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SELECTIVE INHIBITORS OF PROTEIN KINASE C IN A MODEL OF GRAFT-*vs*-HOST DISEASE

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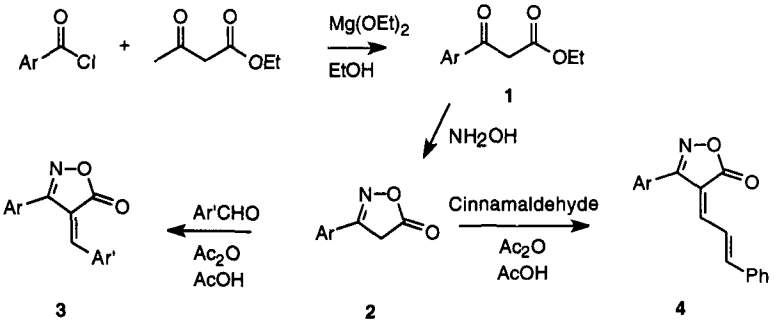
Abstract: A series of 3-aryl-4-arylidene-5-isoxazolones were found to be active as inhibitors of PKC, and in *ex-vivo* and *in vivo* models of graft-versus-host disease.

The protein kinase C (PKC) family of serine/threonine kinases is central to the regulation of numerous cellular functions as a consequence of its role in signal transduction,[1,2,3] and PKC deregulation or over-activity has been implicated in inflammatory diseases, certain cancers, and other disorders.[4,5] In particular, the role of PKC in the regulation of cell differentiation and proliferation suggests that PKC inhibitors might be useful anti-cancer agents.[6,7] These potential utilities have prompted widespread screening of synthetic and natural sources for PKC inhibitors, and a number of natural products have been isolated over the past several years which are indeed very potent PKC inhibitors.[8,9,10] Many of these compounds, and their synthetic and semi-synthetic analogues, are in various stages of evaluation as chemotherapeutic agents. The regulation of cell activation and proliferation within the immune system was of interest to us in that inhibitors of PKC also have the potential to be medically useful immunosuppressive agents.[11]

We report here a series of 3-aryl-4-arylidene-5-isoxazolones **3** and **4** which are selective, micromolar inhibitors of PKC, active against a variety of PKC subtypes but selective for PKC over other protein kinases. This pattern of selectivity, and the ease of synthesis, should make these compounds useful as biochemical tools. The compounds are also active in *ex vivo* and *in vivo* models of Graft-*versus*-Host (GvH) disease.



Scheme 1

The compounds **3** and **4** as a class are known in the literature,[12] and were synthesized by known procedures or modifications thereof (Scheme 1). The key intermediates were the 3-aryl-5-isoxazolones **2**, obtained by condensation of hydroxylamine with the appropriate aroylacetate ester **1**. These were prepared from the corresponding aroyl chloride and the magnesium salt of ethyl acetoacetate,[13] or from diethyl carbonate and an acetophenone lithium enolate.[14] Subsequent condensation of **2** with an aryl aldehyde in the presence of acetic anhydride[15] or other dehydrating agents provided the desired 4-arylidene-5-isoxazolones **3**. Compound **3c**,

bearing a hydroxyl group on Ar, was prepared from the corresponding methoxy compound **3d** by demethylation with boron tribromide. Two compounds bearing a hydroxyl group on Ar' (**3cc** and **3gg**) were obtained as acetates from condensation in the presence of acetic anhydride, and required alkaline hydrolysis as a final step.

The stereochemistry of the exocyclic double bond was not explicitly determined for every compound, but is assumed to be *Z* as shown based on published precedent,[16] and on the results of a nuclear Overhauser effect experiment conducted on compound **3r**. Condensation of **2** (Ar = 2-thienyl) with cinnamaldehyde afforded the cinnamylidene analogue **4**, with the *Z,E* geometry shown based on published NMR coupling constants for related compounds.[17] Melting points and references to known compounds are presented in Table 1. All novel compounds exhibited satisfactory elemental analyses and mass, IR and ^{1}H NMR spectra.

