Simple Aromatics Identified with a NFAT-*lacZ* Transcription Assay for the Detection of Immunosuppressants

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Determination of the mechanism of action of FK506 and cyclosporin A has yielded new molecular targets involved in signal transduction during T cell activation. A common target of FK506 and cyclosporin A is inhibition of activation of the NFAT transcription factor, for which a specific binding region is present in the promoter of the IL-2 gene. A reporter gene assay has been used to screen for agents that interfere with this early step in T cell activation. Simple aromatic compounds that block NFAT-dependent transcription and show *in vitro* immunosuppressive activity were isolated from the broth and mycelia of two *Streptomyces* sp. fermentations. The compounds were active at concentrations that were not directly cytotoxic.

T cell activation is a critical event for initiation and regulation of immune responses and inhibitors of signaling pathways are clinically useful for the treatment of transplant rejection and autoimmunity. Both cyclosporin A and FK506 block the production of IL-2 by disrupting T cell activation pathways involving Ca^{2+} mobilization.[1] Despite similar biological effects, the two drugs are structurally different and bind uniquely to two distinct cytosolic proteins.[2,3] Cyclosporin A binds to cyclophilins and FK506 binds to FKBPs. Cyclophilins and FKBPs are ubiquitous highly conserved proteins that share no primary or tertiary homology. However, both proteins catalyze peptidylpropyl isomerization and may fold proteins *in vivo*. Although binding of the drugs to their respective immunophilins results in inhibition of peptidylproline isomerization this activity is not directly related to immunosuppression.[4] Using constructs in which mRNA production controlled by a specific transcription factor could be measured, it was demonstrated that both FK506 and cyclosporin A inhibited transcription activated by NFAT, a heterodimer that binds to a specific sequence present in the IL-2 promoter region.[5] To find the factors mediating inhibition, affinity chromatography was used to search for proteins that bind FK506-FKBP and cyclosporin A-cyclophilin. Calcineurin, a serine-threonine phosphatase, was found to bind to both complexes and was the major target in tissue and cell extracts.[6] Binding of drug-immunophilin complexes to calcineurin inhibits the phosphatase activity which either directly or indirectly regulates NFAT function by controlling the phosphorylation-dependent nuclear translocation of the cytosolic component of NFAT.[1] Since production of IL-2 is an important early event in T cell activation, inhibition of NFAT-dependent transcription represents a defined functional screen for potential immunosuppressants.

Using a NFAT-*lacZ* β-galactosidase reporter gene construct that has three copies of the NFAT binding region ligated to a minimal IL-2 promoter and the *lacZ* gene we have screened extracts of microbial fermentations for immunosuppressants. Agents that block NFAT-dependent transcription may act at any of the known, or as yet undescribed, early Ca^{2+} dependent signaling pathways in T cell activation. Following 6 hour exposures to phorbol ester and ionomycin, NFAT-dependent expression of β-galactosidase, the product of the *lacZ* gene, is easily detected with 4-methylumbelliferyl β-D-galactoside. Cyclosporin A and FK506 strongly inhibit this response at concentrations that do not result in detectable toxic effects on the cells. In this report we describe the isolation, structure, and biological activity of new substituted aromatics from two different *Streptomyces* sp. that also inhibit NFAT-dependent transcription.

Materials and Methods

Microorganisms

Both cultures described in this report were isolated from soil samples obtained in north central Nigeria. Strain AB 2199J-103 (producer of NFAT-68) was isolated from soil collected from a cashew orchard using the method described by HAYAKAWA *et al.*[7] for obtain-

