human PHA-stimulated PBLs were pretreated with ascending concentrations of NC1153 (0–100 μM) for 2.5 h and then stimulated without (−) or with (+) either 100 nM IL-2 (*top panel*) or 50 nM TNF-α (*bottom panel*). Next, nuclear extracts corresponding to 5 μg of protein were incubated with ^{32}P-labeled oligonucleotide probe corresponding to the PRL-response element of the β-casein gene promoter (*top panel*, *lanes a–f*) or NF-κB DNA binding element (*bottom panel*, *lanes a–f*) or appropriate cold competing probe (*lane g*) and subjected to EMSA analysis. Migrational location STAT5a/b DNA complexes or p50/p65 complexes are indicated by arrow on the *left*. *B*, Nb2 cells were pretreated with ascending concentrations of NC1153, as described in Fig. 2, and stimulated without (−; *lane a*) or with (+) 100 nM PRL (*lanes b–i*) for 10 min at 37°C. Cell lysates were immunoprecipitated (IP) with JAK2 or STAT5a Abs (as depicted) and Western blotted (WB) with anti-phosphotyrosine (αPY) Ab. The same blots were stripped and Western blotted with anti-JAK2 or STAT5a Ab to confirm equal protein loading. *C*, Densitometric analysis of JAK3 (Fig. 2*B*) or JAK2 (Fig. 3*B*) phosphorylated/nonphosphorylated with normalized data plotted on the abscissa for each concentration of NC1153 or vehicle (ordinate). *D*, Various kinases were pretreated with 10 or 50 μM NC1153 and subjected to kinase activity assays as described in *Materials and Methods*. Each kinase (ordinate) was measured for catalytic activity plotted as the percentage of control indicated on the abscissa.

5*A*). Treatment with 160 mg/kg NC1153 prevented rejection, as documented by reduced number of GICs (≈20 × 10^6). When equivalent numbers of GICs (50 × 10^6) were challenged for 10 min with IL-2, untreated recipient cells showed activation and nuclear translocation of STAT5 (Fig. 5*B, lane d*) that was inhibited (Fig. 5*B, lane i*) within NC1153-treated recipients (Fig. 5*B*). Thus, inhibition of signal 3 signaling by NC1153 may reduce clonal expansion of alloreactive T cells and their consequent activities.





Treatment mg/kg/d NC1153	CsA	Ratio	MST ± SD	CI
20	10	2:1	33.6 ± 10.04	0.30
40	5	8:1	28.8 ± 9.87	0.49
40	10	4:1	36.0 ± 10.05	0.36
80	5	16:1	36.6 ± 4.72	0.51

FIGURE 4. In vivo effects of NC1153 on kidney allograft survival. *A*, ACI recipients of Lewis kidney allografts were treated for 7 days with different doses of NC1153 delivered by i.v. or by oral gavage. *B*, ACI recipients of Lewis kidney allografts were treated for 14 days with different doses of NC1153 delivered by oral gavage. *C*, ACI recipients of Lewis kidney allografts were treated for 14 days with 160 mg/kg NC1153 and thereafter three times a week for up to 90 days with 160 or 240 mg/kg NC1153. *D*, ACI recipients of Lewis kidney allografts were treated by oral gavage for 7 days with different doses of NC1153 and/or CsA. d, Day. For additional information, see *Materials and Methods*.

