DUNAIMYCINS, A NEW COMPLEX OF SPIROKETAL 24-MEMBERED MACROLIDES WITH IMMUNOSUPPRESSIVE ACTIVITY

III. IMMUNOSUPPRESSIVE ACTIVITIES OF DUNAIMYCINS

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The immunosuppressive effects of the dunaimycins, a new complex of spiroketal 24-membered macrolides, were compared to cyclosporin A, ascomycin, and rapamycin. Each dunaimycin was a potent inhibitor of the mitogenic response observed in mixed murine splenocyte or human leukocyte cultures, and like immunosuppressive drugs these compounds were relatively less potent inhibitors of the constitutive proliferation of murine EL4 thymoma cells. Dunaimycin D4S showed no selectivity in inhibiting the mitogenic response of spleen cells to concanavalin A, pokeweed mitogen, lipopolysaccharide, or phytohemagglutinin. Cyclosporin A and ascomycin did not inhibit interleukin 2 dependent proliferation, whereas the dunaimycins and rapamycin blocked the uptake of [^{3}H]thymidine in mixed cultures supplemented with exogenous interleukin 2. In addition, dunaimycin D4S had no apparent affinity for cyclosporin A or FK-506 immunophilins. Although the dunaimycins inhibited the activity of Na^+, K^+-ATPase, inhibition of this enzyme appeared insufficient to explain the biological activity of these new macrolides. Over a narrow concentration range, dunaimycin D4S showed *in vivo* immunosuppressive activity in the murine popliteal lymph node hyperplasia model.

Cyclosporin A is an effective immunosuppressant widely used as the primary drug to suppress the rejection of organ transplants.[1] FK-506, a structurally unrelated macrocycle isolated from *Streptomyces tsukubaensis*,[2] is a potent immunosuppressant shown to be effective in human transplant recipients at doses lower than those necessary with cyclosporin A.[3] Rapamycin, originally isolated on the basis of antifungal activity,[4] is structurally related to FK-506 and also shows immunosuppressive activity.[5] Cyclosporin A, FK-506, and rapamycin were found to interact with cytoplasmic proteins that possess peptidyl-prolyl *cis-trans* isomerase (rotamase) activity. Cyclosporin A binds and inhibits the rotamase activity of cyclophilin,[6] whereas FK-506 and rapamycin interact with the FK-506 binding protein (FKBP).[7] The observation that structurally unrelated immunosuppressive agents uniquely inhibit the rotamase activity of distinct cytoplasmic proteins suggested a role for this enzymatic activity in transduction of the mitogenic signal during T cell activation. However, 506BD, a synthetic compound which contains common structural elements of rapamycin and FK-506, inhibits the rotamase activity of FKBP and antagonizes the immunosuppressant activity of FK-506, yet it does not inhibit activation of T cells.[8] In addition, despite their structural similarity, rapamycin and FK-506 display distinct differences in their mechanisms of inhibition of T cell activation.[9,10] Cyclosporin A and FK-506 act early during T cell activation and inhibit the transcription of a limited set of early T cell activation genes, whereas rapamycin appears to alter the response of cells to cytokines. The effects of cyclosporin A and FK-506 on a number of molecular events that occur during T cell activation have been described, yet it is apparent that the mechanisms of action of these immunosuppressive drugs are only partly understood.[11]

Dunaimycins are 24-membered macrocyclic lactones discovered in the fermentation broths of *Streptomyces diastatochromogenes* AB1691Q-321 and AB1711J-452. The dunaimycins are related to, but structurally distinct from, the known actinomycete metabolites cytovaricin, oligomycin, and phthoramycin.[12,13] The discovery, taxonomy, and fermentation of the producing cultures and the isolation and elucidation of structure have been described in separate papers.[14,15] In this report, the *in vitro* immunosuppressive activity of compounds was determined with the murine mixed lymphocyte reaction (MLR) and mixed human leukocyte cultures. In addition, the ability of dunaimycin D4S to inhibit the mitogenic response of murine splenocytes to polyclonal mitogens was assessed. The effects of exogenous interleukin 2 (IL-2), potential for binding to cyclophilin or FKBP, and interactions with immunosuppressive drugs were determined to characterize the mechanism of immunosuppression by dunaimycins. Because the dunaimycins are structurally related to the oligomycins, the ability of these compounds to inhibit the catalytic activity of Na^+, K^+-ATPase was also measured. Finally, the *in vivo* immunosuppressive activity of dunaimycin D4S and oligomycin A were compared to cyclosporin A in the murine popliteal lymph node weight gain assay.

Materials and Methods

Immunosuppressive Agents

The dunaimycins were isolated at Abbott Laboratories as described elsewhere.[14,15] Cyclosporin A, formulated in oil as obtained from Sandoz (Switzerland), was chromatographed on silica gel in 1.5% methanol in chloroform and crystallized. Ascomycin was isolated from the fermentation broth of *Streptomyces hygroscopicus* var. *ascomyceticus* ATCC 14891 purchased from the American Type Culture Collection (U.S.A.) and characterized by spectral methods. Oligomycin A was isolated from the fermentation broth of an uncharacterized streptomycete and characterized by spectral methods at Abbott Laboratories. Concanamycin A was a gift from Prof. HARUYASU KINASHI, Hiroshima University, Japan. Oligomycin B and ouabain were purchased from Sigma Chemical Co. (U.S.A.). [*Mebmt*-β-^{3}H]Cyclosporin A was purchased from Amersham Co. (U.S.A.), and [^{3}H]benzoyl ascomycin was produced at Abbott by a method to be published elsewhere.

