

**CENTER FOR DRUG EVALUATION AND  
RESEARCH**

*APPLICATION NUMBER:*  
**21-560s000**

**MICROBIOLOGY REVIEW(S)**

**MICROBIOLOGY/IMMUNOLOGY REVIEW**  
**DIVISION OF SPECIAL PATHOGEN AND TRANSPLANT PRODUCTS**

**NDA #:** 21-560  
(SDN-089, 092)

**REVIEWER** : Simone M. Shurland  
**CORRESPONDENCE DATE** : 1/22/2010  
**CDER RECEIPT DATE** : 1/28/2010  
**REVIEW ASSIGN DATE** : 1/28/2010  
**REVIEW COMPLETE DATE** : 3/23/2010

**APPLICANT:** Novartis Pharmaceuticals Corporation  
One Health Plaza  
East Hanover, NJ 07936-1080

**DRUG CATEGORY:** Immunosuppressive agent

**INDICATION:** Prevention of kidney rejection in solid organ transplantation

**DOSAGE FORM:** Oral

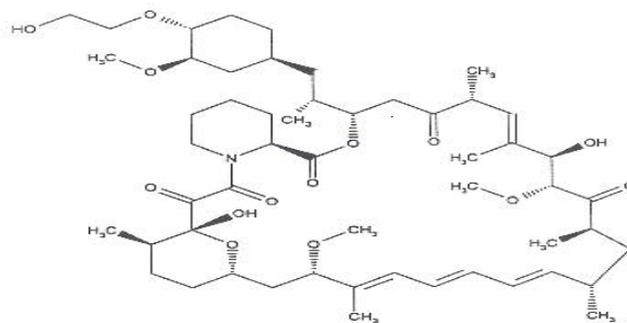
**PRODUCT NAMES:**

a. **PROPRIETARY:** Zortress

c. **NONPROPRIETARY:** RAD; RAD001; Everolimus

d. **CHEMICAL:** 1R, 9S, 12S, 15R, 16E, 18R, 19R, 21R, 23S, 24E, 26E, 28E, 30S, 32S, 35R), -1, 18-DIHYDROXY-12{(1R)-2-[(1S, 3R, 4R)-4-(2-hydroxyethoxy)-3-methoxycyclohexyl]-1-methylethyl}-19, 30-dimethoxy-15, 17, 23, 29, 35-hexamethyl-11, 36-dioxa-4-azatricyclo{30.3.1.0<sup>4,9</sup>} hexatriaconta-16, 24, 26, 28-tetraene-2, 3, 10, 14, 20-pentaone.

**STRUCTURAL FORMULA:**



Everolimus

Molecular Weight: 958.2  
Molecular Formula: C<sub>55</sub>H<sub>83</sub>NO<sub>14</sub>

**SUPPORTING DOCUMENTS:** NDA 21-560, NDA 21-628, NDA 21-561, NDA 21-631, IND 52,003.

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**1. INTRODUCTION**

The subject of this NDA is everolimus, for the prophylaxis treatment of organ rejection in adult patients at low to moderate risk of receiving a kidney transplant. The applicant has re-submitted and updated the label, with proposed changes to *Section 12.1 Mechanism of Action*.

(b) (4)

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**2.3. FDA's version of the labeling**

(b) (4)  
[Redacted]

[Redacted]

[Redacted] (b) (4)

[Redacted]

[Redacted]

**3. RECOMMENDATIONS**

The applicant has accepted all the changes to the labeling. The NDA should be approved with respect to microbiology/immunology.

S.M. Shurland  
Simone M. Shurland  
Microbiologist, DSPTP

**CONCURRENCES:**

DSPTP /Microbiology Team Leader Shukal Bala Signature 3/24/2010 Date

CC:  
DSPTP/ NDA 21-560  
DSPTP/PM/Jacquelyn Smith

Application Type/Number	Submission Type/Number	Submitter Name	Product Name
NDA-21560	ORIG-1	NOVARTIS PHARMACEUTICA LS CORP	CERTICAN (EVEROLIMUS) TABLETS

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/s/

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SIMONE M SHURLAND  
03/24/2010

SHUKAL BALA  
03/24/2010

**MICROBIOLOGY/IMMUNOLOGY REVIEW**  
**DIVISION OF SPECIAL PATHOGEN AND TRANSPLANT PRODUCTS**

**NDA #:** 21-560  
(SDN-054, SDN-061)

**REVIEWER** : Simone M. Shurland  
**CORRESPONDENCE DATE** : 6/30/2009, 8/31/2009  
**CDER RECEIPT DATE** : 7/1/2009, 8/31/2009  
**REVIEW ASSIGN DATE** : 7/13/09; 9/4/2009  
**REVIEW COMPLETE DATE** : 11/18/2009

**SPONSOR:** Novartis Pharmaceuticals Corporation  
One Health Plaza  
East Hanover, NJ 07936-1080

**DRUG CATEGORY:** Immunosuppressive agent

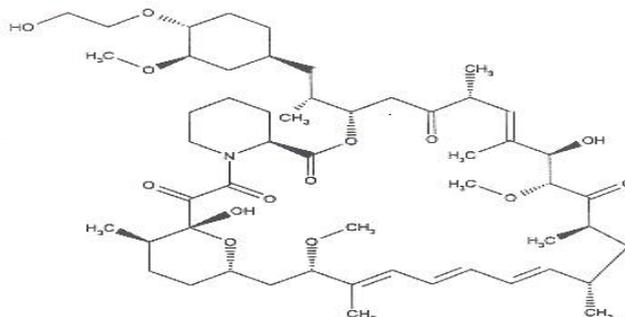
**INDICATION:** Prevention of kidney rejection in solid organ transplantation

**DOSAGE FORM:** Oral

**PRODUCT NAMES:**

- a. **PROPRIETARY:** Zortress
- c. **NONPROPRIETARY:** RAD; RAD001; Everolimus
- d. **CHEMICAL:** 1R, 9S, 12S, 15R, 16E, 18R, 19R, 21R, 23S, 24E, 26E, 28E, 30S, 32S, 35R), -1, 18-DIHYDROXY-12{(1R)-2-[(1S, 3R, 4R)-4-(2-hydroxyethoxy)-3-methoxycyclohexyl]-1-methylethyl]-19, 30-dimethoxy-15, 17, 23, 29, 35-hexamethyl-11, 36-dioxa-4-azatricyclo{30.3.1.0<sup>4,9</sup>} hexatriaconta-16, 24, 26, 28-tetraene-2, 3, 10, 14, 20-pentaone.

**STRUCTURAL FORMULA:**



Everolimus

Molecular Weight: 958.2  
Molecular Formula: C<sub>55</sub>H<sub>83</sub>NO<sub>14</sub>

**SUPPORTING DOCUMENTS:** NDA 21-560, NDA 21-628, NDA 21-561, NDA 21-631, IND 52,003.

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## 1. INTRODUCTION

The subject of this NDA is everolimus for the prophylaxis of organ rejection in adult patients at low to moderate risk of receiving a renal transplant. The applicant is proposing to administer everolimus concurrently with reduced doses of cyclosporine (Neoral) and corticosteroids. Everolimus (AFINITOR) is approved for the treatment of patients with advanced renal cell carcinoma after failure of treatment with sunitinib or sorafenib.

Everolimus is a chemical derivative of the macrolide, rapamycin (a macrolide produced by *Streptomyces hygroscopicus*). The main structural difference between everolimus and rapamycin is that the hydrogen of the 40-hydroxyl group in rapamycin was replaced with a 2-hydroxyethyl group, thus forming an ether bond [40-O-(2-hydroxyethyl)-rapamycin]. The ether bond is metabolically stable, that is everolimus is not converted to rapamycin.

The applicant claims that everolimus immunosuppressive activity is comparable to that of rapamycin. Protein binding studies show that everolimus binds to 75% of plasma proteins in humans compare to 99% in mouse, 92% in rats, and 84% in monkeys. Pharmacokinetic studies, in kidney and heart transplant patients receiving everolimus twice daily with cyclosporine, show that the maximum concentration ( $T_{max}$ ) occurs at 1 to 2 hour post-dose. Table 1 shows that the  $C_{max}$ , AUC averages and  $C_0$  trough blood levels vary depending on the dose. The applicant is proposing to administer everolimus at a starting dose of 0.75 mg twice daily (i.e. 1.5 mg/day) in combination with reduced recommended dose of cyclosporine A. Therapeutic drug monitoring (TDM) of everolimus to target trough levels of 3-8 ng/mL will be done and dose will be adjusted. TDM for cyclosporine will also be done.

Table 1: Pharmacokinetic parameters of everolimus given twice daily

Dose (mg)	$C_{max}$ (ng/mL)	AUC (h. ng/mL)	$C_0$ trough blood levels (ng/mL)
0.75	11.1 ± 4.6	75 ± 31	4.1 ± 2.1
1.5	20.3 ± 8.0	131 ± 59	7.1 ± 4.6

The non-clinical studies were reviewed previously by Dr. Avery Goodwin (*See Immunology Review dated 7-21-2003*). There were no additional non-clinical studies included in the NDA application. The applicant has included the following information for the proposed labeling.

## 2. THE LABELING

(b) (4)

[Redacted] (b) (4)

[Redacted]

[Redacted]

[Redacted] (b) (4)

[Redacted]

**3. RECOMMENDATIONS**

The NDA is approvable pending an accepted version of the labeling.

S.M. Shurland  
Simone M. Shurland  
Microbiologist, DSPTP

**CONCURRENCES:**

DSPTP /Microbiology Team Leader Shukal Bala Signature 11/18/2009 Date

CC:  
DSPTP/ NDA 21-560 (N-010)  
DSPTP/PM/Jacquelyn Smith

Application Type/Number	Submission Type/Number	Submitter Name	Product Name
NDA-21560	ORIG-1	NOVARTIS PHARMACEUTICA LS CORP	CERTICAN (EVEROLIMUS) TABLETS

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SIMONE M SHURLAND  
11/24/2009

SHUKAL BALA  
11/24/2009

## IMMUNOLOGY/MICROBIOLOGY FILING CHECKLIST FOR NDA or Supplement

**NDA Number:** NDA 21560

**Applicant:** Novartis  
Pharmaceuticals Corp

**Stamp Date:** 06/30/2009

**Drug Name:** Certican

**NDA Type:** Re-Submission

On **initial** overview of the NDA application for filing:

	<b>Content Parameter</b>	<b>Yes</b>	<b>No</b>	<b>Comments</b>
1	Is the microbiology information (preclinical/nonclinical and clinical) described in different sections of the NDA organized in a manner to allow substantive review to begin?			NA
2	Is the microbiology information (preclinical/nonclinical and clinical) indexed, paginated and/or linked in a manner to allow substantive review to begin?			NA
3	Is the microbiology information (preclinical/nonclinical and clinical) legible so that substantive review can begin?			NA
4	On its face, has the applicant <u>submitted</u> <i>in vitro</i> data in necessary quantity, using necessary clinical and non-clinical strains/isolates, and using necessary numbers of approved current divisional standard of approvability of the submitted draft labeling?			NA
5	Has the applicant <u>submitted</u> any required animal model studies necessary for approvability of the product based on the submitted draft labeling?			NA
6	Has the applicant <u>submitted</u> all special/critical studies/data requested by the Division during pre-submission discussions?			NA
7	Has the applicant <u>submitted</u> the clinical microbiology datasets in a format which intends to correlate baseline pathogen with clinical and microbiologic outcome?			NA
8	Has the applicant <u>submitted</u> draft/proposed interpretive criteria/breakpoint along with quality control (QC) parameters and interpretive criteria, if applicable, in a manner consistent with contemporary standards, which attempt to correlate criteria with clinical results of NDA/BLA studies, and in a manner to allow substantive review to begin?			NA
9	Has the applicant <u>submitted</u> a clinical microbiology dataset in an appropriate/standardized format which intends to determine resistance development by correlating changes in the phenotype (such as <i>in vitro</i> susceptibility) and/or genotype (such as mutations) of the baseline pathogen with clinical and microbiologic outcome?			NA
10	Has the applicant used standardized or nonstandardized			NA

## IMMUNOLOGY/MICROBIOLOGY FILING CHECKLIST FOR NDA or Supplement

	Content Parameter	Yes	No	Comments
	methods for measuring microbiologic outcome? If nonstandardized methods were used, has the applicant included complete details of the method, the name of the laboratory where actual testing was done and performance characteristics of the assay in the laboratory where the actual testing was done?			
11	Has the applicant <u>submitted</u> draft labeling consistent with current regulation, divisional and Center policy, and the design of the development package?	X		
12	Has the applicant <u>submitted</u> annotated microbiology draft labeling consistent with current divisional policy, and the design of the development package?	X		
13	Have all the study reports, published articles, and other references been included and cross-referenced in the annotated draft labeling or summary section of the submission?	X		
14	Are any study reports or published articles in a foreign language? If yes, has the translated version been included in the submission for review?		X	

**IS THE MICROBIOLOGY SECTION OF THE APPLICATION FILEABLE?** Yes

If the NDA is not fileable from the microbiology perspective, state the reasons and provide comments to be sent to the Applicant.

Please identify and list any potential review issues to be forwarded to the Applicant for the 74-day letter.