ing *Dactylosporangium*. Strain AB 2184C-502 (producer of NFAT-133) was isolated from a corn field soil using a method designed to increase the range of diverse cultures that can be isolated from a soil by plating it on several media differing only by nitrogen source. The control medium was a modified Gause No. 1 agar containing soluble starch (Baker) 0.1%, KNO_3 0.1%, yeast extract (Difco) 0.01%, K_2HPO_4 0.05%, $MgSO_4$ 0.05%, NaCl 0.05%, 1.0 ml of a trace element solution per liter[8] and agar 2.0%. The medium was amended with cycloheximide (50 μg/ml), nystatin (20 μg/ml) and nalidixic acid (30 μg/ml). AB 2184C-502 was isolated from an experimental medium which substituted histidine 0.125% for KNO_3 as a sole nitrogen source. Subcultures of each microorganism were deposited at the National Center for Agricultural Utilization Research, United States Department of Agriculture, 1815 North University Street, Peoria, Illinois, 61604 U.S.A. The accession codes of strains AB 2199J-103 and AB 2184C-502 are NRRL B-16948 and NRRL B-16949, respectively.

Taxonomic Studies

The cultural characteristics of strains AB 2199J-103 and 2184C-502 were examined using the methods and media described by SHIRLING and GOTTLIEB.[9] Incubation for cultural characteristics was at 28°C for 21 days. Analysis of the whole-cell diaminopimelic acid isomer was done by the method of HASEGAWA, *et al.*[10] Color names were assigned to the mycelial and diffusible pigments on the basis of the Inter-Society Color Council-National Bureau of Standards (ISCC-NBS) Centroid Color Charts.[11]

Fermentation

NFAT-68 was produced by submerged fermentation of strain AB 2199J-103 in a medium consisting of glucose monohydrate 2%, molasses 0.5%, Lexein F-152 liquid peptone (Inolex Chemical Co.) 1%, primary yeast 0.5%, $CaCO_3$ 0.2% and Amberlite XAD-16 resin (Rohm & Haas) 5%. Inoculum preparation and operation of the fermentation was as described below for NFAT-133 except that a 42-liter fermentor charged with 30 liters of medium was used. The NFAT-68 fermentor was harvested after 5 days.

NFAT-133 was produced by fermentation in a 22-liter stirred vessel (LH Fermentation) charged with 15 liters of a medium consisting of soluble starch 3%, molasses 2%, spray-dried lard water (Inland Molasses Co.) 1%, brewer's yeast 0.5%, $CaCO_3$ 0.2% and Amberlite XAD-4 resin (Rohm & Haas) 5%. The medium was prepared in distilled water and the pH was adjusted to 7. Sterilization was at 121°C and 1.05 kg/cm^2 for 1 hour. Inoculum for the fermentation was prepared in 2-liter Erlenmeyer flasks containing 600 ml of a medium consisting of glucose monohydrate 1.5%, soy flour 1.5%, yeast extract (Difco) 0.1%, NaCl 0.1% and $CaCO_3$ 0.1% in distilled water. The flasks were seeded at 0.5% with vegetative mycelium from previous inoculum which had been maintained at −70°C. Incubation of the seed flasks was at 28°C for 72 hours on a rotary shaker operated at 225 rpm (5.08 cm stroke). The resulting growth was used at 5% to inoculate the fermentor. During fermentation the temperature was controlled at 28°C, agitation was 250 rpm, the air flow was 0.7 vol/vol/minute and the head pressure was maintained at 0.35 kg/cm^2. Foam was controlled with a silicone antifoam, XFO 371 (Ivanhoe Industries), added initially at 0.01% and then available on demand. The fermentation was harvested on the 5th day.

Structure Determination

Fast atom bombardment mass spectra were measured on a Kratos MS-50 mass spectrometer. Ultraviolet spectra were recorded on a Perkin-Elmer Lambda 3B UV-visible spectrophotometer. Infrared spectra were recorded on a Nicolet model 60SX FT-IR. Nuclear magnetic resonance spectra were acquired on a General Electric GN500 spectrometer.