Murine MLR

The immunosuppressive activity of compounds was assessed with two-way mixed lymphocyte reactions.[16] Balb/c and C57BL/6 mice (Charles River (U.S.A.), female, 17~18 g, 36~42 days old) were sacrificed by cervical dislocation and spleens were asceptically removed. Spleens were homogenized and large debris allowed to settle, before cells were collected by centrifugation (5 minutes, $900 \times g$). Contaminating erythrocytes were lysed by suspension in EDTA-ammonium chloride solution (0.16 M NH_4Cl, 0.01 M $KHCO_3$, and 0.13 mM EDTA), and splenocytes were collected by centrifugation and washed twice in growth medium RPMI-1640 supplemented with 10% fetal bovine serum. Viability of cells was determined by trypan blue exclusion (0.4% trypan blue in 0.15 M NaCl) and cell numbers determined with an electrical particle counter (Model ZBI, Coulter Electronics, U.S.A.). The Balb/c and C57BL/6 spleen cell suspensions were diluted to 2.5×10^6 viable cells/ml and pooled, before mixed cultures (200 μl/well) were established in tissue culture 96-well microtiter plates (96-well flat bottom tissue culture cluster, Costar Co., U.S.A.). After 72-hour incubations at 37°C in a humidified atmosphere of 5% CO_2 in air, increases in proliferation were assessed by cellular uptake of tritiated thymidine. To measure thymidine uptake, 1 μCi of [*methyl*-^{3}H]thymidine (2 Ci/mmol, Amersham Co.) was added per culture. After 4-hour incubations, cells were harvested onto glass fiber filters (G-10, Inotech Biosystems, U.S.A.) by vacuum with a 96-manifold aspirator (Inotech Biosystems). Radioactivity was determined by liquid scintillation counting or direct β-particle counting (Matrix 96, Packard, Meriden, U.S.A.). Agents to be tested were dissolved in 95% ethanol, dispensed into wells of the microtiter plate, and dried under vacuum before cultures were established. The IC_{50} of immunosuppressive compounds was graphically determined after

incorporation of [*methyl*-^{3}H]thymidine was corrected for incorporation in unstimulated cultures and normalized to stimulated untreated cultures: % inhibition = (stimulated cpm − experimental cpm)/(stimulated cpm − unstimulated cpm).

Human Mixed Leukocyte Proliferation

Peripheral blood was drawn into heparin treated tubes and mononuclear cells were isolated by centrifugation over Histopaque 1077 (Sigma). Stimulator cells were suspended at 10^7 cells/ml in complete RPMI-1640 medium (supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 U/ml benzylpenicillin, 50 μg/ml streptomycin, and 50 μM 2-mercaptoethanol) and incubated with 25 μg/ml mitomycin C at 37°C for 30 minutes. After treatment, the stimulator cells were washed three times by centrifugation in complete RPMI-1640 medium. For the assay, responder and stimulator cells were suspended in 200 μl of complete RPMI-1640 medium per well. Responder cells from a single individual were present at a final concentration of 5×10^5 cells/ml. A pool of stimulator cells from 3~4 individuals was used and the cells from each individual were present at a final concentration of 5×10^5 cells/ml. Test compounds were diluted in complete RPMI-1640 medium and added immediately before addition of the stimulator cells. The cultures were incubated for 96 hours at 37°C in a humidified atmosphere of 5% CO_2 in air. After 92 hours of incubation, [*methyl*-^{3}H]thymidine (0.5 μCi/well, E.I. du Pont de Nemours & Co., Inc., U.S.A.) was added. Cultures were collected onto glass fiber filters and washed with water using a PHD harvester (Cambridge Technology, Inc., U.S.A.). The incorporated amount of [*methyl*-^{3}H]thymidine was determined by scintigraphy. The IC_{50} of immunosuppressive compounds was determined by linear regression analysis of the data after normalization as described for the murine MLR.

EL4 Toxicity Assay

EL4 cell cultures (2×10^5 cells/ml, 200 μl volume) were established in microtiter plates, and after 48-hour incubations with agents, cells were enumerated with MTT as previously described.[17]

Mitogen Stimulation

Spleen cells were isolated from C57BL/6 mice and prepared for culture as described above. Optimal concentrations of mitogens (Sigma) were determined in separate experiments, and the concentrations used were 2 μg/ml concanavalin A, 20 μg/ml pokeweed mitogen, 50 μg/ml lipopolysaccharide, or 20 μg/ml phytohemagglutinin. The ability of agents to inhibit mitogen stimulated proliferation was assessed at concentrations that inhibited the MLR by 90%. After 48-hour incubations, uptake of [*methyl*-^{3}H]-thymidine was determined as described for the MLR.

IL-2 Dependent Proliferation

The ability of agents to inhibit IL-2 dependent proliferation was assessed as follows. Mixed spleen cell cultures were established as above and exposed to immunosuppressive agents in the presence of 0, 20, 50, and 100 units of recombinant murine IL-2 (Genzyme Co., U.S.A.). After 72-hour incubations, uptake of [*methyl*-^{3}H]thymidine was measured as described for the MLR.

Immunophilin Binding

Binding of [*Mebmt*-β-^{3}H]cyclosporin A to cyclophilin was assessed with the Sephadex LH-20 method previously described.[6] Briefly, reactions contained 1 μg of the cloned neutral isoelectric form of cyclophilin[18] in 80 μl of Tris buffer (20 mM, pH 7.2) containing 2-mercaptoethanol (5 mM), sodium azide (0.02%) and 7.5% calf serum in wells of a "V-bottom" 96-well microtiter plate. Samples to be tested were added in 10 μl of ethanol and then [^{3}H]cyclosporin A (25,000 cpm/assay, 10 μl volume in ethanol-Tris buffer, 40:60) was added to each reaction mixture and samples were agitated. To separate free cyclosporin A from bound, the samples were applied to 1.8-ml columns of LH-20 in Pasteur pipets. Bound [^{3}H]cyclosporin A was quantitated by measuring the radioactivity in the 0.5 to 1.5-ml eluate. Binding of [^{3}H]benzoyl ascomycin (25,000 cpm/assay) to a human cloned FKBP (1 μg protein/assay) was determined by separation of bound and free [^{3}H]benzoyl ascomycin over Sephadex G-75 columns. The cloning and purification of the human cloned FKBP will be described in a separate report.