*None*

*Simone M. Shurland*

Reviewing Microbiologist

*08/03/2009*

Date

*Shukal Bala*

Microbiology Team Leader

*08/03/2009*

Date

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/s/  
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SIMONE M SHURLAND  
08/03/2009

SHUKAL BALA  
08/03/2009

## IMMUNOLOGY REVIEW

### DIVISION OF SPECIAL PATHOGEN AND IMMUNOLOGIC DRUG PRODUCTS (HFD-590)

**NDA #:** 21-560  
**REVIEWER** : Avery Goodwin  
**CORRESPONDENCE DATE** : 12-01-02  
**CDER RECEIPT DATE** : 12-20-02  
**REVIEW ASSIGN DATE** : 03-25-03  
**REVIEW COMPLETE DATE** : 07-21-03

**SPONSOR:** Novartis Pharmaceuticals Corporation  
East Hanover, N.J. 07936

**SUBMISSION REVIEWED:** Original

**DRUG CATEGORY:** Immunosuppressive agent

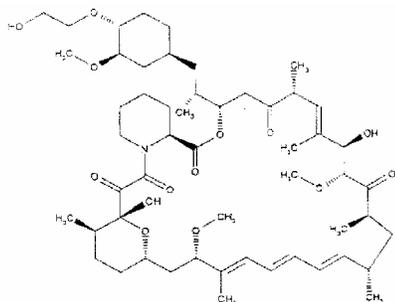
**INDICATION:** Prevention of organ rejection in solid organ transplantation

**DOSAGE FORM:** Oral administration

#### PRODUCT NAMES:

- a. **PROPRIETARY:** Certican™
- b. **NONPROPRIETARY:** RAD; RAD001; Everolimus.
- c. **CHEMICAL:** 1R, 9S, 12S, 15R, 16E, 18R, 19R, 21R, 23S, 24E, 26E, 28E, 30S, 32S, 35R), -1, 18-DIHYDROXY-12{(1R)-2-{(1S, 3R, 4R)-4-(2-hydroxyethoxy)-3-methoxycyclohexyl]-1-methylethyl}-19, 30-dimethoxy-15, 17, 23, 29, 35-hexamethyl-11, 36-dioxa-4-azatricyclo{30.3.1.0<sup>4,9</sup>] hexatriaconta-16, 24, 26, 28-tetraene-2, 3, 10, 14, 20-pentaone.

#### STRUCTURAL FORMULA:



Molecular weight: 958.2  
Empirical Formula: C<sub>55</sub>H<sub>83</sub>NO<sub>14</sub>

**SUPPORTING DOCUMENTS:** IND 52,003

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**1. EXECUTIVE SUMMARY:**

The sponsor seeks approval of RAD for the prevention of organ rejection in renal and heart transplant recipients. The sponsor has recommended that RAD should be used in a regimen concurrently with cyclosporine A (Neoral) and corticosteroids. The mechanism of action of this new chemical entity is reported to be similar to that of rapamycin. The *in vitro* data suggests that RAD, like rapamycin, interacts with FK-506-binding protein 12 (FKBP-12) and inhibits the activation of p70<sup>S6k</sup> kinase.

The *in vitro* studies show that RAD, like rapamycin inhibits (a) lymphoproliferation of mouse and human mononuclear cells, (b) Interleukin-2 (IL-2) and IL-15 dependent proliferation of T cells, and (c) IL-6 dependent proliferation of a B-cell clone. RAD, like rapamycin, was also shown to inhibit the *in vitro* responses against T cell-dependent (sheep erythrocytes) and T cell-independent [trinitro-phenyl-coupled bacterial lipopolysaccharide (TNP-LPS) and (2, 4-dinitro-phenyl)- $\beta$ -Ala-Glt-Gly-Ficoll] antigens. However, cyclosporine A (CysA) was less active. RAD was also shown to interfere with smooth muscle cell proliferation *in vitro*, and intima thickening of rat aorta allograft *in vivo*.

Studies in rats and monkeys suggest that RAD, like rapamycin, is effective at prolonging the survival of orthotopic kidney (rats and monkeys) and heterotopic heart allografts. There is also evidence which suggest that RAD has the potential of preventing chronic rejection in studies involving allogeneic aorta transplantation in rats.

A combination of RAD and CysA was more effective at improving orthotopic kidney and heterotopic heart survival in rats and monkeys. In addition, RAD plus CysA were effective at prolonging lung allograft survival in rats and monkeys; RAD alone was not effective.

**2. BACKGROUND:**

The subject of this NDA is Certican<sup>TM</sup> (everolimus, RAD), for the prevention of organ rejection in renal and heart transplant recipients. The sponsor has recommended that RAD should be administered orally for the entire lifetime of the patient at a daily dose of 3 mg. Moreover, RAD should be used in a regimen concurrently with CysA (Neoral) and corticosteroids.

RAD is a chemical derivative of the immunosuppressive macrolide rapamycin (a macrolide produced by *Streptomyces hygroscopicus*). For the synthesis of RAD, (b) (4)

The main structural change of rapamycin consists of the alkylation of the hydroxyl group at position 40 with a 2-hydroxyethyl group (40-O-(2-hydroxyethyl)-rapamycin). (b) (4)

The sponsor claims that this is a new macrolide rapamycin analog and their initial study showed that this modification resulted in a compound with immunosuppressive activity comparable to that of rapamycin, with enhanced physiochemical properties such as solubility in organic solvents (Sedrani *et al.*, 1998).

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Studies have shown that RAD is stable in human blood at concentrations between 2 and 100 ng/ml at 4 °C, and at room temperature over 48 °C. Protein binding studies show that RAD binds highly (99%) to mouse plasma proteins; 92% in rats; 84% in monkeys; and 75% to humans.

RAD is distributed in its unchanged form in rats, and shows highest allocation in the heart, lungs, kidneys, spleen, thyroid and adrenal gland. The feces represent the primary excretory route in subjects treated with RAD. In renal transplant recipients, RAD administered twice daily (at 0.75 and 1.5 mg bid), together with CysA, attain steady state by day four; with an average steady state maximum blood concentration between 11 and 20 ng/ml. Moreover, RAD had a terminal elimination half-life of 33 hours in renal transplant patients who received an oral dose of RAD (0.038 mg/kg) together with cyclosporine. *In vitro* studies show that cytochrome p450 is the major enzyme involved in biotransformation of RAD; and there are five major metabolites of RAD detected in blood of mice, rats, monkeys, and humans. Four of these metabolites were tested for biological activity in mixed lymphocyte reaction (MLR) assay.

### 3. TRANSPLANTATION IMMUNOBIOLOGY OVERVIEW:

The term transplantation, as the term is used in immunology, refers to the process of transferring cells, tissues, or organs from one site to another. Cells that are antigenically similar are referred to be histocompatible, and as such, do not induce an immunologic response that culminates to rejection. Each year transplant procedures are performed at an increasing rate for conditions such as kidney failure, liver disease, and heart disease and incompatibility may lead to the potential complication of graft rejection. The degree of immune response to a graft varies with the type of graft. Grafts between genetically different members of the same species are referred to as allografts. Allograft rejection may manifest itself either through cell-mediated or humoral immune reaction of the recipient against the major histocompatibility antigens that are present on the membranes of the donor's cell. The antigens are governed by a complex genetic loci referred to as the major histocompatibility complex. The process of graft rejection varies with the type of tissues, and the immune responses involved. Rejection that occurs within 24 hours following transplantation is referred to as hyperacute rejection, and although rare, the process occurs because of the presence of pre-existing host serum antibodies that are specific for antigens of the graft. In acute allograft rejection, T cell activation and antibody production occurs in response to the foreign graft. The acute rejection phase that may follow, usually takes place within 7-10 days following allograft transplantation. In chronic rejection, the rejection episode may take months or years to develop following an acute rejection episode; it involves localized inflammatory reaction called delayed-type-hypersensitivity (DTH) and is often difficult to manage with immunosuppressive agents. Allogeneic transplantation involves continuous immunosuppressive therapy to aid in the suppression of antibody production and cell-mediated immunity. Immunosuppressive agents such as steroids, CysA, tacrolimus and monoclonal antibody administered separately or in combinations have demonstrated some degree of effectiveness in the improvement of transplant tolerance.

The down regulation of T cell antigen receptor expression, T cell anergy (antigen-specific non-reactivity), suppression and the deletion of antigen-reactive T cells have been shown to increase

allograft survival. T cells are activated by cell surface receptors, and co-stimulatory signals presented by antigen presenting cells (APC) such as macrophages, dendritic cells, and B-cells. Proliferation is therefore dependent on a myriad of cellular signals each generated by immune cells.

#### 4. SUMMARY:

The immunosuppressive activity of RAD was measured *in vitro* and *in vivo* and compared with that of other immunosuppressive agents including rapamycin.

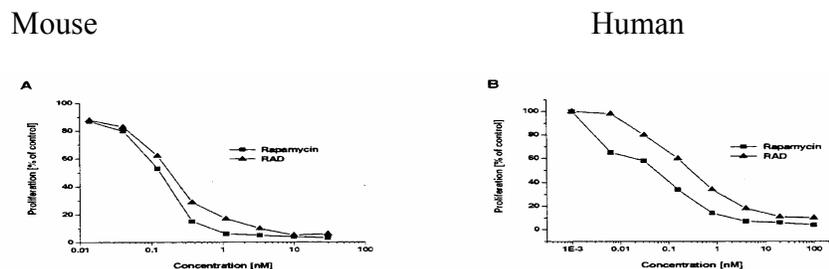
#### 4.1. ACTIVITY *IN VITRO*:

##### 4.1.1. Effect of RAD on lymphoproliferation:

The immunosuppressive activity of RAD was measured by the two-way mixed lymphocyte reaction (MLR) using human peripheral blood mononuclear cells (PBMC) and mouse splenocytes (Report # RD-2000-02013). Murine splenocytes from CBA and BALB/c mice were co-cultured in equal proportion in the absence or presence of various immunosuppressants for 5 days. The activity of RAD was measured by the incorporation of <sup>3</sup>H-thymidine 16 hours before harvesting of the culture. MLR using human PBMC from three donors was performed as described above except that the cultures were incubated for 6 days.

The results in Figure 1 show that RAD, like rapamycin, inhibited lymphoproliferation of mouse and human mononuclear cells in a dose dependent manner at sub-nM levels. The 50% inhibitory concentration (IC<sub>50</sub>) of RAD was calculated to be 0.53 ± 0.71 nM vs. 0.48 ± 0.34 nM (0.51 ± 0.69 µg/ml vs 0.46 ± 0.32 µg/ml) for mouse and human mononuclear cells, respectively (Table 1). RAD was approximately 2 and 4-fold less inhibitory against mouse and human cells compared to rapamycin [IC<sub>50</sub> 0.23 ± 0.27 nM and 0.16 ± 0.17 nM (0.22 ± 0.26 µg/ml and 0.15 ± 0.16 µg/ml) respectively].

Figure 1: Inhibition of mixed-lymphocyte reaction in mice and human.



Microcultures with mixtures of equal numbers of spleen cells from Balb/c and CBA mice (A) or with PBMC from two genetically different human volunteers (B) were set up in the presence of increasing concentrations of RAD and rapamycin, respectively. Shown are the results of one representative experiment each where the two compounds were tested side-by-side. Results are expressed as percent of maximal proliferation (as measured by <sup>3</sup>H-TdR incorporation) in the absence of any inhibitor.

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Table 1: Quantitative comparison of RAD versus rapamycin in mixed-lymphocyte reaction

Compound	Relative IC50 <sup>a)</sup> [absolute IC50]	
	mouse MLR n=4	human MLR n=6
RAD	2.1 ± 0.5 [0.53 ± 0.71nM]	4.1 ± 2.1 ** [0.48 ± 0.34nM]
Rapamycin	1 [0.23 ± 0.27nM]	1 [0.16 ± 0.17nM]

#### 4.1.2. Effect of RAD on B cell response to T-independent and T-dependent antigens:

The effect of RAD, rapamycin and CysA on mouse B cell responses to T-independent (TI) and T-dependent (TD) antigens was investigated using spleen cells from athymic C57BL/6 *nu/nu* and wild-type C57BL/6 mice (Report # RD-2000-02304). Micro-cultures of spleen cell were prepared in IMDM-ATL medium containing 10% FCS. Splenocytes, in the presence and/or absence of different concentration of test compounds (RAD, rapamycin and CysA), TI antigens [STNP-LPS (trinitrophenyl-lipopolysaccharide); and DAGG-Ficoll (N-2, 4-dinitrophenyl-b-Ala-Gly-Gly-AECM-Ficoll)] and TD antigens [SRBC (sheep red blood cells)] were incubated for 4 or 5 days at 37 °C. Following incubation, replicate cultures were pooled, and washed in HBSS. B-cell response was subsequently assessed by plaque forming assays. As depicted in Table 2, all three compounds demonstrated activity against SRBC. RAD showed 4 to 9 fold less activity compared to rapamycin against all three antigens. In comparison to CysA, both RAD and rapamycin were more active against both TI and TD antigens.

Table 2: Inhibition of *in vitro* B cell responses to TI- and TD-antigens

	Relative IC50 <sup>a)</sup> [absolute IC50]		
	TNP-LPS (n = 3)	DAGG-Ficoll (n = 3)	SRBC (n = 4)
RAD	4.9 ± 1.8 <sup>ns</sup> [1.49 ± 1.76nM]	5.5 ± 1.3 ** [0.86 ± 0.18nM]	7.6 ± 1.9 * [1.65 ± 0.84nM]
Rapamycin	1 [0.37 ± 0.50nM]	1 [0.17 ± 0.08nM]	1 [0.23 ± 0.12nM]
Cyclosporin A	- [>3000nM]	- [7.1 ± 3.5nM]	- [25.3 ± 5.6nM] (n = 3)

<sup>a)</sup> Rapamycin was included as a standard for each individual IC50 determination, and results are expressed as relative IC50 values, *i.e.*, as the ratio IC50 of RAD / IC50 of rapamycin. Given are the means ± SD of (n) individual experiments as indicated. Statistical analysis, compared to rapamycin, paired *t*-test: *ns*, not significant; \*, *p*<0.05; \*\*, *p*<0.01.