NFAT Assay

The Fgl-5 Jurkat cell line, obtained from GERALD CRABTREE, Stanford University, has NFAT specific enhancer regions which regulate the activity of the IL-2 promoter on CD-8 and β-gal reporter genes. Cells were maintained in RPMI-1640 with 10% fetal bovine serum and 50 μg/ml hygromycin-B (the plasmid used to transfect the Jurkat cells contained a hygromycin resistance marker) in a humidified atmosphere containing 5% CO_2. Cultures (25 ~ 100 ml, 2×10^5 cells/ml) were established in plastic tissue culture flasks and cells were stimulated the next day by the addition of 2 μM ionomycin and 32 nM phorbol myristate acetate (final concentrations). Cultures were established in 96-well microtiter trays (200 μl/well) and incubated for 5 hours. After the incubation, the cells were pelleted by centrifugation and the culture fluids removed by aspiration. β-Galactosidase reaction medium (180 μl; 100 mM sodium phosphate pH 7.0, 10 mM KCl, 1 mM $MgSO_4$, 0.1% Triton X-100 and 0.5 mM 4-methylumbelliferyl β-D-galactoside) was added to each well and incubated 1 hour at 37°C. The reaction was stopped by the addition of 75 μl of a stop buffer (300 mM glycine and 15 mM EDTA pH 11.3) per well. The fluorescence at 460 nm was measured using 355 nm excitation with a Millipore Cytofluor 2350.

Calcineurin Phosphatase Activity

Bovine brain calcineurin and calmodulin (Sigma) were mixed in reaction buffer (20 mM Tris-acid pH 7.4, 0.1 M NaCl, 6 mM $MgCl_2$, 1 mM $CaCl_2$, 3 mM $MnCl_2$, 0.5 mM dithiothreitol, 0.1% Triton X-100 and 0.1 mg/ml bovine serum albumin (Sigma, type V) with calmodulin in a 10-fold molar excess. Reactions were initiated by mixing 25 μl of the reaction buffer containing the substrate, 4-methylumbelliferyl phosphate (Sigma). Final concentrations of calcineurin, calmodulin, and 4-methylumbelliferyl phosphate were 3 nM, 30 nM, and 250 μM,

respectively. After rapid shaking to mix the reactions, the microplates were incubated for 6 hours in a humidified atmosphere at 37°C. Reactions were stopped by the addition of an alkaline buffer (300 mM Na_2HPO_4, 15 mM EDTA and 15 mM EGTA, pH 11). Fluorescence was measured as described for the NFAT assay.

Mixed Lymphocyte Reaction

The two-way mouse mixed lymphocyte reactions using splenocytes isolated from Balb/c and C57BL/6 mice were carried out as previously described.[12]

β-Galactosidase Assays

The ability of compounds to directly inhibit the reporter gene product, β-galactosidase, was determined as described for the NFAT assay, except that authentic *E. coli* β-galactosidase (Sigma, Grade VIII, final enzyme concentration was 10^{-5} units/ml) was the source of enzyme.

Results

Taxonomy

Analysis of whole-cell hydrolysates of strains AB 2199J-103 and AB 2184C-502 showed that both contained LL-diaminopimelic acid indicating that the cultures have a type I cell wall.[13] Neither culture formed sporangia, sclerotia or zoospores. The substrate mycelium of both isolates was branched and did not fragment. The cultural characteristics of both strains are shown in Table 1.

The mature aerial spore mass of strain AB 2199J-103 was gray or grayish brown on most media. The colony reverse was brown. This culture did not produce soluble pigment in ISP-6 or ISP-7 agars. It colored ISP-1 broth a light brown. The pigment appeared not to be melanoid since these are dark. The spores were spherical to oval, had a smooth surface and formed long spiral chains on ISP-2, ISP-3, ISP-4 and ISP-5 media. They typically measured 0.8 ~ 1.1 μm × 1.0 ~ 1.3 μm in diameter. A spore chain of strain AB 2199J-103 is shown in Fig. 1. The

Fig. 1. Scanning electron micrograph of spore chains of strain AB 2199J-103 grown on ISP-4 agar for 10 days at 28°C.

Bar represents 1 μm.