Sodium-Potassium Dependent ATPase

The inhibitory effects of compounds on the activity of Na^+, K^+-ATPase were determined in Tris buffer (50 mM, pH 7.6) containing NaCl 0.1 M, KCl 15 mM, $MgSO_4$ 5 mM, and EDTA 2 mM.[19] A solution of the compound to be tested (50 μl) was added to 900 μl of the assay buffer containing 5 mM ATP, and reactions were initiated by the addition of 50 μl of a stock solution of porcine cerebral cortex Na^+, K^+-ATPase (0.01 units/reaction, Grade III, Sigma). After 20-minute incubations at 37°C, 500 μl of 10% TCA was added to each tube to stop reactions, and inorganic phosphate was quantitated as described previously.[20]

To assess the type of inhibition of porcine cerebral cortex Na^+, K^+-ATPase by dunaimycins, enzyme reactions were established as described above. The concentrations of dunaimycin D4S ranged from 10 to 50 μM, and substrate concentrations were 1, 0.5, and 0.1 mM ATP. Data were plotted as the reciprocal form of the velocity equation and a replot of $1/Km_{apparent}$ versus the concentration of dunaimycin ($R=0.96$) was used to determine the Ki.[21]

The phosphatase activity of porcine cerebral cortex Na^+, K^+-ATPase was measured with *p*-nitrophenolphosphate as the substrate in the presence of Na^+ and low concentrations of K^+. The reaction buffer consisted of NaCl 100 mM, KCl 2 mM, $MgCl_2$ 20 mM, EDTA 0.2 mM, and histidine 30 mM, pH 7.4. The compound to be tested was dissolved in ethanol and 50 μl of the solution was added to 900 μl of buffer containing the substrate, before reactions were initiated by the addition of 50 μl (0.05 units/assay) of the Na^+, K^+-ATPase preparation. After 20-minute incubations at 37°C, 50 μl of 1.0 M NaOH was added to each reaction, and absorbance was measured at 400 nm.

Dunaimycin Interactions with Known Immunosuppressants

Mixed splenocyte cultures were established and exposed to agents as described above. Preliminary experiments tested serial dilutions of each drug individually, in order to establish the 50% inhibitory concentrations for [*methyl*-^{3}H]thymidine uptake, and concentrations used are indicated in the text. The interactions of immunosuppressive drugs with dunaimycin D4S were analyzed by the median-effect principle using a commercially available software package.[22]

Uptake of [^{3}H]Cyclosporin A

Balb/c splenocytes were isolated as described above and P388 cells were cultured as previously described.[17] Splenocytes (10^8 cells/ml) and P388 cells (10^{12} cells/ml) were incubated with 0.1, 0.5, or 2.0 μg/ml cyclosporin A spiked with tritiated cyclosporin A (specific activity was 1.2, 0.2, and 0.06×10^6 cpm/μg cyclosporin A, respectively) in the presence or absence of dunaimycin D4S (0.1, 0.5, or 2 μg/ml). Uptake of cyclosporin A was determined with a previously described sucrose-cushion centrifugation method.[23] Briefly, a 2-ml sample of each cell suspension was layered on a 1-ml cushion of 0.25 M sucrose in 15-ml centrifuge tubes and centrifuged at $120\times g$ for 10 minutes. Tubes were washed twice with minimal disturbance of the sucrose cushion, the sucrose solution was aspirated, and the cell pellet was suspended in 150 μl of 2% Triton X-100. Radioactivity present in a 100-μl sample of the cell suspension was determined by liquid scintillation counting.

In Vivo Popliteal Lymph Node Hyperplasia

For *in vivo* assessment of immunosuppressive activity, Balb/c spleen cells were prepared as described above for the murine MLR and X-irradiated (2000 rads). Cells were washed and resuspended in DULBECCO's phosphate-buffered saline (PBS, pH 7.2) and 50 μl containing 2.5×10^6 cells were injected subcutaneously on the planter surface of the rear foot of C3H recipient mice.[24] Compounds were dissolved in ethanol to give a final solvent concentration of 1% and diluted in 0.2% carboxymethylcellulose in PBS for intraperitoneal administration in a volume of 10 ml/kg. Animals were dosed on challenge (day 0) and once daily thereafter. On day 4, the mice were killed with CO_2 and the popliteal nodes from the challenged limb were dissected free and weighed. Mean weights from control uninjected mice were subtracted to correct for the baseline unstimulated normal node weight. The mean weight increase from spleen cell challenged control mice given vehicle alone was used to calculate percent inhibition of node weight increase in compound-treated mice.

Results

Cyclosporin A and FK-506 potently inhibit the human and murine MLR, but are not effective inhibitors of the constitutive proliferation of cultured T leukemia cells.[25] Similarly, the dunaimycins inhibited the mitogenic response in mixed murine splenocyte or human leukocyte cultures at ng/ml concentrations, but were relatively poor inhibitors of EL4 cell proliferation (Table 1). In parallel determinations, the ratio of the IC_{50}s for inhibition of the MLR and EL4 cell proliferation by dunaimycin C2 was greater than that observed with cyclosporin A or rapamycin, but less than that of ascomycin. With the exception of dunaimycin D2, concentrations in excess of 1 μg/ml of each dunaimycin were required to inhibit the growth of EL4 cells.

Dunaimycin D4S, at a concentration that inhibited the MLR by 90%, strongly inhibited the mitogenic response stimulated by concanavalin A, pokeweed mitogen, lipopolysaccharide, or phytohemagglutinin. As shown in Table 2, no selectivity for inhibition of B or T cell mitogens was observed for rapamycin, whereas cyclosporin A and ascomycin were less potent inhibitors of proliferation induced by the B cell mitogen lipopolysaccharide.

Cyclosporin A and ascomycin inhibit production of IL-2 by T cells, but do not alter certain responses

Table 1. Inhibition of the murine MLR and EL4 cell proliferation by dunaimycins and immunosuppressive drugs.