Inhibition of PKC (derived from Jurkat cells) *in vitro* was determined by the method of Kaibuchi *et al.*[18] Activity in the *ex vivo* GvH model was determined by measuring popliteal node hyperplasia in rats.[19] Results of both assays are presented in Table 1. We were able to discern a consistent structure-activity relationship from the early results, but applying the information to the synthesis of further analogues did not result in increases in potency beyond an IC_{50} of about 1 μM. Specifically, we observed greater potency when Ar' was an electron-rich aryl or heterocycle (*e.g.* **3t** vs. **3i** vs. **3j**), but extension of the concept to the very electron-rich indole or pyrrole rings (**3n** through **3q**) greatly reduced activity. The few substituents on Ar which we examined (**3c** through **3h**) were either detrimental or had no significant effect, and we concentrated our efforts on the 3-(2-thienyl) compounds because of their relative novelty. Although a hydrophilic substituent on Ar reduced activity in the two examples examined (**3c**, **3h**), hydrophilic substituents on Ar' were generally well-tolerated. The notable exception was compound **3w**, where a carboxylate group greatly reduced the *ex vivo* GvH activity despite the retention of activity in the enzyme assay. The cinnamylidene isoxazolone **4** was one of the more potent analogues, but we did not further explore this class of compound. As with the PKC inhibition, activity in the GvH model seemed to plateau at an IC_{50} of 2-4 μM, with several compounds reaching this level of potency, and again our structural variations were not successful in attaining greater potency.

A subset of the compounds was screened, and found to be inactive, in the NCI's anti-cancer cytotoxicity assays.[20] Compounds **3** and **4** had no activity against the $p56^{lck}$ tyrosine kinase when screened at 50 μM, and no activity was found in standard anti-inflammatory assays, such as adjuvant-induced arthritis in rats.

A variety of PKC subtypes are expressed in Jurkat cells,[21] and the observation of 100% inhibition in a number of our examples suggests that these compounds are inhibitory against all of the PKC subtypes expressed in this T-cell line. (These cells are currently known to express PKCs α, β, δ, and θ.[22]) Given that calphostin C appears to interact with a regulatory site on PKC,[23,24] and that the PKC subtypes differ mainly in their regulatory regions,[25] the activity of the isoxazolones across the various PKC subtypes could indicate a binding site and/or mechanism of action different from that of calphostin C. Structures **3** and **4** incorporate electrophilic functionality, which suggests covalent modification of PKC, but no evidence regarding the kinetics, time-dependence, or irreversibility of inhibition was obtained in these studies.

The compounds exhibited specificity for PKC to the extent that although all of them were tested against PKA, none were inhibitors at concentrations up to 50 μM. To our knowledge, only calphostin C and its analogues[26] show complete selectivity for PKC over PKA. Certain staurosporine analogues[27,28,29,30] have demonstrated selectivity, but are not as readily available as **3** and **4**, while a series of fatty acyl piperidines[31] has been reported to be selective for PKC over PKA, but are not as potent.

The correlation between PKC inhibition and activity in the GvH model is tenuous, since potency values in both assays tended to cluster within experimental errors. There was, however, only one example with good potency in the PKC assay but poor activity in the GvH model (**3w**), perhaps due to poor penetration of intact lymphocytes; otherwise all good PKC inhibitors showed good activity in the GvH experiments. Because of limited resources, we pre-selected compounds for the GvH assay based on their potency in the PKC assay (*e.g.* **3g, 3h, 3n–3q**), which confounds further attempts to correlate the two sets of data.

Table 1. Physical and biological data.