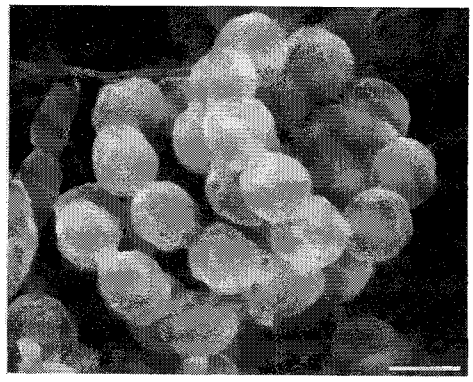


Table 1. Cultural characteristics of strains AB 2199J-103 and 2184C-502.

Medium		AB2199J-103	AB2184C-502
Yeast extract - malt extract agar (ISP 2)	G*:	Abundant	Moderate
	AM:	Medium gray (265)** to greenish white (153)	Bluish gray (191)** to pinkish white (9)
	R:	Strong brown (55)	Light yellowish brown (76)
	SP:	Deep yellowish brown (75)	Light yellowish brown (76)
Oatmeal agar (ISP 3)	G:	Moderate	Moderate
	AM:	Grayish brown (61)	Bluish gray (191)
	R:	Moderate brown (58)	Light grayish yellowish brown (79)
	SP:	Absent	Absent
Inorganic salts - starch agar (ISP 4)	G:	Abundant	Abundant
	AM:	Light brownish gray (63)	Bluish gray (191)
	R:	Moderate yellowish brown (77)	Light yellowish brown (76)
	SP:	Light yellowish brown (76)	Absent
Glycerol - asparagine agar (ISP 5)	G:	Poor	Poor
	AM:	Grayish brown (61)	Light bluish gray (190); no spores
	R:	Moderate brown (58)	Yellowish white (92)
	SP:	Absent	Absent
Peptone - yeast extract iron agar (ISP 6)	G:	Moderate	Moderate
	AM:	Pinkish white (9)	Absent; substrate mycelium is grayish reddish orange (39)
	R:	Moderate orange yellow (71)	Light yellowish brown (76)
	SP:	Absent	Absent
Tyrosine agar (ISP 7)	G:	Moderate	Poor
	AM:	Light grayish brown (60)	Bluish white (189)
	R:	Grayish yellowish brown (80)	Pale orange yellow (73)
	SP:	Absent	Absent

* Abbreviations: G = growth, AM = aerial mycelium, R = reverse, SP = soluble pigment.

** Color and number in parenthesis follow the color standard in KELLY, K. L. & D. B. JUDD: ISCC-NBS Color-Name Charts Illustrated with Centroid Colors. U.S. Dept. of Comm. Suppl. to Cir. 553, Washington, D.C. (1976).

bluish gray on ISP-2, ISP-3 and ISP-4 media. No spores were observed on ISP-5 agar. The colony reverse was light yellowish brown on most media. This culture did not produce melanoid pigment in ISP-6, ISP-7 or ISP-1 media. Long spiral spore chains were formed on ISP-2, ISP-3 and ISP-4 agars. The spores had a hairy surface, were spherical and measured 1.0 μm in diameter. A typical spore chain of strain AB 2184C-502 is shown in Fig. 2.

The whole-cell analyses and morphological characteristics of these two cultures indicate that they both belong to the genus *Streptomyces*. They have been designated *Streptomyces* sp. AB 2199J-103 and *Streptomyces* sp. AB 2184C-502.

Isolation and Structure Determination

Upon completion of fermentation of strain AB 2199J-103, the Amberlite XAD-16 resin and mycelial mass were removed by centrifugation and filtration before the solids were eluted with 14 liters of methanol. The methanol extract was concentrated to yield 72 g of crude material which was triturated sequentially with one liter each of; hexane, EtOAc, Me_2CO, MeOH and H_2O. The MeOH triturate was found to inhibit NFAT dependent transcription and was concentrated to yield 35 g of an oil which was subjected to column chromatography on an XAD-16 resin column developed in an aqueous methanol gradient. NFAT inhibitory fractions which eluted with 25% MeOH were combined and concentrated to yield 2.8 g of oil which was partitioned between EtOAc-EtOH-H_2O (2:1:2). The upper layer from this partition was concentrated to yield 1.6 g of an oil which was subjected to column chromatography on Sephadex LH-20 developed in MeOH to yield 117 mg of pure NFAT-68.