Compound	Mouse MLR IC_{50} (ng/ml)	EL4 cell IC_{50} (ng/ml)	EL4 IC_{50} / MLR IC_{50}	Human MLR IC_{50} (ng/ml)
Dunaimycin:				
A1	120 ±10[a]	1,760±580[b]	15	—
C1	1 ± 0.9	1,930±130	1,930	1.1[c]
C2	0.4± 0.1	1,120±350	2,800	0.20
C2S				—
D2	0.4± 0.2	230± 75	575	0.18
D2S	1 ± 0.9	1,500±200	1,500	0.22
D3	10 ± 8	1,270± 90	130	1.8
D3S	3 ± 1	2,100±550	700	1.2
D4S	3 ± 3	1,570±390	520	0.35
Cyclosporin A	6.5± 0.7	4,300±200	660	5.8
Ascomycin	0.6± 0.3	6,500	10,800	0.13
Rapamycin	1.4± 0.7	260±100	190	0.3

[a] IC_{50} for inhibition of [^{3}H]thymidine uptake in mixed murine splenocyte cultures (mean±SD, $n=3\sim5$ separate determinations).

[b] IC_{50} for inhibition of MTT reduction by cultured EL4 cells (mean±SD, $n=3$ separate determinations).

[c] IC_{50} for inhibition of [^{3}H]thymidine uptake in mixed human leucocyte cultures (mean of 2~4 separate determinations).

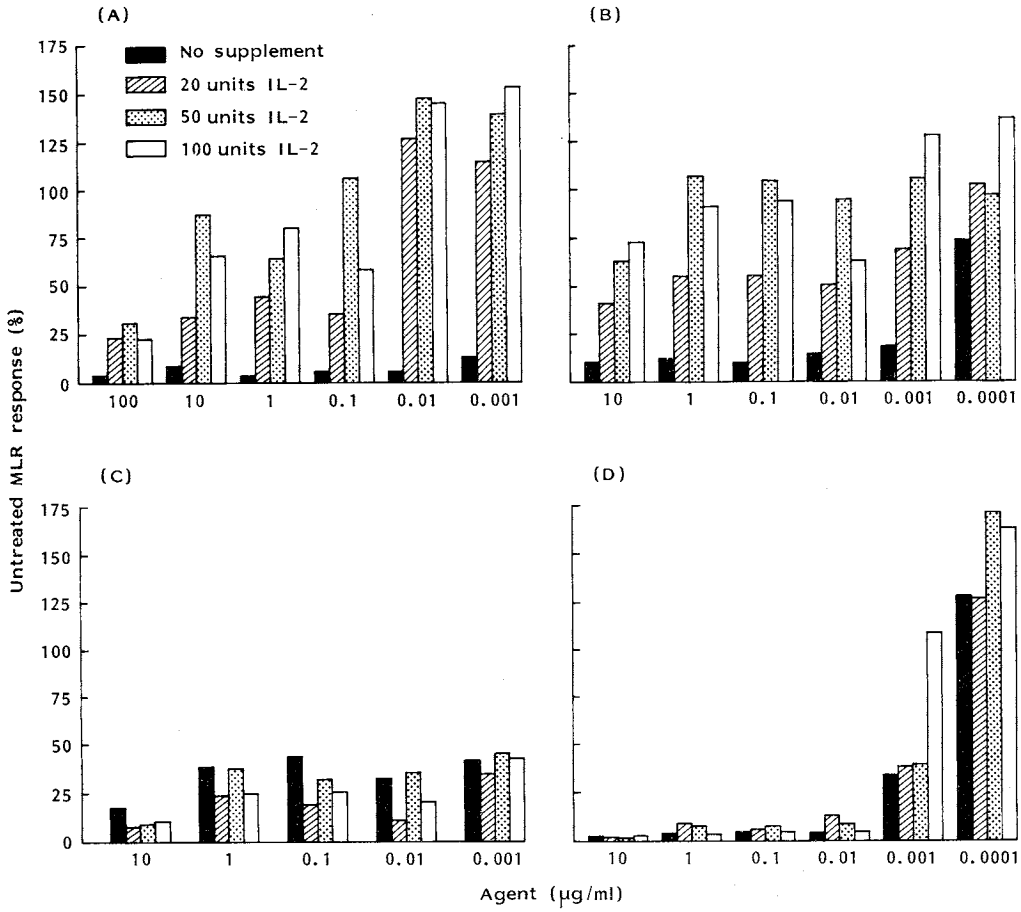
Table 2. Inhibition of the mitogenic response induced by polyclonal mitogens.

Mitogen	Untreated	Dunaimycin D4S	Cyclosporin A	Ascomycin	Rapamycin
None	1,740± 160[a]	316± 16	181± 24	862± 92	351± 79
Con A	44,306±7,530	542± 88	674± 183	1,157± 587	1,959±382
PHA	11,744±3,005	631± 84	938± 265	743± 206	1,009±379
PWM	27,027± 622	531± 68	870± 359	1,475± 568	1,253±274
LPS	29,939±2,132	539±153	7,636±1,941	8,204±2,437	813±172

[a] Uptake of [^{3}H]thymidine (cpm±SD, $n=3$) by spleen cells in the presence of the MLR IC_{90} of agents indicated.

Fig. 1. Uptake of [^{3}H]thymidine by spleen cells in mixed cultures treated with agents in the presence and absence of exogenous recombinant IL-2.

(A) Cyclosporin A, (B) ascomycin, (C) rapamycin, (D) dunaimycin.



to this cytokine.[9,26] The addition of exogenous recombinant IL-2 to mixed splenocyte cultures blocks the ability of cyclosporin A or ascomycin to inhibit uptake of [*methyl*-^{3}H]thymidine, but has no apparent effect on the immunosuppressive activity of rapamycin or dunaimycin D4S (Fig. 1).

Since the known immunosuppressants cyclosporin A, FK-506, and rapamycin bind with high affinity to immunophilins, the capacity of dunaimycins to bind to cyclophilin or FKBP were assessed with competition binding experiments. At the relatively high concentration of 100 μg/ml, dunaimycin D4S inhibited binding of [*Mebmt*-β-^{3}H]cyclosporin A to cyclophilin by 24% and binding of [^{3}H]benzoyl ascomycin to FKBP by 11%. Each of the other seven dunaimycins (100 μg/ml), did not significantly inhibit binding of [*Mebmt*-β-^{3}H]cyclosporin A or [^{3}H]benzoyl ascomycin to their respective immunophilins. In parallel experiments, the IC_{50} of unlabeled cyclosporin A for displacement of radiolabeled drug was 2 ng/ml, and unlabeled FK-506 (100 μg/ml) totally blocked binding of [^{3}H]benzoyl ascomycin to FKBP.