Example	Ar	Ar'	m.p. (°C)	Method	PKC Inhibition IC_{50} (95% c.l.) (μM)	PKC Inhibition % Inhib @ 50 μM	GvH activity IC_{50} (95% c.l.) (μM)	GvH activity % Inhib ± sem @ 10 μM
3a	Ph	Ph	191-193[32]	A	14 (11-17)	-	-	-
3b	Ph	2-furyl	129-132[33]	D	0.9 (.02-2.6)	84%	2.9 (2.3-3.7)	89 ±4.3
3c	2-hydroxyPh	2-thienyl	180-181(d)	A,E	25 (21-29)	-	-	20 ±8.4
3d	2-methoxyPh	2-thienyl	141-144	A	1.2 (1.0-1.4)	100%	7.1 (5.6-9.2)	66 ±5.1
3e	4-methoxyPh	2-thienyl	166-167	A	0.7 (.39-1.1)	100%	11 (7.6-24)	44 ±4.5
3f	2,5-dimethoxyPh	2-thienyl	164-167	A	1.9 (1.4-2.5)	100%	7.9 (5.3-15)	59 ±5.9
3g	4-nitroPh	2-thienyl	269-270(d)	A	-	0%	-	-
3h	4-aminoPh	2-thienyl	(>270)	D	-	0%	-	-
3i	2-thienyl	Ph	130-132	A	15 (14-15)	81%	4.4 (3.0-6.7)	88 ±2.6
3j	2-thienyl	2-thienyl	190-192	A	1.0 (.88-1.1)	100%	3.7 (2.7-4.9)	97 ±4.3
3k	2-thienyl	3-thienyl	166-169	B	2.9 (2.0-4.1)	100%	3.0 (2.2-4.1)	95 ±4.9
3l	2-thienyl	2-furyl	119-120	A	1.4 (.44-2.8)	100%	5.8 (4.6-7.0)	88 ±5.4
3m	2-thienyl	3-furyl	170-173	B	1.7 (0.80-2.8)	100%	3.6 (2.9-4.4)	94 ±2.4
3n	2-thienyl	2-pyrrolyl	176-179	D	-	8.9%	-	-
3o	2-thienyl	3,5-$(Me)_2$-2-pyrrolyl	154-156	A	-	19%	-	-
3p	2-thienyl	3-indolyl	250-253	A	-	48%	-	-
3q	2-thienyl	1-methyl-3-indolyl	223-225	A	-	21%	-	-
3r	2-thienyl	4-fluoroPh	137-139	B	8.8 (8.0-9.8)	98%	4.8 (4.0-5.6)	92 ±2.3
3s	2-thienyl	4-chloroPh	149-152	C	11 (7.3-19)	82%	6.2 (3.0-11)	71 ±2.3
3t	2-thienyl	3-chloroPh	126-128	B	23 (20-26)	24%	-	-
3u	2-thienyl	4-bromoPh	147-150	B	8.4 (5.8-12)	100%	-	91 ±3.3
3v	2-thienyl	4-(Me_2N)Ph	179-180	D	2.1 (.37-4.6)	88%	4.2 (2.2-5.8)	89 ±6.1
3w	2-thienyl	4-(OCH_2CO_2H)Ph	234-235	D	8.2 (3.9-12)	-	100*	-7 ±14
3x	2-thienyl	4-acetoxyPh	172-175	A	11 (7.1-15)	81%	4.5 (2.8-8.2)	65 ±6.5
3y	2-thienyl	4-methoxyPh	130-133	A	1.2 (.26-2.5)	89%	2.5 (1.8-3.2)	96 ±1.8
3z	2-thienyl	3-methoxyPh	80-82	B	22 (17-29)	65%	-	66 ±6.0
3aa	2-thienyl	2,4-dimethoxyPh	231-234	D	>100	35%	-	-
3bb	2-thienyl	3-OMe-4-OAc-Ph	143-145	A	11 (7.6-14)	82%	-	43 ±7.9
3cc	2-thienyl	4-hydroxyPh	204-205	A,F	3.5 (2.6-4.9)	93%	4.2 (2.5-8.0)	87 ±5.9
3dd	2-thienyl	3-hydroxyPh	178-180	D	9.9 (6.2-14)	-	-	58 ±4.8
3ee	2-thienyl	2-hydroxyPh	173-177	D	5.5 (4.1-8.1)		5.7 (5.0-6.7)	87 ±5.3
3ff	2-thienyl	3,4-dihydroxyPh	205-206(d)	D	3.5 (1.4-6.3)	-	3.4 (2.6-4.4)	87 ±3.5
3gg	2-thienyl	2,6-$(Me)_2$-4-OH-Ph	204-206	A,F	5.3 (3.1-8.5)	78%	1.6 (1.2-2.1)	88 ±3.0
3hh	2-thienyl	3,5-$(Me)_2$-4-OH-Ph	259-264(d)	A	2.5 (.86-4.4)	-	2.6 (2.0-3.4)	-
3ii	2-thienyl	3,5-$(MeO)_2$-4-OH-Ph	199-202	A	11 (8.1-15)	-	-	72 ±6.8
3jj	2-thienyl	3,5-$(t\text{-}Bu)_2$-4-OH-Ph	189-192	A	35 (25-59)	-	-	-
4	2-thienyl	PhCH=CH-	171-173	A	1.0 (.40-1.9)	-	3.0 (2.2-3.9)	93 ±1.8
	Staurosporine				.003 (.002-.004)	-		45 ±6.3

