Table 11: Survival of Grafts

GROUP	SPECIES ORGAN	DOSE SCHEDULE	SURVIVAL IN DAYS ( ) DAY LAST DOSED	MEAN±SE SURVIVAL	LATEST CREATININE uMOL/L
1,(n=4)	RAT HEART	50 mg/Kg x 10 days	>100,>100,>100,>100	100	
2,(n=4)	RAT HEART	10 mg/Kg x 10 days	65,77,88,>100	82±7	
3,(n=4)	RAT HEART	2 mg/Kg x 10 days	58,59,59,66	60±2	
4,(n=4)	RAT HEART	1 mg/Kg x 10 days	34,49,52,55	47±5	
5(n=4)	RAT HEART	0.5 mg/Kg x 10 days	19,20,20,35	23±4	
6,(n=5)	RAT HEART	10 mg/Kg days 3-6	15,18,18,19,21	18±1	
7,(n=10)	RAT HEART	NIL	7,7,7,7,7,7,8,8,8,8	7.4±0.2	
8,(n=9)	PIG KIDNEY	2 mg/Kg	4*,48*,49*,50*,55*,63*, >127(43),>147(52),>148(64)	76±17	1410,161,235,176,239 208,216,210
9,(n=7)	DOG KIDNEY	2 mg/Kg days 5-5	9*,10*,14*,14*,15*, 16*,23*	14*2	294,303,1758,1708, 1358,871,541

CAUSE OF DEATH GROUPS 8 AND 9: *REJECTION, *INTERSTITIAL PNEUMONITIS, ‡TOXICITY

Similar observations were made in another pig study (Report # GTR-21843). Treatment with Srl (0.25 to 0.75 mg/kg/day for 30 days, by the oral route) was initiated on the day of transplant and continued for 28 days. The activity of Srl was compared with a combination of CsA (1 mg/kg/day) + azathioprine (2 to 3 mg/kg/day) + prednisone (3 to 4 mg/kg/day). In this study, a low dose (0.25 mg/kg/day) of Srl was more effective in improving survival as compared to the higher dose (0.75 mg/kg/day, n=1) and the triple therapy. The sponsor stated that in the high dose group the animals developed multiple pulmonary and intra-abdominal abscesses. The most common cause of death in the low dose group and triple therapy group was infection of the lung.

In baboons (Report # GTR-19079), treatment with Srl was initiated a day prior to or on the day of transplant. Ten baboons included in the study were treated with 3 different formulations and different regimens of the drug (2 mg/kg). No vehicle control or any approved immunosuppressive agent was used for comparison of activity. Drug activity was evaluated against historical data (i.e., the sponsor stated that untreated baboons with renal transplant die within 7 – 10 days of transplantation). In four animals administered Srl (in acetamide/PEG mixture) by the oral or intravenous route, graft survival varied from 18 – 40 days. These animals were killed when evidence of rejection was observed. Of the 6 animals administered a different formulation of Srl by the oral route, 5 died within 10 days of transplantation. The sixth animal was administered Srl intramuscularly and died on day 25.

Other studies:

The following additional studies have been conducted to demonstrate the activity of Srl in the treatment/prevention of heart, skin, pancreatic and bone marrow graft rejection:

Report no.	Species	Organ Transplant	Allogeneic/ Xenogeneic	Remarks	Effectiveness
GTR-17939	Mouse	Heart	Allograft	Heterotopic transplantation to the dorsal pinna of the ear	Srl prolonged survival whether given pre- or post-transplant
GTR-18690	Rat	Heart	Allograft	Heterotopic transplantation to the abdominal cavity; treatment initiated at the time of transplant for 14 days	Srl prolonged graft survival by 27-times as compared to CsA treated group. A combination of a low dose of Srl with a low dose CsA was more effective in prolonging graft survival
GTR-24148	Rat	Heart	Allograft	Heterotopic transplantation to the ear	Prolongs graft survival at a dose of 0.3 mg/kg (for ≤ 28 days) which produces whole blood trough concentrations of < 1 ng/ml
GTR-17938	Rat	Heart	Allograft	Heterotopic transplant in the neck	Data not shown
GTR-22203	Rat	Heart	Allograft	Heterotopic, intra-abdominal transplant	A combination of 1.5 mg/kg/ day of Srl with CsA (5 mg/kg on day -1 followed by 2.5 mg/kg for about 6 to 8 days improved graft survival (≥123 days)
GTR-31588	Rat	Heart	Allograft	Heterotopic intra-abdominal heart transplant; treatment initiated on day 1 or 4 of transplant for 14 days; untreated graft survival of ≤ 7 days	Low dose of Srl (0.02 mg/kg/ day) + FK506 (0.02 mg/kg/day) showed synergistic effect in the prevention of graft rejection (42 - 50 days) or treatment of acute rejection (28 – 60 days)
GTR-22118	Rabbit	Heart	Allograft	Heterotopic, intra-abdominal transplant	Srl between the doses of 0.05 – 1 mg/kg/day for 60 days improved graft survival. The effective blood concentrations were in the range of 10 - 60 ug/litre. CsA was also effective in this model at higher doses

Report no.	Species	Organ Transplant	Allogeneic/ Xenogeneic	Remarks	Effectiveness
GTR-19470	Cynomolgus Monkey	Heart	Allograft	Heterotopic transplant; Srl administered intramuscularly. A preliminary report	Different doses and regimens for different animals (graft survival varied from 13 - >141 days). Graft survival data in control animals not included; the sponsor stated that the survival time varied from 8-35 days and that day 35 animal is an outlier
GTR-19837	Cynomolgus Monkey	Heart	Allograft	Heterotopic transplant; Srl administered intramuscularly. (This is a final report by the same group as for GTR-19470)	Srl (0.2-7mg/kg) improved graft survival (27 - >100 days). No dose dependent effect. Srl + CsA also effective
GTR-20661	Rat	Heart/Skin	Allograft	Heterotopic cardiac transplant; skin transplant to the lateral thorax	Improved skin graft survival by about 6 days (doses 3 or 10 mg/kg); Cardiac graft survival > 100 days (0.3 – 5 mg/kg for 11 days)
GTR-18691	Rat	Heart/small bowel	Allograft	Heterotopic cardiac transplant to the abdominal cavity;	A dose of ≥0.08 mg/kg/day improved survival of cardiac graft. Small bowel graft survived for 22 – 31 days (about 16 days longer than the untreated group) at a dose of 0.8 mg/kg/day. No known immunosuppressive agent tested for comparison of activity
GTR-22342	Rat	Heart/ Pancreatico-duodenal	Allograft	Heterotopic heart to the abdominal cavity and pancreaticoduodenal graft to the duodenal region; treatment initiated 4 days post-transplantation for 14 days. Survival of cardiac graft measured by palpitation and that of pancreatico-duodenal graft by blood glucose and serum amylase levels	A dose of 0.8 mg/kg improved survival of the cardiac graft (30 - >200 days) as compared to vehicle treated group (<8 days). A combination of a lower dose of Srl with CsA (2 mg/kg/day) also improved survival (13 - > 200 days). Survival of pancreatico-duodenal graft also improved
GTR-18902	Mouse	Skin	Allograft	Dorsal skin; treatment initiated a day after grafting for 6 days	Prolonged survival by about 4-9 days. Srl+CsA exhibited a weak synergistic effect
GTR-19067	Mouse	Skin	Allograft	Dorsal skin; same as GTR-18902	I.P. route of treatment better than the oral route; Srl was not effective in 2nd set of host vs. graft reactions in presensitized animals

Report no.	Species	Organ Transplant	Allogeneic/ Xenogeneic	Remarks	Effectiveness
GTR-31585	Mouse	Skin	Allograft	Dorsal side; 2 models – class I and complete mismatch. Tested in combination with CTLA4 (homologue of CD28, which binds CD80 and CD86) or control antibody (ab) The CTLA4 ab inhibits CD28 (on T-cells) signal by saturating CD80 and CD86 (on APC). Ab was administered from day 0 – 12 whereas drugs were administered every other day for 12 days by the intraperitoneal route	Not effective in complete mismatch model; 0.75 mg/kg Srl in combination with ab improved graft survival in 4/8 mice (73 – 136 days); Higher dose of Srl (1.5 mg/kg) less effective (2/9 mice: >157 days). A combination of Srl with CsA less effective
GTR-31586	Mouse	Skin	Allograft/ Xenograft	Lateral thoracic wall; tested in combination with anti-lymphocyte serum / donor bone marrow cells	CsA effective in prolonging allograft but not xenograft survival; Srl effective in both allograft and xenograft survival
GTR-23223	Mouse	Skin/Bone marrow	Allograft	Tested in combination with anti-lymphocyte serum	Minimal effect in nonthymectomized mice. Effective in anti-lymphocyte serum treated mice
GTR-22023	Mouse	Bone marrow/ spleen cells	Allograft	In graft vs. host disease	Srl prolonged survival of bone marrow transplant; not as effective as antiThy 1.2 + C' treated cells
GTR-22341	Mouse	Bone marrow/ spleen cells	Allograft	In graft vs. host disease	Prolonged survival and prevented graft vs. host disease, however, lead to occurrence of autoimmune-like syndrome (skin ulceration)
GTR-23847	Mouse	T-cell depleted bone marrow	Allograft	In graft vs. host disease	Prolonged graft survival
GTR-22191	Mouse	Islet	Allograft	Transplanted beneath the renal capsule in diabetic mice (streptozotocin induced)	Reduced blood glucose; at high doses (10x) adversely effected glucose homeostasis but not on end organ toxicity
GTR-19549	Rat	Pancreatic/ duodenal	Allograft	In diabetogenic rats	A dose of 0.08 mg/kg/day for 14 days improved survival (>45 days); CsA effective at a dose of 4 mg/kg/day
GTR-22340	Mongreal dog	Purified pancreatic islet	Allograft	Heterotopic intra-spleen graft. Activity measured by levels of blood glucose	Srl (0.05 mg/kg/day) or CsA alone for 30 days not effective in improving graft hyperglycemia. A combination of Srl+CsA effective (about 6 - 7 fold)