The dunaimycins are structurally related to oligomycin A which interacts with the extracellular side of the Na^+, K^+-ATPase at a site different from the cardiac glycosides and vanadate.[27] As shown in Fig. 2, dunaimycin D4S inhibited the ATPase activity of porcine cerebral Na^+, K^+-ATPase with a potency

Fig. 2. Inhibition of porcine cerebral cortex Na^+, K^+-ATPase by dunaimycin D4S and oligomycin A.

■ Dunaimycin D4S, □ oligomycin A.

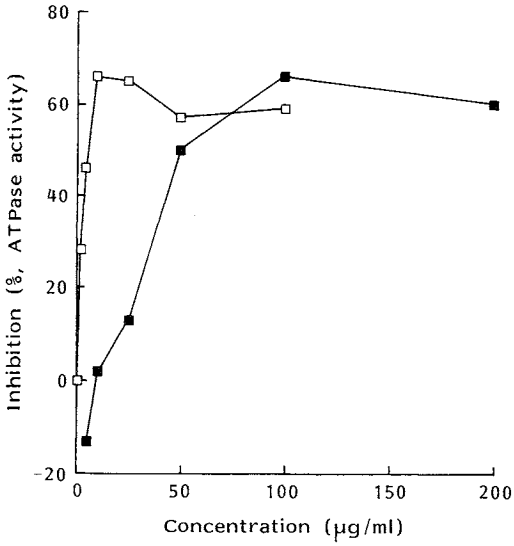


Table 3. Inhibition of porcine cerebral Na^+, K^+-ATPase by dunaimycins.

Compound	ATPase inhibition		Na^+ phosphatase inhibition[c]
	%[a]	IC_{50}[b]	
Dunaimycin A1	9	225	16
Dunaimycin C1	38		38
Dunaimycin C2	34	140	28
Dunaimycin D2	39		30
Dunaimycin D2S	22		21
Dunaimycin D3	22		30
Dunaimycin D3S	15	115	22
Dunaimycin D4S	50	50	30
Oligomycin A	69	7	−44

[a] % inhibition at 50 μg/ml.
[b] IC_{50} value, μg/ml.
[c] % inhibition at 50 μg/ml.

Table 4. Inhibition of the MLR and EL4 cell proliferation by known inhibitors of Na^+, K^+-ATPase.

Compound	MLR IC_{50} (ng/ml)	EL4 IC_{50} (ng/ml)	MLR/EL4 IC_{50} ratio
Ouabain	13,000	350,000	27
Concanamycin A	0.2	0.2	1
Oligomycin A	0.2	80	400
Oligomycin B	0.4	400	1,000

similar to that of oligomycin A, and like oligomycin A appeared to only partially inhibit activity. At concentrations of dunaimycin D4S as high as 200 μg/ml, one third of the total ATPase activity remained. As shown in Table 3, each of the dunaimycins (50 μg/ml) inhibited the ATPase activity of the enzyme, and unlike oligomycin A also inhibited the *p*-nitrophenolphosphatase activity of the enzyme in the presence of Na^+ and low concentrations of K^+. Reciprocal plots of the velocity equation indicated uncompetitive inhibition of Na^+, K^+-ATPase by dunaimycin D4S with an apparent *Ki* of 38 μM. In a parallel experiment, uncompetitive inhibition of porcine cerebral Na^+, K^+-ATPase by oligomycin A with an apparent *Ki* of 22 μM was observed.

The IC_{50}s for inhibition of the MLR and EL4 cell proliferation by known inhibitors of Na^+, K^+-ATPase were quantitated to determine if inhibition of this enzyme was sufficient to produce the toxicity profile observed with dunaimycins. As shown in Table 4, ouabain is a relatively poor inhibitor of both the mitogenic response in mixed splenocyte cultures or proliferation of EL4 cells. Concanamycin A, is a more potent inhibitor of both processes, but no differential toxicity was observed. However, both oligomycins A and B were potent inhibitors of the MLR and were less active in inhibiting the proliferation of EL4 cells.

Mixed murine splenocyte cultures were exposed to dunaimycin D4S in the presence of cyclosporin A or ascomycin to determine if interactions between these agents occurred. From the data shown in Table 5, it appears that combinations of cyclosporin A or ascomycin with dunaimycin D4S resulted in less immunosuppressive effects. The observed inhibition of [*methyl*-^{3}H]thymidine uptake in mixed cultures in the presence of ascomycin or cyclosporin A was less than that expected by simply adding the percent inhibition observed in the presence of each agent alone. More rigorous median-effect analysis of

Table 5. Interactions between dunaimycin D4S and cyclosporin A or ascomycin.

[Dunaimycin D4S][a]	Alone[b]	Cyclosporin A				Ascomycin			
		+0.5 ng/ml		+5.0 ng/ml		+0.5 ng/ml		+5.0 ng/ml	
		Obsd.[c]	Pred.[d]	Obsd.[c]	Pred.[d]	Obsd.[c]	Pred.[d]	Obsd.[c]	Pred.[d]
0.00	0	27	—	74	—	11	—	87	—
0.01	21	−39	48	59	95	−6	32	115	100
0.1	52	−62	79	87	100	−13	63	111	100
1.0	88	90	100	111	100	74	99	87	100
10.0	107	107	100	115	100	98	100	59	95

[a] Concentration of dunaimycin D4S, ng/ml.
[b] % inhibition observed in the presence of dunaimycin D4S.
[c] % inhibition observed in the presence of combinations of dunaimycin D4S and agent indicated.
[d] Predicted % inhibition obtained by adding the % inhibition observed in the presence of dunaimycin D4S or the agent indicated, alone in separate cultures.

Fig. 3. Median-effect calculated combination indices for dunaimycin D4S in combination with ascomycin, cyclosporin A, and rapamycin.

■ Ascomycin, ● cyclosporin A, □ rapamycin.

