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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‡

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 from voiding 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 has shown 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: myelodysplasia and hypercholesterolemia (14). Double-blinded clinical trials confirmed improved therapeutic efficacy by SRL-CsA combinations to prevent allograft rejection (synergism) but also revealed their unique side effects, namely, thrombocytopenia, hypercholesterolemia, and hypocitrullinemia (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 a B7/CD28 interaction that induces production of the TCGF-specific cytokine receptors promotes T cell clonal expansion via G1-S transition (18). Many TCGFs share a common γ-chain (γc), 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-2Rγc recruits and activates the IL-2Rβ-associated JAK1 and γc-associated JAK3 (20, 21). Auto-activation of these enzymes promotes Tyr phosphorylation of IL-2Rβ-chain, attracting other signaling elements, including STAT5a...
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 γc 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 γc 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 allograft survival. However, NC1153 is not metabolized by the cytochrome P450 3A4 isoform, 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.

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 t-glutamine, 5 mM HEPEs, pH 7.3, and penicillin-streptomycin (50 IU/ml and 50 μg/ml, respectively), at 37°C-5% CO2. 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 IL-2Rβ (catalog no. 554-525), and PE-labeled mouse anti-human γc (catalog no. 555-898) Abs. Cell staining and FACSscan were performed as previously described (36).

Proliferation assays

Quiescent human T cells or Jurkat cells (5.0 × 104/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 cultured for 4 h with [3H]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× 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 She Ab (catalog no. 06-203; Upstate Biotechnology) and C termini of human STAT5a (aa 775–794) or STAT5a (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 phospho-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. IPVH 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 described earlier (37). For EMISA analyses (39), 1 μg of 32P-labeled oligo-nucleotide corresponding to the β-casein promoter (5’-agattctagaaacctc-3’) or NF-κB DNA binding element (5’-agttgcggacctgtc-3’) was labeled with [32P]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). 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 mEq NaHCO3, 1 X-100, 100 mM NaCl, 10 mM MgCl2, 3 mM Hepes, and 0.5 mM sodium orthovanadate. Isotope-free tyrosine kinase reactions were initiated by the addition of 100 μM unlabelled 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/√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 control reactions with selective substrates were used to monitor P450 3A4 (ketocazole), 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 isoenzyme activity with a competitive substrate without or with NC1153 × 100.

Rat kidney and spleen transplants

ACI (WF; RT1a) and Lewis (RT1b) 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). Cold ischemia times were 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 (Polym 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 for vascular changes is used for kidney sections stained with H&E. The right femurs were sectioned and stained with H&E, and megakaryocytes in four high-power fields was used to estimate platelet formation.

Results

Selection of NC1153 based on inhibition of TGF-β-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. 1A). 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 γc; Fig. 1B) 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 [3H]thymidine incorporation. NC1153 almost completely abolished uptake of radiolabeled thymidine at 10 μM (IC50 of −2.5 μM) in contrast to its effect on Jurkat T cells void of JAK3 (Fig. 1C). 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 γc cytokines. Indeed, [3H]thymidine incorporation was reduced in a dose-dependent manner by NC1153 (0.01–25 μM) regardless of the JAK3-activating cytokine used (Fig. 1D).

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 Tyr508 and Tyr511 (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. 2A) and then reprobed for total JAK3 protein (bottom blot, Fig. 2A). 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. 2A) (49, 50). These results suggest that blockade of TGCF-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. 2B). NC1153 caused a concentration-dependent loss of Tyr phosphorylation of JAK3 with similar IC50 of 2.5 μM (Fig. 2B, top panel). Because catalytically active JAK3 is required for IL-2-driven Tyr phosphorylation of STAT5a/b (38), both substrates STAT5a (Fig. 2B, middle panel) and STAT5b (Fig. 2B, 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. 2B). 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 Tyr338 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
NC1153 inhibits IL-2R expression of IL-2R

Jurkat T cells or PHA-activated human PBLs were challenged in the presence of 1 nM IL-2 with ascending concentrations of NC1153. DNA synthesis in activated human PBLs were stimulated in the presence of 1 nM IL-2, IL4, IL7 with ascending concentrations of NC1153. DNA synthesis in activated human PBLs were stimulated in the presence of 1 nM IL-2 with ascending concentrations of NC1153.

