CENTER FOR DRUG EVALUATION AND RESEARCH

APPLICATION NUMBER: 021083

MICROBIOLOGY REVIEW(S)

IMMUNOLOGY REVIEW
DIVISION OF SPECIAL PATHOGENS AND IMMUNOLOGIC DRUG PRODUCTS (HFD-590)

NDA #: 21-083

REVIEWER	: Shukal Bala
CORRESPONDENCE DATE	: 12-15-98, 04-08-99
CDER RECEIPT DATE	: 12-15-98, 04-12-99
REVIEW ASSIGN DATE	: 12-28-98, 04-16-99
REVIEW COMPLETE DATE	: 07-26-99

SPONSOR: Wyeth-Ayerst Research
P. O. Box 8299
Philadelphia, PA 19101

SUBMISSION REVIEWED: Original and BI

DRUG CATEGORY: Immunosuppressive agent

INDICATION: Prevention of organ rejection in renal transplant recipients

DOSAGE FORM: Solution for oral administration

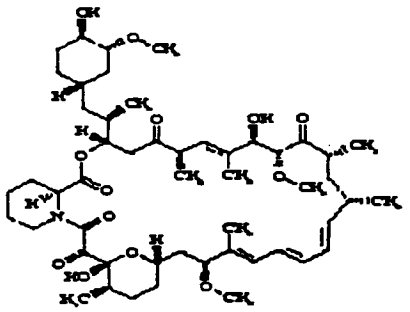
PRODUCT NAMES:

a. **PROPRIETARY:** Rapamune

b. **NONPROPRIETARY:** Sirolimus, AY-022989, rapamycin

c. **CHEMICAL:** (3S,6R,7E,9R,10R,12R,14S,15E,17E,19E,21S,23S,26R,27R,34aS)-9,10,12,13,14,21,22,23,24,25,26,27,32,33,34,34a-hexadecahydro-9,27-dihydroxy-3-[(1R)-2-[1S,3R,4R)-4-hydroxy-3-methoxycyclohexyl]-1-methylethyl]-10,21-dimethoxy-6,8,12,14,20,26-hexamethyl-23,27-epoxy-3H-pyrido[2,1-c][1,4]oxaazacyclohentriacontine-1,5,11,28,29(4H,6H,31H)-pentone

STRUCTURAL FORMULA:



Molecular weight: 914.2
Empirical formula: $C_{51}H_{79}NO_{13}$

SUPPORTING DOCUMENTS: IND [redacted]

BACKGROUND:

The subject of this NDA is Rapamune (sirolimus, rapamycin), for the prevention of organ rejection in renal transplant recipients. The sponsor has recommended that Rapamune be used in a regimen with cyclosporine and corticosteroids.

Sirolimus, like tacromilus, is a macrolide derived from Actinomycetes (*Streptomyces hygroscopicus*). The structure of sirolimus is similar to that of FK-506. It is insoluble in water but soluble in benzyl alcohol, chloroform, acetone, and acetonitrile. The half-life of the drug is about 60 hours.

Immunobiology of transplantation:

Transplantation is a process of transferring grafts (organs, tissues or cells) from one individual to another. Graft rejection is a potential complication of transplantation in which lymphocytes play an important role. Rejection may be mediated by a variety of cell types, which recognize the graft as non-self or foreign and attack it. The process may occur within several different time frames. Hyperacute rejection relies on pre-formed antibodies that activate complement and occurs within a week. Acute rejection employs either antibodies that develop in response to the graft and activate complement (vascular rejection) or is mediated more directly by cells (cellular rejection) including cytotoxic T-cells and macrophages. Chronic rejection is considered to represent a phenomenon of delayed type hypersensitivity. Since antibody production and cell-mediated immunity both depend on functional T cells, a diminution or obliteration of T cell responsiveness has frequently been the goal of drug development for this indication. Steroids, cyclosporine A, tacromilus, monoclonal antibodies, and combinations of these agents have proven to be effective in suppressing cellular (broadly speaking, T cell-dependent) and antibody (T and B cell-dependent) -mediated rejection mechanisms.

For optimal T-cell activation to occur, it is essential that (1) alloantigens be recognized by T-cell receptors, (2) T-cells are costimulated with antigen presenting cells (APC) such as macrophages/dendritic cells/B-cells, and (3) T-cells proliferate and acquire effector functions. Several macrophage (monokines), lymphocyte (lymphokines), and other nonlymphoid cell derived soluble mediators play an important role in the regulation of immune responses and graft rejection. Interleukin (IL) -2 and IL-4, produced by activated T-cells (CD4 and CD8 cells), play an important role in the clonal expansion of T-cells. Recently some growth factors produced by non T-cells such as IL-7 (produced by stromal cells and important for T-cell maturation, proliferation and differentiation) and IL-15 (produced by activated macrophages, muscle cells,

keratinocytes, renal epithelial cells and endothelial cells) were shown to exhibit IL-2 like activities which includes providing support for induction of T- and NK- cell proliferation, cytotoxic T cell function and production of interferon-γ and TNF-α.

SUMMARY:

The immunosuppressive activity of sirolimus (Srl) was measured *in vitro* and *in vivo* and compared with that of other immunosuppressive agents including cyclosporine A (CsA), tacromilus (FK506) and/or cyclophosphamide (CyP).

A. Activity *in vitro*

1. Effect on lymphoproliferation:

Several experiments were done in different laboratories using mononuclear cells (splenocytes or peripheral blood mononuclear cells, PBMC) from mice (Report # GTR-18901, 18914), pigs (Report # GTR-18914) or humans (Report # GTR-18914, 23211, 23211, 23220, 19063, 19070, 20110, 18686) stimulated with various nonspecific agents including phytohemagglutinin (PHA), concanavalin A (ConA), lipopolysaccharide (LPS), pokeweed mitogen (PWM), phorbol myristate acetate (PMA), ionomycin), antibodies to cell surface receptors (CD3, CD28, IgM), allogeneic cells and/or recall antigens (*Staphylococcus aureus* Cowan I, SAC). The lymphoproliferative effect was measured either by incorporation of ^{3}H thymidine (an indicator of DNA synthesis) or ^{35}S methionine (an indicator of protein synthesis). Results of some representative experiments are shown in Tables 1 to 5 and Figures 1 to 8 and discussed below.

The studies showed that Srl was effective in decreasing proliferation induced by ConA, PHA, PWM, LPS, PMA, anti-CD3, anti-CD28, anti-IgM, and allogeneic stimuli but not SAC in mouse, porcine and/or human mononuclear cell cultures. The magnitude of inhibition varied with the concentrations of immunosuppressive agent and mitogen in culture (Tables 1 and 2). Srl also inhibited protein synthesis in unstimulated lymphocytes by about 20% (Table 3). In contrast, the inhibitory effect of 10 nM FK506 on protein synthesis by unstimulated cells was stated to be minimal (4 ± 5 %).

Table 1

EFFECT OF RAPAMYCIN VS. CYCLOSPORIN A ON MURINE SPLENOCYTE PROLIFERATION STIMULATED WITH DIFFERENT MITOGENS

	CPM ± S.E. [a]					
Concentration (M)	0	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Con A 2 µg/ml						
RAPA	76,800 ± 6,268	27,918 ± 4,031	79,958 ± 4,959	85,164 ± 11,944	89,624 ± 11,298	94,502 ± 7,140
CsA	76,800 ±6,768	370 ± 29	12,026 ± 1,909	31,132 ± 3,071	78,200 ± 6,594	84,843 ± 11,252
Con A 0.5 µg/ml						
RAPA	77,712 ± 11,746	7,496 ± 1,004	58,135 ± 6,953	67,376 ± 6,441	61,182 ± 1,132	61,349 ± 4,314
CsA	77,712 ± 11,746	91 ± 19	750 ± 253	6,098 ± 1,835	59,607 ± 18,295	78,326 ± 21,311
LPS 10 µg/ml						
RAPA	49,071 ± 5,101	1,576 ± 690	27,027 ± 3,906	31,700 ± 9,758	32,555 ± 7,744	35,168 ± 9,250
CsA	49,071 ± 5,101	3,482 ± 3,169	35,688 ±11,852	46,255 ± 1,449	49,467 ± 6,459	49,288 ± 9,830

[a] Mean ± S.D. of 3 separate experiments. Triplicate samples were incubated with or without drugs dissolved in ethanol and diluted to the indicated final concentrations in RPMI 1640 medium for a total of 72 h. Proliferation was assessed by incorporation of ^{3}H-TdR (1.0 µCi/ml) during the last 18 hours of incubation. Inhibition of proliferation by drug at 10^{-10} was similar to that by drug at 10^{-9}.