The activity of Srl was also tested in some of the autoimmune disease models. These studies are summarized below:

Report #	Species	Model	Effectiveness
GTR-19064	Mouse	MRL/MpJ/lpr/lpr (MRL/l) > 6 weeks old; activation and proliferation of T-cells important in the progression of the disease (SLE); lymphoadenopathy of double negative (CD4/CD8) cells. Srl and CsA administered 3 times a week at a dose of 12 – 12.5 and 25 mg/kg by the oral route	Srl was more effective than CsA in improving survival and decreasing serum anti-DNA ab; Both Srl and CsA decreased urine albumin
GTR-19065	Mouse	MRL/l; Srl and CsA administered 3 times/week for 2 months at a dose of 12.5 and 25 mg/kg by the oral route	Srl reduced spleen weight and lymph node size, lymphoid hyperplasia in the spleen, lymph nodes, thymus; also reduced mononuclear cell infiltration in the lung, liver and kidneys and vacuolation in the cortex of adrenals and anti-DNA antibodies; *ex vivo* proliferation to ConA was restored and responsiveness of splenocytes (from treated mice) to PHA, LPS and PMA also increased but to a lesser degree; production of IL-2 increased in ConA stimulated cell cultures from Srl treated mice. CsA was less effective
GTR-24897	Mouse	MRL/l; Srl (5–10 mg/kg) and CsA (6–12 mg/kg) administered at lower doses were administered 3 times a week by gavage	A combination of Srl with CsA improved survival, decreased anti-DNA antibodies, decreased urinary albumin, decreased spleen and lymph node weights, reduced lymphocytic infiltration in the lungs, kidneys, thymus, lymph nodes and increased *ex vivo* lymphoproliferation to ConA and LPS, production of IL-2 in ConA stimulated splenocytes
GTR-20795 GTR-19061	Mouse	Nonobese diabetic (NOD) murine model (MrKTACfBR)– autoimmune model of type I insulin dependent diabetes mellitus (IDDM)	Srl (≥6 mg/kg, 2 times a week for 4 months) in 8 – 9 week old mice suppressed incidence of diabetes; however, in older mice (18 – 20 weeks i.e., immediately after the onset of diabetes) Srl was not effective (data not shown); Srl was effective in preventing the onset of diabetes 41 weeks after discontinuation of treatment although animals died of natural causes; In one study CsA was used for comparison of activity. A comparison of the activity of Srl with CsA showed that like Srl, CsA suppressed the onset of the incidence of diabetes until day 110 but was not effective if administered later (≥ 131 days)
GTR-19421	Mouse	Collagen induced arthritis (T-cell mediated autoimmune disorder) can be potentiated by IL-1; mice were immunized with bovine type II collagen and challenged with LPS on day 21; treatment with Srl (0.25 – 50 mg/kg) administered from days 18 – 35	Treatment with Srl (5 mg/kg) reduced the incidence of arthritis (affected paw) on day 42 and 98 (2 months after discontinuation of treatment). CsA was less effective
GTR-23214	Rat	Collagen induced arthritis; immunosuppressive agents administered on the day of induction of arthritis	Administration of a single dose (30 mg/kg) of Srl on day 0 reduced the incidence of arthritis, serum antibody levels and delayed type hypersensitivity; a combination of Srl with FK506 was more effective in reducing the incidence of arthritis. The effectiveness of Srl was less or lost when administered on day 4 or 8/12/16 respectively

Report #	Species	Model	Effectiveness
GTR-19243	Mouse	Methylated bovine serum albumin (MBSA) in CFA was administered into the abdominal wall followed by a challenge into the right paw on day 7 to induce hypersensitivity; treatment with immunosuppressive agents initiated either one day prior to sensitization or on the day of challenge by the oral route.	Srl reduced 24 hour paw edema when administered 1 day prior to challenge (ED_{50}=4.7 mg/kg); however, no effect observed when administered 24 hours prior to sensitization; CsA was stated to be effective at a higher dose.
	Rat	Arthritis was induced by injection of heat killed *Mycobacterium butyricum*; Immunosuppressive agents were administered from day 0 to 15 or 16 to 29.	Srl effective in reducing edema. The ED_{50} values on the higher side in rats with established arthritis as compared to those developing arthritis.
	Rat	Experimental allergic encephalomyelitis (EAE) was induced by injection of isologous spinal cord and *M. tuberculosis* emulsion into the hind foot pad; immunosuppressive agents were administered by the oral route from day 0 – 14	Srl at a dose of 9 mg/kg reduced paralysis
GTR-20113	Rat	Arthritis was induced by injection of heat killed *Mycobacterium butyricum*; Immunosuppressive agents were administered from day 0 to 15 in 7 doses (10 mg/kg)	Srl effective in reducing edema
GTR-22347	Mouse	12-O-tetradecanoyl phorbol-13-acetate (TPA) induced ear edema (a model for psoriasis); immunosuppressive agents administered orally or topically	Oral route less effective than the topical route in reducing edema and neutrophil influx. ED_{50} values by the topical route varied from 71 to 126 ug/ear.
GTR-25311	Rat	Autoimmune myocarditis (induced by immunization with porcine cardiac myosin); treatment with Srl from day 0 – 14 by the intraperitoneal route.	Srl prevented the development of pathology; ECG profiles were normal
GTR-22201	Rat	Autoimmune uveoretinitis (EAU) induced by retinal S antigen into the hind foot pad; treatment with Srl initiated either on the same day or 7 days after immunization for 14 days	Srl at a dose 1 mg/kg inhibited the development of EAU when initiated on day 1 or day 7. This was associated with decreased infiltration of the cells in the lymph nodes, decreased antigen specific lymphoproliferation and serum antibody levels
GTR-23208	Rat	Balloon injury and thickening of arterial intimal walls lead to vascular narrowing in 2 weeks. Effect of immunosuppressive agents is thought to be mediated via inhibition of some factors produced by platelets which are responsible for smooth muscle cell proliferation and is not T-cell mediated; however, the production of these factors by smooth muscle cells, endothelial cells etc. is regulated by cytokines produced by macrophage and T-cells	Srl (1.5 mg/kg) + mycophenolate (40 mg/kg), administered at the time of injury for 14 days, were effective in inhibiting smooth muscle cell proliferation and arterial wall thickening when measured on day 14; the effectiveness was lost when measured on day 44. CsA and FK506 not effective in any of the experimental design
GTR-23209	Rat	Similar to GTR-23208; in addition done in rats with artery transplant [progressive, continuous injury requires a longer time (over a month) to produce intimal thickening comparable to that of balloon caused injury]. Immunohistochemistry done to determine the cell type, cytokine levels	Results not shown. However, it was stated that treatment with Srl (1.5 mg/kg) did not alter the arterial thickening or the expression of IL-2R, class II antigen or cytokine mRNA expression. Higher dose of Srl (6 mg/kg followed by 3 mg/kg) reduced intimal thiokening and infiltration of T-cells, macrophages, IL-2R and cytokine expression

Report #	Species	Model	Effectiveness
GTR-32668	Pig	**Coronary balloon angioplasty**: SrI administered by the intramuscular route at a dose of 0.5 mg/kg on day 3 to 0 before injury followed by 0.25 mg/kg for 14 days	SrI reduced intimal thickening as measured 2 weeks after discontinuation of treatment; such an intimal inhibition was shown to be due to decreased fibrocellular proliferative response and not residual thrombus or hematoma; No correlation observed between drug levels in the blood and decrease in intimal thickening
GTR-32669	Pig	**Coronary balloon angioplasty** (by the same group as GTR-32668); SrI administered by the oral route at a dose of 1 mg/kg on day 3 to 0 before injury followed by 0.4 to 1.0 mg/kg for 14 days; intimal thickening measured at 6 months after injury	Treatment with SrI administered up to a day before injury decreased intimal wall thickening as measured at 22 weeks after discontinuation of treatment

Treatment with immunosuppressive agents can increase susceptibility to infection. The studies conducted to measure the effect of SrI in altering the susceptibility to infection are as follows:

Report #	Species	Model	Effectiveness
GTR-19068	Mouse	*Candida albicans*: immunosuppressive agents [SrI, 20 mg/kg; CsA 50 mg/kg; CyP 50 mg/kg] administered for 6 days by the intraperitoneal route	Mice were infected with *C. albicans* 24 hours before the last dose with the immunosuppressive agents. Untreated mice die within 8 days of infection with a median of 4.5 days; mice treated with SrI, CsA or CyP died with a median survival of 2.5, 2 or 1 days respectively. SrI improved survival compared to CsA or CyP treated mice when mice were infected 30 – 40 minutes before the last dose (such an effect was stated to be probably due to some anti-Candida activity of the drug). Similar observations were made when different doses of SrI were administered 5 days pre- and 5 days post-infection. The effect of treatment with CsA for 5 days followed by infection on day 6 and treatment with different doses of SrI initiated 30 minutes post-infection for 5 days was compared with mice treated with CsA alone. Results showed that SrI at a dose of 4 mg/kg or 16 mg/kg to be effective in improving survival; mice treated with CsA alone prior to infection died within 4 days In the allograft skin recipient mice with *C. albicans* infection, SrI improved survival as compared to CsA treated mice
GTR-19069	Mouse	*Pseudomonas aeruginosa*: SrI, CsA or CyP administered intraperitoneally (1 – 20, 50 or 5 mg/kg respectively) or orally (1 – 20, 100 or 5 mg/kg respectively) once daily for 6 days	SrI and CsA did not alter susceptibility when administered intraperitoneally. By the oral route CsA did not alter susceptibility, whereas in mice treated with SrI a modest increase in susceptibility was observed. CyP increased susceptibility to infection irrespective of the route of drug administration.
GTR-23216	Mouse	Cytomegalovirus infection with MCMV (Smith strain): latent and acute infection model	SrI did not activate the latent virus (measured by plaque forming cells) under the experimental conditions tested; however, the mortality increased in the group of mice treated with SrI as compared to the untreated mice. CsA treated mice also exhibited increased mortality but to a lesser degree

It is of note that prolonged exposure to immunosuppressive drugs was not tested. Also, in most of the studies the measure of susceptibility was based on survival and not microbiological burden.