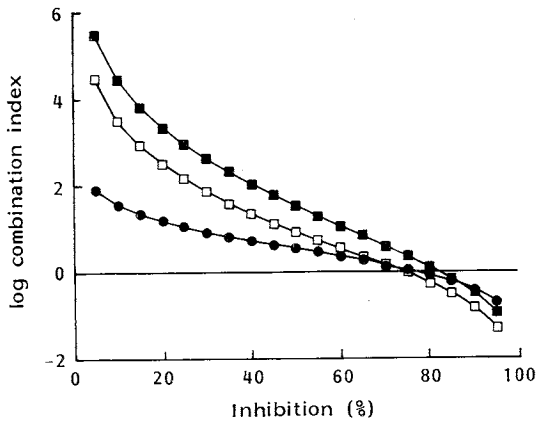
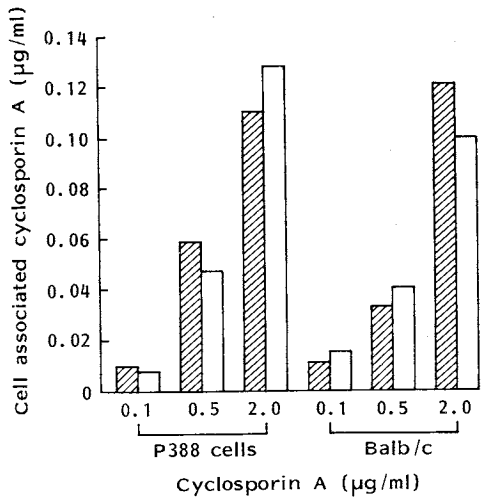


Table 6. Inhibition of popliteal lymph node hyperplasia in mice following allogeneic spleen cell challenge.

Compound	Daily dose (mg/kg, ip)	Node weight % inhibition	Lethality (Mice, dead/total)
Cyclosporin A	100	108	1/10
	30	76	0/10
	10	34	0/10
	3	31	0/10
	1	−15	0/10
Oligomycin A	1.0	—	9/10
	0.3	66	0/30
	0.1	29	0/30
	0.03	−2	0/10
Dunaimycin D4S	3.0	—	10/10
	1.0	54	0/10
	0.3	4	0/10

Fig. 4. Uptake of [^{3}H]cyclosporin A by Balb/c splenocytes or P388 cells in the presence and absence of dunaimycin D4S.

Presence: closed bars, absence: open bars.



The concentration of dunaimycin D4S was the same as indicated for cyclosporin A.

interactions indicated antagonism between dunaimycin D4S and cyclosporin A, ascomycin, and rapamycin (1 to 0.95, 0.8, and 1.2 molar ratios, respectively). Shown in Fig. 3 are the calculated combination indices (CI) for these agents as a function of percent inhibition of [*methyl*-^{3}H]-thymidine uptake in mixed splenocyte cultures. Log CI values less than 0 (a CI of 1 indicates simple additive effects) indicate synergism, whereas values greater than 0 indicate antagonism. At

percent inhibition values less than 80, it is apparent that combinations of dunaimyci D4S and cyclosporin A or ascomycin were antagonistic. However, the model predicted synergism at hig levels of suppression of the MLR by combinations of immunosuppressant drugs and dunaimycin D4S. [edian effect analysis of the interaction of dunaimycin D4S with cyclosporin A (1:9.5), ascomycin (1 8.0), and rapamycin (1:12) also showed antagonism when the MLR was inhibited from 10 to 80%, wi synergism at higher levels of inhibition.

Uptake of [^{3}H]cyclosporin A in the presence and absence of dunaimycin)4S was assessed to determine if competition at the level of cellular transport or binding could ex lain the antagonism between the two agents. As shown in Fig. 4, uptake of [^{3}H]cyclosporin A by alb/c splenocytes or cultured P388 cells was not significantly affected by the presence of dunaimycin D. .

The immunosuppressant activity of the dunaimycins, as described above wi ı *in vitro* assays has been further demonstrated in an *in vivo* model. Table 6 shows results from testing lunaimycin D4S and oligomycin A in comparison to cyclosporin A in a murine model of lymphop)liferation. All three compounds inhibited popliteal node hyperplasia in response to allogeneic challeng consistent with their *in vitro* effects on T cell proliferation, with ED_{50} values of 10, 0.2 and 0.9 m kg/day, respectively. However, unlike cyclosporin A, which showed relatively little toxicity at doses in t immunosuppressive range, both oligomycin A and dunaimycin D4S were almost uniformly fatal at dc es less than an order of magnitude greater than ED_{50} levels.

Discussion

The dunaimycins are new spiroketal 24-membered macrolide antibiotics with immunosuppressive activity. Certain representatives of the dunaimycin class inhibited the mitogenic response of mouse splenocytes or human leukocytes in mixed cultures with a potency similar to that of immunosuppressive drugs, but were relatively poor inhibitors of the proliferation of cultured EL4 cells. Dunaimycin D4S was not selective in inhibiting B or T cell activation, as no differential inhibition of the mitogenic response by lectins specific for B or T cells was observed. The *in vitro* mechanism of immunosuppression by the dunaimycins was apparently different from that of cyclosporin A or FK-506 because the activity of dunaimycin D4S was not antagonized by exogenous IL-2, and these compounds had no apparent affinity for immunophilins in competition binding experiments. The dunaimycins antagonized the immunosuppressive activity of cyclosporin A, ascomycin and rapamycin at percent inhibition values less than 80. However, uptake of cyclosporin A by splenocytes or a cultured tumor cell line was not altered by the presence of dunaimycin D4S, suggesting that antagonism between these agents was unrelated to effects at the level of cellular transport or competition for intracellular binding of cyclosporin A. However, competition for uptake or binding by a small subset of cells present in the unfractionated spleen cell preparations can not be ruled out. Dunaimycin D4S and oligomycin A displayed *in vivo* immunosuppressive activity in the murine popliteal lymph node hyperplasia model, but the therapeutic index was poor in comparison to cyclosporin A.