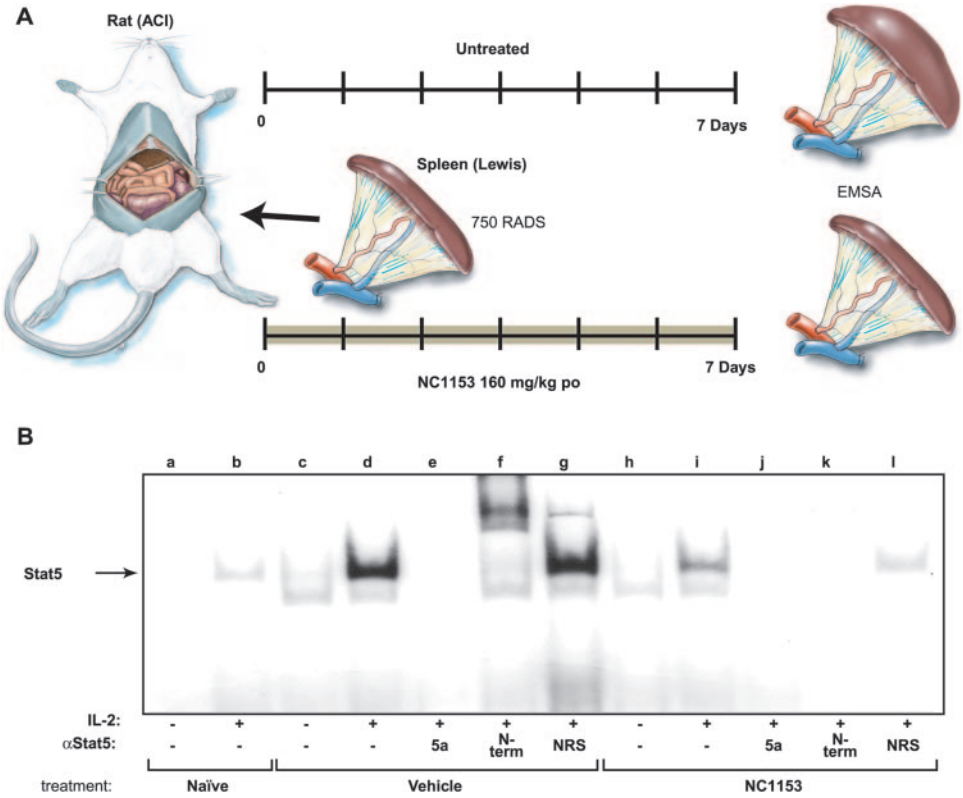


FIGURE 5. NC1153 blocks IL-2-inducible STAT5 DNA binding in vivo of splenocytes isolated from Lewis to ACI spleen transplants. *A*, Schematic representation of Lewis to ACI irradiated spleen transplants treated with vehicle (*top*) or 7-day course with NC1153 (*bottom*). *B*, Spleens were harvested on day 7 from vehicle- or NC1153-treated animals, and splenocytes were isolated, resuspended in medium (RPMI 1640), and then stimulated for 10 min at 37°C in the absence (−) or presence (+) of 100 nM IL-2. Nuclear extracts were then prepared for naive (*lanes a* and *b*), vehicle-treated (*lanes c* and *g*), or NC1153-treated (160 mg/kg p.o.) animals (*lanes h–l*) and incubated with a ^{32}P-labeled probe corresponding to the β-casein gene promoter (*lanes a–l*). STAT5 complexes were supershifted with antisera specific to dimerize the hetero-STAT5a/b complex to STAT5a (*lanes e* and *j*) or STAT5a/b N terminus (N-term; *lanes f* and *k*) or normal rabbit serum (NRS; *lanes g* and *l*). Migrational location of nonsupershifted (without Ab) STAT5a/b DNA complexes are indicated by an arrow on the *left*. A representative gel from each sample treatment set is shown ($n = 3$).

NC1153 does not display nephrotoxicity, myelosuppression, and lipotoxicity

We have evaluated the potential toxicities produced by NC1153 alone compared with those produced by CsA or SRL alone. In addition, the potential interactive toxicities between the NC1153-CsA vs SRL-CsA combinations also were evaluated. Our previous work in the salt-depleted rats revealed that SRL alone caused myelosuppression and lipotoxicity, whereas the SRL/CsA combination significantly aggravated CsA-induced nephrotoxicity (14). Our present results documented that a 28-day daily oral administration of NC1153 (160 or 240 mg/kg) did not produce renal dysfunction or lipotoxicity (Fig. 6*A*, *a–f*). Both serum creatinine and creatinine clearance confirmed that the addition of SRL to CsA significantly increased CsA-induced nephrotoxicity (Fig. 6*A*, *a* and *b*). In contradistinction, addition of 160 or 240 mg/kg NC1153 to the same CsA protocol had no impact on CsA-induced nephrotoxicity (Fig. 6*A*, *a* and *b*). As shown in Fig. 6*B*, these results were confirmed by histological examination; NC1153 (*a*) or SRL (*b*) monotherapy caused no changes in kidneys, whereas CsA alone caused increased glomerular cellularity accompanied by modest (<25%) thickening of vessels, focal tubular dilation, and significantly increased interstitial fibrosis (*c*). A combination of SRL-CsA caused more pronounced damage, with severe tubular damage and thickening of the walls of small arterioles (>75%) and perivascular infiltrates than that observed in the CsA group (Fig. 6*Bd*). In contrast, rats exposed to combined NC1153-CsA therapy displayed identical changes to those observed in CsA alone group (Fig. 6*Be*). Furthermore, although NC1153 did not affect lipid metabolism, SRL increased levels of total cholesterol (Fig. 6*Ac*), serum LDL-cholesterol (Fig. 6*Ae*), and serum HDL-cholesterol (Fig. 6*Af*). Therapy with CsA, SRL, NC1153, CsA-SRL, or CsA-NC1153 had no effect on blood hematocrit (Fig. 6*Ag*) and hemoglobin (Fig. 6*Ah*) levels. Furthermore, although NC1153 alone did not alter femoral bone marrow cellularity (Fig. 6*Ca*), SRL alone produced mild myelosuppression (Fig. 6*Cb*), as evidenced by histological examination. Again, two-drug CsA-SRL therapy significantly worsened cellularity (Fig. 6*Cd*), whereas NC1153-CsA combination showed no changes (Fig. 6*Ce*). Thus, targeting of JAK3 via NC1153 has no traditional side effects produced by current immunosuppressants and does not potentiate toxicities associated with CsA when used in combination. Most importantly, NC1153 produces no effects on hematopoiesis, supporting the notion for lack of in vivo JAK2 inhibition.

Oral bioavailability and intestinal drug absorption is often modified by drug-metabolizing enzymes (60). The most prevalent metabolizing enzyme for CsA and SRL is cytochrome P450 3A4 (3). Therefore, we examined whether activity of P450 3A4 and other P450 isomers modify NC1153 (Fig. 7). Our results showed that NC1153 did not affect the metabolic activity of four cytochrome P450 isomers for their well-characterized control substrate (3A4, 1A2, 2C8, and 2C9; Fig. 7, *A–D*), but did compete for substrate of two other P450 isomers (2D6 and 2C19; Fig. 7, *E* and *F*). These results indicate that NC1153 is not competing with CsA for the P450 3A4 enzyme. Overall, our results suggest that NC1153 is not