Effect of *in vivo* administration of sirolimus on immune responses:

Several studies were conducted to measure the effect of Srl administration on cellular and antibody responses in animals with skin, heart or kidney transplant. For example, the ability of mononuclear cells to respond to allogeneic cells was tested by MLR using PBMC before and 30 – 35 days (i.e., last day of treatment) after orthotopic renal transplant in pigs (Report # GTR-21843). Results showed development of tolerance upon transplantation.

The effect of Srl on skin graft survival and production of anti-tetanus (anti-TT) antibodies was measured in rats (Report # GTR-22204). Skin allografts and immunizations with TT were performed on days 1, 10, 31, 116 and 137. Treatment with Srl was administered from day 17 to 45. IgG antibodies, TT specific antibodies and cytotoxic antibodies (using lymph node cells from the donor rats as the target cells) were measured at different time points for 6 months. Results in Figures 23 to 25 indicate that the impact of Srl on serum antibody (IgG and antiTT) levels were reversed (Figures 23 and 25) after discontinuation of treatment. Alloantigen specific cytotoxic antibody levels (Figure 24) in rats treated with Srl remained lower than in the vehicle treated control animals.

It is of note that in the experiments described above the survival of skin graft (on the dorsal side) on days 1 and 10 was similar in all the rats (Figure 26). The skin grafts conducted on days 31 (during treatment) or 116 (after 2 months of discontinuation of therapy) were shown to survive significantly longer in Srl treated rats than the untreated group. Skin graft survival on day 137 (i.e., 3 months after discontinuation of treatment) was similar in the 2 groups.

Figure 23

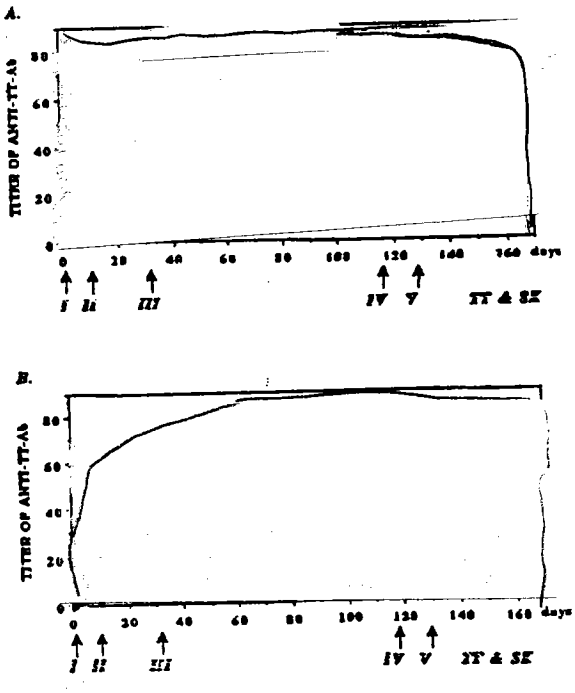


Figure 24

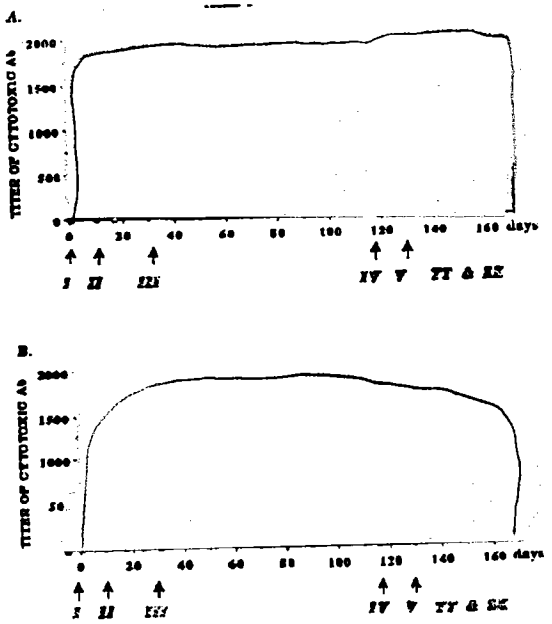


Figure 23. RAPA's effect on in vivo anti-TT Ab Production. WFU rats were immunized i.m. with TT on day 1, 10, 31, 116 and 137, and were grafted with BUF skin on these same dates. The time of TT immunization and skin grafting were indicated with arrows and marked as containing TT and SK. On day 17 and 31, 14-day osmotic pumps vehicles (Fig. 23A) or RAPA (Fig. 23B) were planted twice with connection to lumbar veins. The duration of vehicle or RAPA delivery was indicated with open bars. The serum anti-TT IgG was measured with ELISA. Samples were in duplicate. Each group consisted of 5 rats in the beginning. One rat from each group died of anesthesia on day 116 during the bleeding procedure, and the Ab titre curves of these two terminated on that day.

Figure 24. RAPA's effect on in vivo cytotoxic Ab Production. The same two groups of rats treated with RAPA or vehicle, as described in detail in Fig.23, were employed. The serum titres of specific cytotoxic Ab against donor-strain (BUF) were measured with a modified panel reaction assay. Samples were in triplicate.

Figure 25

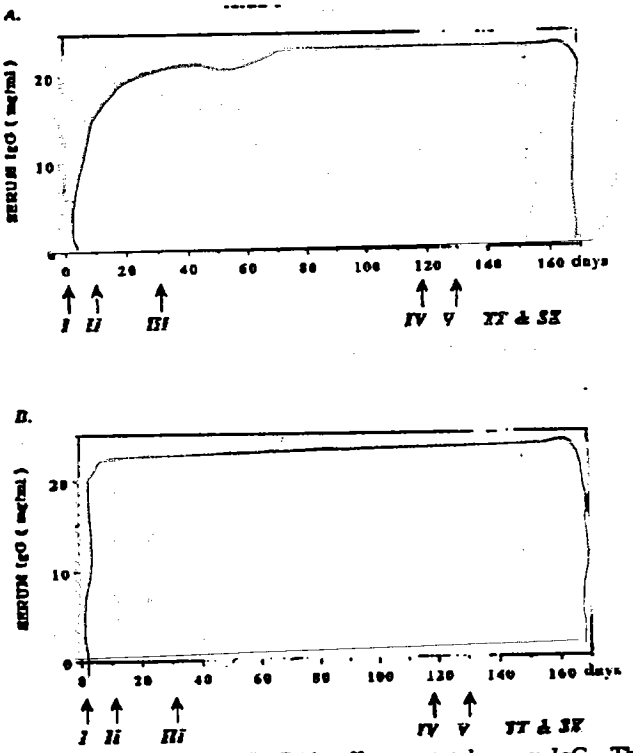


Figure 26

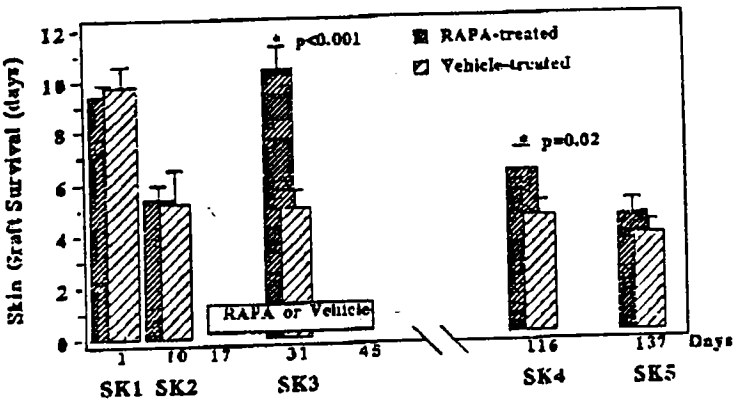


Figure 25. RAPA's effect on total serum IgG. The same two groups of rats as detailed in Fig. 23, were employed. Serum IgG levels were measured with ELISA. Samples were in duplicate.

Figure 26. RAPA's effect on survival of skin allograft. WFU rats were grafted with BUF skin 5 times on different days as indicated in Figure 23. RAPA (0.8 mg/kg/day) or vehicles were given i.v. from day 17 to day 45. MST of the skin grafts of each group (n=5) were shown. The statistically significant difference in MST between the two groups was indicated with asterisks, and p values were given.

In rats with a heterotopic heart transplant to the abdominal cavity on day 197 (i.e., 5 months after discontinuation of treatment) the survival of the grafts was similar in the treated and untreated rats thereby indicating that the immunosuppressive effect of Srl was no longer detectable at that time point.

Also the ability of lymph node cells to respond to allogeneic (as measured by mixed lymphocyte reaction) and ConA stimulation was examined 5 months after discontinuation of treatment. Results show that the ability of cells to respond to ConA or allogeneic stimulation was similar in Srl and the vehicle treated groups. The *ex vivo* activity of lymph node cells at earlier time points was not tested. Also, no known immunosuppressive agent was used as a comparator in any of the experiments described above.