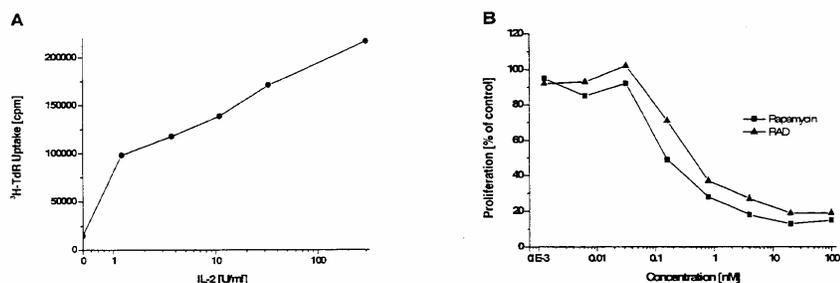
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**4.1.3. Effect of RAD on lymphoproliferation in the presence of exogenous cytokines:**

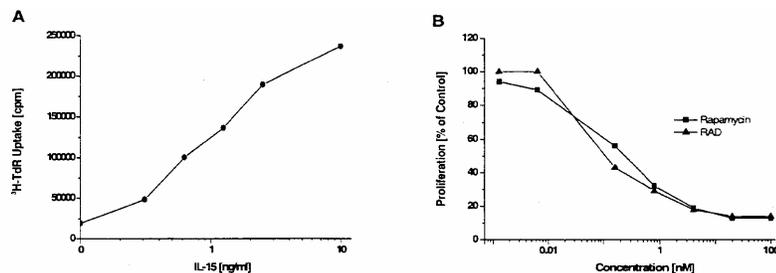
The effect of RAD on lymphoproliferation was measured in the presence of exogenous IL-2 and IL-15 using human **CD4 cells** (Report # RD-2000-02013). Briefly, purified CD4 cells (purified by magnetic cell sorting;  $\geq 90\%$  were CD3 and CD4 positive) were cultured in the presence or absence of different concentrations of IL-2 and IL-15 and immunosuppressive drugs for 5 days. The lymphoproliferation was measured by incorporation of  $^3\text{H}$ -thymidine 16 hours before termination of culture. The results in Figures 2 and 3, show that IL-2 and IL-15 dependent T-cell proliferation to be decreased in the presence of increasing concentrations of RAD and rapamycin. The inhibition profile of both test compounds was similar, and although not statistically significant, the RAD  $\text{IC}_{50}$  values were 2-fold higher than rapamycin (Table 3).

Figure 2: Inhibition of IL-2 stimulated CD4 positive T-cells by RAD and Rapamycin.



(A) CD4-positive T cells were cultivated in the presence of increasing concentrations of human IL-2 and cell proliferation measured after 5 days of cell culture by determining  $^3\text{H}$ -thymidine incorporation after an additional 16-hour cultivation period. (B) CD4-positive cells from the same preparation, stimulated with 20 units of IL-2, were cultivated in the presence of increasing concentrations of either RAD or rapamycin. Cell proliferation was assessed as in (A); results are expressed as percent of maximal proliferation in the absence of any inhibitor. CD4-positive T cells were obtained by removing B cells, monocytes/ macrophages, and NK cells from the total PBMC population by means of magnetic beads coated with appropriate antibodies.

Figure 3: Inhibition of IL-15 stimulated CD4 positive T-cells by RAD and Rapamycin.



As Figure 3.2-1, using CD4-positive T cells from the same cell preparation. (A) Titration of IL-15. (B) Cell proliferation stimulated with 2ng/ml IL-15 in the presence of increasing concentrations of either RAD or rapamycin.

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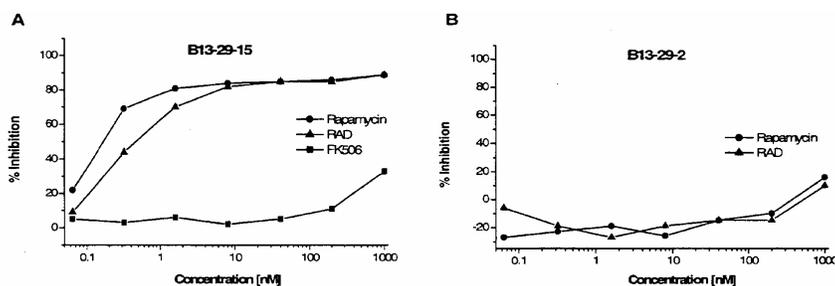
Table 3: Inhibition of T-cell growth factor-stimulated proliferation of human CD4-positive T-cells.

Compound	Relative IC <sub>50</sub> <sup>a)</sup> [absolute IC <sub>50</sub> ]	
	IL-2 stimulation n=4	IL-15 stimulation n=3
RAD	2.0 ± 0.8 <sup>ns</sup> [1.1 ± 1.0nM]	2.0 ± 1.6 <sup>ns</sup> [1.2 ± 1.4nM]
Rapamycin	1 [0.54 ± 0.38nM]	1 [0.47 ± 0.25nM]

<sup>a)</sup> Relative IC<sub>50</sub>: ratio IC<sub>50</sub> of RAD / IC<sub>50</sub> of rapamycin. Given are the means ± SD for n independent experiments as indicated; in each individual experiment RAD and rapamycin were tested side-by-side. Statistical analysis compared to rapamycin (paired t-test): ns, not significant.

The effect of RAD on **B-cell proliferation**, in the presence of exogenous IL-6, was measured using B-cell hybridoma cell lines (Report # RD-2000-02012). This cell line is strictly dependent on IL-6 for growth. RAD was compared with rapamycin for its ability to block IL-6 stimulated proliferation of a rapamycin-sensitive (B13-29-15) and a rapamycin-resistant (B13-29-2) B-cell subclone. Briefly, the cells were incubated for 5 days in the presence or absence of RAD, or rapamycin. B-cell proliferation was measured by incorporation of <sup>3</sup>H-thymidine after an additional 16-hour incubation period. The results in Figure 4A show that rapamycin and RAD were effective in inhibiting IL-6 stimulated, rapamycin-sensitive B-cell subclone. As expected, no such inhibition was observed with the rapamycin resistant B cell subclone (B13-29-2). The immunosuppressive effects of RAD on B13-29-15 cells were less than that of rapamycin on B13-29-15 cells. RAD was 2.5 fold less active than rapamycin (IC<sub>50</sub> values for rapamycin were 0.28 ± 0.19 nM vs. 0.68 ± 0.48 nM for RAD or 0.245 µg/ml ± 0.174 vs. 0.65 µg/ml ± 0.46, respectively) (Table 4). In addition, the chemically related immunosuppressive macrolide, FK506, shows no effect on inhibition of B13-29-15 cells (Figure 4A). This may be possible due to a different mechanism of action of FK506.

Figure 4: Inhibition of (A) B13-29-15 and (B) B13-29-2 proliferation.



Cells of the rapamycin-sensitive and -resistant B cell hybridomas B13-29-15 (A) and B13-29-2 (B), respectively, were cultured in the presence of increasing concentrations of either RAD, rapamycin or FK506. <sup>3</sup>H-thymidine was added on day 5 of the culture and the radioactivity incorporated into the DNA, reflecting cell proliferation, measured after an additional 16 hours of cultivation. Given are the percent inhibition with respect to a control culture in the absence of any inhibitor. RAD and rapamycin were tested in one single experiment; the results with FK506 were obtained in an independent experiment.

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Table 4: Inhibition of B13-29-15 and B13-29-2 proliferation

Compound	Relative IC <sub>50</sub> <sup>a)</sup> [absolute IC <sub>50</sub> ]	
	B13-29-15 n=5	B13-29-2 <sup>b)</sup> n=3
RAD	2.5 ± 0.7 * [0.68 ± 0.48nM *]	- >1000 nM
Rapamycin	1 [0.28 ± 0.19nM]	- >1000 nM

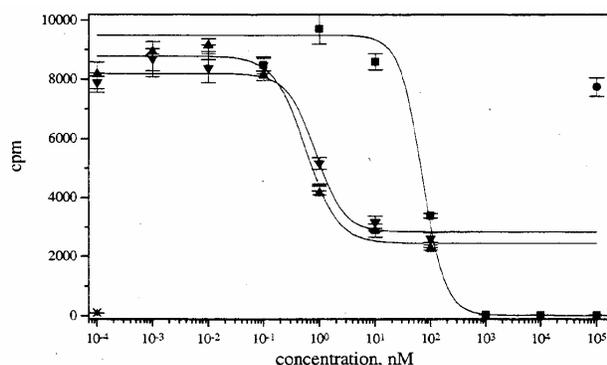
<sup>a)</sup> Rapamycin was included as a standard for each individual IC<sub>50</sub> determination, and results are expressed as relative IC<sub>50</sub> values, *i.e.*, as the ratio IC<sub>50</sub> of RAD / IC<sub>50</sub> of rapamycin. Given are the means ± SD of n individual experiments as indicated. Statistical analysis, compared to rapamycin, paired t-test: \* p<0.05.

<sup>b)</sup> Highest concentration of compounds tested: 1000nM

#### 4.1.4. Effect of RAD on smooth muscle cell proliferation:

The effect of RAD on bovine vascular smooth muscle cell (SMC) proliferation was measured using aortic cells (Report # RD-2000-02435). Briefly, aortic cells were incubated for three days without serum to arrest cell-growth. The serum-deprived cells were then cultured for three days in fresh medium containing 10% fetal calf serum and different concentrations of the respective anti-proliferative compounds (adriamycin, rapamycin and RAD). Following serum deprivation, cells were allowed to incubate in the presence of fresh media and <sup>3</sup>H-thymidine for 24 hours. RAD and rapamycin were less effective at eliciting complete inhibition when compared to adriamycin. In the presence of 1000 nM adriamycin complete blockage of SMC proliferation was observed, a phenomenon not seen with RAD nor rapamycin (Figure 5). IC<sub>50</sub> values of 1.5 ± 1.7 nM for rapamycin, and 2.0 ± 1.4 nM for RAD (1.37 ± 1.55 µg/ml vs. 1.91 ± 1.33 µg/ml, respectively) were calculated. Adriamycin had an IC<sub>50</sub> of 50.3 ± 9.7 nM (Table 5).

Figure 5: Inhibition of SMC proliferation



Dose-response for inhibition of SMC proliferation by rapamycin (▲), RAD (▼) or adriamycin (■). Thymidine incorporation in the presence (●) or absence of 10 % FCS (\*) is also indicated.

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Table 5: Inhibition of SMC proliferation.

SMC proliferation (relative IC <sub>50</sub> )	
Compounds	n= 3
adriamycin	50 ± 9.7 nM
RAD	2.0 ± 1.4 nM
rapamycin	1.5 ± 1.7 nM

#### 4.1.5. Effect of metabolites on lymphoproliferation:

The immunosuppressive activity of 4 of the 5 metabolites of RAD, identified in humans, was measured *in vitro* by MLR (Report # RD-2001-01459). Two of the HPLC purified metabolites 46-hydroxy-RAD, and 24-/25-hydroxy-RAD were obtained from incubations of <sup>3</sup>H-RAD with human and monkey liver microsomal preparations in the presence of potassium phosphate buffer (pH 7.4) NADPH, and a NADPH regeneration system. The two metabolites were assessed *in vitro* by the two-way MLR using mouse splenocytes. Their *in vitro* activities were compared side-by-side to that of RAD and rapamycin. Since both metabolites were radio labeled with <sup>3</sup>H-thymidine to aid in their identification, an alternative, non-radioactive colorimetric assay, using tetrazolium salt, was employed to measure cell proliferation. For this, equal numbers of spleen cells from inbred CBA and BALB/c mice were co-cultured in equal proportion in the absence or presence of test compounds for 4 days. The degree of cellular activation was measured by the addition of tetrazolium salt followed by an additional incubation period of 5 hours. Based on IC<sub>50</sub> values, rapamycin was more effective than RAD in achieving 50% inhibition of cell proliferation in mouse MLR assay (Table 6). Moreover, the two metabolites were about 566-fold (46-hydroxy-RAD) and 91-fold (24-/25-hydroxy-RAD) less active than RAD (Table 6).

Table 6: Inhibition of mouse MLR by RAD metabolites.

	IC50	
	[ng/ml]	relative <sup>a)</sup>
Rapamycin	0.1 / 0.3	1
RAD	0.4 / 0.7	4 / 2.3
46-hydroxy-RAD	140 / 550	1400 / 1833
24- / 25-hydroxy-RAD	35 / 70	350 / 233

a) relative IC<sub>50</sub>: ratio IC<sub>50</sub> of test sample / IC<sub>50</sub> of rapamycin

In a similar experiment, the immunosuppressive activity of the other two, HPLC purified, opened-ring, degradation products (b) (4) (Figure 6), was assessed by the two-way MLR by the incorporation of <sup>3</sup>H-thymidine. The products were obtained by heating RAD, in a mixture of ethanol and 0.1M ammonium acetate buffer (pH 8), to 40 °C for three days. Both products are metabolites of RAD that are found in human blood and their activity in

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MLR was compared to that of rapamycin but not RAD. Murine splenocytes from inbred CBA and BALB/c mice were co-cultured in equal proportion in the absence or presence of the test compound for 4 days. Compound activity was measured by the incorporation of <sup>3</sup>H-thymidine 16 hours before harvesting of the culture. The result of the study showed that (b) (4) (b) (4) were less active than rapamycin, 124- and 288-fold respectively (Table7). RAD was not used as a comparator in this study. However, based on previous mouse MLR experiments, the activity of RAD is 2-fold less active in the mouse MLR assays as compared to rapamycin and based on that premise, the sponsor speculates that the degradation products would be 60- to 145-fold less active than RAD.

Figure 6: Chemical structure of (b) (4).



Table 7: Inhibition of mouse MLR by RAD metabolites.

	IC50 <sup>a)</sup>	
	[nM]	Relative <sup>b)</sup>
Rapamycin	0.75 ± 0.4 (n=3)	1
(b) (4)	97.5 ± 60* (n=6)	124 ± 42
(b) (4)	212.8 ± 103** (n=6)	288 ± 29

<sup>a)</sup> Given are the means ± S.D. of n IC50 determinations. <sup>b)</sup> relative IC50: ratio IC50 of test sample / IC50 of rapamycin. Statistical analysis (paired t-test): \* p<0.01; \*\* p<0.005

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**4.2. ACTIVITY IN VIVO:****4.2.1. Effect of RAD on graft survival:****4.2.2. Renal graft**

The effect of RAD in prolonging the survival of an orthotopic kidney transplant was measured in rats (Report # RD-2000-01533) and primates (Report # RD-97-03851).