The dunaimycins inhibited porcine Na^+, K^+-ATPase and *p*-nitrophenylphosphatase activities. Uncompetitve inhibition with respect to ATP and partial inhibition at relatively high concentrations indicates that dunaimycin D4S interacts with the Na^+, K^+-pump in a manner similar to that of oligomycin A. Oligomycin A decreases the rate of turnover of ion transport and the hydrolysis of a phosphoenzyme intermediate by blocking conformational changes in the enzyme required to complete the pump cycle, but does not completely inhibit the enzyme.[28] In the presence of Na^+ plus K^+, fractional inhibition increases with the ATP concentration, and with saturating ATP concentrations (>3 mM) maximal inhibition (64%) reported for oligomycin A (2.5 μg/ml) is similar to results obtained in this study.[29] Dunaimycin D4S was also a potent, but incomplete inhibitor of the ATPase activity. In addition, the dunaimycins inhibited the phosphatase activity of the Na^+, K^+-pump as assessed by hydrolysis of *p*-nitrophenylphosphate in the

presence of Mg^{2+}, Na^+, and low concentrations of K^+.[30] This activity is thought to parallel the terminal hydrolysis of enzyme bound phosphate in the final steps of the Na^+, K^+-ATPase reaction, and is not inhibited by oligomycin A. Instead, activating effects of oligomycin A have been reported. The stimulatory effects are not understood, but inhibition of this activity by dunaimycins suggests that these macrolides interact somewhat differently than oligomycin with the Na^+, K^+-pump.

Electrophysiologic and functional studies have demonstrated that human T and B lymphocytes possess voltage-gated K^+ channels[31~33] and that agents that block these channels (verapamil, quinine, 4-aminopyridine, and tetraethylammonium ion) inhibit mitogenesis.[34] Kinetic studies indicate that 4-aminopyridine and tetraethylammonium ion interfere with early events of T cell activation, and exogenous IL-2 blocked the ability of either compound to inhibit generation of cytotoxic lymphocytes. The activity of these compounds and the general importance of ionic fluxes for signal transduction of the mitogenic response in a number of experimental models[35] has led to the suggestion that K^+ channels are important in transmembrane control of the T lymphocyte activation process. The importance of K^+ flux for the mitogenic response suggests that disruption of membrane potential by the dunaimycins, through inhibition of the Na^+, K^+-pump, could explain the *in vitro* selectivity of the compounds. However, no correlation between potency for inhibition of the porcine Na^+, K^+-ATPase and uptake of [^{3}H]thymidine in stimulated spleen cell cultures was observed, and other inhibitors of Na^+, K^+-ATPase structurally unrelated to the dunaimycins did not show preferential inhibition of the mitogenic response in comparison to inhibition of proliferation of EL4 cells. Others have questioned the specificity of the inhibitory effects of K^+ channel blockers, and have suggested that the effects of these agents result from inhibition of transport of essential metabolites across cell membranes.[36] Hence, it remains possible that the dunaimycins inhibit the ability of stimulated cells to divide, rather than specifically disrupting signal transduction of mitogenic stimuli.

References

1) BRITTON, S. & R. PALACIOS: Cyclosporin A — Usefulness, risks and mechanism of action. Immunol. Rev. 65: 5~22, 1982
2) KINO, T.; H. HATANAKA, M. HASHIMOTO, M. NISHIYAMA, T. GOTO, M. OKUHARA, M. KOHSAKA, H. AOKI & H. IMANAKA: FK-506, a novel immunosuppressant isolated from a *Streptomyces*. I. Fermentation, isolation, and physico-chemical and biological characteristics. J. Antibiotics 40: 1249~1255, 1987
3) STARZEL, T.; J. FUNG, R. VENKATARAMMAN, S. TODO, A. J. DEMETRIS & A. JAIN: FK 506 for liver, kidney, and pancreas transplantation. Lancet 1989-II: 1000~1004, 1989
4) SEHGAL, S. N.; H. BAKER & C. VÉZINA: Rapamycin (AY-22,989), a new antifungal antibiotic. II. Fermentation, isolation and characterization. J. Antibiotics 23: 727~732, 1975
5) MARTEL, R. R.; J. KLICIUS & S. GALET: Inhibition of the immune response by rapamycin, a new antifungal antibiotic. Can. J. Physiol. Pharmacol. 55: 48~51, 1977
6) HANDSCHUMACHER, R. E.; M. W. HARDING, J. RICE, R. J. DRUGGE & D. W. SPEICHER: Cyclophilin: A specific cytosolic binding protein for cyclosporin A. Science 226: 544~547, 1984
7) HARDING, M. W.; A. GALAT, D. E. UEHLING & S. L. SCHREIBER: A receptor for the immunosuppressant FK506 is a *cis-trans* peptidyl-prolyl isomerase. Nature 341: 758~760, 1989
8) BIERER, B. E.; P. K. SOMERS, T. J. WANDLESS, S. J. BURAKOFF & S. L. SCHREIBER: Probing immunosuppressant action with a nonnatural immunophilin ligand. Science 250: 556~559, 1990
9) DUMONT, F. J.; M. J. STARUCH, S. L. KOPRAK, M. R. MELINO & N. H. SIGAL: Distinct mechanisms of suppression of murine T cell activation by the related macrolides FK-506 and rapamycin. J. Immunol. 144: 251~258, 1990
10) DUMONT, F. J.: M. R. MELINO, M. J. STARUCH, S. L. KOPRAK, P. A. FISCHER & N. H. SIGAL: The immunosuppressive macrolides FK-506 and rapamycin act as reciprocal antagonists in murine T cells. J. Immunol. 144: 1418~1424, 1990
11) SIGAL, N. H.; J. J. SIEKIERKA & F. J. DUMONT: Observations on the mechanism of action of FK-506. A pharmacologic probe of lymphocyte signal transduction. Biochem. Pharmacol. 40: 2201~2208, 1990
12) ŌMURA, S.: Chapter 13. Production, structure, and biological properties of macrolide-like antibiotics. *In* Macrolide Antibiotics. Chemistry, Biology, and Practice. *Ed.*, S. ŌMURA, pp. 509~552, Academic Press, 1984
13) ŌMURA, S.; T. TANAKA, K. HISATOME, S. MIURA, Y. TAKAHASHI, A. NAKAGAWA, H. IMAI & H. B. WOODRUFF: Phthoramycin, a new antibiotic active against a plant pathogen, *Phytophthora* sp. J. Antibiotics 41: 1910~1912, 1988
14) KARWOWSKI, J. P.; M. JACKSON, M. L. MAUS, W. L. KOHL, P. E. HUMPHREY & P. M. TILLIS: Dunaimycins, a new complex of spiroketal 24-membered macrolides with immunosuppressive activity. I. Taxonomy of the producing organisms, fermentation and antimicrobial activity. J. Antibiotics 44: 1312~1317, 1991