Table 2

EFFECT OF RAPAMYCIN VS. CYCLOSPORIN A ON MURINE SPLENOCYTE PROLIFERATION STIMULATED WITH DIFFERENT MITOGENS

	IC_{50} (µM) [a]	
MITOGEN	RAPAMYCIN	CYCLOSPORIN A
Con A, 2 µg/ml	8.23 ± 1.71	0.17 ± 0.02
Con A, 0.5 µg/ml	2.00 ± 0.50	0.03 ± 0.02
LPS, 10 µg/ml	1.76 ± 0.07	1.77 ± 1.11

[a] Mean IC_{50} ± S.E. for 3 separate experiments. Triplicate samples were incubated with or without drugs dissolved in ethanol and diluted to the appropriate concentrations in RPMI 1640 medium for a total of 72 hours. Proliferation was assessed by incorporation of ^{3}H-TdR (1.0 µCi/ml) during the last 18 hours of incubation.

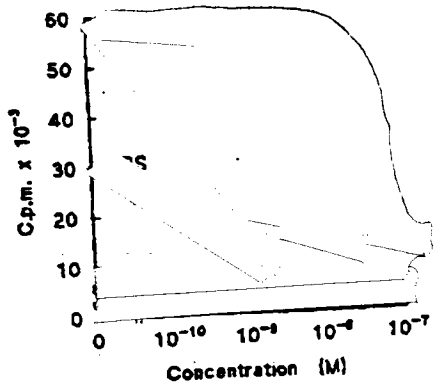
Table 3. Inhibition of lymphocyte protein synthesis by rapamycin. Porcine peripheral blood lymphocytes were incubated with the concentrations of rapamycin indicated and with and without 5-10 ug/ml Con A, and the percentage inhibition of the rate of [^{35}S]-methionine into protein after 24 h in unstimulated cultures or in response to addition of Con A calculated. The values shown are mean percentage inhibition ± S.D., with the number of experiments included indicated in brackets.

Inhibition of lymphocyte protein synthesis by rapamycin

[Rapamycin]	Unstimulated % inhibition	Con A response % inhibition
0.1 nM	18 ± 4% (6)	37 ± 8% (16)
1 nM	22 ± 3% (7)	52 ± 4% (17)
10 nM	21 ± 5% (18)	57 ± 4% (24)
100 nM	19 ± 6% (8)	59 ± 4% (7)

In one comparative study Srl was shown to be less effective (≤ 48-fold) than CsA in inhibiting ConA induced lymphoproliferation (Table 1). The effect of Srl on LPS induced lymphoproliferation was similar to that of CsA (Table 1). In another study (Report # GTR-18914) using mouse splenocytes, Srl was shown to inhibit ConA, LPS or anti-IgM induced lymphoproliferative responses (Figure 1). Although the sponsor stated that CsA and FK506 resistant LPS induced responses were sensitive to Srl, no data to support this claim were included in the report.

Figure 1. Effect of rapamycin on the responses of murine spleen cells to Con A, LPS and anti-IgM. Cells were incubated without mitogen (■) or with 2 ug/ml Con A (o), 20 ug/ml LPS (●), 40 ug/ml goat-anti–mouse IgM (mu chain specific) (-). Activation was assessed by determination of the rate of incorporation of [^{35}S]-methionine into protein at 44 h.



The activity of Srl was also compared with FK506 using porcine and human PBMC (Report # GTR-18914). Using porcine PBMC, Srl inhibited lymphoproliferation of ConA induced cells (Figure 2 and Table 3). However, using human PBMC Srl was more effective than FK506 in inhibiting protein synthesis by PHA or TPA + anti-CD28 antibody stimulated cells (Figure 3). The inhibition of activity by Srl and FK506 on anti-CD3 antibody or TPA stimulated cells was comparable. The sponsor stated that CsA was also used for comparison, however, no data were shown in the report.

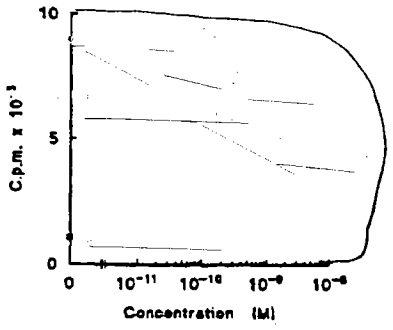


Figure 2a

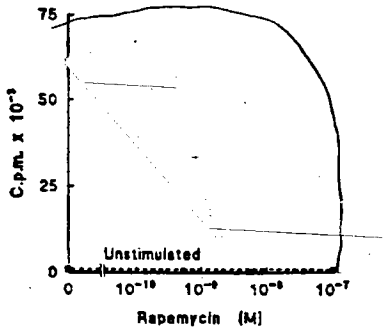


Figure 2b

Figure 2. Effect of rapamycin and FK-506 on the activation of porcine peripheral blood lymphocytes by ConA. Lymphocytes were incubated with 5 ug/ml Con A at the concentrations of rapamycin (•) and FK–506 (o) indicated. Activation was assessed by determination of the rate of incorporation of [^{35}S]-methionine into protein at 24 h (Fig. 2a) or the rate of incorporation of [^{3}H]-thymidine into DNA at 48 h (Fig. 2b).

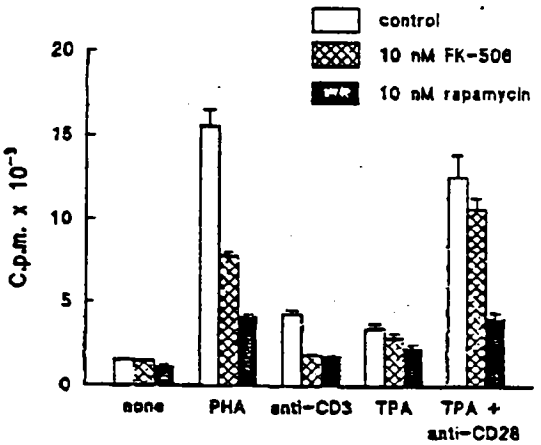
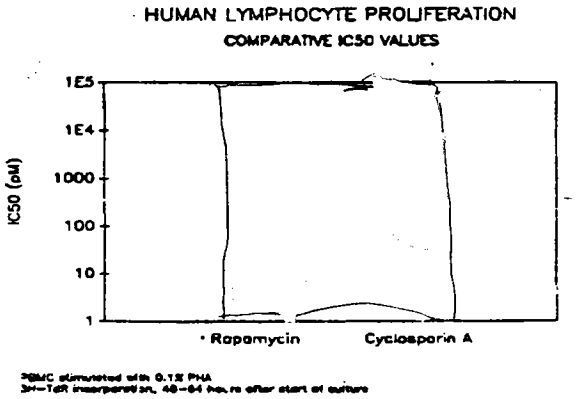


Figure 3. Comparison of the effects of rapamycin and FK-506 on the activation of human peripheral blood lymphocytes. Lymphocytes were incubated without mitogen; with 10 ug/ml PHA; 2.5 ng/ml anti-CD3 (OKT3); 20 ng/ml TPA; or 20 ng/ml TPA + 1 ug/ml anti-CD28 (9.3). Activation was assessed by determination of the rate of incorporation of [^{35}S]-methionine into protein at 46 h.

In another study, human PBMC were activated with PHA or allogeneic cells and incubated with either Srl or CsA (Report # GTR-19063). In the presence of a suboptimal concentration of PHA, the lymphoproliferation in the presence of Srl was highly variable among cells from different individuals with IC_{50} values ranging from 7 pM to 50 nM (Figure 4). The immunosuppressive effect of CsA was less variable with IC_{50} values in the range of 8 to 63 nM (Figure 4). Also, monocyte depleted lymphocytes were more sensitive to the inhibitory activity of Srl, whereas the inhibitory effect of CsA was not altered by removal of thrombin aggregated monocytes.

Figure 4



Inter-person variability was also observed in another study (Report # GTR-18686). PBMC from 7 donors were tested to determine the immunosuppressive effect of Srl on PHA induced proliferation. The IC_{50} values varied from 5 pg/ml to 100 ng/ml. The impact of the drug on activation by allogeneic cells in a mixed lymphocyte reaction showed similar variability.

The immunosuppressive effect of Srl on PMA induced lymphoproliferation was compared to that of FK506 or CsA. PMA can stimulate lymphocytes directly through activation of protein kinase C (Ca^{++} independent pathway), whereas PHA activates cells through a calcium dependent pathway. Human PBMC were stimulated with a calcium ionophore (A23187), PHA or PMA (Report # GTR-19070). Results showed that both Srl and FK-506 inhibited the lymphoproliferative response induced by A23187 and PHA. In general, inhibition was greater with FK-506 compared to Srl. Although the inhibition was highly variable from individual to individual, results in Table 4 show that Srl was effective in inhibiting PMA induced stimulation, whereas FK-506 was not.

In another study, purified human T-cells were stimulated with PMA, anti-CD28 and/or anti-CD3 antibodies (Report # 23220). Results in Figure 5 show that Srl inhibited the PMA, anti-CD28 and/or CD3 antibody induced T-cell proliferation in a dose dependent manner. This effect was observed in the presence or absence of APC (Figure 5a and 5b).