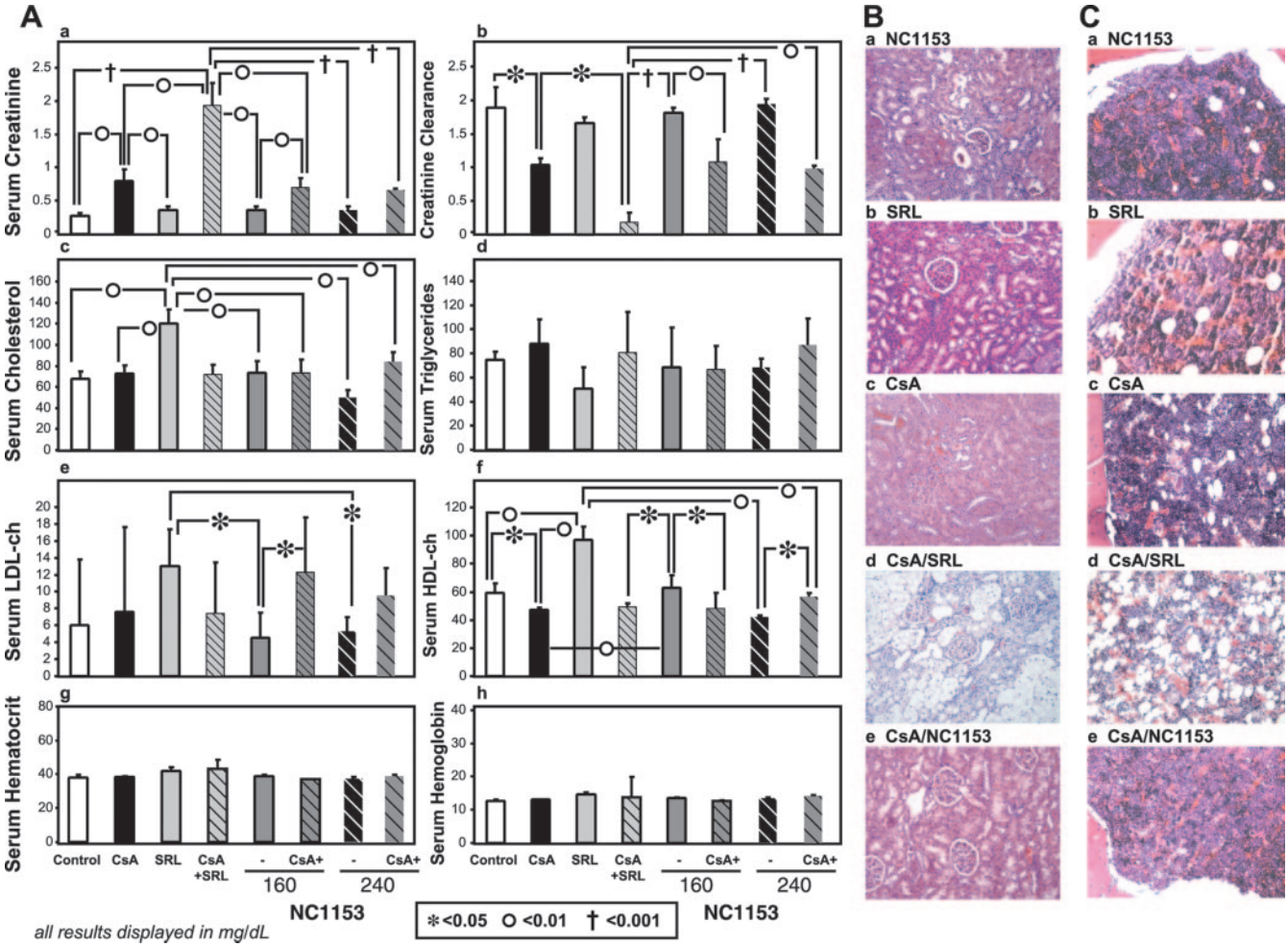


FIGURE 6. NC1153 lacks in vivo toxicities displayed by CsA or SRL. *A*, Rats were fed a low-salt diet 7 days before therapy and during 28-day therapy with 160 or 240 mg/kg NC1153 alone, 10 mg/kg CsA alone, 1.6 mg/kg SRL alone, or at the same doses of CsA/SRL or CsA/NC1153 combinations; following 24-h urine collection, blood samples were collected at day 28; serum creatinine and creatinine clearances, as well as serum cholesterol, triglycerides, LDL-cholesterol (LDL-ch), and HDL-cholesterol (HDL-ch) were measured as plotted on the abscissa. Kidneys (*B*) and femoral bones (*C*) harvested at day 28 from therapeutic groups were H&E stained and evaluated for morphology. For additional details, see *Materials and Methods*.

toxic compared with CsA or SRL and is metabolically cleared via a distinct cytochrome P450 enzymes.

Discussion

One fundamental problem with current immunosuppressants is the promiscuous expression of their target molecules. JAK3 has the distinct advantage of being almost exclusively expressed in T, B, and NK cells. Our present results demonstrate that a Mannich base, NC1153, inhibits TCGF-induced T cell growth (IC_{50} ~2.5 μM; Fig. 1*C*) likely by selectively disrupting at least JAK3-dependent effector molecules (Fig. 2). Consequently, NC1153 blocks activation of JAK3 substrates, including STAT5a and STAT5b, as assessed by phosphotyrosine Western blots (Fig. 2*B*) and their DNA binding activity (Fig. 3*A*), but also their subsequent downstream effectors, Shc, Ser/Thr kinases, p44/ERK1, and p42/ERK2 (Fig. 2*C*). Although NC1153 was equally effective in blocking T cell proliferation by IL-2, IL-4, or IL-7 (Fig. 1*C*), the drug did not appear to inhibit several effectors, including NF-κB and several distinct kinases (Fig. 3).

In vivo therapy with NC1153 reduced GICs' response to IL-2-dependent signals (Fig. 5*B*). Extended treatment with NC1153 alone resulted in permanent acceptance of kidney allografts (Fig. 4*C*), whereas a short-term combination of NC1153/CsA therapy produced synergistic effects (CI = 0.3–0.5; Fig. 4*D*). Thus, NC1153 may represent a new class of selective JAK3 inhibitors compared with AG490 (25), PNU156804 (26), or CP-690,550 (27) and others. Our in vivo results documented that NC1153 uncouples T cell activity without causing major toxic effects over this measured time period that include nephrotoxicity, myelodepression, and lipotoxicity (Fig. 6).