In another study (Report # GTR-23213; details of the experimental design shown in Figure 27), heterotopic heart transplant to the abdominal cavity of rats, pretreated with Srl (0.8 mg/kg/day for 14 days) 6 months prior to transplant, was rejected in ≤ 7 days. However, when treatment with Srl was initiated at the time of transplant the heart survived the period of observation i.e., ≥ 6 months. In such tolerized rats, another heterotopic transplant of a different allotype (than the original donor) conducted 6 months later did not survive beyond day 8 of transplantation. It is of note though that a second heterotopic transplant of the same allotype as the first one survived over 60 days post-transplant. The first heterotopic transplant also survived the period of observation. If the heart of the first heterotopic transplant was removed 4 to 5 weeks before the second transplant of the same type, the unresponsiveness was lost and the graft was rejected in ≤ 10 days. These results indicate that the persistence of alloantigen exposure is essential for the maintenance of the long-term allograft.

Similar observations were made in experiments where single orthotopic kidney transplantation was conducted and the contralateral kidney removed (Report # GTR-23213). Srl was administered at the time of transplantation and continued for 14 days. Six to 7 months later, the transplanted rats were used for a heterotopic cardiac transplant. Results showed that the rats with kidney allografts and treated with Srl survived for > 6 months. Heterotopic cardiac transplant, with the same allotype as the kidney, done over 7 months after withdrawal of Srl treatment survived > 60 days. However, heterotopic heart transplant of different allotype was rejected in ≤ 8 days. These results indicate that the persistence of alloantigens was essential for the maintenance of the long-term allograft and that tolerization was not organ specific.

Antibody and cellular responses were examined in some of the tolerized and nontolerized transplant rats. Low titres of cytotoxic antibodies (using lymph nodes as the target cells) were detected in the sera of tolerized rats 6 months post transplant. A second heterotopic transplant

did not alter the level of antibodies against the donor allotype specific lymph node target cells (Figure 28A) or the frequency of target specific cytotoxic cells on day 7 (Figure 29). However, in another group of rats the cytotoxic antibody titres decreased after the removal of the primary transplant and increased after the second transplant (Figure 28B). Frequency of antigen specific target cells increased after cardiac transplantation in naive rats. Results also showed that the production of IL-2 in an MLR assay was reduced in the presence of lymph node cells from rats treated with Srl i.e., tolerized rats (Figure 30). Such an effect was observed irrespective of whether the cells were used as responder or target cells.

Figure 27. Experimental schemes. A. The effect of persistence of alloantigen on the rejection response, on the production of cytotoxic Ab and on the frequency of cytotoxic cell precursors. B. The organ specificity of the tolerance, and the IL-2 production by lymphocytes from the tolerized rats. The recipients were all WFu rats. The day of last heart transplantation was designated as day 1. The 14-day RAPA administration was indicated by rectangles. The time of heart transplantation (HTx), kidney transplantation (KTx), and removal of the tolerized BUF hearts was indicated by upward arrows. The donor strain was indicated in the parentheses. The time points of limiting dilution assays, of one-way mixed lymphocyte reactions, and of bleedings was indicated by downward arrows.

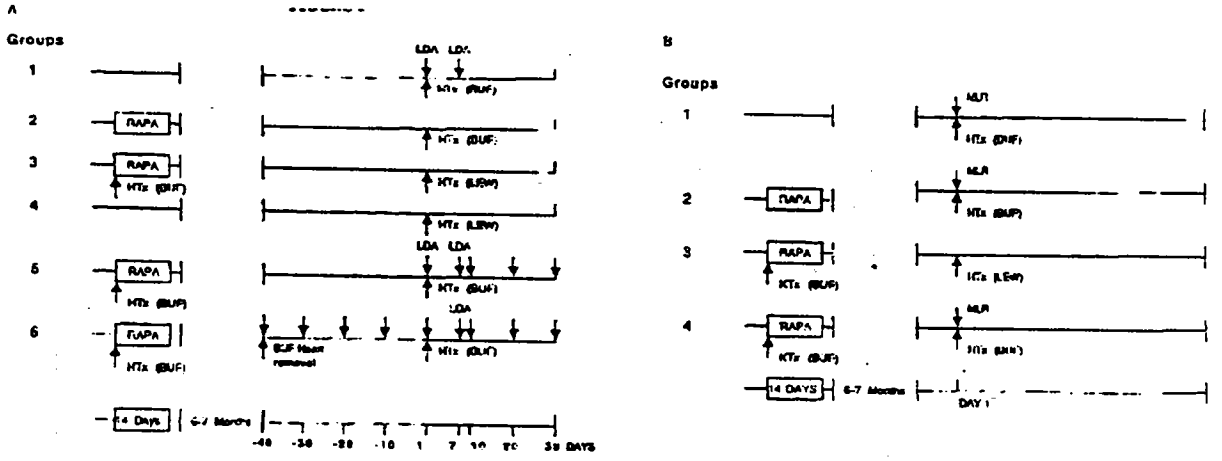
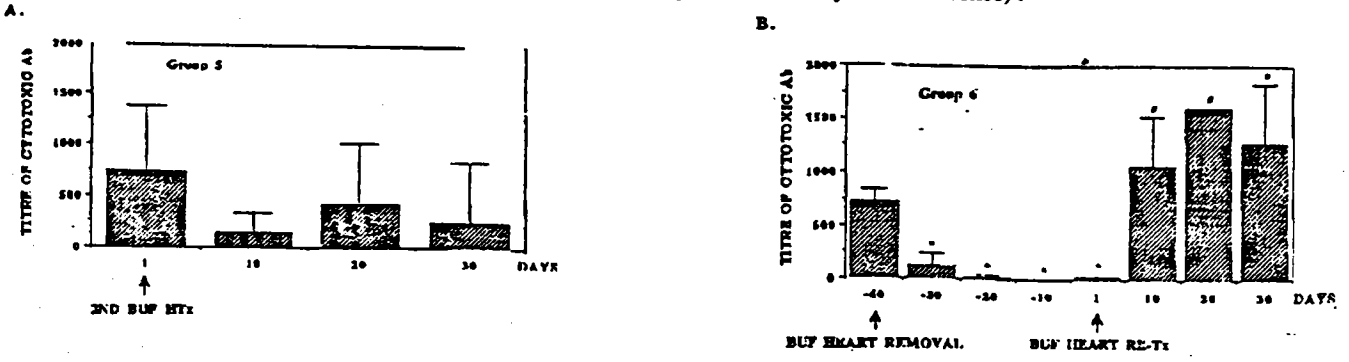


Figure 28. The effect of antigen persistence on the production of cytotoxic Ab. Detailed experimental scheme is depicted in Fig. 27A for Groups 5 and 6 (n = 4 per group). The rats were bled at 10–day intervals as indicated in Fig. 27A, and the serum levels of BUF-specific cytotoxic Ab were assayed. A. BUF-specific cytotoxic Ab in the rats with a tolerized BUF heart (Group 5). B. BUF-specific cytotoxic Ab in the rats which had the tolerized BUF heart removed and then retransplanted (Group 6). The day of the transplantation of the second BUF heart (2ND BUF HTx, Group 5) or BUF heart retransplantation (BUF HEART RE–Tx, Group 6) is designated as day 1. The data are plotted as means ±1 SD. Statistically significant differences ($p<0.01$; paired Student *t tests*) were indicated with asterisks (*) (using the value of day -40 as a reference) or number signs (#) (using the value of day 1 as a reference).



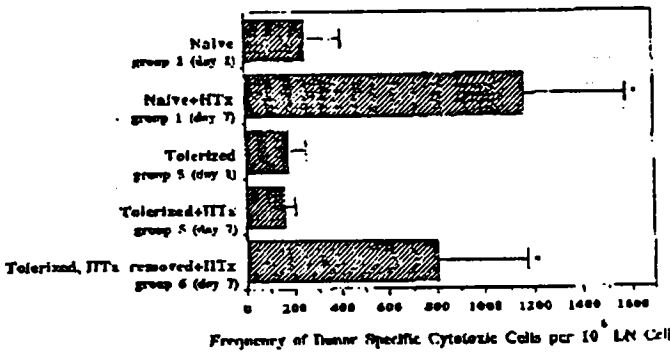
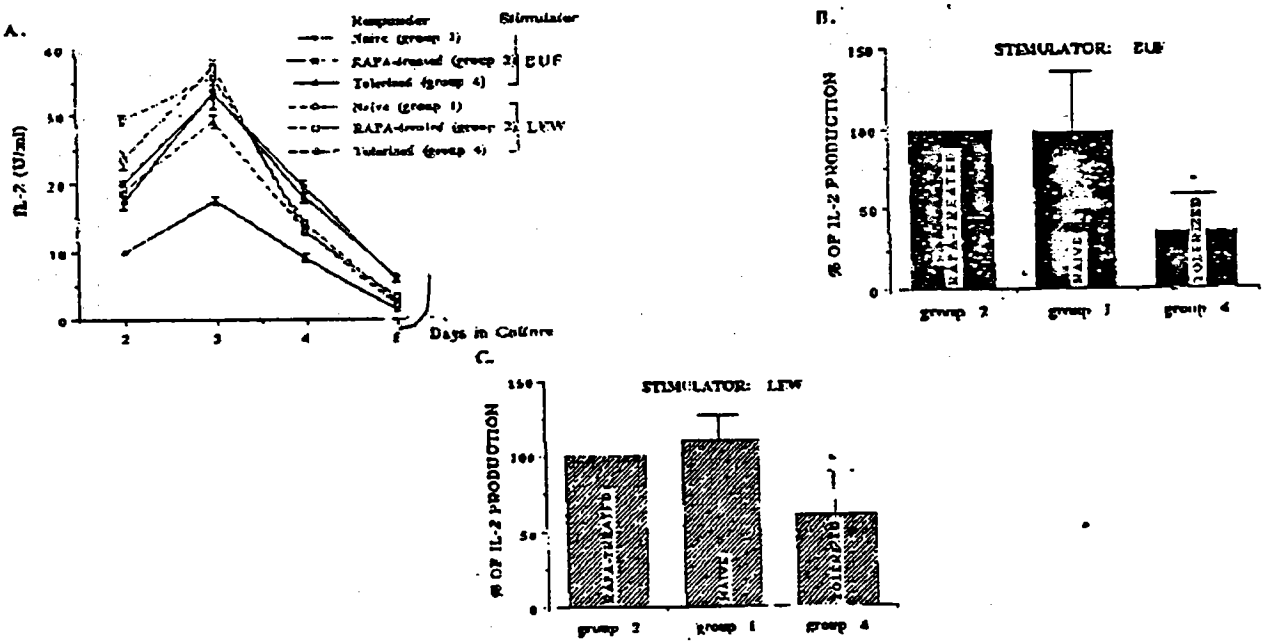


Figure 29. Frequency of donor-specific cytotoxic cell precursors (fDSCP) in rats with or without persistent alloantigen. A limiting dilution assay in conjunction with a ^{51}Cr release assay for cytotoxic cells was performed using recipient-strain (WFU) LN cells as effector cells and donor-strain (BUF) LN cells as target cells. The detail experimental design is depicted in Fig. 27B (Groups 1, 5 and 6). The mean ± 1 SD of three independent experiments is plotted, and statistically significant differences using the value of Group 1 (day 1) as a reference are indicated with asterisks (P<0. 01, paired Student t tests).