A fast atom bombardment positive ion mass spectrum of NFAT-68 gave an M+H ion at 350, indicating a molecular weight of 349. A ^{13}C NMR spectrum contained 18 carbon signals with 19 attached protons. A ^{14}N NMR spectrum contained one N signal at δ 135.77 ppm (relative to formamide) indicative of an *N*-acetate. Analysis of data from COSY, HMBC and HMQC experiments suggested the presence of a 1,2,3,5-substituted aromatic ring (see Table 2, C-8~C-13) and a branched chain ester moiety (C-1~C-7 and C-14~C-15). Attachment of the branched chain ester and functional groups to the aromatic moiety was determined by analysis of NOE[14] experiments in CD_3OD and d_6-DMSO and by considerations of chemical shifts for the aromatic ring carbons as shown in **1**.

Upon completion of fermentation of strain AB 2184C-502, the XAD-4 resin and mycelial mass were removed by centrifugation and filtration before the solids were eluted with 12 liters of MeOH. The MeOH eluate was concentrated to yield 55 g of material which was then triturated sequentially with one liter volumes each of; hexane, EtOAc, Me_2CO, MeOH and H_2O. Activity was observed in the aqueous triturate which was concentrated to yield 46 g of solid material which was subjected to chromatography on a column of SP-207 eluted with an aqueous methanol gradient. Active fractions from this column were combined and concentrated to yield 5.7 g of material which was then subjected to flash reversed phase C-18 chromatography developed with an aqueous methanol step gradient. Active material eluted with 25% methanol was concentrated to yield 320 mg of material which was subjected to flash reversed phase chromatography on bonded-phase amino packing eluted with an

Fig. 2. Scanning electron micrograph of spore chains of strain AB 2184C-502 grown on ISP-4 agar for 10 days at 28°C.

Bar represents 1 μm.

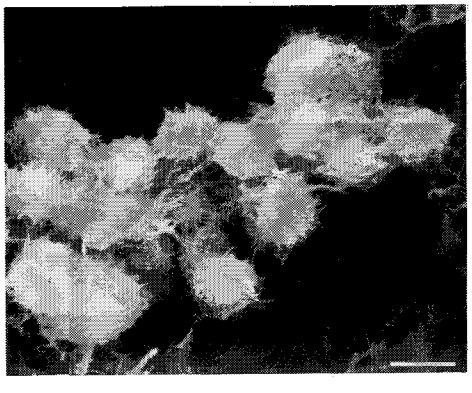
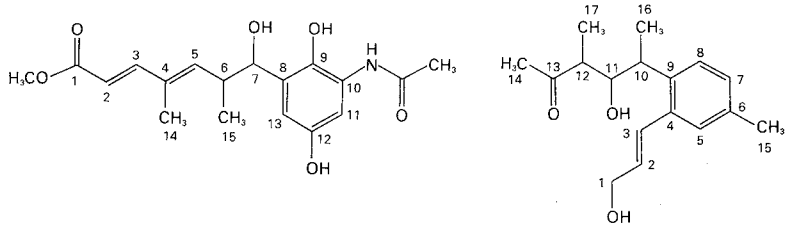


Table 2. NMR assignments for NFAT-68 (in CD_3OD).