15) HOCHLOWSKI, J. E.; M. M. MULLALLY, G. M. BRILL, D. N. WHITTERN, A. M. BUKO, P. HILL & J. B. MCALPINE: Dunaimycins, a new complex of spiroketal 24-membered macrolides with immunosuppressive activity. II. Isolation and elucidation of structures. J. Antibiotics 44: 1318~1330, 1991
16) BRADLEY, L. M.: Mixed lymphocyte responses. *In* Selected Methods in Cellular Immunology. *Eds.*, B. B. MISHELL & S. M. SHIIGI, pp. 162~164, W. H. Freeman and Company, 1980
17) BURRES, N. S.; S. SAZESH, G. P. GUNAWARDANA & J. J. CLEMENT: Antitumor activity and nucleic acid binding properties of dercitin, a new acridine alkaloid isolated from a marine *Dercitus* species sponge. Cancer Res. 49: 5267~5274, 1989
18) HOLZMAN, T. F.; D. A. EGAN, R. EDALJI, R. L. SIMMER, R. HELFRICH, A. TAYLOR & N. S. BURRES: Preliminary characterization of a cloned neutral isoelectric form of the human peptidyl prolyl isomerase cyclophilin. J. Biol. Chem. 256: 2474~2479, 1991
19) HENSENS, O. D.; R. L. MONAGHAN, L. HUANG & G. ALBERS-SCHÖNBERG: Structure of the sodium and potassium ion activated adenosinetriphosphatase inhibitor L-681,110. J. Am. Chem. Soc. 105: 3672~3679, 1983
20) MARTIN, J. B. & D. M. DOTY: Determination of inorganic phosphate — modification of isobutyl alcohol procedure. Anal. Chem. 21: 965~967, 1949
21) SEGEL, I. H.: Enzyme Kinetics. pp. 136~143, John Wiley & Sons, 1975
22) CHOU, J. & T.-C. CHOU: Dose effect analysis with microcomputers: quantitation of ED_{50}, LD_{50}, synergism, antagonism, low-dose risk, receptor ligand binding and enzyme kinetics. IBM-PC Series, Elsevier-Biosoft. Cambridge, UK, Elsevier, 1988
23) HAIT, W. N.; J. M. STEIN, A. J. KOLETSKY, M. W. HARDING & R. E. HANDSCHUMACHER: Activity of cyclosporin A and a non-immunosuppressive cyclosporin against multidrug resistant leukemic cell lines. Cancer Communications 1: 35~43, 1989
24) TWIST, V. W. & R. D. BARNES: Popliteal lymph node weight gain assay for graft-versus-host reactivity in mice. Transplantation 15: 182~185, 1973
25) KINO, T.; H. HATANAKA, S. MIYATA, N. INAMURA, M. NISHIYAMA, T. YAJIMA, T. GOTO, M. OKUHARA, M. KOHSAKA, H. AOKI & T. OCHIAI: FK-506, a novel immunosuppressant isolated from a *Streptomyces*. II. Immunosuppressive effect of FK-506 *in vitro*. J. Antibiotics 40: 1256~1265, 1987
26) HOOTON, J. W. L.; C. L. MILLER, C. D. HELGASON, R. C. BLEACKLEY, S. GILLIS & V. PAETKAU: Development of precytotoxic T cells in cyclosporin-suppressed mixed lymphocyte reaction. J. Immunol. 144: 816~823, 1990
27) SKOU, J. C.: Overview: The Na, K-Pump. Methods in Enzymol. 156: 1~25, 1988
28) SKOU, J. C.: The effect of pH, of ATP and of modification with pyridoxal 5-phosphate on the conformational transition between the Na^+-form and K^+-form of the $(Na^+ + K^+)$-ATPase. Biochim. Biophys. Acta 688: 369~380, 1982
29) ROBINSON, J. D.: Effects of oligomycin on the $(Na^+ + K^+)$-dependent adenosine triphosphatase. Mol. Pharm. 7: 238~246, 1971
30) ASKARI, A. & D. KOYAL: Studies on the partial reactions catalyzed by the $(Na^+ + K^+)$-activated ATPase. II. Effects of oligomycin and other inhibitors of the ATPase on the *p*-nitrophenylphosphatase. Biochem. Biophys. Acta 225: 20~25, 1971
31) DECOURSEY, T. E.; K. G. CHANDY, S. GUPTA & M. CAHALAN: Voltage-gated K^+ channels in human T lymphocytes: a role in mitogenesis? Nature 307: 465~468, 1984
32) DOS REIS, G. A.; A. F. NÓBREGA & P. M. PERSECHINI: Stage-specific distinctions in potassium channel blocker control of T-lymphocyte activation. Int. J. Immunopharm. 10: 217~226, 1988
33) AMIGORENA, S.; D. CHOQUET, J.-L. TEILLAUD, H. KORN & W. H. FRIDMAN: Ion channels and B cell mitogenesis. Molecular Immunol. 27: 1259~1268, 1990
34) SHARMA, B.: Inhibition of the generation of cytotoxic lymphocytes by potassium ion channel blockers. Immunology 65: 101~105, 1988
35) SOLTOFF, S. P. & L. C. CANTLEY: Mitogens and ion fluxes. Ann. Rev. Physiol. 50: 207~223, 1988
36) SCHELL, S. R.; D. J. NELSON, H. A. FOZZARD & F. W. FITCH: The inhibitory effects of K^+ channel-blocking agents on T lymphocyte proliferation and lymphokine production are "nonspecific". J. Immunol. 139: 3224~3230, 1987