Presently used CaN (CsA and FK506) and mTOR (SRL and everolimus) inhibitors target molecules that are ubiquitously expressed, thereby producing many side effects. Indeed, CaN inhibitors produce nephrotoxicity, neurotoxicity, and diabetogenicity (3, 4). Although the exact mechanism of CsA-induced nephrotoxicity is not fully understood, multiple components show significant changes such as increased vascular resistance, causing reduced renal blood flow (61, 62), elevated reactive free radicals causing oxidative stress (63, 64), as well as up-regulated expression of TGF-β (65), vasoconstrictive stimuli, angiotensin II receptors (66), and NO synthases (67), all potential contributors to the kidney malfunction. Furthermore, CsA was reported to promote Fas-mediated apoptosis of cultured renal tubular cells in vitro (68), an effect that was blocked by peptide inhibitors of caspases 3, 8, and

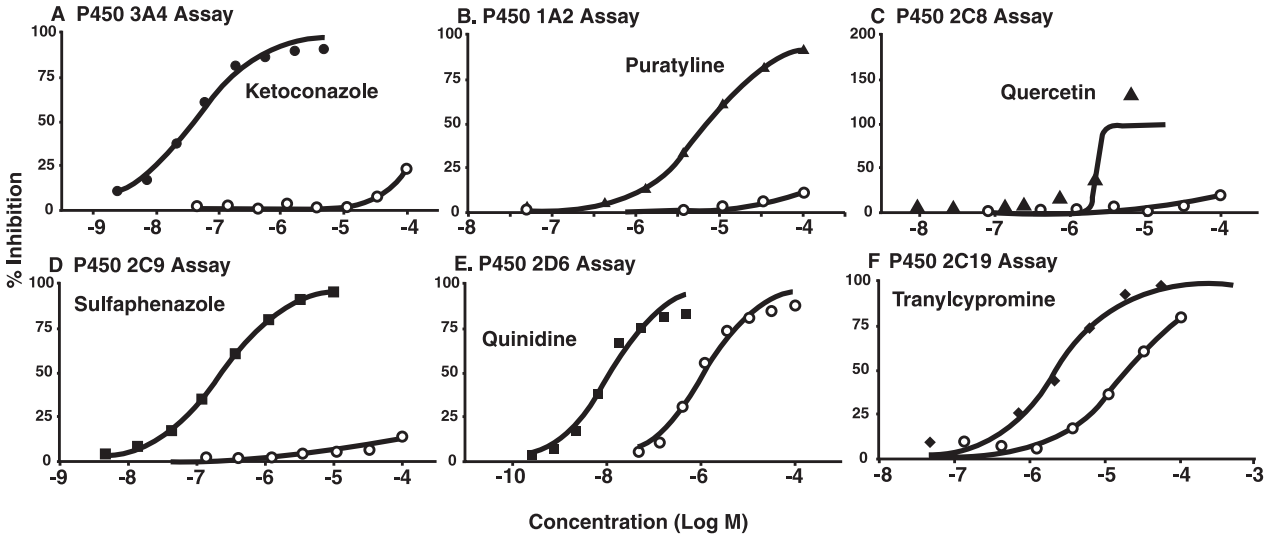


FIGURE 7. NC1153 does not affect activity of P450 3A4 enzyme. Effects of NC1153 on the metabolism of known substrates by their corresponding P450 enzymes, including 3A4 (*A*), 1A2 (*B*), 2C8 (*C*), 2C19 (*D*), 2D6 (*E*), and 2C9 (*F*), were measured in human microsomes with different concentrations of NC1153 (−8 to −4 log M) plotted on the ordinate. Selective blockades of P450 3A4 (ketoconazole), 1A2 (puratyline), 2C8 (quercetin), 2C19 (tranylcypromine), 2D6 (quinidine), and 2C9 (sulfaphenazole) were monitored for their ability to compete for the metabolic conversion of measurable substrate to product in the presence of the possible competitive substrate, NC1153. The percent inhibition in metabolic conversion of substrate to product for each P450 enzymes in the absence (●) or presence (○) of NC1153 is plotted on the abscissa. For additional details, see *Materials and Methods*.

9 (69). Thus, vasoconstriction and increased apoptosis are likely responsible for CsA-induced nephrotoxicity. Moreover, mTOR inhibition by SRL ablates cytokine-mediated growth not only in T and B cells but also in many other cells, resulting in myelosuppression and hyperlipidemias (12, 14, 70, 71).

Simultaneous disruption of two distinct T cell signaling pathways holds the potential for synergistic inhibitory effects. The most dramatic proof of this concept is provided by the two-drug interaction analysis. Indeed, the addition of NC1153 to CsA produced a synergistic therapeutic effect by extending the survival of kidney allografts, as documented by CI values of 0.3–0.5 (Fig. 4). Most importantly, when NC1153 was combined with CsA, it did not aggravate CsA-induced nephrotoxicity. In contrast, SRL/CsA combination was synergistic for therapeutic (CI = 0.3–0.6) (12) and nephrotoxic effects (14). The present experiments confirmed that CsA alone is nephrotoxic, whereas SRL alone is lipotoxic, and that the two-drug combination worsened nephrotoxicity. Previously published studies revealed that both CsA and SRL are metabolized by the P450 3A4 and that this predisposition contributes to their toxicities (3). In our present results, NC1153 is not likely to be a substrate of P450 3A4 (Fig. 7), and this may explain the lack of interaction between two drugs in CsA-induced toxicity. These experiments further support the model that blockade of JAK3 in conjunction with signal 1 can yield synergistic therapeutic qualities without toxic side effects.