Carbon number	^{13}C NMR shift (mult)	Attached ^1H NMR shift
1	169.7 (Q)	
1-OCH_3	51.9 (CH_3)	3.70 (s, 3H)
2	146.2 (CH)	5.72 (d, 1H, J=15.7 Hz)
3	151.4 (CH)	7.22 (br d, 1H, J=15.7 Hz)
4	133.7 (Q)	
5	116.1 (CH)	5.84 (br d, 1H, J=9.9 Hz)
6	40.8 (CH)	3.01 (mult, 1H)
7	75.1 (CH)	4.80 (d, 1H, J=7.2 Hz)
8	133.2 (Q)	
9	140.3 (Q)	
10	127.8 (Q)	
10-NAc	172.5 (Q)	
	23.3 (CH_3)	2.15 (s, 3H)
11	109.3 (CH)	6.80 (d, 1H, J=2.9 Hz)
12	150.9 (Q)	
13	112.5 (CH)	6.57 (d, 1H, J=2.9 Hz)
14	15.9 (CH_3)	1.63 (br s, 3H)
15	12.4 (CH_3)	1.02 (d, 3H, J=6.6 Hz)

Fig. 3. Structures of NFAT-68 and NFAT-133.



(1) NFAT-68 (2) NFAT-133

aqueous acetonitrile step gradient. Active material from this column eluted with 30% acetonitrile, was concentrated to yield 59 mg of material and was subjected to chromatography on Sephadex LH-20 developed with EtOH-H_2O (1 : 1). Active fractions from this column were combined to yield 2.1 mg of pure NFAT-133.

An ammonium ion DCI mass spectrum of NFAT-133 gave a highest mass peak of m/z 294 $[M+NH_4]^+$ and a fast atom bombardment positive ion mass spectrum gave a highest mass peak at m/z 299 $[M+Na]^+$ suggesting a molecular weight of 276. A ^{13}C NMR spectra indicated 17 unique carbon atoms with 22 attached protons consistent with a molecular formula of $C_{17}H_{24}O_3$, containing 2 exchangeable protons. ^{13}C and ^1H NMR spectra (see Table 3) suggested the presence of a 1,2,4-trisubstituted benzene moiety with coupled proton signals at δ 7.24 (C-5), 7.07 (C-7) and 7.17 (C-8). HMBC[15] and HMQC[16] experiments defined the moieties attached to this aromatic ring as shown in **2**.

Strain AB 2184C-502 also yielded the previously reported compound conglobatin[17] which had an IC_{50} of 0.63 μg/ml in the NFAT-dependent transcription assay.

Table 3. NMR assignments for NFAT-133 (in CD_3OD).

Carbon number	^{13}C NMR shift (mult)	Attached ^1H NMR shift
1	63.7 (CH_2)	4.24 (dd, 1H, J=5.5, 1.8 Hz)
2	132.8 (CH)	6.16 (dt, 1H, J=15.6, 5.5 Hz)
3	129.3 (CH)	6.92 (dt, 1H, J=15.6, 1.8 Hz)
4	137.2 (CH)	
5	128.6 (Q)	7.24 (d, 1H, J=1.8 Hz)
6	137.0 (Q)	
7	129.7 (CH)	7.07 (dd, 1H, J=7.8, 1.8 Hz)
8	128.0 (CH)	7.17 (d, 1H, J=7.8 Hz)
9	140.2 (Q)	
10	39.8 (CH)	3.08 (dq, 1H, J=9.0, 6.8 Hz)
11	76.6 (CH)	4.20 (dd, 1H, J=9.0, 3.7 Hz)
12	51.2 (CH)	2.34 (qd, 1H, J=7.0, 3.7 Hz)
13	214.2 (Q)	
14	28.4 (CH_3)	2.05 (s, 3H)
15	21.0 (CH_3)	2.25 (s, 3H)
16	19.2 (CH_3)	1.26 (d, 3H, J=6.8 Hz)
17	9.7 (CH_3)	0.92 (d, 3H, J=7.0 Hz)