In the rat kidney transplant model (using either DA or BN rats as donor and Lewis rats as recipients; or Wistar/FxFisher344 F1 rats as donors and Wistar/F rats as recipients), one recipient kidney was removed and a contra-lateral nephrectomy was performed one week later. RAD and rapamycin were administered orally, on a daily basis during the first 14 days following transplantation, and graft survival was measured by the survival of the recipient animals.

In the Wistar/FxFisher (F1) into Wistar/F rat model, the untreated controls showed macroscopic signs of severe acute rejection at 7 days after transplantation. In the RAD and rapamycin treatment arm, animals that received a dose of 0.25 mg/kg/day for 14 days following transplantation, showed signs of rejection, however, histological signs of rejection were less severe when compared to the untreated control (Table 8). Animals who received a higher dose of compound (0.5 or 1.0 mg/kg/day for 14 days) showed long term survival of greater than 100 days following the 14-day treatment period. Histological assessment showed that these animals had significantly less severe signs of rejection as compared to either of the treatment group (Table 8). The activity of RAD appears to be similar to that of rapamycin at prolonging graft survival.

Table 8: The effect of RAD and rapamycin in Wistar/FxFisher (F1) into Wistar/F kidney transplantation.

Compound	Dose (mg/kg/d)	Treatment period	Nr of animals	Survival (d)	Severity of rejection (score)
Control			4	7*,7*,7*,7*	5,5,5,5
RAD	0.25	14 days	10	7*(n=6), 20,>51, >100, >100	3,3,4,4,4,4, 3,1, 1,1
	0.5	14 days	5	>100(n=5)	1,1,1,1,1
	1.0	14 days	3	>100(n=3)	1,1,1
Rapamycin	0.25	14 days	7	7*(n=6),12	1,2,3,3,3,4,3
	0.5	14 days	5	7*,>100(n=4)	3,0,1,1,1
	1.0	14 days	3	>100(n=3)	1,1,1

Survival represent times till death or sacrifice; \*, animal killed at nephrectomy because of macroscopic signs of rejection; >, animal killed for histology with apparent good health status with functioning kidney graft.

Histology data are presented in the same order as data presented in the column "Survival", with scores of cellular rejection as follows: score 0, normal kidney architecture, no signs of rejection; score 1, infiltration of renal parenchyma, without signs of rejection; score 2, cellular rejection with slight destruction of renal parenchyma; score 3, cellular rejection with moderate tissue destruction; score 4, cellular rejection with severe tissue destruction; and score 5, end-stage rejection with almost complete destruction of the graft. In case of vessel changes indicative of transplant vasculopathy, the histologic score is underlined.

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In the DA-to-Lewis rat model, untreated controls demonstrated signs of rejection and severe tissue destruction 7 days following transplantation. Animals that received Neoral (5.0 mg/kg and 7.5 mg/kg/day) for 14 days showed long-term survival (Table 9). Animals that received a dose of 5.0 mg/kg/day of RAD showed long-term survival rates (>100 days) in two of the three animals (Table 9).

Table 9: Effect of RAD, rapamycin and Neoral, in DA into Lewis kidney transplant model.

Compound	Dose (mg/kg/d)	Treatment period	Nr of animals	Survival (d)	Severity of rejection (score)
Neoral	2.5	14 days	3	7*,7*,7*	5,4,4
	5.0	14 days	2	24,>100	4,4
	7.5	14 days	6	>90,>90,>91, >100(n=3)	3,3,3, 1,2,1
RAD	1.0	Continuous	3	11,13,18	3,3,4
	2.5	Continuous	4	14,15,18,18	2,3,3,3
	5.0	Continuous	3	42,>100,>100	3,1,1
Rapamycin	1.0	Continuous	3	7*,15,>100	4,3,3
	2.5	Continuous	3	20,26,>100	3,3,3

In the BN-to-Lewis rat study animals treated with Neoral (5 mg/kg/day) for 14 days demonstrated long term survival (57 to >100 days) with no signs of rejection in most of the treatment animals. Animals that received a dose of 2.5 mg/kg/day of either RAD or rapamycin for 14 days showed survival rates of 26-100 days and 35-83 days, respectively (Table 10).

Table 10: Effect of RAD, rapamycin and Neoral in BN/ Lewis rat transplant model.

Compound	Dose (mg/kg/d)	Treatment period	Nr of animals	Survival (d)	Severity of rejection (score)
Control			2	7*,7*	5,5
Neoral	5.0	14 days	3	57,>100(n=5)	4,1,1,1,1,1
RAD	1.0	14 days	5	20,20,25,37,>78	-,4,4,3,3
	2.5	14 days	5	26,>80,>100(n=3)	4,2,2,2,3
Rapamycin	1.0	14 days	3	18,22,26	-,4,4
	2.5	14 days	3	35,>83,>83	4,1,4

In another study, the activity of RAD, and rapamycin at prolonging the survival of kidney orthotopic allograft was measured in cynomolgus monkeys. The experiments were carried out in two steps:

In the first step (dose finding study) recipients began receiving RAD at a high daily dose (11 mg/kg) one day prior to transplantation, followed by a reduction of the dose in a step-wise

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manner by 50% every two weeks or until rejection occurred (Table 11). The dose of RAD required for graft survival was determined to be approximately 1.25 mg/kg/day.

Table 11: Survival days in dose finding study: relation to dose.

Group	Survival with histology-proven rejection (day)	RAD dose
Controls	4, 6, 6, 8	
RAD	34, 35, 37	2.5 mg/kg/d day 0-14, 1.25 mg/kg/d day 15-28 0.63 mg/kg/d from day 29
	48	2.5 mg/kg/d day 0-14, 1.25 mg/kg/d day 15-28, 0.63 mg/kg/d day 29-42, 0.31 mg/kg/d from day 43
	38 (died)	10 mg/kg/d day 0-7, 7.5 mg/kg/d day 8-18, 5 mg/kg/d day 19-22, 2.5 mg/kg/d day 23-36, 1.25 mg/kg/d from day 37
	76	10 mg/kg/d day 0-20, 5 mg/kg/d day 21-31, 2.5 mg/kg/d day 32-45, 1.25 mg/kg/d day 46-64, 0.63 mg/kg/d from day 65

All animals in this study were sacrificed except for the animal on RAD that died at day 38 after transplantation.

The second step investigated the efficacy of RAD at a dose of 0.75 mg/kg/day, and 1.5 mg/kg/day (a dose above and below the 1.25 mg/kg/day survival dose). The experimental design was the same as described above. Four to eight days following transplantation, the placebo controls were all sacrificed because of rejection. However, animals that received a dose of 0.75 mg/kg/day of RAD, demonstrated a median kidney allograft survival of 27 days, whereas a dose of 1.5 mg/kg/day was effective at extending graft survival for 59 days (Table 12). Graft histology revealed moderate to severe rejection in these animals (Table 13). In the rapamycin arm, a dose of 0.75 mg/kg/day lead to a 43 day graft survival and animals that received a 1.5 mg/kg/day dose of rapamycin resulted in a 56 day graft survival. The activity of both RAD and rapamycin appears to be similar. In both RAD and rapamycin treatment groups the sponsor noted that there was a persistent decrease in absolute number of lymphocytes (Table 13).

Table 12: Survival days in efficacy study.

Group	Survival with histology-proven rejection (day)	Median Survival (day)
RAD 0.75 mg/kg/d	8, 8, 22, 27, 36, 70, <u>91</u>	27
RAD 1.50 mg/kg/d	28, 34, 48, 69, 76, <u>85</u>	59
Rapamycin 0.75 mg/kg/d	5, 29, 39, <u>43</u> , 53, 57, 103	43
Rapamycin 1.50 mg/kg/d	8, 11, 35, 43, 68, 103, <u>103</u> , 103	56

Survival dates underlined indicate that animal had died.

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Table 13: Hematological parameters in efficacy study:

<b>Hematologic Parameters in Efficacy Study</b>				
	<b>RAD 0.75 mg/kg/d</b>	<b>RAD 1.50 mg/kg/d</b>	<b>Rapamycin 0.75 mg/kg/d</b>	<b>Rapamycin 1.50 mg/kg/d</b>
<i>White blood cell counts</i>				
Day 0	9.7±1.4	9.4±1.9	8.0±2.1	8.2±2.0
Day 14	14.0±7.0	8.7±3.3	8.5±3.1	12.7±9.4
Day 28	11.2±2.8	8.1±3.1	8.7±4.0	12.0±7.0
Day 56	4.0±0.5	5.7±3.1	9.6 (n=1)	7.9±6.7
<i>Lymphocyte counts</i>				
Day 0	4.8±0.5	5.1±1.4	4.2±2.0	3.3±1.2
Day 14	3.4±1.6	2.4±0.8	2.0±1.1	2.4±1.5
Day 28	2.3±1.9	1.7±0.3	2.3±0.8	2.0±0.9
Day 56	1.2±0.6	2.2±1.3	1.3 (n=1)	2.5±0.8
<i>Neutrophil counts</i>				
Day 0	4.6±1.0	3.9±1.4	3.6±3.0	4.6±1.9
Day 14	8.8±5.0	5.3±2.5	5.6±2.4	9.4±9.5
Day 28	8.1±3.6	5.5±3.2	5.2±3.5	9.0±6.6
Day 56	2.4±0.1	2.2±1.3	7.3 (n=1)	4.6±5.3
<i>Platelet counts</i>				
Day 0	360±60	360±120	330±70	360±80
Day 14	510±190	500±210	560±180	600±50
Day 28	390±70	420±290	390±130	420±120
Day 56	340±170	270±150	350 (n=1)	310±40

#### 4.2.3. Heart graft:

The efficacy of RAD at prolonging the survival of heterotopic heart transplants was evaluated in rats (Report # RD-2000-01533). Hearts from DA rats were transplanted onto the abdominal cavity of Lewis rats. Immunosuppressants were administered at the same day of the surgery at a daily dose of 1.0, 2.5, and 5.0 mg/kg for RAD and rapamycin, and 2.5 and 5.0 mg/kg for Neoral. The assessment of graft function was made by graft beat and rejection was concluded when the graft stopped beating. In untreated animals, graft rejection was observed 6-8 days after transplantation. However, in the treatment group, Neoral, at a dose of 2.5 mg/kg/day, and 5.0 mg/kg/day, prolonged graft survival from 10-23 days, and >100 days, respectively. RAD and rapamycin were less effective at prolonging graft survival (Table 14).

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Table 14: The effect of RAD, rapamycin and Neoral on herterotopic rat heart transplantation.

Compound	Dose (mg/kg/d)	Treatment period	Nr of animals	Survival (d)	Severity of rejection (score)
Neoral	2.5	Continuous	3	10,10,11,12,13,14, 21,23	4,4,3,3,4,4, 4,4
	5.0	Continuous	3	>100 (n=3)	0,1,0
RAD	1.0	Continuous	3	12,14,14	5,4,4
	2.5	Continuous	8	13,14,18,18,22 25,27,28	4,4,4,4,3 3,4,3
	5.0 <sup>a</sup>	Continuous	2	22,33	3,3
Rapamycin	1.0	Continuous	3	12,15,27	3,3,3
	2.5	Continuous	4	25,31,33,>81	3,3,3,1
	5.0 <sup>a</sup>	Continuous	2	22,32	3,3

Survival represents times till death or sacrifice; >, animal killed for histology with apparent good health status and with a functioning heart graft. Histology data are presented in the same order as data presented in the column "Survival", with scores of cellular rejection as follows: score 0, normal heart architecture, no signs of rejection; score 1, infiltration of myocyte parenchyma, without signs of rejection; score 2, cellular rejection with slight destruction of myocyte parenchyma; score 3, cellular rejection with moderate tissue destruction; score 4, cellular rejection with severe tissue destruction; and score 5, end-stage rejection with almost complete destruction of the graft. In case of vessel changes indicative of transplant vasculopathy, the histologic score is underlined. <sup>a</sup>Treatment initiated two weeks before transplantation.

#### 4.2.4. Effect of RAD and Neoral on intimal wall thickening:

The effect of RAD on intimal wall thickening was investigated in rat aorta transplant model (Report # RD-2000-01288). Transplantation procedures were done using DA rats as donors and Lewis rats as recipients. Under appropriate conditions a piece of aorta about 1 cm in length, was removed from the donor animal and transferred into the recipient. RAD was administered at a dose of 0.31, 0.63 and 1.25 mg/kg/day, and Neoral was administered at a dose of 2.5, 5.0, and 7.5 mg/kg/day. Although the total length of the study was 8 weeks, the sponsor did not mention the time of initiation of drug administration. Based on the results, oral administration of RAD did not appear to completely prevent intima thickening of rat aorta grafts. However, a dose-dependent reduction was observed at 0.31, 0.63, and 1.25 mg/kg/day. Neoral was shown to decrease intima thickening at lowest dose tested and appeared to completely prevent intimal thickening at 7.5 mg/kg/day (Table 15).

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Table 15: The effect of RAD or Neroal on aorta parameters in allogeneic transplantation.

**Effect of RAD or Neroal (single compound treatment) on aorta parameters in allogeneic transplantation**

Compound (mg/kg/d)	Own aorta	Grafted aorta					
		Media+ Intima ( $\mu$ m)	Media ( $\mu$ m)	Intima ( $\mu$ m)	Media (%)	Intima (%)	
Placebo	98 $\pm$ 15	135 $\pm$ 37	73 $\pm$ 15	63 $\pm$ 30	56 $\pm$ 12	44 $\pm$ 12	
RAD	0.31	94 $\pm$ 18	127 $\pm$ 61	79 $\pm$ 9	48 $\pm$ 62	71 $\pm$ 22**	29 $\pm$ 22**
	0.63	101 $\pm$ 14	94 $\pm$ 17**	81 $\pm$ 11**	13 $\pm$ 3**	87 $\pm$ 12**	13 $\pm$ 12**
	1.25	108 $\pm$ 20	96 $\pm$ 21**	85 $\pm$ 15**	11 $\pm$ 11**	90 $\pm$ 9**	10 $\pm$ 9**
Neroal	2.5	100 $\pm$ 22	143 $\pm$ 49	90 $\pm$ 13**	53 $\pm$ 47	68 $\pm$ 19*	32 $\pm$ 19*
	5.0	95 $\pm$ 17	99 $\pm$ 23**	93 $\pm$ 20**	6 $\pm$ 9**	95 $\pm$ 8**	5 $\pm$ 8**
	7.5	81 $\pm$ 18**	94 $\pm$ 18**	94 $\pm$ 18**	1 $\pm$ 2**	99 $\pm$ 3**	1 $\pm$ 3**

Data presented are mean values (each value is the average of 5 measurements in one longitudinal and one transversal section)  $\pm$ SD. Statistical significance of difference from placebo-treated control: \*  $p$ <0.05, \*\*  $p$ <0.01.