Several studies have already revealed that the JAK3 Tyr kinase is essential for the function of T cells (28, 29). Only retroviral introduction of JAK3 into JAK3-deficient mice restored normal T cell development (72) as the JAK3/STAT5a/b signaling cascade likely regulates "survival" genes in mature T cells (49, 73). As presented herein, NC1153 abolished IL-2-driven T cell proliferation by inhibition of JAK3-mediated autokinase activity and likely STAT5a/b-mediated T cell survival regulated by several TCGFs. Our present in vivo results suggest that blockade of JAK3 prevents allograft rejection but may also have implication within donor-specific transplantation tolerance (Fig. 4*C*), as confirmed by the acceptance of donor-type heart allografts and rejection of third-party heart allografts. Although different protocols induce tolerance in rats, it is suggested that IL-2-mediated signaling is necessary for generation of regulatory T cells to maintain the tolerant state (74). Our ongoing studies suggest that purified T cells from tolerant recipients transferred tolerance to irradiated recipients; hence, JAK3 inhibition by NC1153 does not necessarily prevent generation of regulatory T cells (S. M. Stepkowski and R. A. Kirken, unpublished observation). Whether this event might be due to alloreactive T cell depletion is yet unknown. However, in vitro exposure of lymphocytes to NC1153 (>24 h) did induce T cell death, as measured by TUNEL assays (data not shown). This pattern of apoptosis, although more protracted, occurred following antisense specific depletion of "survival factors" STAT5a/b in lymphoid cells (73). This evidence further supports the model that JAK3/STAT5a/b can protect activated T cells against cell death. An ongoing study is underway to address these issues.

In conclusion, NC1153 selectively disrupts γ_c-cytokine pathways and JAK3 kinase activity as opposed to a limited pool of other kinases, which should not be considered an exhaustive study. NC1153 prevents allograft rejection and can induce transplantation tolerance. The combination of NC1153 and CsA produces therapeutic synergism to protect kidney allograft survival. Moreover, NC1153 lack toxicities associated with CsA and SRL alone or in combination. Thus, NC1153 may represent a novel class of molecules with potential for clinical immunosuppression without toxicities associated with currently used agents.

Acknowledgments

We thank Wendy Mohon and Scott Holmes for the skilled preparation of the manuscript and figures. We thank Drs. Hallgeir Rui and Henry Strobel for helpful comments.

Disclosures

The authors have no financial conflict of interest.

References

1. Kahan, B. D. 2000. Efficacy of sirolimus compared with azathioprine for reduction of acute renal allograft rejection: a randomised multicentre study. *Lancet* 356: 194–202.
2. Gourishankar, S., and P. F. Halloran. 2002. Late deterioration of organ transplants: a problem in injury and homeostasis. *Curr. Opin. Immunol.* 14: 576–583.