Figure 30. IL-2 production in vitro by the lymphocytes of tolerized rats upon alloantigen stimulation. The experimental scheme is depicted in detail in Fig. 27B. LN cells from naive (Group 1), previously RAPA-treated (Group 2) or tolerized (i.e. carrying a BUF kidney, Group 4) WFU rats were stimulated with BUF or LEW LN cells, and IL-2 production in the supernatants was measured. A. Kinetics of IL-2 production. Data are from one representative experiment out of four. B. IL-2 production upon donor-strain BUF stimulation. C. IL-2 production upon third-party LEW stimulation. In Fig. B and C, the IL-2 production by previously RAPA-treated- group is considered as 100 %, and data are compiled from 4 independent experiments. Data are all plotted as mean ± 1 SD. Statistically significant differences ($p<0.01$) are indicated with asterisks (Student t test, unpaired). The difference between the third columns (Group 4) in Fig. B and C is also statistically significant ($p<0.01$).



In another study (Report # GTR-22342) prior sensitization with 2 skin allografts and treatment with Srl initiated 14 days prior or 14 days post heterotopic cardiac transplant to the abdominal cavity was shown to improve the survival of the heart graft (Figure 31). In another group of rats, prior sensitization was conducted with 3 skin allografts on days 1, 10 and 31, treatment with Srl administered from days 17 to 45, and serum IgG and cytotoxic antibodies (using lymph node cells as the target cells) measured at regular intervals from days 1 to 52. Results showed a decrease in the cytotoxic antibodies in rats treated with Srl (0.8 mg/kg/day) as compared to the vehicle treated animals (Figure 32). This suppression was maintained until 3 weeks after the last dose (i.e., the last time point tested).

Figure 31

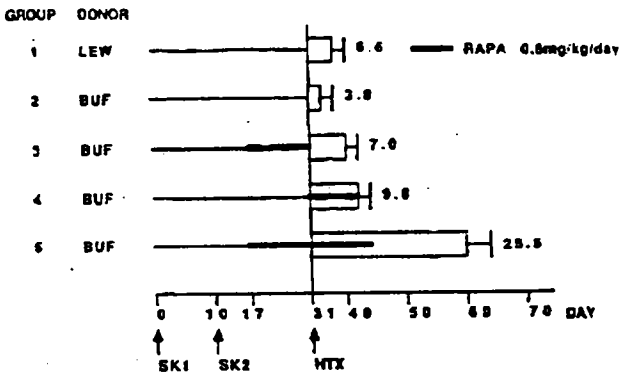
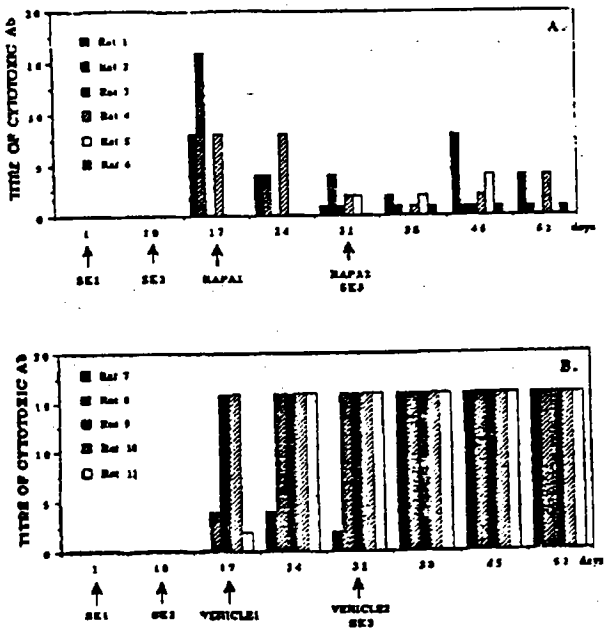


Figure 32



In the studies described above no attempts were made to correlate alterations in cell function (antibody or cellular) with cell number. In a small study (Report # GTR-19837) conducted in cynomolgus monkeys no alterations in absolute T-cell numbers and ConA induced lymphoproliferation were observed at 3-weeks post-transplant as compared to the pretransplant values. The effect of Srl on B-cell number is less clear. The number of B-cells were, shown to be decreased in monkeys treated with either Srl (2 mg/kg) alone or in combination with CsA. It should be noted that no change in B cell numbers was observed in other dose groups (0.5/1 mg/kg or 7 mg/kg). The number of animals treated with different doses was in the range of 3 to 5.

Srl was shown to increase the production of TGF-β *ex vivo* using splenocytes and lymph node cells from mice treated with Srl for 14 days and stimulated with anti-CD3 antibodies *in vitro* (*Dodge et al.*, 1999, Am. Soc. Transplantation, 1999, 67: Abstract # 175).

The direct effect of Srl on IL-7 induced lymphoproliferation was not tested. Studies by Li *et al.*, 1998 (J. Immunol. 161: 890) using IL-2 and IL-4 double knockout mice were shown to reject islet allografts and this rejection was attributed to the presence of T-cell growth factors (which include IL-7 and IL-15, as measured by PCR) secreted by cells other than T-cells within the grafts. Since Srl was shown to improve graft survival in this double knockout murine model, the sponsor has concluded that Srl suppresses T lymphocyte activation and proliferation that occurs in response to antigenic and IL-7 stimulation. Intragraft IL-7 or IL-15 cytokine levels in mice treated with Srl were not measured.

Cells as vehicle for drug administration:

Srl binds to erythrocytes and lymphoid cells and can serve as a vehicle for drug administration. Rats with heterotopic neonatal cardiac allograft to the ear were given erythrocytes containing Srl (180 ug/kg/day) by the intravenous route for 7 days (Report # GTR-24547). The cells were obtained from the blood of the same strain of rats as the recipient and incubated with Srl for 30 minutes at 37°C then washed prior to administration. Results showed that treatment with Srl improved survival (Table 12). Similar observations were made in another experiment (Report # GTR–24943) using splenocytes (from the same strain of rats as the donor) containing Srl (102 ug/kg/day, prepared as above) for administration of the drug by the intravenous route on days 4 and 13 post-transplant. Results showed an improvement in the survival of the graft. These studies suggest that erythrocytes and lymphoid cells may serve as a reservoir for Srl, which may be present in different parts of the body including the site of transplant. Also, the drug may become available intermittently. The factors that maintain equilibrium/transfer of the drug in different compartments of the body and across different cell types is not known.

Table 12

Dose	Vehicle	Route	Mean Survival (days)
100 µg/kg/14d	Phenol	P.O.	9.0
250 µg/kg/14d	Phenol	P.O.	10.6
900 µg/kg/14d	Phenol	P.O.	15.6
1.5 mg/kg/14d	Phenol	P.O.	20.9
2.0 mg/kg/14d	Phenol	P.O.	28.0
900 µg/kg/14d	I.V. 5	I.P.	27.4
180 µg/kg/7d	RBCs	I.V.	28.1
102 µg/kg/2d	Splenocytes	I.V.	17.0

Anti-fungal Activity:

Activity against *Candida albicans* was investigated *in vitro* and *in vivo* (Report # GTR-M-73-17). The minimum inhibitory concentrations against *Candida* species (*C. albicans* and non albicans) ranged between <0.02 – >10 ug/ml. The *in vitro* antifungal activity decreased in the presence of serum and at low pH. No significant *in vitro* activity was observed against dermatophytes (*Trichophyton mentagrophytes* and *Microsporum gypseum*).

In vivo, Srl was shown to be effective in improving survival of mice infected with systemic *C. albicans* infection (ED_{50} = 10 mg/kg) when administered by the subcutaneous route. The oral route of drug administration was less effective. In a murine model of vaginal candidiasis, Srl showed some inhibitory activity at a dose of 50 mg/kg.

No antibacterial (gram positive and gram negative) activity of Srl was observed (Report # GTR-M-73-17).