#### 4.2.5. Effect on lung grafts:

The effect of RAD, and Neroal was evaluated in the rat lung transplant model (Hausen *et al.*, 1999). In the unilateral lung transplant model, Lewis rats received Brown Norway rat lungs followed by daily drug doses: (A) RAD at 2.5 mg/kg; (B) Neroal at 7.5 mg/kg. Animals were assessed by daily body weight measurements, chest radiographs, drug trough levels (high performance liquid chromatography/mass spectrometry) and blinded scoring of graft histology up to the time of necropsy at 21 days postoperative. Animals that received vehicle control or RAD showed severe chest opacity of the left lung by 7 days and 21 days, respectively (Table 16). Animals that received Neroal monotherapy resulted in mild opacity by 21 days following surgery (Table 16).

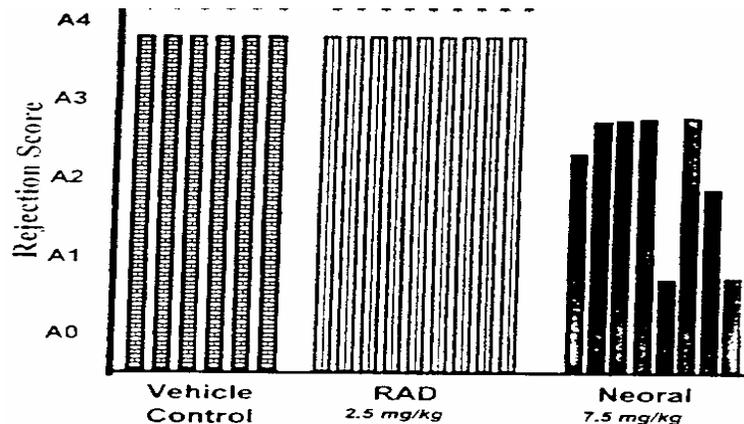
Table 16: Chest radiograph scores.

Postoperative day	Control	RAD 2.5 mg/kg/day	Neroal 7.5 mg/kg/day
1	1.5 (1, 4)	3 (2, 4)	3 (1, 4)
7	4 (4, 4)	2 (1, 4)	2 (1, 4)
14		4 (3, 4)	1.5 (1, 4)
21		4 (4, 4)	2 (1, 4)

Median (range) score of chest radiograph opacification. 1=no opacification of the left chest cavity, 2=mild opacification, 3=moderate opacification, 4=severe opacification of the left chest cavity. One-way ANOVA with post hoc tests: postoperative day 7: control vs. SDZ RAD,  $P$ <0.05; control vs. Neroal,  $P$ <0.03; control vs. combined simultaneously administered group,  $P$ <0.003; control vs. combined staggered administered group,  $P$ <0.0001; postoperative day 14 and 21: SDZ RAD vs. Neroal,  $P$ <0.001; SDZ RAD vs. combined simultaneously administered group,  $P$ <0.0001; SDZ RAD vs. combined staggered administered group,  $P$ <0.0001; Neroal vs. combined staggered administered group,  $P$ <0.023.

Histological evaluations revealed that animals that received vehicle control or RAD resulted in severe rejection by postoperative days 14 and 21 respectively (Figure 7). Animals treated with Neoral for 21 days showed moderate rejection at the time of death (Figure 7).

Figure 7: Comparison of acute rejection score of individual rats.



Comparison of the histology scores of acute lung rejection in individual rats. \*Hemorrhagic infarction. Kruskal Wallis one-way ANOVA on ranks:  $P < 0.001$ , RAD vs. Neoral+RAD (sim);  $P < 0.002$ , RAD vs. Neoral+RAD (stag). All other comparisons were not significant.

The effect of RAD and Neoral was evaluated in cynomolgus monkey lung transplantation model (Hausen *et al.*, 2000). Thirty-nine cynomolgus monkeys received mismatched unilateral allogeneic lung transplants. Animals were administered a single oral dose of immunosuppressants daily for the total observation period of 28 days. However, the sponsor does not state the exact time of drug administration. The result of the study showed that animals treated with vehicle, Neoral (day 1-7: 150 mg/kg/day; day 8-28: 100 mg/kg/day) or RAD (1.5 mg/kg/day) showed severe rejection (Figure 8). One monkey in the high Neoral treatment arm and one from the vehicle control arm was euthanized due to bilateral bacterial pneumonia. Three animals who received RAD at high doses had to be euthanized before study completion. RAD did not appear to have a significant effect at prolonging lung transplantation in primates (Figure 9).

Figure 8: Comparison of acute rejection scores in non-human primates.

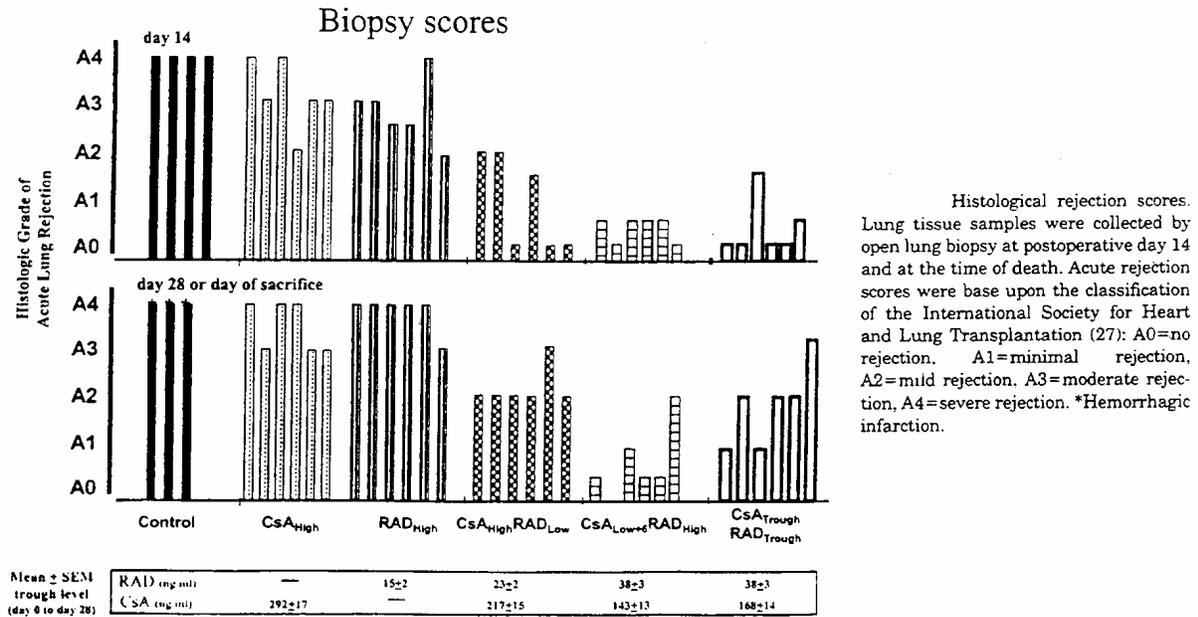
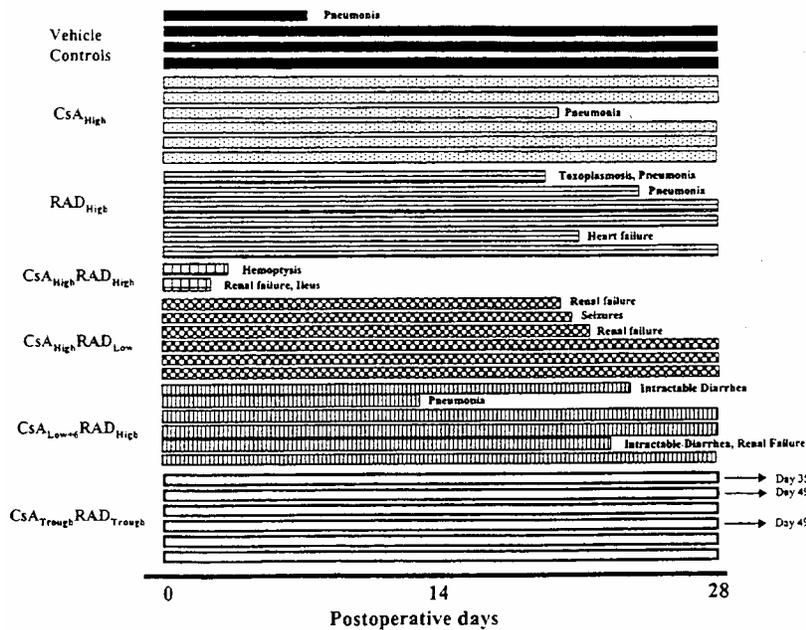


Figure 9: Non-human primate survival in treatment groups.



Animal survival in the treatment groups. The causes of death or reasons for early termination are given for animals that failed to reach the end of the observation period.

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**4.2.6. Graft vs. host reaction:**

The effect of RAD and rapamycin on localized graft-versus-host reaction was investigated in rats (Report # RD-2000-01289). Briefly, spleen cells, from donor animals [male Wistar/F (RT1<sup>u</sup>)], were prepared and injected subcutaneously in the right footpad of recipient animals [male (Wistar/F x Fisher344)F<sub>1</sub>(RT1<sup>u,1</sup>)] and the left footpad was left untreated. RAD and rapamycin were administered orally at the same time as allogeneic cell injection. Drug administrations were done at a dose of 1.0 and 3.0 mg/kg of body weight. Animals were sacrificed one week later and the left and right popliteal lymph nodes were removed, weighed, and compared to nodes from animals that received no compound. The result of the study shows that both RAD and rapamycin inhibited the swelling of lymph nodes compared to control animals (Table 17). Greater than 50% decrease in the weight of the lymph nodes was observed in rats treated with RAD or rapamycin at 1.0 mg/kg.

Table 17: Effect of RAD on lymph node parameters.

Compound and dose (mg/kg/d)	Lymph node				
	Weight Left (mg)	Weight Right (mg)	Weight difference (mg)	Percent inhibition	
Control	5.9±1.3	39.9±4.9	34.0±4.9		
RAD	1.0	5.3±0.7	19.5±8.5**	14.2±8.8**	58±26**
	3.0	5.0±0.6	12.9±3.9**	7.9±3.6**	77±11**
Rapamycin	1.0	4.7±1.1	17.8±8.8**	13.1±8.3**	61±24**
	3.0	4.7±0.7	16.2±4.6**	11.5±4.2**	66±12**

Data presented are mean values ±SD. Statistical significance of the difference between treatment group and controls: \*  $p < 0.05$ , \*\*  $p < 0.01$ .

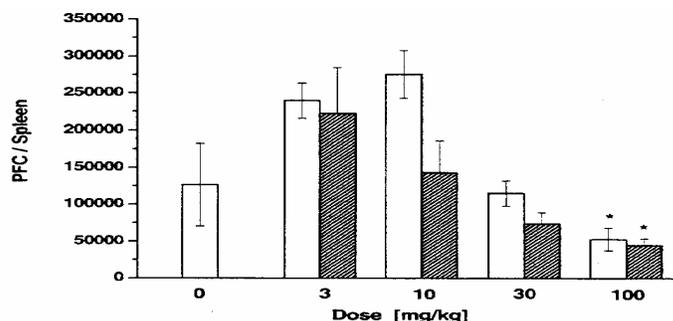
**4.2.7. Effect of RAD on B cell responses:**

The ability of RAD, rapamycin, and CysA to inhibit B cell responses *in vivo* was investigated in mice (Report # RD-2000-02304). Mice (6 week old C57BL/6 male, 3-4 months old C57BL/6 *nu/nu* female and 4 week old OF<sub>1</sub> female) were intravenously immunized with DAGG-Ficoll (N-2, 4-dinitrophenyl-b-Ala-Gly-Gly-AECM-Ficoll), TNP-LPS (trinitrophenyl-lipopolysaccharide), and SRBC (sheep red blood cells) followed by the administration of immunosuppressive therapy (CysA, RAD, or rapamycin). Spleen cells from immunized animals were removed 4-5 days following immunosuppressive therapy and IgM forming B-cells measured by plaque assay. In addition, serum IgG antibodies against DNP antigens were measured by enzyme-linked immunosorbent assay (ELISA). As depicted in Figure 10, treatment with RAD and rapamycin appears to enhance the humoral immune response at a dose of 3 mg/kg. However, at a higher dose of 100 mg/kg both RAD and rapamycin appear to significantly inhibit DNP specific IgM response.

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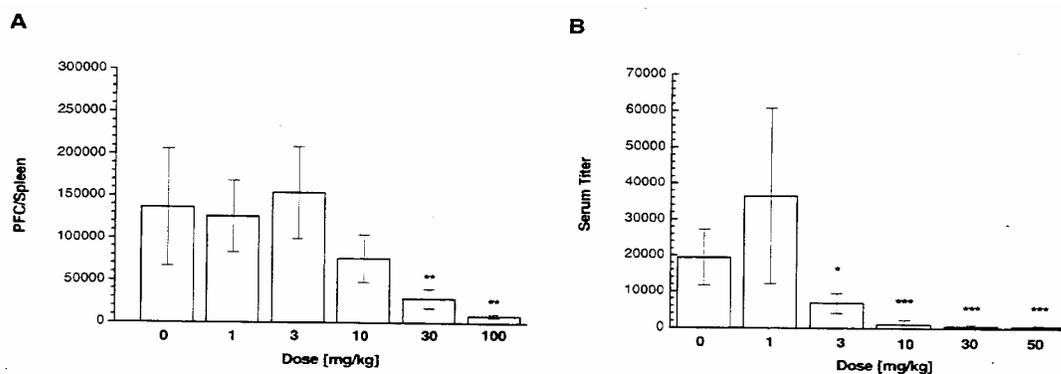
Figure 10: Inhibition of mouse humoral immune response to TNP-LPS.