Table 4

Effect of FK 506 and RAPA on lymphocyte proliferation induced by PMA

PBL	Proliferation cpm±S.D.	Drug	%Inhibition of proliferative response Drug (ng/ml)					
			1	0.5	0.1	0.05	0.01	0.001
AZ	107,463±6,912	FK	1.3					
		RAPA	82	78	63	47	26	7
Y	128,645±5,888	FK	8.4					
		RAPA	52	51	42	28	9	2
RR	70,080±7,575	FK	2					
		RAPA	44	28	15	1	0.1	0.1
ML	97,805±4,510	FK	0.6					
		RAPA	75	60	24	12	1	0.3

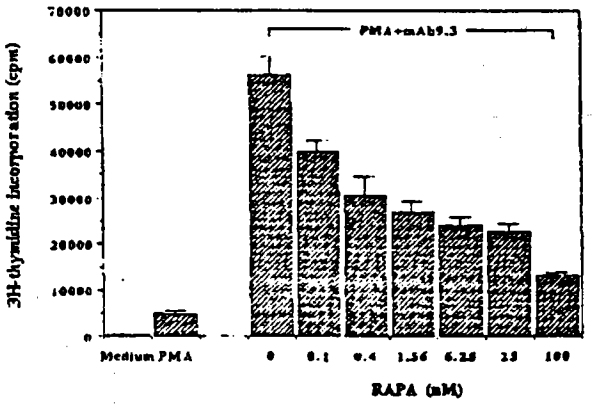


Figure 5a

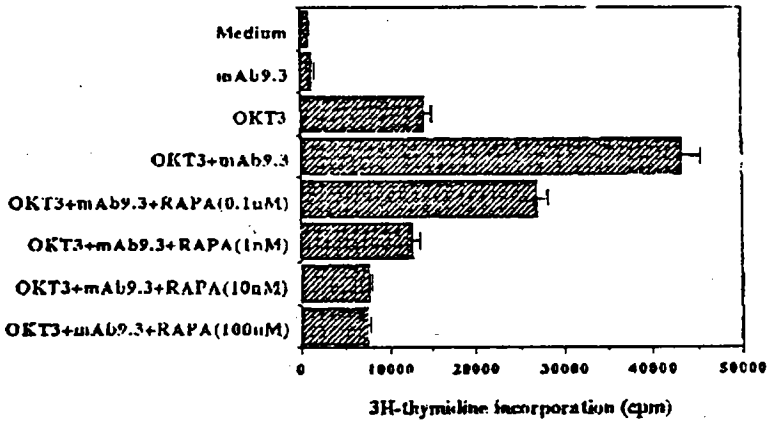


Figure 5b

Figure 5a: RAPA inhibits T–cell proliferation triggered by the CD28 pathway. Highly purified T cells were incubated with PMA (1 ng/ml), αCD28 (mAb 9.3, 50ng/ml), or PMA plus αCD28 in the absence or presence of RAPA (0.1 nM to 100 nM) for 3 days. The cells were pulsed with ^{3}H–thymidine for 6 h before harvest. Samples were in triplicate. Samples with αCD28 alone had ^{3}H-thymidine uptake of less than 1000 cpm.

Figure 5b: RAPA inhibits T-cell proliferation triggered by a combination of CD3 and CD28 pathways. PBMC were incubated with αCD28 (clone 9.3, 50 ng/ml), αCD3 (OKT3, 50 ng/ml) or a combination of both for 3 days in the absence of presence of RAPA (0.1 nM to 100 nM). The cells were pulsed with ^{3}H-thymidine for 6 h before harvest.

Similar observations were made in another study (Report # GTR-23211) wherein the immunosuppressive effect of Srl and CsA on lymphoproliferation was measured using human PBMC stimulated with optimal concentrations of various stimulants (PHA, PMA, ionomycin). Results in Figure 6 show that Srl inhibited cell proliferation in response to all stimulants ($p < 0.001$) with the exception of PMA+ionomycin, whereas CsA inhibited all ($p < 0.001$) with the exception of PMA.

Figure 6. The effect of addition of rapamycin (blank bars; 10 ng/ml) or CsA,(hatched bars; 1 ug/ml) on the proliferation of PBMC in response to either PHA, PMA, ionomycin (ION), PHA/PMA (P/P) or PMA/ionomycin (P/I). Results are expressed as the ratio of cpm in cultures with immunosuppressants to the cpm in control cultures using the same cells and the same stimulant, and are presented as the mean ± 1 SEM of 9 experiments.

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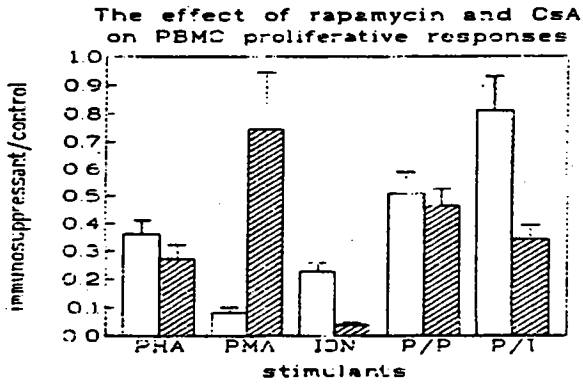

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Srl was also shown to inhibit (> 65%) the proliferation of 4 EBV transformed cell lines, whereas FK506 was not effective at even 10-fold higher concentrations. Daudi (human Burkitt's lymphoma) and HL60 (peripheral blood) cells exhibited intermediate sensitivity (29 and 64% respectively) to Srl but not to FK506. K562 (human chronic myelogenous leukemia) and HUT 78 (human cutaneous T-cell lymphoma) cell lines were not sensitive to either of the immunosuppressive agents.

Similar observations were made using 10 adult T-cell leukemia/lymphoma (ATL) cell lines or primary cell cultures. ATL is believed to result from infection by HTLV-1 and the transformed T-cells are characterized by uncontrolled proliferation in the absence of antigenic stimulation and IL-2, and significantly greater expression of high affinity IL-2 receptor on the cell surface. Cells were cultured for 24 hours in the presence or absence of Srl or CsA (Report # GTR-20110). Lymphoproliferation was measured by incorporation of ^{3}H thymidine following incubation for an additional 24 hours. Results showed that 8/10 cell lines or primary cell cultures were inhibited by ≥ 50% at a concentration of ≥ 100 nM Srl. CsA below 100 nM was not effective in inhibiting lymphoproliferation in these cell lines (data not shown).

The time kinetics of various immunosuppressive agents was tested using porcine PBMC (Report # GTR-18914). Results showed that both Srl and FK506 inhibited ConA induced lymphoproliferation when added up to 24 hours post stimulation (Figure 7). A delay of up to 4 hours did not appear to alter the inhibitory effect of Srl (as shown by mean cpm). In contrast, a delay in the addition of FK506 did appear to have an effect on its activity, as evidenced by the increase in cpm. In the absence of raw cpm data or an indicator of measurement of variability it is difficult to conclude that the differences in mean cpm are meaningful. The sponsor also compared the time kinetics of FK506 with CsA, however, these data were not included in the report.

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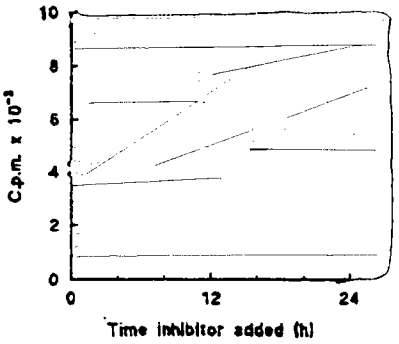

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Figure 7. Effect of delayed addition of rapamycin and FK-506. Porcine lymphocytes were incubated with 10 ug/ml Con A and 10 nM rapamycin (•) or FK506 (o) added at the times indicated thereafter. Activation was assessed by determination of the rate of incorporation of [^{35}S]-methionine into protein at 28 h.

In another study (Report # GTR-18686) the lymphoproliferative response of human PBMC to PHA was shown to be inhibited even when Srl was added at 48 hours of cultures. However, the inhibition was greater when added at the time of initiation of culture. In contrast, CsA was only effective if added at the time of initiation of cultures (Figure 8). These results indicate that the inhibitory effect of Srl on T-cell proliferation was not cell cycle restricted whereas the effect of CsA was. The inhibitory effect of Srl on PWM induced lymphoproliferation was less (≤ 49%). Srl inhibited the proliferative response by 32 – 49 % when added at baseline (0 hour). However, the inhibitory effect decreased when the drug was added at later time points (Table 5).