3. Kelly, P., and B. D. Kahan. 2002. Review: metabolism of immunosuppressant drugs. *Curr. Drug Meta.* 3: 275–287.
4. Kahan, B. D., P. Keown, G. A. Levy, and A. Johnston. 2002. Therapeutic drug monitoring of immunosuppressant drugs in clinical practice. *Clin. Ther.* 24: 330–350.
5. Bassel-Duby, R., and E. N. Olson. 2003. Role of calcineurin in striated muscle: development, adaptation, and disease. *Biochem. Biophys. Res. Commun.* 311: 1133–1141.
6. Feske, S., H. Okamura, P. G. Hogan, and A. Rao. 2003. Ca^{2+}/calcineurin signalling in cells of the immune system. *Biochem. Biophys. Res. Commun.* 311: 1117–1132.
7. Bennett, W. M., A. DeMattos, M. M. Meyer, T. Andoh, and J. M. Barry. 1996. Chronic cyclosporine nephropathy: the Achilles' heel of immunosuppressive therapy. *Kidney Int.* 50: 1089–1100.
8. Kahan, B. D. 1989. Cyclosporine. *N. Engl. J. Med.* 321: 1725–174.
9. Kim, D. H., and D. M. Sabatini. 2004. Raptor and mTOR: subunits of a nutrient-sensitive complex. *Curr. Top. Microbiol. Immunol.* 279: 259–270.
10. Chen, J. 2004. Novel regulatory mechanisms of mTOR signaling. *Curr. Top. Microbiol. Immunol.* 279: 245–257.
11. Stepkowski, S. M., L. Tian, K. L. Napoli, R. Ghobrial, M. E. Wang, T. C. Chou, and B. D. Kahan. 1997. Synergistic mechanisms by which sirolimus and cyclosporine inhibit rat heart and kidney allograft rejection. *Clin. Exp. Immunol.* 108: 63–68.
12. Stepkowski, S. M., K. L. Napoli, M. E. Wang, X. Qu, T-C. Chou, and B. D. Kahan. 1996. Effects of the pharmacokinetic interaction between orally administered sirolimus and cyclosporine on the synergistic prolongation of heart allograft survival in rats. *Transplantation* 62: 986–994.
13. Napoli, K. L., M. E. Wang, S. M. Stepkowski, and B. D. Kahan. 1998. Relative tissue distributions of cyclosporine and sirolimus after concomitant peroral administration to the rat: evidence for pharmacokinetic interactions. *Ther. Drug Monit.* 20: 123–133.
14. Podder, H., S. M. Stepkowski, K. L. Napoli, J. Clark, R. R. Verani, T. C. Chou, and B. D. Kahan. 2001. Pharmacokinetic interactions augment toxicities of sirolimus/cyclosporine combinations. *J. Am. Soc. Nephrol.* 12: 1059–1071.
15. Boutin, Y., D. Leitenberg, X. Tao, and K. Bottomly. 1997. Distinct biochemical signals characterize agonist- and altered peptide ligand-induced differentiation of naive $CD4^+$ T cells into Th1 and Th2 subsets. *J. Immunol.* 159: 5802–5809.
16. Kuo, C. T., and J. M. Leiden. 1999. Transcriptional regulation of T lymphocyte development and function. *Annu. Rev. Immunol.* 17: 149–187.
17. Kane, L. P., J. Lin, and A. Weiss. 2000. Signal transduction by the TCR for antigen. *Curr. Opin. Immunol.* 12: 242–249.
18. Dallman, M. J., K. J. Wood, K. Hamano, A. R. Bushell, P. J. Morris, M. J. Wood, and H. M. Charlton. 1993. Cytokines and peripheral tolerance to alloantigen. *Immunol. Rev.* 133: 5–18.
19. Appleman, L. J., and V. A. Boussiotis. 2003. T cell anergy and costimulation. *Immunol. Rev.* 192: 161–180.
20. Leonard, W. J. 1996. STATs and cytokine specificity. *Nat. Med.* 2: 968–969.
21. Zhou, Y. J., K. S. Magnuson, T. P. Cheng, M. Gadina, D. M. Frucht, J. Galon, F. Candotti, R. L. Geahlen, P. S. Changelian, and J. J. O'Shea. 2000. Hierarchy of protein tyrosine kinases in interleukin-2 (IL-2) signaling: activation of *syk* depends on Jak3; however, neither *Syk* nor *Lck* is required for IL-2-mediated STAT activation. *Mol. Cell Biol.* 20: 4371–4380.
22. Nelson, B. H., and D. M. Willerford. 1998. Biology of the interleukin-2 receptor. *Adv. Immunol.* 70: 1–81.
23. Bromberg, J., and J. E. Darnell Jr. 2000. The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* 19: 2468–2473.
24. Lin, J. X., and W. J. Leonard. 2000. The role of Stat5a and Stat5b in signaling by IL-2 family cytokines. *Oncogene* 19: 2566–2576.
25. Behbod, F., R. A. Erwin-Cohen, M. E. Wang, B. W. Trawick, X. Qu, R. Verani, B. D. Kahan, S. M. Stepkowski, and R. A. Kirken. 2001. Concomitant inhibition of JAK3 and calcineurin-dependent signaling pathways synergistically prolongs the survival of rat heart allografts. *J. Immunol.* 166: 3724–3732.
26. Stepkowski, S. M., R. A. Erwin-Cohen, F. Behbod, M. E. Wang, X. Qu, N. Tejpal, Z. S. Nagy, B. D. Kahan, and R. A. Kirken. 2002. Selective inhibitor of Janus tyrosine kinase 3, PNU156804, prolongs allograft survival and acts synergistically with cyclosporine but additively with rapamycin. *Blood* 99: 680–689.
27. Changelian, P. S., M. E. Flanagan, D. J. Ball, C. R. Kent, K. S. Magnuson, W. H. Martin, B. J. Rizzuti, P. S. Sawyer, B. D. Perry, W. H. Brissette, et al. 2003. Prevention of organ allograft rejection by a specific Janus kinase 3 inhibitor. *Science* 302: 875–878.
28. Thomis, D. C., and L. J. Berg. 1997. Peripheral expression of Jak3 is required to maintain T lymphocyte function. *J. Exp. Med.* 185: 197–206.
29. Thomis, D. C., W. Lee, and L. J. Berg. 1997. T cells from Jak3-deficient mice have intact TCR signaling, but increased apoptosis. *J. Immunol.* 159: 4708–4719.
30. Russell, S. M., J. A. Johnston, M. Noguchi, M. Kawamura, C. M. Bacon, M. Friedmann, M. Berg, D. W. McVicar, B. A. Witthuhn, O. Silvennoinen, et al. 1994. Interaction of IL-2R β and γ c chains with Jak1 and Jak3: implications for XSCID and XCID. *Science* 266: 1042–1045.
31. Uckun, F. M., B. A. Roers, B. Waurzyniak, X. P. Liu, and M. Cetkovic-Cvrlje. 2002. Janus kinase 3 inhibitor WHI-P131/JANEX-1 prevents graft-versus-host disease but spares the graft-versus-leukemia function of the bone marrow allografts in a murine bone marrow transplantation model. *Blood* 99: 4192–4199.
32. Kuhn, B., W. Jacobsen, U. Christians, L. Z. Benet, and P. A. Kollman. 2001. Metabolism of sirolimus and its derivative everolimus by cytochrome P450 3A4: insights from docking, molecular dynamics, and quantum chemical calculations. *J. Med. Chem.* 44: 2027–2034.