Groups of five mice each per dosage group were immunized i.v. with 30 $\mu$ g TNP-LPS each. They received, on four consecutive days (starting on the day of immunization), either the indicated oral dose of RAD (open bars) and rapamycin (hatched bars), respectively. Control mice (0) received only the solvent, *i.e.*, solvent G / corn oil. Mice were sacrificed on day 5 and the number of DNP-specific IgM-producing B cells (plaque-forming cells, PFC) determined by means of a modified Jerne plaque assay. Given are the means for 5 animals/group  $\pm$  SD. Statistical analysis, compared to control (One-Way ANOVA): \*,  $p < 0.05$ .

In response to DAGG-Ficoll, RAD appears to dose dependently inhibit both the IgM and IgG antibody response of C57BL/6 mice (Figure 11).

Figure 11: Inhibition of mouse humoral immune responses to DAGG-Ficoll.



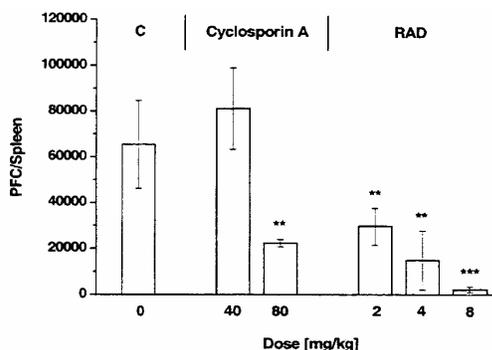
Groups of five mice each per dosage group were immunized i.v. with 10 $\mu$ g DAGG-Ficoll each. (A) They received, on four consecutive days (starting on the day of immunization), either the indicated oral dose of RAD, dissolved in solvent G / corn oil, or the solvent only. Mice were sacrificed on day 5 and the number of DNP-specific IgM-producing B cells (plaque-forming cells, PFC) determined by means of a modified Jerne plaque assay. (B) In contrast to the experiment in (A), mice were treated with an experimental microemulsion formulation of RAD, or the vehicle only. A 2% microemulsion concentrate (batch X142-0694, YL8-344), diluted with 0.9% NaCl was used. Mice were bled on day 6 and the DNP-specific serum IgG antibody titer determined by means of a specific ELISA. Given are the means for 4 animals/group  $\pm$  SD. Statistical analysis, compared to control (One-Way ANOVA): \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

Treatment with RAD resulted in a dose dependant reduction in the number of SRBC-specific IgM-producing B cells (Figure 12). More specifically, it was determined that the oral dose necessary to obtain a 50% decrease in the number of plaque-forming cells (PFCs) was 2 mg/kg. A higher dose of RAD (8 mg/kg/day) resulted in complete suppression of anti-SRBC response. The comparator drug, CysA, at 80 mg/kg/day (the highest dose tolerated by mice) resulted in 70% inhibition. Rapamycin was not used as a comparator.

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Figure 12: Inhibition of the mouse anti-SRBC immune responses.



Groups of five mice each per dosage group were immunized i.v. with  $5 \times 10^7$  sheep red blood cells (SRBC). They received, on four consecutive days (starting on the day of immunization), either the indicated oral dose of cyclosporin A and RAD, respectively, or the solvent (solvent G / corn oil) only (C). Mice were sacrificed on day 5 and the number of SRBC-specific IgM-producing B cells (plaque-forming cells, PFC) determined by means of the Jerne plaque assay. Given are the means for 5 animals/group  $\pm$  SD. Statistical analysis, compared to control (One-Way ANOVA): \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

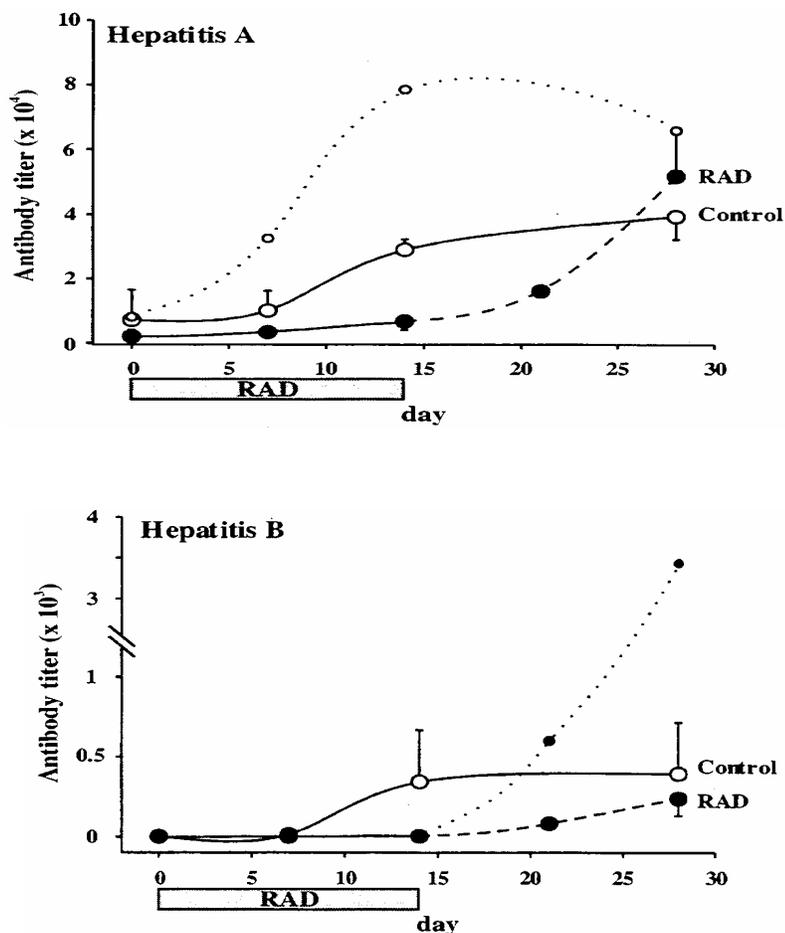
#### 4.2.8. Effect of RAD on antibody response to hepatitis A/B vaccination:

The effect of RAD on antibody response to hepatitis A and B vaccination was investigated in cynomolgus monkeys (Report # RD-98-03918). The experiment was conducted in eight animals from 3-6 years of age. The control group included four female animals that received vaccination only, and the study group consisted of one female and three males that received vaccination and RAD treatment. All animals were vaccinated (with 720 antigen-units of inactivated hepatitis A virus and 20  $\mu$ g recombinant HbsAG in a volume of 1.0 ml) on day 0 with the experimental group receiving an oral dose of RAD (1.5 mg/kg/day). Blood was collected from all animals on days 0, 7, 14, and 28. In addition, blood was collected on day 21 from monkeys treated with RAD. Antibody response to hepatitis A and B was determined using the HAVAB and AUSAB (Abbot AG, Cham, Switzerland) kits. The HAVAB kit is based on a microparticle-enzyme immunoassay in which antibody in the test solutions competes with alkaline-phosphatase conjugated reference anti-HAV antibody in binding to HAV-coated microparticles. The AUSAB kit is also based on a microparticle-enzyme immunoassay in which anti-HBs antibody in the test solution, after binding to HbsAg-coated microparticles, is detected by biotin-labeled HbsAg. The result of the study revealed that animals in the study group that were subjected to vaccination and an oral dose of RAD for 14 days failed to develop a response to HAV/HBV. Animals were able to mount an effective immune response once RAD treatment was discontinued (Figure 13). One of the RAD treated animals displayed a reduction in body weight (loss of 0.6 kg) during the 14-day period and white blood cell count. Following the discontinuation of treatment, the values returned to normal. No adverse changes were observed in the other animals.

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Figure 13: Antibody response to hepatitis A and B vaccine in cynomolgus monkeys.



Hepatitis A graph, controls: the solid line presents mean data without those from animal #90, which showed very high antibody levels, and the dotted line represents the average of all four animals. Hepatitis B graph, RAD treatment: the solid/dashed line presents mean data without those from animal #30, which showed very high antibody levels after day 14, and the dotted line presents the average of all four animals.

### 4.3. DRUG COMBINATION:

#### 4.3.1. *In vitro*:

#### 4.3.2. Effect of RAD plus cyclosporine-A on lymphoproliferation:

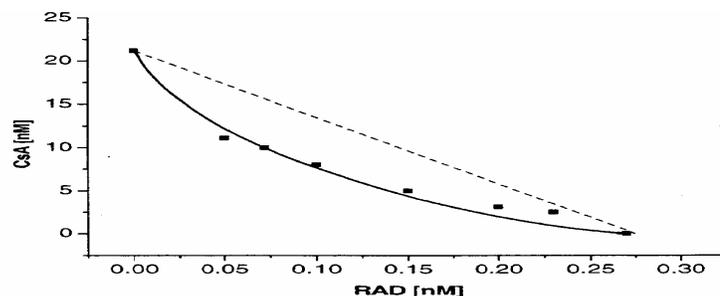
The effect of RAD and CysA on lymphoproliferation was investigated *in vitro* by MLR (Report # RD-2000-02014). Splenocytes from 8-10 week old CBA and BALB/c mice were mixed and incubated in the absence or presence of either RAD or CysA. Following four days of incubation, <sup>3</sup>H-thymidine was added and incubated for an additional 16 hours. The degree of cellular proliferation was measured by the incorporation of <sup>3</sup>H-thymidine. The result in Figure 14 shows

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a non-linear dose response curve of the 70% inhibitory concentration ( $IC_{70}$ ) values. The graph was generated by plotting values obtained from each compound when used alone, plotted against values obtained from combinations with a fixed concentration of the other compound. The result suggests that the combine action of both drugs is greater than the sum of their effect individually.

Figure 14: MLR showing synergism between RAD and CysA.



Isobologram analysis of the interaction between RAD and CsA in the MLR: plotted are the concentrations of each compound necessary to achieve 70% inhibition of cell proliferation when tested either alone or in combination with various concentrations of the other compound.

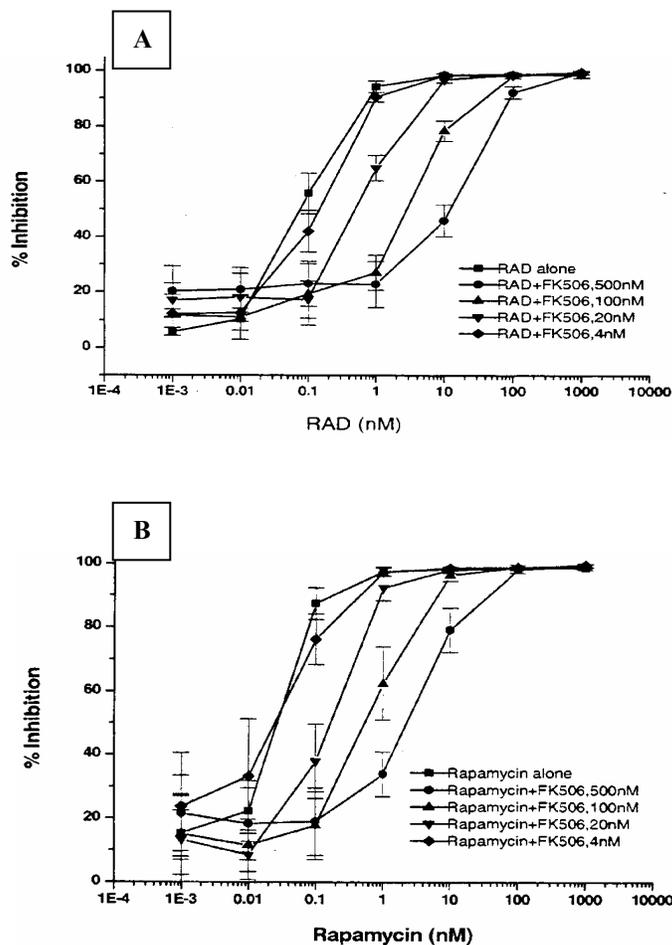
#### 4.3.3. Effect of RAD plus FK506 on lymphoproliferation.

The effect of RAD plus FKBP-12 on lymphoproliferation was investigated using B-cell hybridoma subclones (B13-29-15) (Report # RD-2000-02011). B13-29-15 cells were cultured in the presence of IL-6 and increasing concentrations of RAD, or rapamycin, either alone or in the presence of fixed concentrations (ranging from 4 to 500 nM) of FK506. Cell proliferation was measured following three days of cultivation and cell proliferation was quantified by the addition of  $^3H$ -thymidine five hours before harvesting. Results in Figure 15 show that in the presence of increasing concentrations of FK506 (at concentrations that do not significantly affect B12-29-15 proliferation) the dose-inhibition curves obtained with RAD for inhibition of IL-6 dependent B12-29-15 proliferation are progressively shifted to the right. This is indicative of competitive antagonism of the antiproliferative effect of RAD by FK506 and a 100- to 200-fold molar excess of FK506 was found to completely abolish the inhibitory effects of RAD (Figure 15A). Similar results were observed with rapamycin (Figure 15B), and reported by Dumont *et al.*, 1990. Such an antagonism may be due to higher affinity of FK506 to the binding domain or the presence of limited receptors *in vitro*.

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Figure 15: Effect of a combination of FK506 and (A) RAD, or (B) rapamycin on B cell proliferation.



#### 4.3.4. *In vivo*:

#### 4.3.5. The effect of RAD plus Neoral on renal transplantation:

The effect of combination of oral formulation of RAD, or rapamycin, and Neoral was evaluated in the BN/Lewis rat orthotopic kidney transplant model (Report # RD-2000-01533). Graft survival was measured by the survival of the recipient animals. RAD, in combination with Neoral, achieved long-term survival without histological signs of rejection. The minimal effective dose was determined to be 1.0 mg/kg for RAD and 1.0 mg/kg for Neoral or 0.5 mg/kg for RAD and 2.0 mg/kg Neoral (see section 6.1.1.). A combination of low doses of RAD plus CysA was more effective in prolonging graft survival than either drug alone (Table 18).