Figure 8

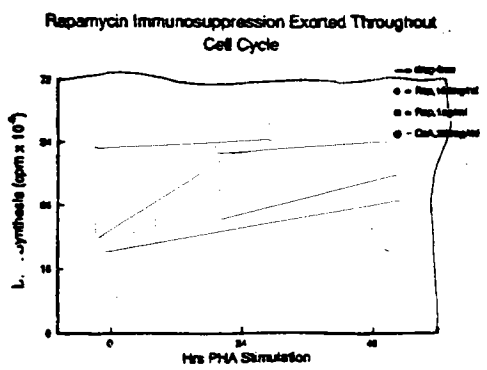


Table 5: Effect of rapamycin on PWM induced lymphoproliferation

DRUG ADDED	IMMEDIATELY	AFTER 24 HRS	AFTER 48 HRS
1. DRUG-FREE	34,974 ± 100 CPM	-	-
2. 0.1 NG/ML	23,774 ± 464 CPM	27,979 ± 432	34,591 ± 700
3. 10 NG/ML	17,595 ± 82 CPM	27,708 ± 900	33,188 ± 2006
4. 100 NG/ML	18,902 ± 66 CPM	29,657 ± 150	32,144 ± 811

THE EFFECT OF RAPAMYCIN DOSE ON PWM-STIMULATED B-CELL PROLIFERATION WAS MEASURED. RAPAMYCIN WAS ADDED AT THE ONSET OF CULTURE (G_0 PHASE OF THE CELL CYCLE), 24 HRS AFTER CULTIVATION (G_1 PHASE OF THE CELL CYCLE) OR 48 HRS AFTER CULTIVATION (POST-MITOSIS G_1). MITOGENESIS WAS MEASURED BY UPTAKE OF ^{3}H-THYMIDINE (CPM ± SD) AFTER 72 HRS CULTIVATION.

2. Effect on production of cytokines or expression of cytokine receptors:

The effect of Srl on production of cytokines [IL-2, IL-1β, tumor necrosis factor-α (TNF-α), or transforming growth factor-β (TGF-β)] or expression of cytokine receptors [IL-2 and IL-4 receptors (R)] was measured by standard procedures. For measurement of IL-2, murine splenocytes were stimulated with ConA in the presence or absence of drug for 24 hours (Report # GTR-18901). Supernatants were collected and used for measurement of levels of IL-2 by a bioassay using CTLL-2 cells (an IL-2 dependent T-cell line). The actual concentrations of the IL-2 produced were not reported. Results expressed as calculated IC_{50} values showed CsA to be > 50 fold more potent than Srl in inhibiting IL-2 production in this model (Table 6). Studies by Powell *et al.*, 1999 (J. Immunol. 162: 2775) using a CD4 Th1 clone (A.E7) showed that Srl did not alter the production of IL-2 (Figure 9). These Srl treated cells were shown to be unresponsive to costimulation with anti-T-cell receptor and anti-CD28 antibodies. Upon stimulation the production of IL-2 by these cells was not altered, although the production of IFN-γ and IL-3 was decreased.

Table 6

EFFECT OF RAPAMYCIN VS. CYCLOSPORIN A ON SPLENOCYTE INTERLEUKIN -2 PRODUCTION

	IC_{50} (μM)[a]	
MITOGEN	RAPAMYCIN	CYCLOSPORIN A
Con A 2 μg/ml	2.70 ± 0.80	0.046 ± 0.018
Con A 0.5 μg/ml	1.50 ± 1.30	0.014 (N=1)

[a]Mean IC_{50} ± S.E. for 3 separate experiments. Triplicate samples were incubated with mitogen, and with or without drugs dissolved in ethanol and diluted to the appropriate concentrations in RPMI 1640 medium for a total of 24 hours. Tissue culture supernatants were then incubated with CTLL-2 cells for 20-24 hours and the cultures pulsed for an additional 8 hours with ^{3}H-TdR (1 μCi/ml). IC_{50} values were calculated by determining the concentration of drug required to inhibit by 50% the maximal ^{3}H-TdR incorporation in non-drug-treated control cultures.

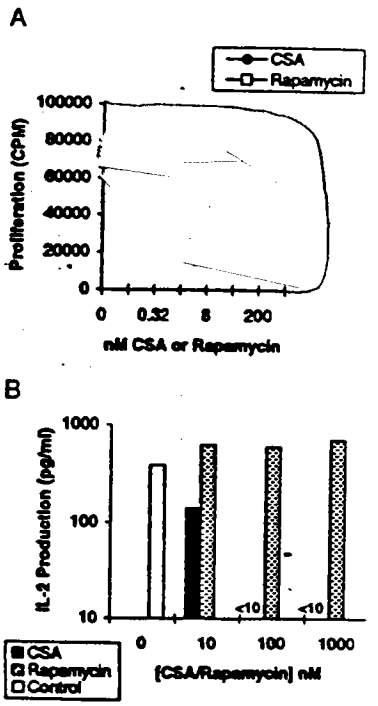


Figure 9. A, Rapamycin but not CSA inhibits proliferation of A.E7 cells to exogenous IL-2. A.E7 cells were incubated with 50 U/ml of IL-2 in the presence of increasing doses of either CSA or rapamycin. After 48 h, [3H] thymidine was added to each well and the cells were harvested and assayed for proliferation after an additional 16 h of culture. Comparable data were obtained in three other experiments. B, CSA but not rapamycin inhibits IL-2 production. A.E7 cells were incubated overnight with plate-bound anti-TCR and soluble anti-CD28 in the presence of increasing doses of either CSA or rapamycin. Supernatant fluids were harvested and assayed for IL-2 by ELISA.

Effect of Srl on the production of IL-1β and TNF-α (measured by ELISA) by human monocytes stimulated with LPS was examined after incubation for 24 hours *in vitro* (Report # GTR-19062). Results in Table 7 show that Srl and CsA [even at 10-fold higher concentration (uM) than those shown to inhibit T-cell responses] do not significantly alter the levels of either of the monokines measured.

Table 7

EFFECT OF COMPOUNDS ON MEASUREMENT OF IL-1β AND TNFα IN ELISA[1]

Compound	Conc	IL-1β[2]	TNFα[2]
Rapamycin	10 μM	99.7 ± 8.6	79.2 ± 4.6
	1 μM	112.9 ± 9.9	98.8 ± 8.3
CsA	10 μM	93.9 ± 12.3	96.6 ± 5.1
	1 μM	100.5 ± 8.5	100.2 ± 13.8
SKF86002	10 μM	96.8 ± 2.2	89.6 ± 9.3
	1 μM	100.1 ± 8.6	105.6 ± 20.3
Dexamethasone	10 μM	93.5 ± 10.0	91.7 ± 4.9
	1 μM	101.6 ± 7.8	92.5 ± 7.4

[1] measured in presence of LPS
[2] % control ± SD

A recent study by Dodge *et al.*, 1999 (Am. Soc. Transplantation, 1999, 67: Abstract # 175) showed that Srl increased the production of transforming growth factor-β (TGF-β). This effect was demonstrated using mouse splenocytes stimulated with anti-CD3 antibodies. Similar observations were made using human PBMC stimulated with ConA or anti-CD3 antibodies (Khanna, 1999, Am. Soc. Transplantation, 1999, 67: Abstract # 207). However, the production of TGF-β in the presence of Srl was less as compared to CsA.

Flow cytometric measurements of IL-2 and IL-4 receptor (R) expression were also taken using PHA or anti-CD3 induced human PBMC in the presence or absence or Srl (Report # GTR-23220). The results showed that incubation with Srl at a 10 nM concentration over a period of 3 days lead to a moderate decrease in the expression of IL-2R and IL-4R on the surface of activated PBMC. The cell viability was not altered. In another study (Barten *et al.*, 1999, Am. Soc. Transplantation, 67: Abstract # 202) using rat whole blood, the expression of CD25 (IL-2R), CD71 (transferrin receptor), CD11a (LFA-1) and CD54 (ICAM) were reduced upon incubation with Srl or CsA (Table 8). The Srl IC_{50} values were lower than those of CsA.

Table 8

	IC_{50} nM							
Drug	CD25		CD71		CD11a		CD54	
	Con A	P+28	Con A	P+28	Con A	P+28	Con A	P+28
RAP	7.8	0.7	6.4	0.1	9.9	1.2	8.5	0.2
CsA	577	721	502	257	622	257	628	255
MPA	895	1031	1115	1068	904	1592	1076	2342
A77	10739	43087	2998	6470	3447	41244	12193	48116

In another study (Report # GTR 23211), the immunosuppressive effect of Srl and CsA on the release of soluble IL-2R (sIL-2R) was measured by enzyme linked immunosorbent assay (ELISA) using human PBMC stimulated with optimal concentrations of PHA, PMA, or ionomycin. Results in Figure 10 show that Srl inhibited the release of sIL-2R in response to all stimulants including PMA (an effect similar to that observed on lymphoproliferation, see Figure 6). CsA was less effective than Srl against PHA or PMA induced sIL-2R release but was more effective against ionomycin induced stimulation.