33. Sattler, M., F. P. Guengerich, C. H. Yun, U. Christians, and K. F. Sewing. 1992. Cytochrome P-450 3A enzymes are responsible for biotransformation of FK506 and rapamycin in man and rat. *Drug Metab. Dispos.* 20: 753–761.
34. Kirken, R. A., R. A. Erwin, D. Taub, W. J. Murphy, F. Behbod, L. Wang, F. Pericle, and W. L. Farrar. 1999. Tyrphostin AG-490 inhibits cytokine-mediated JAK3/STAT5a/b signal transduction and cellular proliferation of antigen-activated human T cells. *J. Leukocyte Biol.* 65: 891–899.
35. Dimmock, J. R., M. Chamankhah, A. Seniuk, T. M. Allen, G. Y. Kao, and S. Halleran. 1995. Synthesis and cytotoxic evaluation of some Mannich bases of alicyclic ketones. *Pharmazie* 50: 668–671.
36. Pericle, F., R. A. Kirken, V. Bronte, G. Sconocchia, L. DaSilva, and D. M. Segal. 1997. Immunocompromised tumor bearing mice show a selective loss of STAT5a/b expression in T and B lymphocytes. *J. Immunol.* 159: 2580–2585.
37. Yamashita, H., J. Xu, R. A. Erwin, W. L. Farrar, R. A. Kirken, and H. Rui. 1998. Differential control of the phosphorylation state of proline-juxtaposed serine residues Ser^{725} of Stat5a and Ser^{730} of Stat5b in prolactin-sensitive cells. *J. Biol. Chem.* 273: 30218–30218.
38. Kirken, R. A., H. Rui, M. G. Malabarba, O. M. Z. Howard, M. Kawamura, J. J. O'Shea, and W. L. Farrar WL. 1995. Activation of JAK3, but not JAK1, is critical for IL-2-induced proliferation and STAT5 recruitment by a COOH-terminal region of the IL-2 receptor β-chain. *Cytokine* 7: 689–700.
39. Tian, L., S. M. Stepkowski, X. Qu, M. E. Wang, M. Wang, J. Yu, and B. D. Kahan. 1997. Cytokine mRNA expression in tolerant heart allografts after immunosuppression with cyclosporine, sirolimus or brequinar. *Transpl. Immunol.* 5: 189–198.
40. Kirken, R. A., H. Rui, G. A. Evans, and W. L. Farrar. 1993. Characterization of an interleukin-2 (IL-2)-induced tyrosine phosphorylated 116-kDa protein associated with the IL-2 receptor β-subunit. *J. Biol. Chem.* 268: 22765–22770.
41. Rodrigues, A. D. 1999. Integrated cytochrome P450 reaction phenotyping: attempting to bridge the gap between cDNA-expressed cytochromes P450 and native human liver microsomes. *Biochem. Pharmacol.* 57: 465–480.
42. Chou, T.-C. 1991. The median effect principle and the combination index for quantitation of synergism and antagonism. In *Synergism and Antagonism in Chemotherapy*. T.-C. Chou and D. Rideout, eds. Academic Press, San Diego, pp. 61–102.
43. Chou, J., and T.-C. Chou. 1987. Dose-effect analysis with microcomputers: quantitation of ED50, LD50, synergism, antagonism, low-dose risk, receptor-ligand binding and enzyme kinetics. In *Biosoft.* Cambridge.
44. Schrader, B., and G. Steinhoff. 1993. Models of inflammatory cascade reactions by adhesion molecules. In *Cell Adhesion Molecules in Human Organ Transplants*. G. Steinhoff, ed. R. G. Lands, Austin, pp. 71–86.
45. Boyd, M. 1995. The NCI in vitro anticancer drug discovery screen. In *Anticancer Drug Development Guide: Preclinical Screening, Clinical Trials, and Approval.* B. Teicher, ed. Humana Press, Totowa, pp. 23–42.
46. Malabarba, M. G., H. Rui, H. H. Deutsch, J. Chung, F. S. Kalthoff, W. L. Farrar, and R. A. Kirken. 1996. Interleukin-13 is a potent activator of JAK3 and STAT6 in cells expressing interleukin-2 receptor-γ and interleukin-4 receptor-α. *Biochem. J.* 319: 865–872.
47. Malabarba, M. G., R. A. Kirken, H. Rui, K. Koettnitz, M. Kawamura, J. J. O'Shea, F. S. Kaltoff, and W. L. Farrar. 1995. Activation of JAK3, but not JAK1, is critical to interleukin-4 (IL4) stimulated proliferation and requires a membrane-proximal region of IL4 receptor α. *J. Biol. Chem.* 270: 9630–9637.
48. Zhou, Y. J., E. P. Hanson, Y. Q. Chen, K. Magnuson, M. Chen, P. G. Swann, R. L. Wange, P. S. Changelian, and J. J. O'Shea. 1997. Distinct tyrosine phosphorylation sites in JAK3 kinase domain positively and negatively regulate its enzymatic activity. *Proc. Natl. Acad. Sci. USA* 94: 13850–13850.
49. Moriggl, R., D. J. Topham, S. Teglund, V. Sexl, C. McKay, D. Wang, A. Hoffmeyer, J. van Deursen, M. Y. Sangster, K. D. Bunting, et al. 1999. Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. *Immunity* 10: 249–259.
50. Higuchi, M., H. Asao, N. Tanaka, K. Oda, T. Takeshita, M. Nakamura, J. Van Snick, and K. Sugamura. 1996. Dispensability of Jak1 tyrosine kinase for interleukin-2-induced cell growth signaling in a human T cell line. *Eur. J. Immunol.* 26: 1322–1327.
51. Evans, G. A., M. A. Goldsmith, J. A. Johnston, W. D. Xu, S. R. Weiler, R. Erwin, O. M. Z. Howard, R. T. Abraham, J. J. O'Shea, W. C. Green, and W. L. Farrar. 1995. Analysis of interleukin 2 dependent signal transduction through the SHC/GRB2 adapter pathway-interleukin-2-dependent mitogenesis does not require SHC phosphorylation or receptor association. *J. Biol. Chem.* 270: 28858–28863.
52. Howard, O. M. Z., R. A. Kirken, G. G. Garcia, R. H. Hackett, and W. L. Farrar. 1995. Structural domains of IL-2 receptor β critical for signal transduction: kinase association and nuclear complex formation. *Biochem. J.* 306: 217–224.
53. Salcini, A. E., J. McGlade, G. Pelicci, I. Nicoletti, T. Pawson, and P. G. Pelicci. 1994. Formation of Shc-Grb2 complexes is necessary to induce neoplastic transformation by overexpression of Shc proteins. *Oncogene* 9: 28272836.
54. Gotoh, N., A. Tojo, and M. Shibuya. 1996. A novel pathway from phosphorylation of tyrosine residues 239/240 of Shc, contributing to suppress apoptosis by IL-3. *EMBO J.* 15: 6197–6204.
55. Darnell, J. E. Jr. 1997. STATs and gene regulation. *Science* 277: 1630–1635.
56. Cheung, J. Y., and B. A. Miller. 2001. Molecular mechanisms of erythropoietin signaling. *Nephron* 87: 215–222.
57. Lacombe, C., and P. Mayeux. 1999. The molecular biology of erythropoietin. *Nephrol. Dial. Transplant.* 14: 22–28.
58. Wendling, F., and W. Vainchenker. 1998. Thrombopoietin and its receptor. *Eur. Cytokine Netw.* 9: 221–231.