Mechanism of Action:

The mechanism by which Srl exhibits immunosuppressive activity was evaluated by several different investigators, including Terada *et al.* (J. Cellular Physiol., 1993 154: 7), Nourse *et al.* (Nature, 1994, 372: 570), and Sabatini *et al.* (J Biol. Chem. 1995, 270: 20875). Also, several review articles are available on the subject (Dumont and Su, 1996; Life Sciences 58: 373; Abraham and Wiederrecht, 1996, Ann. Rev. Immunol. 14: 483; Abraham, 1998, Current Opinion in Immunol. 10: 330; Sehgal, 1997, Report # GTR-31712). Overall, these studies showed that Srl, like FK506, binds to intracellular proteins called immunophilins or FK506 binding proteins (FKBP) which are present within the cytosol and exhibit peptidyl-prolyl-cis-trans isomerase (PPIase) activity. Some of the FKBPs to which Srl and FK506 have been shown to bind include FKBP12, FKBP13, and FKBP25. FKBP12 is ubiquitous. Its functions include control of Ca^{++}efflux from the endoplasmic reticulum (ER) and interactions with type I TGF-β receptors. FKBP13 and FKBP25 are present in the ER of mast cells and within the nucleus, respectively. Srl was shown to exhibit greater binding to FKBP25 than to FK506. However, the immunosuppressive activity of these drugs is considered to be due to binding to FKBP12. FK506 inhibits a calcineurin (calcium dependent phosphatases) dependent pathway that is mediated through activation of the T cell receptor (TCR). Signal transduction through CD28 [a T-cell surface molecule which binds B7-1 (CD80) and B7-2 (CD86) on the surface of APC] is not inhibited by FK506 or CsA but is inhibited by Srl which appears to act primarily on a noncalcineurin-dependent pathway. The inhibitory effect of Srl is more in the G_1 to S phase of the cell cycle whereas that of FK506 and CsA is on early cell activation events (G_0 to G_1).

The complex of FKBP:Srl binds to the kinase mammalian target of rapamycin (mTOR). This target is also known as RAFT1, RAPT1, or FRAP. The precise role of mTOR has not been established. It is thought to have protein/lipid kinase activities and to regulate several signal transduction pathways (Figures 33 and 34). The steps in protein synthesis which were shown to be inhibited by Srl include: (1) phosphorylation and activation of 70 KDa S6 protein kinase ($p70^{s6k}$), (2) a cytokine triggered protein kinase cascade leading to the phosphorylation of the eukaryotic initiation factor 4E (eIF-4E) binding protein, PHAS-1, (3) a cyclin dependent kinase (cdk) 2-cyclin E complex, and (4) IL-2 induced expression of bcl-2. The activation of $p70^{s6k}$ and cdk enzymes, expression of bcl-2 and phosphorylation of PHAS-1 play an important in controlling the cytokine induced mitogenic response and G_1 to S phase transition.

The $p70^{s6k}$ or its isoform ($p85^{s6k}$) are phosphorylated and activated within minutes of T-cell activation with IL-2, PMA, or anti-CD28 antibodies. Srl was shown to suppress T-cell activation by these stimuli. The immunosuppressive effects of FKBP:Srl:mTOR were not observed in a cell free system. The suppression of $p70^{s6k}$ activity may represent an effect on an early event of the cytokine induced response. In addition to the effect of Srl on inhibition of $p70^{s6k}$ using s6 ribosomal protein as a substrate, the drug also inhibits $p70^{s6k}$ dependent cAMP-responsive element (CRE) modulator phosphorylation. This phosphorylation occurs in the late G1/S phase of the cell cycle. It was also shown that the activity of mTOR, including activation of $p70^{s6k}$, depended upon the concentration of nutrients such as amino acids (Iiboshi *et al.*, 1999, J. Biol. Chem. 274: 1092).

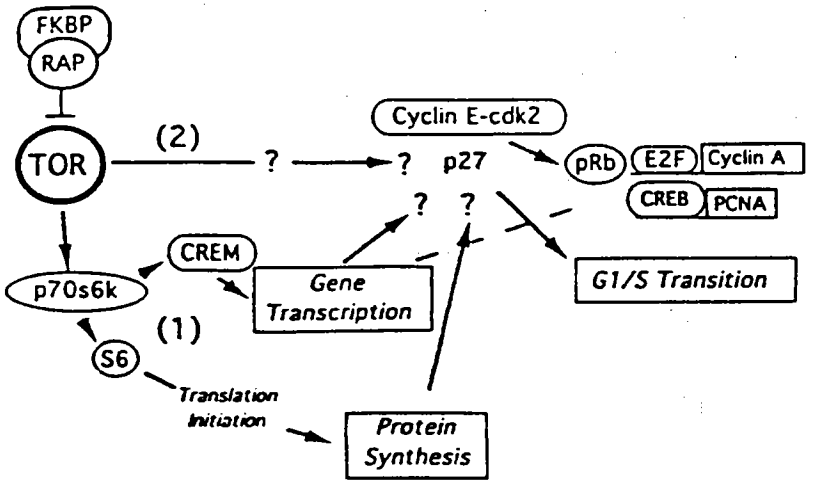


FIG 33

"mTOR-dependent signaling". This model postulates that mTOR acts as the proximal sensor of incoming signals from cytokine/growth factor receptors. Signal transmission promoting entry of cells into the proliferation cycle through activation of G1/S transition might operate downstream of mTOR via either a linear pathway (1) or bifurcating pathways (2).

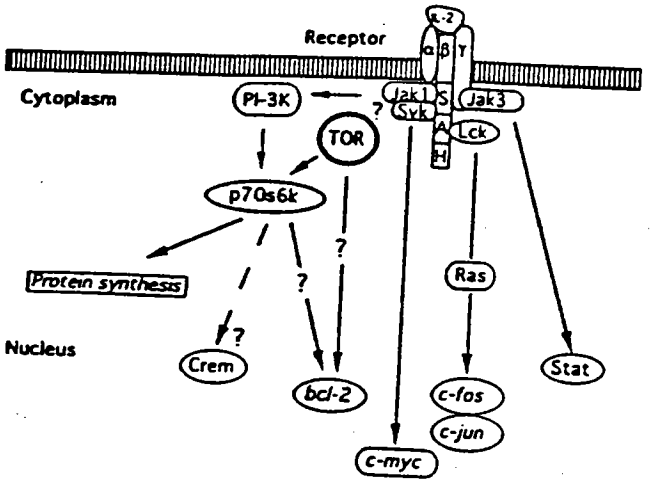


FIG. 34

Multiple signaling pathways cooperate in mediating IL-2R-induced stimulation of early proto-oncogene expression. This model is based on data by Miyazaki et al (173) and postulates that the rapamycin-sensitive pathway leading to *bcl-2* induction involves mTOR. Note that it is not known whether *bcl-2* induction depends on $p70^{s6k}$ activity or whether CREMτ (105,107)plays any role in IL-2R signaling. The putative connections of these early events with the activation of G1/S transition through p27 and cdk2-cyclin E regulation remain to be unraveled.

The enzyme cdk2 belongs to the family of serine/threonine kinases, which are important in cell activation in the progression from mid to the late G_1 phase of the cell cycle. Cyclin E is considered to be an essential partner with cdk enzymes for cells to progress from G_1 to the S phase. Cyclin A also activates cdk2 during the S phase. In lymphoid cells, Srl, like TGF-β prevents activation of the cdk2-cyclin E complex although both cdk2 and cyclin E are present and form complexes. Srl also inhibits the cyclin A dependent function of cdks. In an osteosarcoma cell line Srl was shown to inhibit the cyclin D1-cdk complex (present in the early G_1 phase). However, it is unclear whether mTOR directly alters $p70^{s6k}$ and cdk2 or it inhibits cdk2 indirectly by inhibiting $p70^{s6k}$ (Figure 33).

It is known that the serine rich region of the β chain of the IL-2R is essential for proliferation of T-cells in the presence of IL-2. This region is linked to induction of the bcl-2 gene. Srl was shown to inhibit the expression of bcl-2 mRNA thereby inhibiting of signal transduction through the IL-2 R. Several cytokines, including IL-2, IL-3, IL-6, IL-7, IL-15 are known to exhibit activity by binding at least partially through IL-2R (probably the γ-chain). Srl was shown to inhibit lymphoproliferative responses in the presence of IL-2, IL-4 and IL-15 (for details see pages 13 to 16).

It is also of note that the different cells may have a different level of dependence on phosphorylation and activation of $p70^{s6k}$ for protein synthesis. Consequently the antiproliferative effect of Srl will vary from cell to cell. Studies using NIH3T3 (fibroblast) cells stimulated with growth factors or mitogens showed that Srl did not inhibit the phosphatidyl (PI) 3-kinase dependent activation of D-

type cdks including $p70^{s6k}$ which is essential for transition of these cells from the G_1 to S phase cell cycle (Takuwa *et al.*, 1999, Mol. Cell. Biol. 19: 1346). This observation provides support for the concept that the dependence on mTOR for cell activation and proliferation may vary from cell to cell.

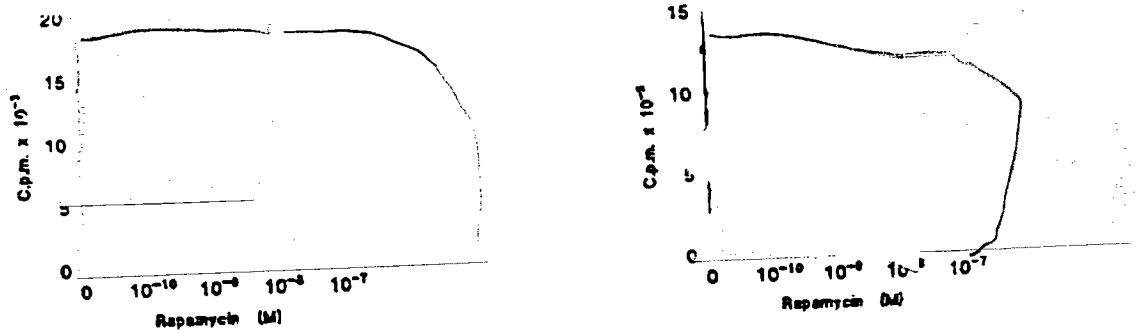
Some other cell types in which Srl was shown to alter enzyme activity (the steps which are considered to be downstream of mTOR) include (1) insulin stimulated human embryonic kidney cells [phosphorylation of PHAS-1 by Srl which inhibited the binding of PHAS-1 to eIF-4E (Brunn *et al.*, 1997, Science, 277: 99)], (2) fibroblast and rat hepatoma cells [Srl inhibited activation of $p70^{s6k}$ (Chung *et al.*, 1992 Cell, 69:1227; Price *et al.*, 1992, Science, 257: 973)], and (3) rat smooth muscle cells [Srl inhibited $p34^{cdk2}$ leading to a delay in the rise of cyclin D1 activity (Marx *et al.*, 1994, Circulation Research, 76: 412)].