Immunosuppressant Activity

Exposure to phorbol ester (P), as an activator of protein kinase C, and ionomycin (I), as a Ca^{2+} ionophore, stimulates NFAT-dependent transcription (Fig. 4A).[5] Both agents are required for expression of β-galactosidase since production in the presence of either activator alone is not significantly different from background levels. As shown in Fig. 4B, expression of β-galactosidase can be detected after 3 hours of stimulation (closed bars) and a strong response (10-fold background levels, hatched bars) was detected after 6 hours. Under these conditions, cyclosporin A and the FK506 analog, ascomycin inhibited NFAT-dependent transcription of the β-galactosidase reporter gene with IC_{50} values of 0.6 and 0.1 ng/ml, respectively. Cytotoxicity, as measured by MTT reduction or ability to exclude vital stains was not observed until cells were exposed to 1 μg/ml concentrations of either agent. Using this assay to screen for immunosuppressants resulted in the identification of two *Streptomyces* sp. fermentation extracts that inhibited NFAT-dependent transcription. Subsequent bioassay guided isolations resulted in the identification of NFAT-68 and NFAT-133. Each compound inhibited NFAT-dependent transcription with IC_{50} concentrations slightly less than 1 μg/ml (Table 4). These compounds were apparently selective inhibitors of NFAT-dependent transcription since marked cytotoxicity was not observed unless cells were exposed to concentrations 7 to 60-fold higher. The compounds also inhibited antigen-dependent activation of T cells in a two way mixed lymphocyte reaction. Neither NFAT-68 or NFAT-133 inhibited β-galactosidase directly or inhibited the phosphatase activity of calcineurin (Table 4).

Fig. 4. NFAT-dependent expression of β-galactosidase.

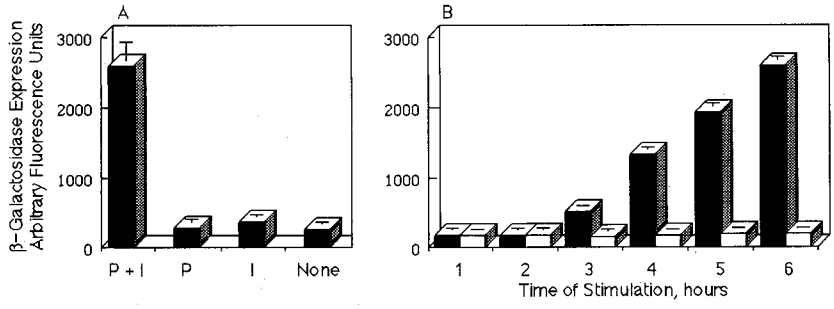


Table 4. Biological activity of NFAT-68 and NFAT-133.

Sample	NFAT assay	Jurkat toxicity	MLR assay	Lymphocyte toxicity	Calcineurin phosphatase	β-Galactosidase activity
NFAT-68	0.22±0.06	14±2	0.65±0.21	4±1	>50	>50
NFAT-133	0.70±0.05	5±1	0.3±0.1	12±5	>50	>50

IC_{50} in assay, μg/ml.

Discussion

The mixed lymphocyte reaction is considered the *in vitro* correlate of graft rejection and has been widely used to screen for immunosuppressants. Assessing effects of agents on the antigen-dependent mitogenic response of splenocytes is the least mechanistically restrictive screen for immunosuppressants and was used to discover FK506.[18] Despite these obvious advantages, the mixed lymphocyte reaction is a time-consuming laborious assay that requires the use of animals. In contrast, the NFAT-*lacZ* β-galactosidase reporter gene construct assay is relatively rapid and only requires cell culture facilities. However, compounds active in this assay would be expected to act on early Ca^{2+}-dependent steps of T cell activation in a manner similar to that of cyclosporin A or FK506.

Simple aromatic compounds from the fermentation broths of *Streptomyces* sp. were discovered with the NFAT-*lacZ* β-galactosidase reporter gene construct assay. These compounds inhibited NFAT-dependent transcription at concentrations that were not apparently toxic to the cells. As expected from the design of the screen, the compounds inhibited the mixed lymphocyte reaction with similar potency. These compounds may provide insight into signalling pathways of early T cell activation.

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