* Single datapoint

In an *in vivo* modification of the GvH model, compound **3hh** had an ED_{50} of 93 mg/kg when administered *i.p.* once daily for three days. (The 95% confidence limits for this ED_{50} were 66-220 mg/kg, due to only 53% inhibition being observed at 100 mg/kg, the highest dose tested.) Compound **3b** was more active (100% inhibition observed at 50 mg/kg/day), but was significantly toxic (2/5, 3/5, and 5/5 animals dead at 25, 50, and 100 mg/kg/day). Cyclophosphamide had an ED_{50} of 3.3 (2.5-4.2) mg/kg *p.o.* in this assay.

In summary, we have identified a class of PKC-specific protein kinase inhibitors, which are active in *ex-vivo* and *in vivo* models of GvH disease. Because of their selectivity, bioactivity, and ease of synthesis, the compounds described may be of use as tools in biochemical and pharmacological research, and they could serve as a starting point for the development of treatments for GvH complications in organ or bone marrow transplantation.

Experimental procedures

Synthesis (Representative examples; yields were not optimized.)

Method A. 3-(2-Thienyl)-4-(3,5-dimethyl-4-hydroxyphenyl)methylidene-2-isoxazolin-5-one (**3hh**):

3-(2-Thienyl)-2-isoxazolin-5-one[34] (2.51 g, 15.0 mmol) and 3,5-dimethyl-4-hydroxybenzaldehyde (2.25 g, 15.0 mmol) were refluxed for 30 min in acetic acid (5 mL) and acetic anhydride (10 mL). The product was precipitated by addition of water and recrystallized from DMF/water to provide 2.25 g (50%) of **3hh** as a yellow powder, mp 259-264 °C (dec.). ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.22 (6H, s), 7.33 (1H, m), 7.78 (1H, d, J=3.3 Hz), 7.89-7.90 (2H, m), 8.26 (2H, s), 10.07 (1H, br) *Anal.* Calcd for $C_{16}H_{13}NO_3S$: C, 64.20; H, 4.38; N, 4.68. Found: C, 64.15; H, 4.35; N, 4.74. IR (KBr, cm^{-1}) 3435 (s, OH) 1726 (s, C=O). m/z 300 (MH^+).

Method B. 3-(2-Thienyl)-4-(4-fluorophenyl)methylidene-2-isoxazolin-5-one (**3r**):

3-(2-Thienyl)-2-isoxazolin-5-one (1.30 g, 7.78 mmol) and 4-fluorobenzaldehyde (965 mg, 7.78 mmol) were dissolved in 10 mL of THF, and NaH (31 mg of a 60% suspension in oil, 0.78 mmol) was added. When gas evolution ceased, anhydrous Na_2SO_4 (ca. 1 g) was added. The mixture was refluxed for 2 hr, filtered, and the filtrate evaporated. The residue was recrystallized from EtOAc/hexane to provide 1.03 g (48%) of **3r** as yellow needles, mp 137-139 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.36 (1H, m), 7.46 (2H, t, J_{HH}=J_{HF}=8.7Hz), 7.90 (1H, d, J=3.7Hz), 7.94 (1H, d, J=5.0 Hz), 8.22 (1H, s), 8.53 (2H, dd, J_{HH}=8.7 Hz, J_{HF}=5.8 Hz). *Anal.* Calcd for $C_{14}H_8FNO_2S$: C, 61.53; H, 2.95; N, 5.12. Found: C, 61.62; H, 2.73; N, 5.04. IR (KBr, cm^{-1}) 1752 (s, C=O). m/z 274 (MH^+). The *Z* configuration was assigned based on the ^{1}H NOE difference spectrum: Irradiation of the methylidene proton (δ 8.22) produced significant and equivalent enhancements of the thienyl H-3 (δ 7.90) and phenyl H-2 (δ 8.53) signals.

Method C. 3-(2-Thienyl)-4-[(4-chlorophenyl)methylidene]-2-isoxazolin-5-one (**3s**).