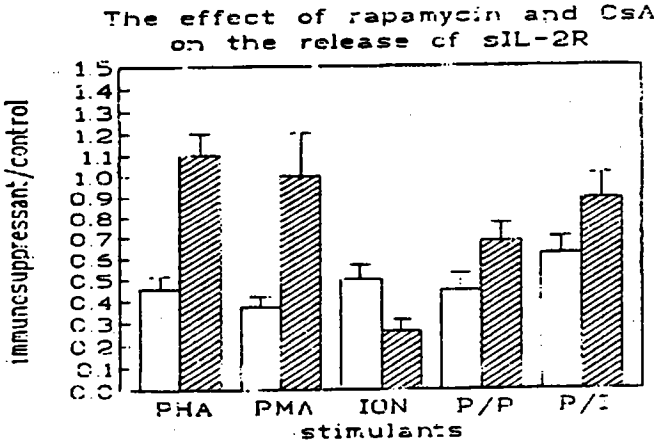


Figure 10. The effect of addition of rapamycin (blank bars; 10 ng/ml) or CSA (hatched bars; 1 ug/ml) on the release of sIL-2R (units/ml) by PBMC in response either to PHA, PMA, ionomycin (ION), PHA/PMA (P/P) or PMA/ionomycin (P/I). Results are expressed as the ratio of sIL-2R counts in cultures with immunosuppressants to the sIL-2R counts in control cultures using the same cells and the same stimulant, and are presented as the mean ± 1 SEM of 9 experiments.

3. Effect of exogenous cytokines on T-cell proliferation:

The effect of Srl on lymphoproliferation in the presence of various cytokines (IL-1β, IL-2, IL-4, IL-12 and IL-15) was tested *in vitro* by standard procedures.

Effect of Srl on mouse thymocyte proliferation obtained from C_3H/HeJ mice (known to be resistant to LPS) was studied by stimulating with PHA + IL-1β in the presence or absence of drug in a medium containing RPMI 1640 with 5% fetal calf serum followed by incubation for 72 hours (Report # GTR-18812). Lymphoproliferation was assessed by incorporation of ^3H- thymidine added at 66 hours of incubation. Results showed that Srl was effective in inhibiting thymocyte proliferation. Srl was more effective than CsA and prednisolone at a concentration of $10^{-9} - 10^{-8}$ M. At higher concentrations, however, the effect was variable.

In another study (Report # GTR-19479) human thymocytes, obtained from 7 pediatric patients (age newborn to 13 months), undergoing cardiac surgery, did not proliferate in response to PHA + IL-1 β. However, addition of PHA + IL-2 resulted in significant lymphoproliferation. In the presence of Srl or CsA this thymocyte proliferation was inhibited by up to 70% although cell viability was not altered. The calculated IC_{50} value for Srl and CsA was 10 pM and 1 uM, respectively.

In another experiment (Report # GTR-18914) IL-2 induced proliferative responses in an IL-2 dependent porcine T-cell line were shown to be inhibited by Srl but not by FK506 (Figure 11).

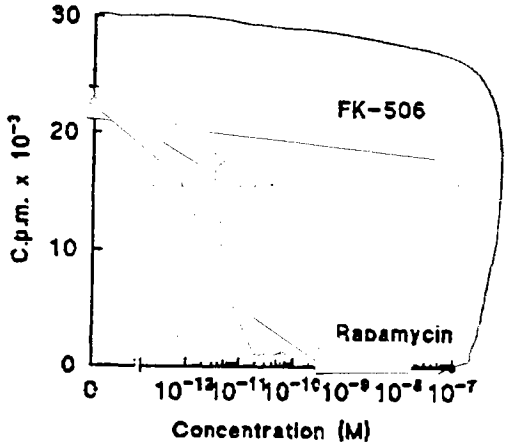


Figure 11. Effect of rapamycin and FK-506 on IL–2 driven proliferation of a porcine IL-2–dependent T cell line. Cells at 2 x 10^4/ml were cultured with 50 units/ml of Cetus recombinant human IL-2 and activation was assessed by determination of the rate of ^{3}H-thymidine incorporation into DNA at 48 h.

The direct effect of Srl on CTLL cells was also determined in the presence of exogenous IL-2 (Report # GTR-18901). Results in Table 9 show that in the presence of 25 and 6.2 units/ml of IL-2 Srl appears to be a more potent inhibitor of IL-2 induced CTLL proliferation (2.4 and 1.75 times respectively) compared to CsA. However, in the presence of 12.5 units/ml of IL-2, the activity of Srl is comparable to CsA.

Table 9

DIRECT EFFECT OF RAPAMYCIN VS. CYCLOSPORIN A ON CTLL-2 CELL PROLIFERATION

IL-2 CONCENTRATION (U/ml)	IC50 (nM)*	
	RAPAMYCIN	CYCLOSPORIN A
25	0.7 ± 0.4	1.7 ± 0.4
12.5	1.1 ± 0.6	1.4 ± 0.2
6.2	0.4 (n=1)	0.7 (n=1)

*Mean IC_{50} ± S.E. for 2 separate experiments. Triplicate wells of CTLL-2 cells were incubated with or without drugs dissolved in ethanol and diluted to the appropriate concentrations in RPMI 1640 medium for a total of 28 hours, and pulsed with ^{3}H-TdR during the last 8 hours of incubation.

Similar observations were made by Powell *et al.*, 1999 (J. Immunol. 162: 2775) using a $CD4^+$ Th_1 clone (A.E7). Results in Figure 9A show that Srl inhibited the proliferation of cells in the presence of exogenous IL-2 but did not alter the production of IL-2 (Figure 9B). CsA decreased the production of IL-2 but exhibited no effect on the proliferation of cells in the presence of exogenous IL-2 (Figure 9).

Srl was shown to inhibit IL-4 induced responsiveness of human PBMC and purified T-cells stimulated with either anti-CD3 or anti-CD28+PMA (Figure 12; Report # 23220).

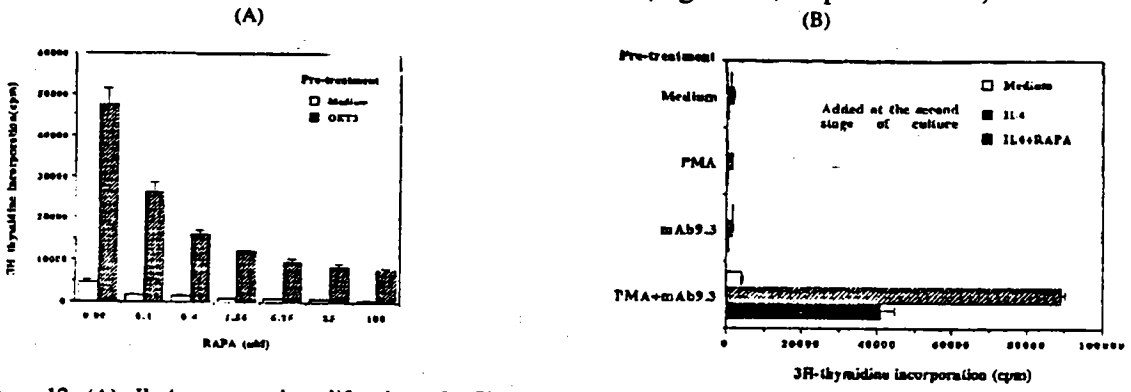


Figure 12: (A): IL-4-augmented proliferation of αCD3-primed PBMC is RAPA sensitive. PBMC were stimulated with αCD3 (OKT3, 50 ng/ml) or culture in plain medium for 3 days. The cells were then washed and recultured in the presence of IL-4 (15 ng/ml) for an additional 3 days. RAPA at different concentrations was added to the second stage culture. The cells were pulsed for 6 h before harvest. Without IL-4 in the second stage, the ^{3}H-thymidine uptake was less than 1000 cpm for the αCD3- or medium-preincubated cells. (B): IL-4 induces proliferation of pure T cells pretreated with PMA plus αCD28 and the proliferation is RAPA sensitive. Highly purified T cells were incubated with PMA (1 ng/ml), αCD28 (clone 9.3, 50ng/ml), PMA plus αCD28, or plain medium for 3 days. After wash, the cells were incubated in medium for 24h and were washed again. These cells were then stimulated with IL-4 (15ng/ml) for an additional 2 days in the absence or presence of RAPA (10 nM). The culture was pulsed with 3H-thymidine for 6h before harvest.

In another study (Report # GTR-31402), the direct effect of IL-2 or IL-15 was measured using human PBMC stimulated with PHA. PHA + IL-2 activated cells were washed and Srl or CsA + IL-2 or IL-15 added. Results in Figure 13A show that Srl inhibited both IL-2 and IL-15 mediated cell proliferation. In this study CsA was less effective in inhibiting these IL-2 or IL-15 dependent responses (Figure 13B).

A study by Bertagnolli *et al.*, 1994 (Transplantation 58: 1091) showed that Srl inhibited IL-12 mediated lymphoproliferation of activated lymphocytes using long term cultured T-cell lines stimulated with PHA, alloantigens or anti-CD3 antibody. Fresh cells were also shown to proliferate in the presence of IL-12 but the impact of Srl on this cell type was not measured.