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. 3A, top panel). Protein nuclear extracts (5 μg/well) from 10 to 100 μM NC1153-treated PHA-activated T cells were mixed with a 32P-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. 3A, 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. 3A, bottom panel). TNF-α induced binding of p50/p65 NF-κB components to a 32P-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 γc-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), whose counterparts of Shc, the same cell lysates were separated on 10% SDS-PAGE and Western blotted with antiphosphotyrosine Abs that likely recognize activated Tyr239, Tyr240, and Tyr257 sites on this adapter molecule. As shown in Fig. 2C, 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 The202 and Tyr204 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/p42) activity. Thus, NC1153 subsequently disrupts MAPK activation in intact IL-2-activated T cells.

FIGURE 1. NC1153 inhibits γc-induced proliferation of T cells. A, Human PHA-activated PBLs (5.0 × 10^6 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 [3H]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 γc 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.
NC1153 promotes allograft survival.

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

Untreated ACI (RT1a) recipients rejected Lewis (RT1l) kidney allografts at a MST of 8.8 ± 0.5 days (Fig. 4A). 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. 4A). 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. 4B). 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. 4C). Because long-surviving recipients accepted donor-type LEW (≥100 days; n = 3) and rejected third-party Buffalo (RT1b) 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. 4D). 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 × 10⁶) of highly sensitized graft-infiltrating cells (GICs) on day 7 postgrafting (Fig. 4). In order to control for nonspecific effects of NC115 on c-cytokine expression, we repeated experiments with II-2 (10 min) in the presence of NC1153 (Fig. 3C). Finally, selectivity of NC1153 was further documented by using assays for multiple kinases (Fig. 3D). 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 Ca and protein kinase A). These results suggest that NC1153 selectively inhibits γc-cytokines driven by activation of JAK3, without affecting several related signaling molecules.
Treatment with 160 mg/kg NC1153 prevented rejection, as documented by reduced number of GICs ($20 \times 10^6$). When equivalent numbers of GICs ($50 \times 10^6$) were challenged for 10 min with IL-2, untreated recipient cells showed activation and nuclear translocation of STAT5 (Fig. 5B, lane d) that was inhibited (Fig. 5B, lane i) within NC1153-treated recipients (Fig. 5B). Thus, inhibition of signal 3 signaling by NC1153 may reduce clonal expansion of alloreactive T cells and their consequent activities.

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 32P-labeled oligonucleotide probe corresponding to the PR3-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 with (−) 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 (PY) 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. 2B) or JAK2 (Fig. 3B) 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.

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.
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. 6a, a–f). Both serum creatinine and creatinine clearance confirmed that the addition of SRL to CsA significantly increased CsA-induced nephrotoxicity (Fig. 6a, 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. 6a, a and b). As shown in Fig. 6b, 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. 6bd). In contrast, rats exposed to combined NC1153-CsA therapy displayed identical changes to those observed in CsA alone group (Fig. 6Be). Furthermore, although NC1153 did not affect lipid metabolism, SRL increased levels of total cholesterol (Fig. 6Ac), serum LDL-cholesterol (Fig. 6Ac), and serum HDL-cholesterol (Fig. 6Ae). Therapy with CsA, SRL, NC1153, CsA-SRL, or CsA-NC1153 had no effect on blood hematocrit (Fig. 6Ag) and hemoglobin (Fig. 6Ah) levels. Furthermore, although NC1153 alone did not alter femoral bone marrow cellularity (Fig. 6Ca), SRL alone produced mild myelosuppression (Fig. 6Cb), as evidenced by histological examination. Again, two-drug CsA-SRL therapy significantly worsened cellularity (Fig. 6Cd), whereas NC1153-CsA combination showed no changes (Fig. 6Ce). 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

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**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, Splenectomies 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 a32P-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 (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).
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 TGF-induced T cell growth (IC50 2.5 μM; Fig. 1C) 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. 2B) and their DNA binding activity (Fig. 3A), but also their subsequent downstream effectors, Shc, Ser/Thr kinases, p44/ERK1, and p42/ERK2 (Fig. 2C). Although NC1153 was equally effective in blocking T cell proliferation by IL-2, IL-4, or IL-7 (Fig. 1C), 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. 5B). Extended treatment with NC1153 alone resulted in permanent acceptance of kidney allografts (Fig. 4C), whereas a short-term combination of NC1153/CsA therapy produced synergistic effects (CI = 0.3–0.5; Fig. 4D). 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...
Most importantly, when NC1153 was combined with CsA, it did produced a synergistic therapeutic effect by extending the survival of mice (Fig. 4C). Indeed, the addition of NC1153 to CsA produced a synergistic inhibitory effect in vitro exposure of lymphocytes to NC1153 (Fig. 4B). 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.

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 (paratyline), 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. 4C), 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 deletion 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 γc 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.

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Disclosures
The authors have no financial conflict of interest.

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