C. Resistance:

Studies using yeast and mammalian cells showed that the complex of FKBP-Srl binds to mTOR at a region containing a serine residue. A mutation in this serine residue was shown to confer resistance to Srl.

D. Drug Interaction:

The combination of Srl with CsA or FK506 was shown to increase the inhibitory effect on ConA induced *in vitro* lymphoproliferative responses of porcine cells (Figure 35) compared to the activity of either drug alone.



(A) (B)

Figure 35. A: Interaction of rapamycin and cyclosporin A.. Porcine lymphocytes were incubated without mitogen (o) or with 5 ug/ml ConA (□). Where indicated 30 nM (●), 100 nM (■) or 1 uM (-) CSA and rapamycin at the concentrations shown were added together with Con A. B. Interaction of rapamycin and FK-506. Porcine lymphocytes were incubated without mitogen (o) or with 5 ug/ml ConA (□). Where indicated 1 nM (●) , 10 nM (■) or 100 nM (-) FK506 and rapamycin at the concentrations shown were added together with Con A. Activation was assessed by determination of the rate of incorporation of [^{35}S]-methionine into protein at 24 h.

Similar observations were made using a combination of Srl and CsA to inhibit lymphoproliferative responses to PHA (Report # GTR-18686).

Studies showed that a combination of CsA with Srl lead to a synergistic response (combination index < 1) not only against PHA but also OKT3 and allogeneic induced stimulation *in vitro* (Report # GTR-18690). Use in a combination lead to a lowering of the dose of the individual drugs. The dose reduction index (DRI) varied from 10 to 10^4 depending on the stimulant used. For example DRI, was lower for PHA stimulated cells than for OKT3 or allogeneic stimulated cells. Inter-person variability was also observed. It should be noted that the raw data were not included in the submission.

The effect of a combination of Srl+CsA on cytotoxic T-lymphocyte (CTL) activity was measured using ^{51}Cr labeled PHA stimulated target cells (Report # GTR-18690). The results show that CsA was about 8-times more effective in inhibiting the cytolytic activity as compared to Srl, but the combination exhibited a synergistic cytotoxic effect. In the presence of exogenous IL-2, a combination of CsA with Srl decreased the frequency of CTL cells to a greater extent than either drug alone.

In another experiment (Report # GTR 18690), cloned T-cell lines (MH60.BSF-2 and CTLL-2) were used to test the inhibitory effect of Srl in the presence of IL-6 and IL-2 respectively. Here again, a combination of CsA with Srl was shown to exhibit synergistic inhibitory effect. Inhibition of IL-6 dependent proliferation was shown to be noncompetitive; whereas that of IL-2 dependent proliferation was competitive.

In mast cells, FK506 was shown to partially prevent the inhibitory effect of Srl (Figure 19). This antagonism would suggest competitive binding to the same intracellular binding protein (Report # GTR 20129). FK506 by itself did not exhibit any inhibitory effect in the model. In contrast CsA + Srl showed an additive effect in inhibiting mast cell proliferation.

An antagonistic interaction between Srl and FK506 was also shown *in vitro* using murine T-cell lymphoma (YAC-1) cells stimulated with IL-1 or IFN-γ. Similar observations were made at the molecular level using a combination of Srl with FK506 to inhibit selected enzyme activities (e.g., $p70^{s6k}$).

In contrast to the *in vitro* results, the combination of Srl with FK506 *in vivo* does not produce antagonism in a variety of different models, but rather had synergistic effect in prolonging the allograft survival as compared to either agent alone. The abundance of FKBP-12 receptors *in vivo* may prevent competitive inhibition of the 2 agents for the receptor sites.

CONCLUSIONS:

The sponsor seeks approval of Srl for the prevention of renal graft rejection. Srl, like CsA and FK506, is an immunosuppressive agent.

Studies *in vitro* showed that Srl inhibited the ConA, PHA, LPS, PWM, PMA, anti-CD3, anti-CD28, anti-IgM, and/or allogeneic induced lymphoproliferation of mouse, porcine and human lymphocytes as measured by ^{3}H-thymidine or ^{35}S-methionine uptake. However, Srl had no inhibitory effect on SAC induced lymphoproliferation. It is also of note that the inhibition by Srl, like FK506 or CsA, was incomplete. The magnitude of inhibition observed was dose dependent and varied with the concentration of mitogen used in culture.

Srl between the concentrations of 0.1 nM to 100 nM inhibited proliferation (as measured by the rate of protein synthesis) of unstimulated porcine cells by about 20%, whereas FK506 was stated to have no effect.

The anti-lymphoproliferative activity of Srl is similar but not identical to that produced by CsA or FK506. The most distinctive difference was the effect of Srl on PMA induced stimulation. The inhibition of human PBMC stimulated with PMA was greater with Srl than CsA. These observations were based on either the measurement of the rate of DNA synthesis or the release of sIL-2R. Other differences were evident from the *in vitro* studies. The inhibitory effect of Srl on LPS induced responses of mouse splenocytes was similar to CsA, whereas the immunosuppressive activity against ConA induced responses was less ($\leq$ 48 fold) than that of CsA. The inhibitory effect of Srl was similar to that of FK506 on ConA induced lymphoproliferation of porcine PBMC. In contrast, using human PBMC, Srl was more effective than FK506 in inhibiting protein synthesis in PHA or TPA+antiCD28 antibody stimulated cells. The anti-proliferative activity of Srl and FK506 on anti-CD3 antibody or TPA stimulated cells was comparable. Also, simple washing of the cells *in vitro* did not reverse the inhibitory activity of Srl or FK506 indicating either tight binding to the cell surface or internalization of the drug. Similar observations were reported using CsA although data were not included in the NDA.

Time kinetic studies showed that the inhibitory effect of FK506 and Srl on the rate of protein synthesis by ConA stimulated porcine PBMC progressively decreased over time. In contrast to FK506 induced suppression which appeared to occur during the first 8 hours of stimulation the inhibitory effect of Srl appeared to last until about 12 to 24 hours of prestimulation with the mitogen. In contrast, CsA was only effective when added at the time of initiation of cultures.

Suppression of lymphoproliferative responses may result from alterations in the production of cytokines, expression of cell surface receptors or signaling events. The effect of Srl on the production of IL-1β, TNF-α, TGF-β, and IL-2 was measured. Srl did not alter the production of IL-1β and TNF-α by human monocytes *in vitro*. Srl increased the production of TGF-β *in vitro* and/or *ex vivo* using murine splenocytes or human PBMC stimulated with anti-CD3 antibodies or ConA *in vitro*. A possible role for TGF-β in the immunosuppressive effect of Srl cannot be ruled out.

The effect of Srl on the production of IL-2 was shown to be variable. For example, Srl decreased the production of IL-2 by ConA stimulated splenocytes *in vitro*. The IC_{50} values were > 50-fold higher for Srl compared to CsA in this study. Similar observations were made *ex vivo* using lymph node cells from rats treated with Srl as responder or target cells in a MLR assay. In contrast, production of IL-2 by CD4 TH_1 cells was not altered in the presence of Srl *in vitro*. These differences in the inhibitory effect of Srl on IL-2 production could be attributed to the heterogeneity of the cell population in splenocyte or lymph nodes vs. the homogenous cells present in CD4 TH_1 clone or to the concentrations of IL-2 produced by the cells in culture. In one of the experiments the IC_{50} values of Srl and CsA were shown to vary with the concentrations of exogenous IL-2 in culture.

Srl, unlike FK506 or CsA, was shown to inhibit *in vitro* lymphoproliferation in the presence of exogenous IL-2. This inhibition of IL-2 dependent activity could be attributed to the decreased binding of IL-2 to IL-2R on the cell surface that is important for T-cell proliferation and several effector functions. Srl also inhibited the release of soluble IL-2R. Such alterations in the IL-2 dependent activity by Srl possibly leads to interference with the signal transduction pathway activated by the IL-2/IL-2R complex.

Several other cytokines such as IL-4, IL-7, IL-9, and IL-15 can also bind to IL-2R. Srl decreased lymphoproliferation in the presence of IL-4 and IL-15 *in vitro* in addition to IL-2. In the presence of IL-12, Srl decreased lymphoproliferative responses of long term cultured T cell lines. The effect of Srl on IL-12 dependent proliferation of fresh cells was not tested.

The effect of Srl on IL-1β dependent proliferation of thymocytes was unclear. For example, Srl inhibited murine thymocyte proliferation in the presence of PHA+IL-1β but had no effect on the proliferation of thymocytes obtained from pediatric patients. Human thymocyte proliferation was, however, inhibited in the presence of PHA + IL-2. These differences may be due to the differences in the stages of cell maturation.

Srl, like CsA, decreased antibody production *in vitro* when stimulated with PWM or SAC. This inhibition of antibody response was shown to be due to the effect of Srl on T-cells since preincubation of T-cells with the drug prior to mixing with the B-cells lead to inhibition of IgG production.

Srl, like CsA, exhibited a modest inhibitory effect on NK, LAK and ADCC activities *in vitro*. The effective concentrations were about 10-100 fold higher than that required to inhibit T cell proliferation. FK506 was reported to have little effect on NK and ADCC activities.