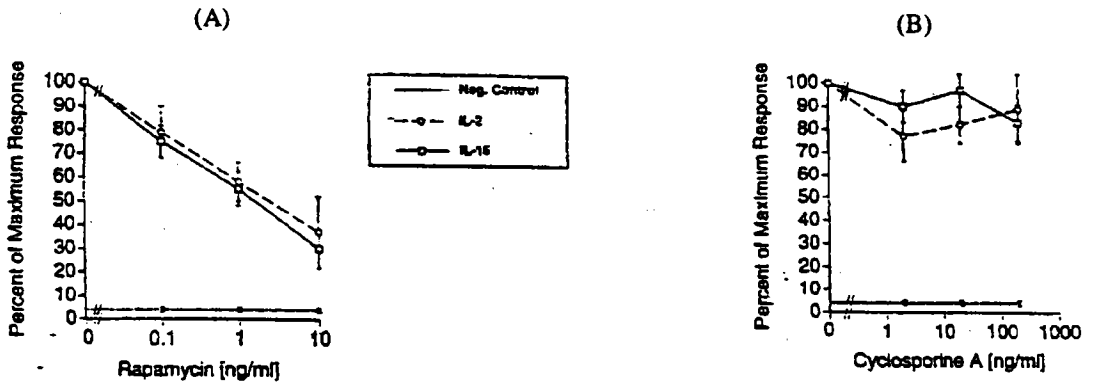


Figure 13: PHA stimulated PBMC were incubated with increasing concentrations of rapamycin for 15 minutes at 37°C followed by stimulation with IL-2 (o---o) or IL-15 (□---□) for 38h in a standard proliferation assay. Control reflect cells not treated with rapamycin. Error bars are mean ± standard deviation. Rapamycin blocks IL-2 and IL-15 induced cell proliferation (A) whereas cyclosporine A does not (B).

4. Effect on Antibody production:

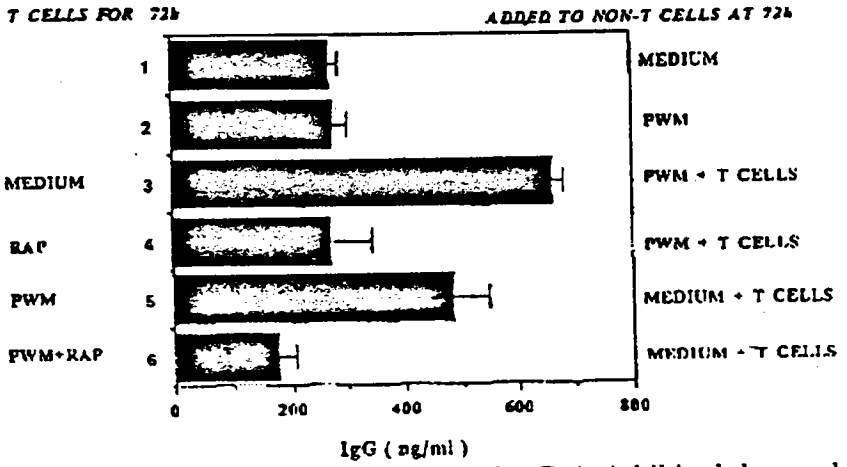
The effect of Srl on antibody production *in vitro* was measured using human PBMC from normal individuals incubated with or without pokeweed mitogen for 7 days (Report # GTR-19237). Antibodies (IgG, IgM, and IgA) were measured by [redacted] Results showed that CsA and Srl inhibited antibody production by both resting and activated cells. Srl was about 1000-fold more potent that CsA in this model system.

PBMC from SLE patients produce about 5 to 10 fold higher levels of antibodies in culture as compared to normal individuals. Srl was effective in inhibiting the antibody responses (IgG, IgM, and IgA) using cells from SLE patients, whereas CsA was not. The effect of Srl on production of IgE antibodies was measured by culture of PBMC from SLE patients with IL-4 for 14 days. Both CsA and Srl suppressed this IgE production, however, Srl was 1000-times more potent than CsA.

In another experiment, the time kinetics of the effect of Srl on IgG production by PBMC cultured for 12 days was investigated (Report # GTR-19237). Using unstimulated cells, Srl was effective in inhibiting IgG production if added by day 6 of culture. CsA was effective if added by day 4 of culture. However, in cultures stimulated with PWM, both Srl and CsA were effective only when added at day 0. The effect was shown to be due to a direct impact of Srl on T-cells. Inhibition of the IgG response could also be observed when isolated T-cells were treated with the drug and then added to the non T cell fraction (Figure 14).

Figure 14

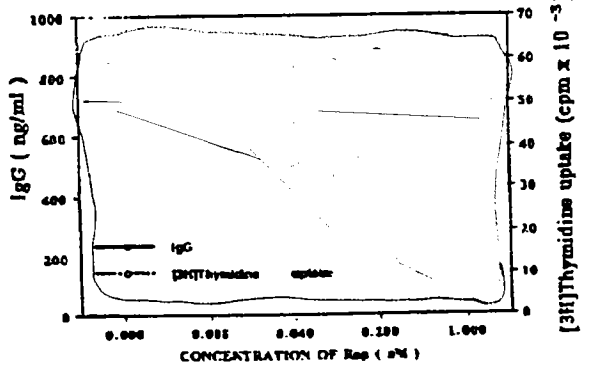
PRE-TREATMENT OF T CELLS WITH RAP SUPPRESSES IgG



Srl (0.2 to 1 nM i.e., 0.18 to 0.9 ng/ml), unlike CsA, inhibited the production of IgG and IgM antibodies by B-cells stimulated with SAC antigen in the presence of IL-2 (Figure 15). However, there was no effect on the proliferative response of B-cells (as measured by the incorporation of ^{3}H thymidine).

Figure 15

NO ANTIPROLIFERATIVE EFFECT OF RAP ON SAC+IL2-ACTIVATED B CELLS



In another study (Report # GTR-22193) the effect of Srl on SAC induced proliferation in the presence or absence of IL-2 and/or IL-6 was measured. Purified B-cells ($CD19^{+}$) were stimulated with SAC for 48 hours followed by incubation with Srl or CsA in the presence or absence of IL-2/IL-6 (IL-6 activates B-cells). Results showed that Srl suppressed the production of IgM antibodies but exhibited no significant inhibitory effect on B-cell proliferation. In the presence of

IL-2+IL-6, both Srl and CsA exhibited about 50% inhibition of B-cell proliferation as compared to the drug free cultures (Figure 16A). The effect of Srl and CsA on production of IgM was different (Figure 16B). Srl was effective in inhibiting the production of IgM antibodies, whereas CsA enhanced the production of IgM antibodies.

Figure 16A

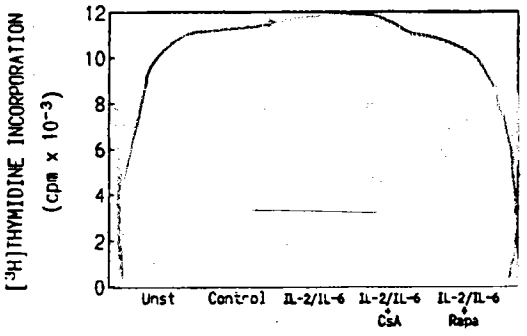
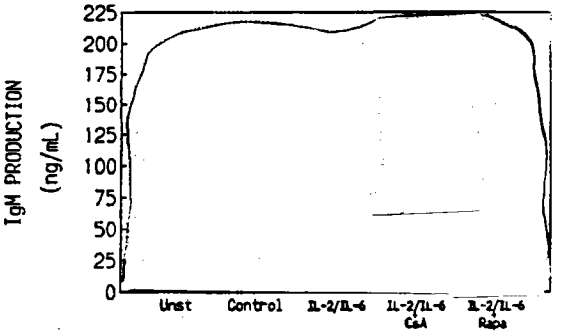


Figure 16B



5. Effect on natural killer (NK), lymphokine activated killer cell (LAC) and antibody dependent cellular cytotoxicity (ADCC) activities:

The effect of Srl on NK, LAK and ADCC activities was measured using human PBMC or adherent cell depleted PBMC. Cells were preincubated with Srl, washed and tested for NK, LAK or ADCC activities by standard procedures (Report # GTR-20649). All measurements were made at different ratios of effector:target cells.

The effect on NK cell activity was measured using ^{51}Cr labeled K562 cells as the targets. Adherent cell depleted peripheral blood mononuclear cells were preincubated overnight with Srl then washed and tested for activity against K562 cells. Results showed that Srl at a concentration of $\geq$ 10 nM inhibited NK cell cytotoxicity. Lower concentrations of Srl had no significant effect. The maximal inhibitory effect was about 31%. In the absence of preincubation of cells and drug no inhibitory effect was observed.