3-(2-Thienyl)-2-isoxazolin-5-one (1.11 g, 6.64 mmol), 4-chlorobenzaldehyde (930 mg, 6.62 mmol), acetic acid (1 mL), and 4Å molecular sieves (ca. 2 g) were combined in 10 mL anhydrous THF. The mixture was refluxed for 1 hr, cooled, neutralized with saturated aqueous $NaHCO_3$, and filtered through Celite™. The aqueous layer was then extracted with EtOAc, and the combined organic solutions were dried over $MgSO_4$, filtered, and evaporated to give a yellow solid. Recrystallization from EtOAc/hexane provided **3s** (850 mg, 44%) as yellow needles, mp 149-152 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.36 (1H, m), 7.67 (2H, d, J=8.5Hz), 7.90 (1H, d, J=3.4Hz), 7.94 (1H, d, J=5.0 Hz), 8.22 (1H, s), 8.41 (2H, d, J=8.5 Hz). *Anal.* Calcd for $C_{14}H_8ClNO_2S$: C, 58.04; H, 2.78; N, 4.83. Found: C, 58.04; H, 2.62; N, 4.67. IR (KBr, cm^{-1}) 1748 (s, C=O). m/z 290 (MH^+).

Method D. 3-(2-Thienyl)-4-[(2,4-dimethoxyphenyl)methylidene]-2-isoxazolin-5-one (**3aa**).

3-(2-Thienyl)-2-isoxazolin-5-one (1.34 g, 8.02 mmol), 2,4-dimethoxybenzaldehyde (1.33 g, 8.00 mmol), acetic acid (ca. 100 mg), and anhydrous Na_2SO_4 (ca. 1 g) were combined in anhydrous THF (10 mL), and the mixture was stirred at reflux. The product precipitated out of solution as it was formed. The solids were collected by filtration, washed with water, and the residue recrystallized from DMF/water to provide **3aa** (2.31 g, 92%) as a yellow powder, mp 231-234 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ 3.92 (3H, s), 3.94 (3H, s), 6.72 (1H, d, J=1.5Hz), 6.79 (1H, dd, J=9.0, 1.5 Hz), 7.37 (1H, m), 7.68 (1H, d, J=3.6 Hz), 7.94 (1H, d, J=5.0 Hz), 9.00 (1H, d, J=9.0 Hz). *Anal.* Calcd for $C_{16}H_{13}NO_4S$: C, 60.94; H, 4.16; N, 4.44. Found: C, 60.96; H, 4.08; N, 4.38. IR (KBr, cm^{-1}) 1741 (s, C=O). m/z 316 (MH^+).

Method E. Boron tribromide (2.1 mmol) was added dropwise to a solution of **3d** (540 mg, 1.9 mmol) in dichloromethane (25 mL) at 0 °C, and the mixture was then warmed to room temperature. Water was added, and the organic layer was isolated, dried ($MgSO_4$), filtered, and evaporated. The residue was recrystallized from hexane-ethyl acetate to provide 320 mg (62%) of **3c** as a yellow powder, mp 180-181(dec.)

Method F. 2,6-Dimethyl-4-hydroxybenzaldehyde (980 mg) was condensed with 3-(2-thienyl)-2-isoxazolin-5-one (1.09 g, 6.52 mmol) by method A. The product (330 mg after chromatography) was dissolved in methanol (10 mL), potassium carbonate (ca. 500 mg) was added, and the mixture refluxed for 2 hr. Aqueous citric acid was added, the product extracted with ethyl acetate, and the extracts filtered through a short silica column. Recrystallization from hexane-ethyl acetate provided 300 mg (15%) of **3gg** as a yellow powder, mp 204-206.

Enzyme assays

Protein Kinase C from Jurkat cells was assayed by the published procedure.[35] Briefly, Jurkat cells were lysed in 20 mM TRIS HCl (pH 7.5), 0.25 M sucrose, 5 mM EGTA, 2 mM EDTA, 1 mM PMSF, and 10 μg/mL of leupeptin and aprotinin, at 10^8 cells/mL, sonicated at 4 °C and centrifuged at 100,000xg for 1 hr at 4 °C. The supernatant was applied to a DEAE column equilibrated in TRIS buffer, washed with two bed volumes of buffer, and eluted with 100 mM NaCl in TRIS buffer. Pooled active fractions were stored at -20 °C. PKC assay reactions contained 20 mM TRIS, pH 7.5, 10 mM $MgCl_2$, 160 μg/ml histone (type II-S), 20 μg/mL phosphatidylserine, 4 μg/mL diolein, and 10 μM γ-^{32}P-ATP ($1.5x10^6$ cpm/nmol). Reactions were run for 12 min at 30 °C, stopped by addition of 0.5 ml 25% TCA, and BSA (250 μL of 2 mg/mL solution) was added. The samples were filtered through GF/F filters presoaked in 25% TCA. The filters were then washed 3x with cold 5% TCA, and the ^{32}P was counted.