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Table 18: Effect of combinations of RAD and Neoral in BN into Lewis kidney transplantation.

RAD	0.5	Continuous	3	14,14,14	4,4,5
Neoral	1.0				
RAD	1.0	Continuous	3	>48,>100,>100	1,1,1
Neoral	1.0				
RAD	2.0	Continuous	3	>43,>79,>100	1,1,1
Neoral	1.0				
RAD	0.5	Continuous	3	>100,>100,>100	1,1,1
Neoral	2.0				
RAD	1.0	Continuous	3	>42,>100,>100	1,1,1
Neoral	2.0				

Survival represents times till death or sacrifice.

#### 4.3.6. The effect of RAD, in combination with Neoral, on heart transplant:

The activity of RAD, in combination with Neoral, was evaluated for prolonging the survival of heterotopic heart transplant in rats (Report # RD-2000-01533). Male DA rats were used as donors and male Lewis rats as recipient. Treatment with immunosuppressive agents was initiated on the day of the transplantation and termination of treatment was set at 100 days irrespective of graft function. Graft function was assessed by graft beat and graft rejection was concluded when the graft was unable to function. The study shows that lower doses of either RAD (2mg/kg/day), or rapamycin combined with Neoral proved more effective at prolonging graft survival than either drug alone (Table 19).

Table 19: Effect of RAD, rapamycin and CysA, in combination with Neoral in rat heterotopic heart transplantation.

Compound	Dose (mg/kg/d)	Treatment period	Nr of animals	Survival (d)	Severity of rejection (score)
RAD	0.5	Continuous	3	18,18,19	3,3,3
Neoral	1.0				
RAD	1.0	Continuous	5	18,>93,>93,95,>105	3,1,2,3,1
Neoral	1.0				
RAD	2.0	Continuous	3	>91,>91,>92	2,2,1
Neoral	1.0				
RAD	1.0	Continuous	3	>78,>92,>106	1,1,1
Neoral	2.0				
RAD	2.0	Continuous	3	>66,>105,>106	1,1,1
Neoral	2.0				
Rapamycin	0.5	Continuous	3	18,18,18	4,4,4
Neoral	1.0				
Rapamycin	1.0	Continuous	3	15,21,24	4,3,3
Neoral	1.0				
Rapamycin	1.0	Continuous	3	>60,>99,>115	1,1,1
Neoral	2.0				
Rapamycin	2.0	Continuous	3	>65,>81,>100	1,1,2
Neoral	2.0				

Survival represents times till death or sacrifice; >, animal killed for histology with apparent good health status and with a functioning heart graft. Histology data are presented in the same order as data presented in the column "Survival", with scores of cellular rejection as follows: score 0, normal heart architecture, no signs of rejection; score 1, infiltration of myocyte parenchyma, without signs of rejection; score 2, cellular rejection with slight destruction of myocyte parenchyma; score 3, cellular rejection with moderate tissue destruction; score 4, cellular rejection with severe tissue destruction; and score 5, end-stage rejection with almost complete destruction of the graft. In case of vessel changes indicative of transplant vasculopathy, the histologic score is underlined. <sup>a</sup>Treatment initiated two weeks before transplantation.

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The effect of RAD in combination with Neoral on intimal thickening was investigated in rat aorta transplantation (Report # RD-2000-01288). Transplantation procedures were done as explained in section 6.1.3. As demonstrated in Table 20, the inclusion of Neoral (2.5 mg/kg/day) was shown to have a greater effect at decreasing intima thickening than either drug alone.

Table 20: The effect of RAD and Neoral on aorta parameters in allogeneic transplantation.

**Effect of combinations of RAD and Neoral on aorta parameters in allogeneic transplantation**

Compound (mg/kg/d)	Own aorta Media (µm)	Grafted aorta				
		Media+ Intima (µm)	Media (µm)	Intima (µm)	Media (%)	Intima (%)
RAD 0.31 Neoral 2.5	92±19	98±22 <sup>**Nr</sup>	93±17 <sup>**R</sup>	5±9 <sup>**NR</sup>	96±7 <sup>**NR</sup>	4±7 <sup>**NR</sup>
RAD 0.63 Neoral 2.5	85±16 <sup>**nR</sup>	101±14 <sup>**N</sup>	101±14 <sup>**NR</sup>	0±1 <sup>**NR</sup>	100±1 <sup>**NR</sup>	0±1 <sup>**NR</sup>
RAD 1.25 Neoral 2.5	102±21	89±14 <sup>**N</sup>	88±13 <sup>**</sup>	1±3 <sup>**NR</sup>	99±3 <sup>**NR</sup>	1±3 <sup>**NR</sup>

Data presented are mean values (each value is the average of 5 measurements in one longitudinal and one transversal section) ±SD. Statistical significance of difference from placebo-treated control; \*\*  $p < 0.05$ , \*\*\*  $p < 0.01$ : from RAD at the same dose; <sup>r</sup>  $p < 0.05$ , <sup>R</sup>  $p < 0.01$ : from Neoral at the same dose; <sup>n</sup>  $p < 0.05$ , <sup>N</sup>  $p < 0.01$ .

#### 4.3.7. The effect of RAD in combination with Neoral on lung transplantation:

The effect of combination of oral formulation of RAD, and Neoral was evaluated in the allograft lung transplant model in rats (Hausen *et al.*, 1999). Lewis recipient received Brown Norway lungs followed by daily doses of (a) RAD at 2.5 mg/kg in combination with Neoral at 7.5 mg/kg, or (b) RAD at 2.5 mg/kg followed by the administration of Neoral 6 hours later. The animals were assessed by daily body weight measurements, chest radiographs, drug trough levels (high performance liquid chromatography/mass spectrometry) and blinded scoring of graft histology on day 21. Animals that received RAD and Neoral combination therapy showed very mild to no chest opacity of the left lung on day 21, when compared with animals in the monotherapy treatment group (Table 21). Chest radiographs of both combination treatment groups also appeared to be normal at day 21 and animals that received RAD and Neoral in combination treatment demonstrated mild rejection at treatment end (Figure 16). A combination of RAD plus CysA was more effective in improving lung graft survival than either drug alone.

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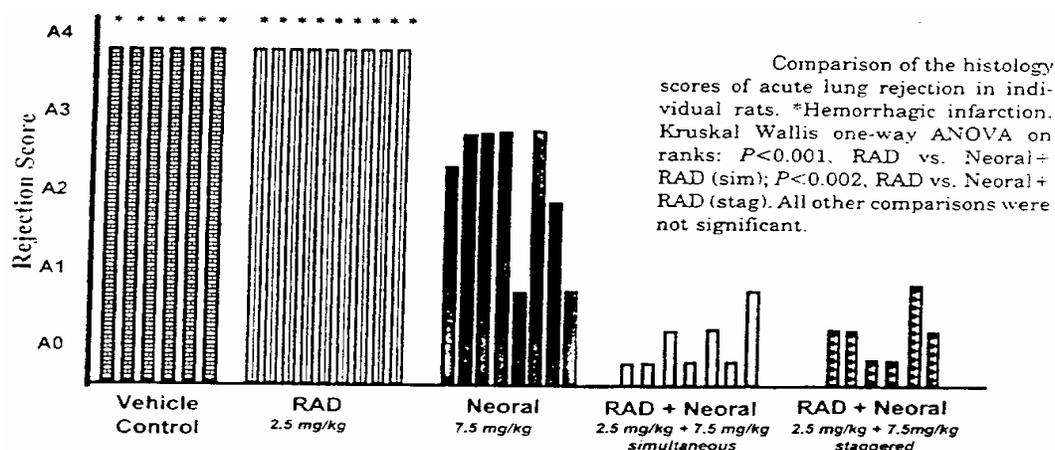
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Table 21: Chest radiograph scores.

Postoperative day	Control	RAD 2.5 mg/kg/day	Neoral 7.5 mg/kg/day	RAD+Neoral 2.5 mg/kg/day+7.5 mg/kg/day Simultaneous administration	RAD+Neoral 2.5 mg/kg/day+7.5 mg/kg/day Staggered administration
1	1.5 (1, 4)	3 (2, 4)	3 (1, 4)	2 (1, 3)	2 (1, 3)
7	4 (4, 4)	2 (1, 4)	2 (1, 4)	1 (1, 3)	1 (1, 2)
14		4 (3, 4)	1.5 (1, 4)	1 (1, 2)	1 (1, 1)
21		4 (4, 4)	2 (1, 4)	1 (1, 2)	1 (1, 1)

Median (range) score of chest radiograph opacification. 1=no opacification of the left chest cavity. 2=mild opacification. 3=moderate opacification, 4=severe opacification of the left chest cavity. One-way ANOVA with post hoc tests: postoperative day 7: control vs. SDZ RAD,  $P<0.05$ ; control vs. Neoral,  $P<0.03$ ; control vs. combined simultaneously administered group,  $P<0.003$ ; control vs. combined staggered administered group,  $P<0.0001$ ; postoperative day 14 and 21: SDZ RAD vs. Neoral,  $P<0.001$ ; SDZ RAD vs. combined simultaneously administered group,  $P<0.0001$ ; SDZ RAD vs. combined staggered administered group,  $P<0.0001$ ; Neoral vs. combined staggered administered group,  $P<0.023$ .

Figure 16: Comparison of acute rejection score of individual rats.



The effect of combinations of oral formulation of RAD, and Neoral was evaluated in cynomolgus monkey lung transplantation model (Hausen *et al.*, 2000). The co-administration of Neroal (150/100 mg/kg/day) and RAD (1.5 mg/kg/day) resulted in the early death of two study animals. Analysis of 2 dead animals showed RAD blood levels 5-fold higher than animals receiving monotherapy. Improved graft outcome was observed in animals that received Neroal at 150/100 mg/kg/day and RAD at 0.3 mg/kg/day than those that were dosed with Neroal at 150/100 mg/kg/day in combination with RAD at 1.5 mg/kg/day (Figure 17). Side effects of this treatment include renal failure in two animals and seizures in one animal (Figure 18). Combine therapy with low dose Neroal (50 mg/kg/day) and high dose RAD (1.5 mg/kg/day administered 6 hours apart) resulted in acute rejection in four of six grafts. The side effects of the staggered treatment of low dose Neroal combine with high dose of RAD was reported as moderate diarrhea (Figure 18).

Figure 17: Comparison of acute rejection scores in non-human primates.

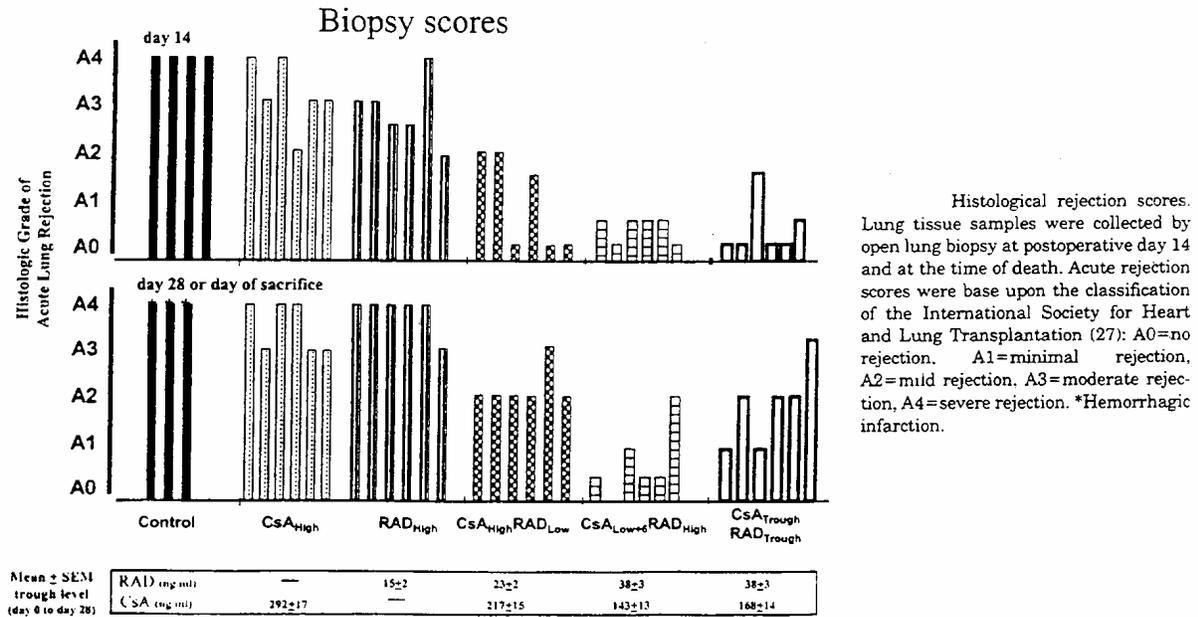
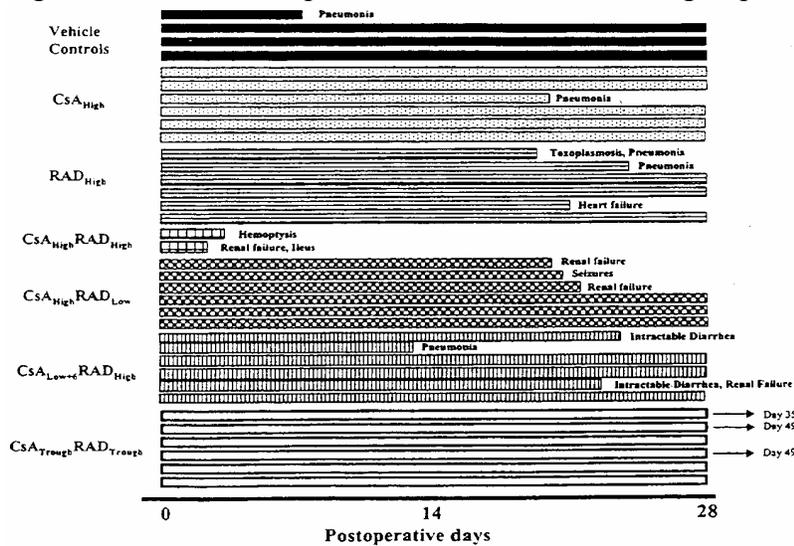


Figure 18: Non-human primate survival in treatment groups.