NK resistant ^{51}Cr labeled Daudi cells were used for measurement of LAK cell activity. Nonadherent PBMC were preincubated with IL-2 for 48 hours in the presence or absence of Srl and then tested for cytotoxicity. The results showed that Srl at a concentration of $\geq$ 10 nM inhibited the LAK-cell mediated cytotoxicity. It was also shown that Srl in the presence of IL-2 and IL-4 (known to inhibit LAK activity) further decreased the LAK activity (Figure 17) thereby indicating that Srl does not antagonize the effect of IL-2 and IL-4 on LAK cells.

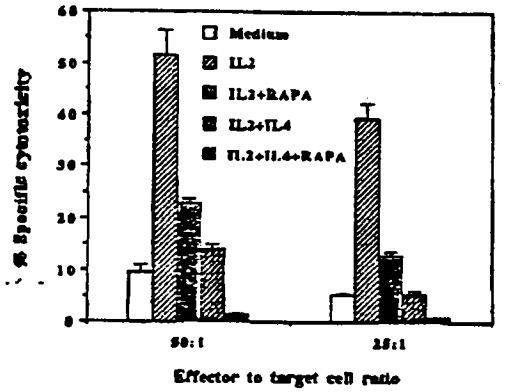
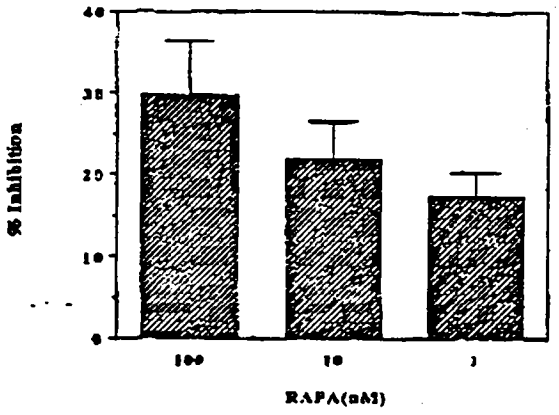


Figure 17: Synergistic inhibitory effect of RAPA and IL-4 on IL-2 induced LAK activity. Nonadherent PBMC were cultured for 48 h with IL-2 (50 U/ml) in the presence of RAPA (100 nM) or IL-4 (15 ng/ml), or both. Cells were then washed and assayed for LAK activity against Daudi cells. The experiment was repeated 3 times and similar results were obtained.

For the measurement of ADCC activity, human PBMC were incubated with ^{51}Cr labeled chicken erythrocytes coated with anti-erythrocytic antibodies (used as target cells) in the presence of different concentrations of Srl. The results in Figure 18 show a significant inhibition of ADCC at all the concentrations tested (≥ 1 nM). Unlike NK- or LAK- cell activity, preincubation of Srl with PBMC did not increase the inhibitory effect of the drug on ADCC activity. CsA was stated to be effective when present during the preincubation phase.

Figure 18



6. Effect on nonlymphoid cells:

The effect of Srl on nonlymphoid cells, which include keratinocytes, mast cells, fibroblasts and hepatocytes was examined *in vitro*.

(a) Keratinocytes:

The effects of Srl on keratinocyte proliferation, cell cycle progression and expression of HLA-DR were studied using human foreskins (obtained from a nursery) or skin carcinoma cell line. Using the human keratinocyte cell line (A-431), Srl (10 nM), FK-506 (10 nM), and CsA (1000 nM) were not effective in inhibiting proliferation at the concentrations tested (Report # GTR-20129).

In another study (Report # GTR-22338), fresh skin tissues were trypsinized, epidermal cells washed and incubated with keratinocyte growth medium then grown to confluence. Trypsinized cells were incubated with or without Srl or CsA for 4 days at 37°C and cell proliferation measured by incorporation of ^{3}H-thymidine (18 hours incubation). Results showed that Srl inhibited keratinocyte proliferation in a dose dependent fashion and was more potent in this model than CsA.

The immunosuppressive effect of Srl on the cell cycle was measured by propidium iodide staining. Results showed variable activity in the presence or absence of serum. For example, in serum free medium, Srl blocked progression from S into G_2/M phase thereby indicating a proliferative, poorly differential basilar state. In contrast, in the presence of serum cells accumulated in G_2/M phase with a relative reduction in the proportion of G_0/G_1 and S phases. The relevance of this finding to the *in vivo* situation is not known.

It was also observed that Srl did not alter the expression of HLA DR in response to stimulation with IFN-γ.

(b) Mast cells:

The effect of Srl on mast cell proliferation was measured in the presence of IL-3 (Report # GTR-20129). Srl was shown to inhibit proliferation of an IL-3 dependent mast cell line, PT 18, in the presence of IL-3 for 30 hours. These cells were shown to be more sensitive to Srl in the presence of rIL-3 (Figure 19B) compared to studies conducted with IL-3 obtained from WEHI cells (Figure 19A).

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Similar observations were made using a rat basophilic leukemia (RBL 2H3) or WEHI cells. Since these cells produce IL-3, the addition of exogenous IL-3 was not required. It should be noted that the anti-proliferative activity of these drugs in this model could be mediated through other mechanisms separate from or in addition to the absence of cytokines.

Figure 19A

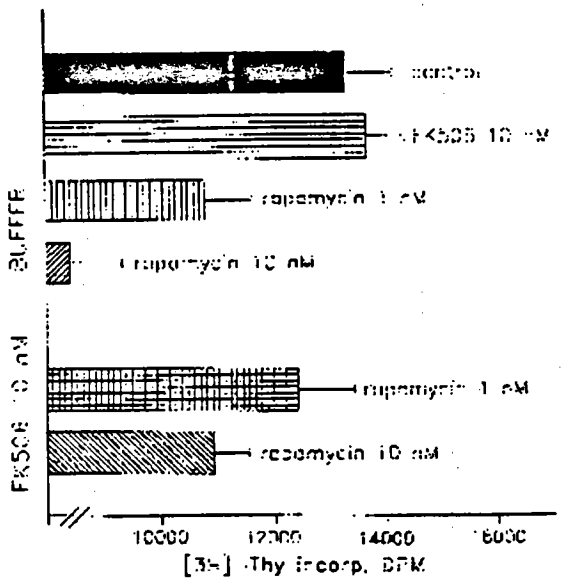
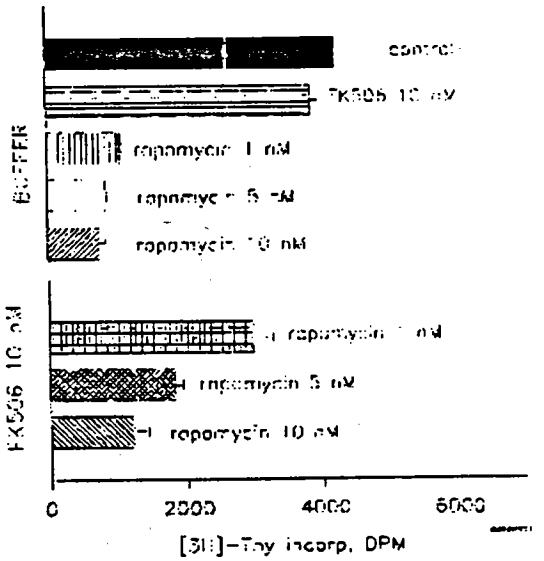


Figure 19B



(c) Hepatocytes:

The effect of Srl on hepatocyte (obtained from Fischer F344 rats) proliferation was measured in the presence or absence of epidermal growth factor (Report # GTR-20655). In this study Srl inhibited hepatocyte proliferation but FK506 and CsA did not. Washing of the cells after 3 hours of incubation followed by incubation for up to 48 hours did not alter the inhibitory effect of Srl suggesting that it may be irreversible.

The inhibitory effect of Srl on hepatocytes may reflect a decrease in the expression of TGF-β. *In vivo*, administration of Srl (0.1 mg/kg) for ≥ 4 days was shown to decrease the expression of TGF-β but had no effect on albumin or glyceraldehyde-3-phopsphate dehydrogenase.

(d) Fibroblasts:

Srl inhibited insulin induced differentiation of 3T3-L1 fibroblasts into adipocytes (Withers *et al.*, 1997, Biochem. Biophysical Res. Comm. 241: 704).

(e) Tumor cells:

Srl inhibited proliferation of selected tumor cell lines including neuroblastoma and glioblastoma cells (Hosoi *et al.*, 1998 Mol. Pharmacol. 54: 815).

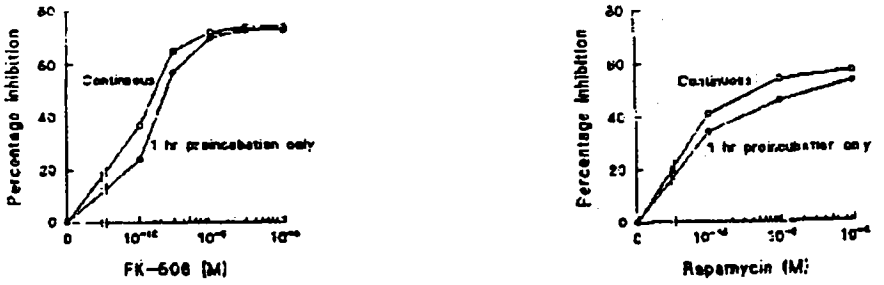
(f) Muscle cells:

Srl inhibited insulin induced stimulation of glycogen synthase in muscle cells.