Protein Kinase A was assayed with the commercially available kit from GIBCO-BRL.

p56^{lck} Tyrosine Kinase was prepared by the method of Casnellie.[36] Phosphorylation reactions contained 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 1 mM sodium vanadate, and 0.05 mM γ-^{32}P-ATP ($1.5x10^6$ cpm/nmol) in a volume of 40 μL. The substrate was the SRC peptide (Ile-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg-Gln-Gly), at 100 μg/sample. Reactions were run for two minutes at 30 °C and stopped by addition of cold 3.2% TCA (155 μL). BSA (20 μL of 10 mg/mL solution) was added, and the mixture was centrifuged 10 min at 13000xg. Aliquots (70 μL) were placed on phosphocellulose filters, and washed 5x with 75 mM H_3PO_4 before quantitating in a liquid scintillation counter.

GvH assays.

Spleens from male Lewis rats, 6-12 weeks old, were placed in Hank's Balanced Salt Solution (HBSS) without Ca, Mg, or phenol red. Spleens were washed in cold HBSS a few times by transferring to fresh baths, then minced, partially crushed, and the resulting tissue suspended in HBSS. The suspension was filtered through sterile cotton gauze, and the filtrate transferred to 50 mL polypropylene tubes and centrifuged (5 min @ 1200 rpm). The pellet was triturated with 5-10 mL of lysing buffer (NH_4Cl 8.26 mg/mL, $KHCO_3$ 1 mg/mL, Na_4 EDTA 0.037 mg/mL) and transfered to new tubes. After 7 min, the volume was brought to 50 mL with HBSS and the suspension was spun as before. The supernatant was discarded, and the pellet washed twice more with HBSS. Depending upon the number of spleens prepared, the pellet was resuspended in 10-40 mL of HBSS. The cells were counted with a Coulter counter, and viability was checked by Trypan Blue exclusion. For *ex vivo* experiments, the lymphocytes were exposed to the test compounds (in DMSO) at concentrations ranging from 0.2 to 10 μM.

A regional GvH reaction was induced in 6-12-week-old male Lewis x Brown Norway (LBNF1 hybrid) rats by injecting 0.5 mL of Lewis rat lymphocytes (10^7 cells/mL) into each hind footpad, using a 23 gauge needle to prevent damage to donor cells. Each compound was examined in five animals (ten paws injected). A syngeneic control group of ten LBNF1 rats were injected with the same concentration of LBNF1 lymphocytes, while ten vehicle (allogeneic) control animals received Lewis lymphocytes exposed to DMSO alone. Recipient LBNF1 rats were weighed before injection of the lymphocyte grafts, and again prior to sacrifice 7 days following injection. On the seventh day following lymphocyte inoculation, animals were sacrificed with CO_2, and the popliteal node was surgically removed from each hind leg. Nodes were frozen in dry ice and stored at -70 °C until weighed.

In the *in vivo* modification, compounds were administered i.p. once daily in DMSO solution (DMSO dose 1 mL/kg), for three days beginning on the day of lymphocyte innoculation. Five animals were used at each dose.

Calculations: Left and right node weights were averaged for each animal, and the node/body weight ratio (NBR) was calculated for each animal.

Adjusted Control NBR = (Allogeneic group average NBR) - (Syngeneic group average NBR)

Adjusted Treated NBR = (Individual treated animal NBR) - (Syngeneic group average NBR)

% Inhibition = 100 x [(Adjusted Control NBR) - (Adjusted Treated NBR)]/(Adjusted Control NBR)

Individual percent inhibitions for each treatment group were averaged and standard error calculated. Average values for percent inhibition at each concentration were then used to calculate the IC_{50} for each compound.

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