Srl also inhibited the proliferation and/or differentiation of some of the nonlymphoid cells which included hepatocytes, fresh keratinocytes (but not established cell lines), mast cells (in the presence of IL-3), fibroblasts, neuroblastoma and glioblastoma cells, and muscle cells. It is also of note that the activity of the drug may vary with the culture conditions. For example, in serum-free medium, Srl blocked the cell cycle of mast cells at G_0/G_1, while in serum-containing medium cells accumulated in G_2/M. Keratinocyte HLA-DR expression in the presence of IFN-γ was not diminished by incubation with Srl. Srl had no inhibitory effect on the proliferation of A-431 cells (a human epidermoid tumor cell line). Similarly, K562 (human chronic myelogenous leukemia) and HUT 78 (human cutaneous T-cell lymphoma) cell lines were not sensitive to either of the immunosuppressive agents.

Srl was shown to improve graft survival in several different animal models. The *in vivo* activity of Srl was measured in mice, rats, dogs, pigs, monkeys and/or baboons with allogeneic renal, heart, skin, pancreatic or duodenal grafts. In the models of orthotopic renal transplant in rats, pigs and baboons with the contralateral kidney removed, treatment with Srl prolonged survival. However, Srl exhibited toxicity in dogs and a survival benefit could not be measured under the experimental conditions tested. A combination of Srl with CsA improved survival in dogs.

Srl was effective in improving the survival of heterotopic heart allograft (dorsal pinna or intra-abdominal) in mice, rats, rabbits and cynomolgus monkeys. A combination of a low dose of Srl with a low dose of CsA or FK506 was more effective in improving graft survival compared to the individual drugs.

Srl was also effective in improving skin (mouse, rats), pancreatic- duodenal (rats) and bone marrow (mice) allografts in rodents. A combination of Srl with CsA was shown to be more effective in the majority of the studies compared to either drug alone. Srl was also effective in improving skin xenograft survival in one study. Srl was not effective in improving pancreatic islet graft survival in dogs, but in combination with CsA did produce improved graft survival.

In addition to the effect on graft survival, the sponsor showed the effectiveness of Srl in suppression of immune mediated events (as measured by improved survival) in rodents with autoimmune disorders such as SLE (mice), collagen or *M. butyricum* induced arthritis (mice and rats), hypersensitivity (mice), non obese diabetic model of autoimmune type I insulin dependent diabetes mellitus (in 8 to 9 week old mice but not in older mice), experimental allergic encephalomyelitis (rats), myocarditis (rats), and uveoretinitis (rats). In an animal model of non obese of autoimmune type I insulin dependent diabetes mellitus, Srl was effective in preventing the onset of diabetes 41 weeks after discontinuation of treatment.

Srl also reduced thickening of arterial intimal walls induced by balloon injury or coronary balloon angioplasty (rats and pigs) when administered a day before injury. Studies in rats showed that the activity of Srl was dose dependent and a decrease in intimal wall thickening was observed on day 14 (during treatment). This effect was not observed a month after discontinuation of treatment in rats. In pigs, however, the effect of Srl lasted for 5 to 6 months after drug withdrawal. CsA and FK506 had no effect at this time point.

Treatment with immunosuppressive agents can increase susceptibility to infection. *In vivo* administration of Srl was shown to decrease survival of mice infected with *C. albicans* and CMV. Mice treated with CsA and CyP were more susceptible to infections with *C. albicans, P. aeruginosa* and CMV as compared to Srl.

Studies showed that treatment with Srl prolonged graft survival in presensitized hosts. For example, in rats with 2 prior skin allografts, treatment with Srl was shown to improve graft survival compared to the vehicle treated animals. This effect was also observed 70 days after drug withdrawal but not after 3 months. Survival of the graft in a presensitized host was shown to be allotype specific but not organ specific. The persistence of alloantigens was shown to be essential for preventing graft rejection. These observations were based on heterotopic model of heart transplant.

Studies *in vivo* showed that treatment with Srl lead to the development of unresponsiveness as measured by MLR and antibody responses in rats and pigs. The reduced antibody response (serum IgG and antiTT antibodies) was reversed by 3 months after discontinuation of therapy in rats. The reversal of cytotoxic antibody response appeared to be incomplete. In another experiment, Srl was shown to induce allo-specific tolerance which lasted for about 6 months after discontinuation of therapy and was associated with a decrease in IL-2 production in a MLR assay or a reduction in the frequency of cytotoxic cells and serum cytotoxic antibody titres. No attempts were made to correlate alterations in serum antibody titres, alloantigenic stimulation or IL-2 production with changes in cell number. Experiments in cynomolgus monkeys indicated no effect of Srl on T-cell

number or ConA induced proliferation *ex vivo* using PBMC. However, the effect on B-cell number is less clear. Although a 2 mg/kg dose was shown to decrease B-cell numbers, other lower and higher doses showed no effect. Unfortunately the duration of treatment and the time of testing after discontinuation of treatment were not specified.

A combination of Srl with CsA showed increased inhibition of lymphopropliferative responses *in vitro* and graft survival *in vivo*. A combination of Srl with FK506 showed conflicting results *in vitro* varying from increased inhibition to antagonism. In contrast to the *in vitro* findings, no antagonism was observed *in vivo* using a combination of Srl with FK506. A combination of Srl with FK506 was shown to be more effective than either agent alone. The abundance of FKBP-12 receptors *in vivo* may prevent competitive inhibition of the 2 agents for their receptor.

In general the preclinical studies showed that Srl inhibited lymphoproliferative responses induced by IL-2 or to stimulation via the CD3/TCR, CD2, or CD28 pathways. The mechanism of action of FK506, CsA and Srl apparently involve distinct pathways, since Srl blocked the intracellular response to IL-2 while FK506 and CsA had no effect.

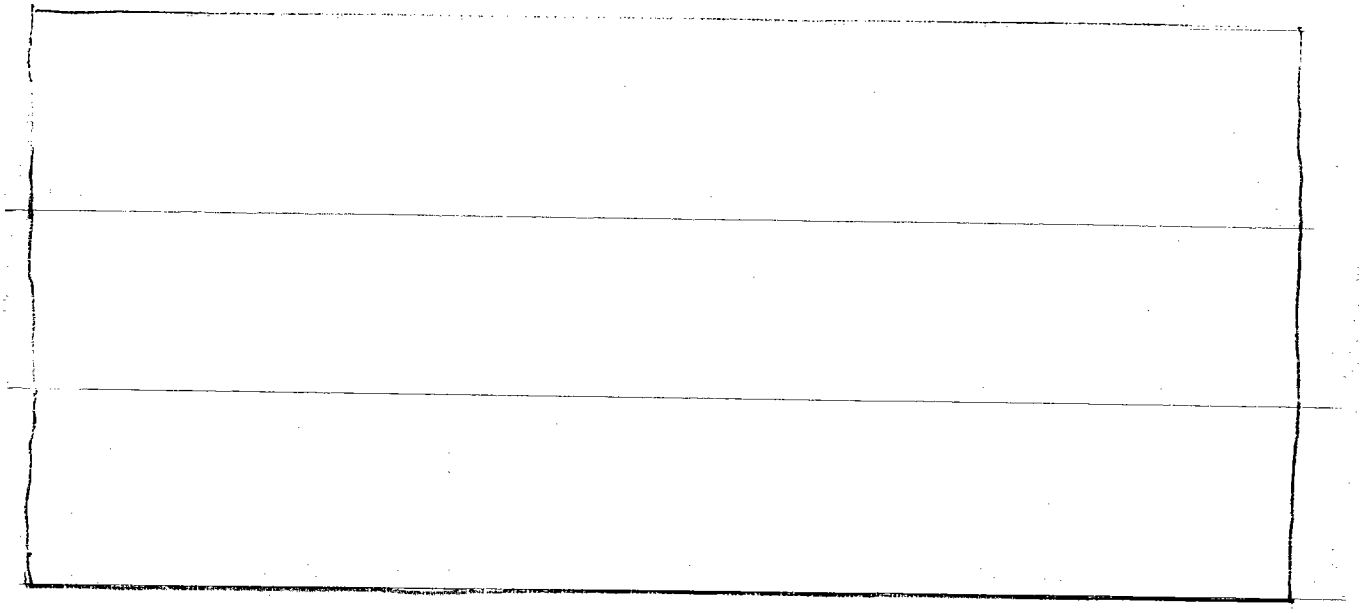
The mechanism by which Srl exhibits antilymphoproliferative responses was investigated mainly using T-cells. Studies showed that Srl binds to FKBP, an immunophilin present in the cytosol and found in all eukaryotic cells thereby indicating that these agents should affect signaling in all cell types. Indeed, studies showed that Srl inhibited proliferation of nonlymphoid cells as well as lymphoid cells.

Some of the FKBPs to which Srl was shown to bind include FKBP12, FKBP13 and FKBP25. However, the immunosuppressive activity of Srl is considered to be due to binding to FKBP12. The complex of FKBP and Srl binds to the kinase mTOR which regulates several signal transduction pathways including phosphorylation and activation of $p70^{s6k}$, a cytokine triggered protein kinase cascade leading to phosphorylation of eIF-4E, cdk2-cyclin E complex, and an IL-2 induced expression of bcl-2. Inhibition of mTOR inhibits transition of cells from G_1 to the S phase. It is of note though that protein synthesis in different cells may have different levels of dependence on phosphorylation and activation of $p70^{s6k}$. Therefore, the antiproliferative effect of Srl may vary from cell to cell.

3 Pages

DRAFT

LABELING



RECOMMENDATIONS:

This NDA is approvable with respect to immunology pending an acceptable version of the label.

/S/
Shukal Bala
Immunologist, HFD-590

CONCURRENCES:
HFD-590/Deputy Dir. /S/ Signature 8/26/99 Date
HFD-590/MicroTL /S/ Signature 26 Aug 99 Date
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