7. **Reversibility of the immunosuppressive effect of sirolimus:**

The immunosuppressive effect of Srl could be measured even after a brief period of exposure to the drug *in vitro* followed by repeated washings (Report # GTR-19569). In this experiment porcine PBMC were preincubated with Srl or FK506 for 1 hour at 37°C, washed, then stimulated with ConA. No significant difference in the inhibitory effect of either drug was observed in the preincubated cultures compared to cultures where drug was present continuously (Figure 20). The sponsor also stated that the immunosuppressive activity of FK-506 or Srl was not reduced when cells were preincubated with the drugs for an hour, then washed and incubated for an additional 24 hours in drug free medium before addition of ConA (data not shown). The washing procedure removed unbound drug but not that bound to the cell surface or internalized. The effect of CsA was reported to be reversible in these experiments (data not shown).

Figure 20



The sponsor stated that Srl, like FK-506 can be absorbed by porcine lymphocytes and erythrocytes (data not shown). PBMC were 10-times more efficient than erythrocytes in taking up the drug. Autologous erythrocytes preincubated with Srl or FK-506 inhibited the ConA induced responses. The inhibitory effect was greater when erythrocytes were added to PBMC cultures compared to the erythrocytic lysates. This effect was not due to increased number of erythrocytic cells in culture since addition of drug free erythrocytes to the culture did not alter the activity of Srl.

Similar observations were made in another study (Report # GTR-18914) using porcine PBMC. Srl, like FK-506, was shown to bind to lymphocytes and erythrocytes at 37°C (but not at 0°C). The binding was very rapid, occurring within 10 minutes of exposure. Also, the binding of Srl to the cell can be prevented by pre-incubation with cells such as erythrocytes and lymphocytes. This effect was thought to be due to the binding of Srl to FK506 binding protein (FKBP).

These studies indicate that the reversibility of the immunosuppressive effect of the drug depends on the presence of cytoplasmic receptor protein, FKBP at the cellular level. Simple washing of the cells was not effective in reversing the immunosuppressive effect of Srl.

8. Effect of storage on lymphoproliferative activity of sirolimus:

The anti-lymphoproliferative effect of Srl stock solution stored for about 20 weeks at –20°C was not altered compared to the fresh solution (Figure 21) using fresh ConA-induced porcine lymphocytes (Report # GTR-18914).

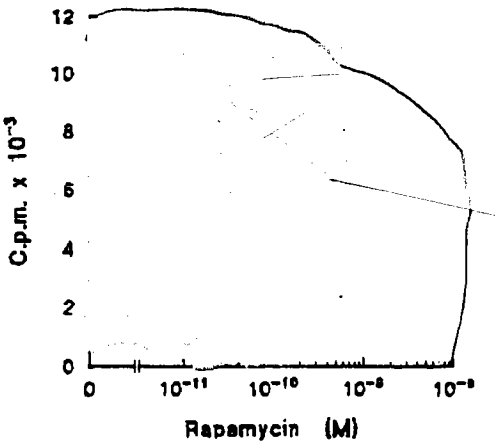


Figure 21. Comparison of the effects of fresh and stored rapamycin. Rapamycin was either freshly made up (0) or from a stock 1 mM solution in ethanol stored for 20 weeks at –20°C before use (•). Inhibition of the ConA response of porcine lymphocyte was assessed by determination of the rate of incorporation of [^{35}S]-methionine into protein at 24 h.

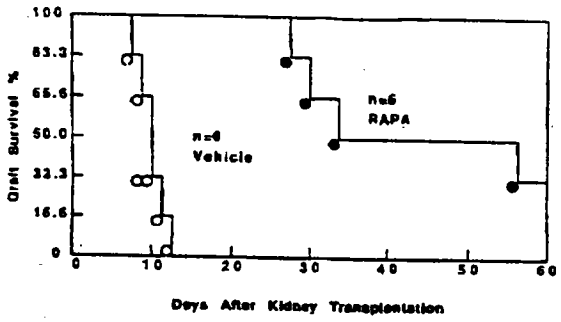
B. Activity in *in vivo*
Effect of sirolimus on renal graft survival/organ transplantation:

The effectiveness of Srl in prolonging the survival of an orthotopic kidney transplant was measured in rats (Report # GTR-18691, 22342), dogs (Report # GTR-17938, 20661), pigs (Report # GTR-17938, 25118, 21843) and baboons (Report # GTR-19079). All experiments were conducted in animals with orthotopic renal transplant and the contralateral kidney removed. In a majority of the studies the graft survival was measured by survival of the recipient animals.

In rats, a dose of ≥ 0.08 mg/kg/day for 14 days was 5 to 10 fold more effective in improving survival compared to the vehicle treated group (Report # GTR-18691). No known immunosuppressive agent was used for comparison of activity. The time of initiation of treatment was not specified.

Similar observations were made in another study (Report # GTR-22342) in rats. Treatment with Srl initiated 4 days post-transplant for 14 days was effective in improving survival (Figure 22).

Figure 22



Group	Survival Days	MST±SD
1 Vehicle	6, 8, 10, 10, 11, 12	16.8±1.4
2 RAPA 0.8 mg/kg/d	27, 30, 34, 57, >60, >60	44.7±15.8

In beagle dogs (Report # GTR-20661) treatment with Srl was initiated immediately after grafting. Results showed that Srl doses of 0.3 to 1.5 mg/kg daily or weekly was not effective in improving the survival of dogs compared to the untreated control group. Administration of Srl at these doses appeared to be toxic to the animals. The sponsor has stated, however, that no histological evidence of graft rejection was observed (vasculitis at low dose of Srl). A combination of Srl (0.3 mg/kg) + CsA (2.5 mg/kg) improved survival in 3 of 5 dogs.

In another study (Report # GTR-20111) conducted in mongrel dogs, treatment with a combination of a low dose of Srl + CsA for 7 days prolonged survival (Table 10).

Table 10

Effects of Immunosuppressive Drugs on Renal Allograft Survival in Mongrel Dogs[a]

Immunosup. Regimen						
CsA[b]	RAPA[c]	E-HAg[d]	n[e]	Individual Survivals (Days)	MST±SD[f]	p[g]
0	0	0	6	6,7,8,9,9	7.6+1.2	
+	0	0	5	11,17,21,24,25	19.6±5.7	0.003
+	0.05	0	4	1*,21,(>29),(>38)	29.3±8.5	0.0096
+	0.05	+	6	13*,14,76,78,81,127	65.4±28.7	0.0019
+	0.50	+	5	10*,11*,11*,17*,20*[h]	13.8±4.4	0.026
+	0.05	0	4	1,21,>70,>77		

a Dogs were transplanted with kidneys from unrelated donors and then underwent bilateral native nephrectomy at day 7. Hosts were followed by serum creatinine values and by host survival.
b. Hosts received CsA 20 mg/kg/day every day po for 60 days beginning on day of transplant.
c. Hosts received either 0.5 or 0.05 mg/kg/day RAPA by bolus iv injection beginning on day of transplant and for 6 days thereafter.
d. Hosts received 5 mg/kg 3M KCl extracted donor histocompatibility antigen by bolus iv injection beginning on the day of transplant and for 6 days thereafter.
e. n is the number of hosts in each group.
f. Mean survival time (MST) ± standard deviation (SD).
g. Significance ($p < 0.05$) between control (no therapy) and experimental groups was calculated using Gehan's survival test.
h All deaths from gastrointestinal complications: at autopsy none of the kidneys showed evidence of rejection.

Similar observations were made in another dog study (Report # GTR-17938) wherein different doses (0.25 to 3 mg/kg) of Srl were administered. A dose of 2 mg/kg from days 3 to 5 was reportedly not effective in improving graft survival (Table 11) but the data provided were incomplete. The sponsor stated that all animals exhibited ulcers of the gastro-intestinal tract (from mouth to the colon).

A study (Report # GTR-17938) in large white pigs showed that a 2 mg/kg dose of Srl started on the day of orthotopic kidney transplant improved survival (Table 11).

In another study (Report # GTR-25118) pigs were treated with Srl (0.1 to 2 mg/kg/day) by the oral route. The treatment was initiated on the day of transplant and continued for 28 days. The activity of the drug was compared to a combination of CsA (1 mg/kg/day) + azathioprine (2 mg/kg/day) + prednisone (3 mg/kg/day). Srl at a dose ≥ 1 mg/kg (plasma trough concentration of 9.3 ng/ml) prolonged survival by about 15 days. The triple therapy regimen was less effective in this model.