59. Kirken, R. A., H. Rui, M. G. Malabarba, and W. L. Farrar. 1994. Identification of interleukin-2 receptor-associated tyrosine kinase p116 as novel leukocyte-specific Janus kinase. *J. Biol. Chem.* 269: 19136–19141.
60. Hesselink, D. A., R. H. van Schaik, I. P. van der Heiden, M. van der Werf, P. J. Gregoor, J. Lindemans, W. Weimar, and T. van Gelder. 2003. Genetic polymorphisms of the CYP3A4, CYP3A5, and MDR-1 genes and pharmacokinetics of the calcineurin inhibitors cyclosporine and tacrolimus. *Clin. Pharmacol. Ther.* 74: 245–254.
61. English, J., A. Evan, D. C. Houghton, and W. M Bennett. 1987. Cyclosporine-induced acute renal dysfunction in the rat: evidence of arteriolar vasoconstriction with preservation of tubular function. *Transplantation* 44: 135–141.
62. Mason, J. 1990. The pathophysiology of Sandimmune (cyclosporine) in man and animals. *Pediatr. Nephrol.* 4: 554–574.
63. Baud, L., and R. Ardaillou. 1993. Involvement of reactive oxygen species in kidney damage. *Br. Med. Bull.* 49: 621–629.
64. Serino, F., J. Grevel, K. L. Napoli, B. D. Kahan, and H. W. Strobel. 1994. Oxygen radical formation by the cytochrome P450 system as a cellular mechanism for cyclosporine toxicity. *Transplant. Proc.* 26: 2916–2917.
65. Shin, G. T., A. Khanna, R. Ding, V. K. Sharma, M. Lagman, B. Li, and M. Suthanthiran. 1998. In vivo expression of transforming growth factor-β1 in humans: stimulation by cyclosporine. *Transplantation* 65: 313–318.
66. Avdonin, P. V., F. Cottet-Maire, G. V. Afanasjeva, S. A. Loktionova, P. Lhote, and U. T. Ruegg. 1999. Cyclosporine A up-regulates angiotensin II receptors and calcium responses in human vascular smooth muscle cells. *Kidney Int.* 55: 2407–2414.
67. Vaziri, N. D., Z. Ni, Y. P. Zhang, E. P. Ruzics, P. Maleki, and Y. Ding. 1998. Depressed renal and vascular nitric oxide synthase expression in cyclosporine-induced hypertension. *Kidney Int.* 54: 482–491.
68. Healy, E., M. Dempsey, C. Lally, and M. P. Ryan. 1998. Apoptosis and necrosis: mechanisms of cell death induced by cyclosporine A in a renal proximal tubular cell line. *Kidney Int.* 54: 1955–1966.
69. Ortiz, A., C. Lorz, M. Catalan, A. Ortiz, S. Coca, and J. Egido. 1998. Cyclosporine A induces apoptosis in murine tubular epithelial cells: role of caspases. *Kidney Int.* 68(Suppl.): S25–S29.
70. Groth, C. G., L. Backman, J. M. Morales, R. Calne, H. Kreis, P. Lang, J. L. Tourine, K. Claesson, J. M. Campistol, D. Durand, et al. 1999. Sirolimus (rapamycin)-based therapy in human renal transplantation: similar efficacy and different toxicity compared with cyclosporine. Sirolimus European Renal Transplant Study Group. *Transplantation* 67: 1036–1042.
71. Murgia, M. G., S. Jordan, and B. D. Kahan. 1996. The side effect profile of sirolimus: a phase I study in quiescent cyclosporine-prednisone-treated renal transplant patients. *Kidney Int.* 49: 209–216.
72. Bunting, K. D., M. Y. Sangster, J. N. Ihle, and B. P. Sorrentino. 1998. Restoration of lymphocyte function in Janus kinase 3-deficient mice by retroviral-mediated gene transfer. *Nat. Med.* 4: 58–64.
73. Behbod, F., Z. S. Nagy, S. M. Stepkowski, J. Karras, C. R. Johnson, W. D. Jarvis, and R. A. Kirken. 2003. Specific inhibition of Stat5a/b promotes apoptosis of IL-2-responsive primary and tumor-derived lymphoid cells. *J. Immunol.* 171: 3919–3927.
74. Sakaguchi, S. 2004. Naturally arising $CD4^+$ regulatory T cells for immunologic self-tolerance and negative control of immune responses. *Annu. Rev. Immunol.* 22: 531–562.