Animal survival in the treatment groups. The causes of death or reasons for early termination are given for animals that failed to reach the end of the observation period.

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**4.4. ANTIFUNGAL ACTIVITY:**

The *in vitro* anti-fungal activity of RAD against six strains of *Candida* species (including a multidrug-resistant clinical isolate: NFI 2023), *Cryptococcus neoformans*, *Aspergillus*, *Saccharomyces cerevisiae* and *Trichophyton* (listed in Table 22) was investigated (Report # RD-1999-03303). Minimum inhibitory concentrations were determined by broth macro-dilution assays in accordance to the NCCLS M27-A guidelines. The results of the study show that RAD was active against 2 *C. albicans* strains including strain NFI 2023, and to a lesser extent *Cryptococcus neoformans*, but showed no activity against single strains of *Aspergillus fumigatus* and *Trichophyton mentagrophytes* (Table 23 and 24). The comparator drug, Amphotericin B, showed higher activity against the test strains. No *in vivo* study was conducted. The antibacterial activity of the drug was not measured.

Table 22: Fungal strains used in *in vitro* study

Fungal species	Strain No.	Origin
<i>Trichophyton mentagrophytes</i>	NFI 0158	NFI collection screening isolate
<i>Aspergillus fumigatus</i>	NFI 0159	NFI collection screening isolate
<i>Candida albicans</i>	NFI 0124	NFI collection screening isolate
<i>Candida albicans</i>	NFI 2023	Clinical isolate multidrug-resistant
<i>Candida glabrata</i>	NFI 2901	ATCC 90030
<i>Candida krusei</i>	NFI 2902	ATCC 6258
<i>Candida parapsilosis</i>	NFI 2903	ATCC 22019 (NCCLS QC strain)
<i>Candida tropicalis</i>	NFI 2905	ATCC 750 (NCCLS reference strain)
<i>Cryptococcus neoformans</i>	NFI 3102	ATCC 36556
<i>Saccharomyces cerevisiae</i>	NFI 3201	ATCC 9763

Table 23: Antifungal activity of RAD in comparison with amphotericin B (MIC values in µg/ml)

Fungus	Strain	SDZ RAD			Amphotericin B
		MIC <sub>80%</sub>	MIC <sub>100%</sub>	MFC	MIC <sub>100%</sub>
<i>T. mentagrophytes</i>	NFI 0158	>128	>128	>128	1
<i>A. fumigatus</i>	NFI 0159	>128	>128	>128	0.5
<i>C. albicans</i>	NFI 0124	1	2	>128	0.125
<i>Cr. neoformans</i>	NFI 3102	1	>128	>128	1
<i>S. cerevisiae</i>	NFI 3201	>128	>128	>128	0.25

Table 24: Anti-Candida activity of RAD in comparison with amphotericin B (MIC values in µg/ml)

Fungus	Strain	SDZ RAD			Amphotericin B	
		MIC <sub>80%</sub>	MIC <sub>100%</sub>	MFC	MIC <sub>100%</sub>	MFC
<i>C. albicans</i>	NFI 2023	1	2	16	4	8
<i>C. glabrata</i>	NFI 2901	1	1	>128	0.125	0.125
<i>C. krusei</i>	NFI 2902	>128	>128	>128	0.25	0.25
<i>C. parapsilosis</i>	NFI 2903	1	2	8	0.5	0.5
<i>C. tropicalis</i>	NFI 2905	1	2	16	0.25	0.25

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**4.5. MECHANISM OF ACTION:****4.5.1. Effects of RAD on the FK506 binding protein FKBP-12.**

The *in vitro* binding of RAD to the FK506 binding protein, FKBP-12 was examined (Report # RD-2000-01777). Rapamycin and FK506 were used as comparators. Binding was measured by first coating microtiter plates with FK506 conjugated to BSA. Biotinylated FKBP-12 was allowed to bind, competitively to the immobilized FK506, in the absence or presence of RAD or rapamycin. Bound biotinylated FKBP-12 was assessed spectrophotometrically at 405nm by first incubating with streptavidin-alkaline phosphate conjugate followed by an incubation period in the presence of p-nitrophenyl phosphate. Results in Table 25 show that the relative IC<sub>50</sub> of RAD versus FK506 was 2.0 ± 0.4 nM (1.91 ± 0.38 µg/ml). The relative IC<sub>50</sub> value of rapamycin was 0.8 ± 0.3 nM (0.73 ± 0.27 µg/ml). The binding of RAD to FKBP-12 was approximately 2-3 folds less than that of rapamycin.

Table 25: Binding of RAD, rapamycin and FK506 to FKBP-12

Compound	Relative IC <sub>50</sub> <sup>a)</sup> (absolute IC <sub>50</sub> ) n=3
FK506	1 (1.0 ± 0.2 nM)
Rapamycin	0.8 ± 0.3 <sup>ns</sup> (0.8 ± 0.3 nM <sup>ns</sup> )
RAD	2.0 ± 0.4 * <sup>†</sup> (2.0 ± 0.5 nM * <sup>†</sup> )

<sup>a)</sup> FK506 was used as a standard in every individual test (microtiter plate); the results are expressed as relative IC<sub>50</sub>, *i.e.*, as ratio of IC<sub>50</sub> of test compound to IC<sub>50</sub> of FK506. Given are the means of n IC<sub>50</sub> determinations ± S.D. Statistical analysis, paired t-test; *versus* FK506: *ns*, not significant, \**p*<0.05; *versus* rapamycin: <sup>†</sup>*p*<0.05.

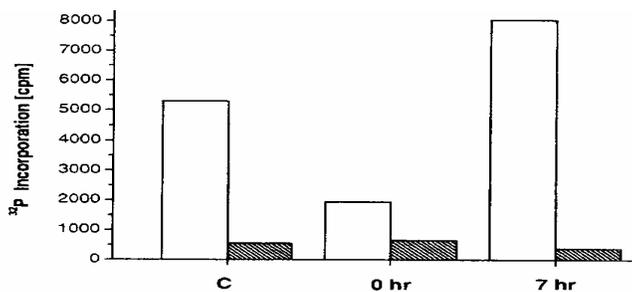
**4.5.2. Effect of RAD on p70<sup>S6k</sup> kinase activity:**

The activity of RAD was compared to that of rapamycin for its ability to inhibit IL-6 induced p70 S6 kinase phosphorylation in B cell hybridoma (B13-29-15) cells lines *in vitro* (Report # RD-2000-02151). The B13-29-15 cell line is characterized by its dependence on IL-6 for its proliferation and sensitivity to rapamycin and RAD. To determine the effects of IL-6 on p70 S6 kinase activation, cells were continuously grown in the presence of 0.2 ng/ml of IL-6. Kinase activity was assayed by first collecting lysate supernatant containing 50 or 100 µg of total protein then by measuring the activity of <sup>32</sup>P incorporation into S6 using purified ribosomal 40S subunits from rat liver as substrates. IL-6 was shown to induce and sustain p70 S6 kinase activity in IL-6-dependent B cell hybridoma (Figure 19). RAD, like rapamycin was capable of inhibiting p70 S6 kinase activity dose dependently and complete inhibition was reported at concentrations as low as 1 nM (0.96 µg/ml) (Figure 20).

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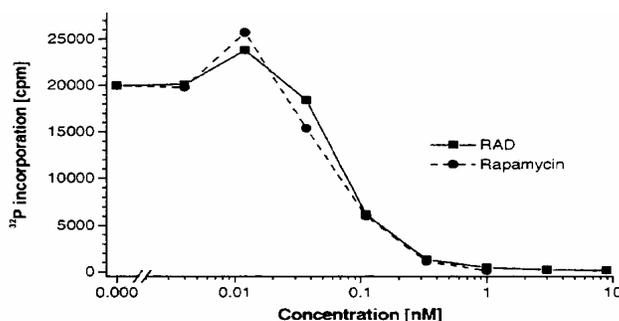
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Figure 19. Induction of p70 S6 kinase by IL-6.



B13-29 cells were either continuously grown in the presence of 0.2ng/ml IL-6 (C), or the cells were washed and then kept for 24 hours in a medium free of IL-6 before adding IL-6 again. To assay for p70 S6 kinase activity, cell lysate supernatant samples were immunoprecipitated with a specific anti-p70 S6 kinase antibody and  $^{32}\text{P}$ -incorporation into purified ribosomal 40S subunits, used as substrate, measured. p70 S6 kinase activity was determined either immediately prior to (0 hr), or 7 hours after re-addition of IL-6 (7 hr) in cells cultured in the absence (open bars) or presence of 100nM rapamycin (hatched bars).

Figure 20. Inhibition of p70 S6 kinase by RAD.

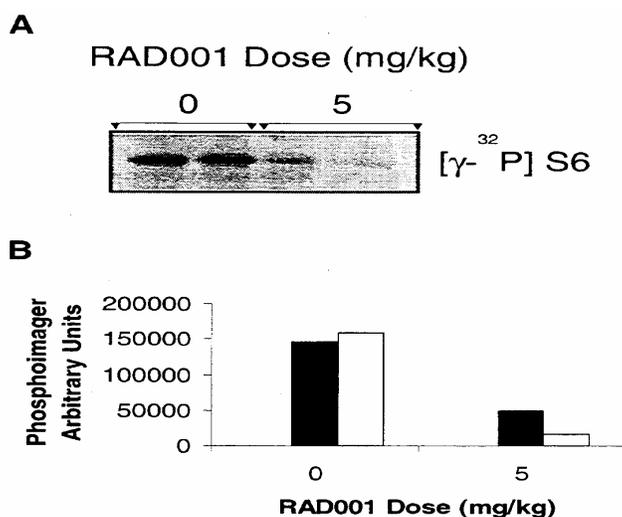


Increasing concentrations of RAD or rapamycin were added to 10ml aliquots of B13-29-15 cells ( $1 \times 10^5$  /ml) which were continuously grown in the presence of 0.2ng/ml IL-6. After 1 hour cultivation at  $37^\circ\text{C}$ , cells were quickly chilled and harvested. p70 S6 kinase was precipitated from the cell lysate supernatants (containing 50 $\mu\text{g}$  total protein) using the p70 S6 kinase-specific polyclonal antibody M5. The S6 kinase activity was then determined with these immunoprecipitates using purified ribosomal 40S subunits as substrate. After the kinase reaction, the 40S ribosomal proteins were electrophoretically separated on an SDS polyacrylamide gel, the S6-containing band cut-out from the gel and incorporation of  $^{32}\text{P}$  into the S6 band quantified by Cerenkov counting [see ref. (8,9)].

In another study, the effect of RAD on the enzyme p70<sup>S6k</sup> was studied in rat peripheral blood lymphocytes (Report RD-2000-02545). A microemulsion of 2% w/v RAD in Neoral vehicle, or RAD-free Neoral vehicle was prepared and given to male Sprague-Dawley rats that were divided into two experimental groups. The control group received Neoral vehicle (5 mg/kg) and the experimental group received RAD (5 mg/kg) in Neoral vehicle. Two hours following treatment, the rats were sacrificed and the peripheral lymphocytes were extracted and prepared for p70 S6 kinase assay. For kinase assay, total protein was extracted and incubated with  $\gamma$ - $^{32}\text{P}$  phosphate and 20 $\mu\text{g}$  of rat liver 40S ribosomal subunits. The level of phosphorylation was determined in the presence or absence of RAD by  $\gamma$ - $^{32}\text{P}$  phosphate incorporation into S6. Phosphorylated S6

was resolved by electrophoresis (Figure 21). Treatment with RAD at a dose of 5mg/kg resulted in the down regulation of the level of protein phosphorylation (Figure 21A). Furthermore, phosphoimager quantification of the gel obtained in Figure 21 showed an increase in the  $\gamma$ - $^{32}\text{P}$  phosphate signal at the 5mg/kg dose (Figure 21B). These sets of experiments were repeated with similar results. Rapamycin was not used as a comparator in this experiment; however, similar findings were reported by Chung, *et al.*, 1992.

Figure 21: Effect of RAD on p70<sup>s6k</sup> kinase activity.



Male Sprague-Dawley rats were treated by gavage with a single administration of RAD001 (5 mg/kg) or Neoral vehicle, sacrificed after 2 hr and peripheral blood lymphocytes prepared, extracted and p70<sup>s6k</sup> activity measured using 40S ribosomal subunits as an *in vitro* substrate. Panel A: autoradiograph of [ $\gamma$ - $^{32}\text{P}$ ]phosphate incorporation into S6 protein [ $\gamma$ - $^{32}\text{P}$ ] S6). Panel B: phosphoimager quantification of panel A. Black and white bars represent duplicate p70<sup>s6k</sup> assays performed at each dose level. Averages of these values are presented in the text.

The studies show that RAD, like rapamycin, binds to FKBP-12 and inhibits p70<sup>s6k</sup> kinase activity. It is known that the rapamycin-FKBP-12 complex binds to a key regulatory kinase, mTOR and affects cell cycle (Heitman *et al.*, 1991). However, there is not enough evidence to show that the binding of FKBP-12-RAD complex is similar to that of FKBP-12-rapamycin in altering mTOR signaling, and the cascades of protein interaction, which leads to the control of cell cycle.

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**5. CONCLUSIONS:**

The sponsor seeks approval of RAD, a macrolide immunosuppressant, for the prophylaxis of cardiac and renal graft rejection in adult patients.

The *in vitro* immunosuppressive effect of RAD was compared with rapamycin in the MLR, using mouse and human derived mononuclear cells. RAD, like rapamycin, was capable of dose dependently inhibiting T cell proliferation in both mouse and human mononuclear cells. The immunosuppressive activity of RAD appears to be comparable to that of rapamycin. Therefore, the different mechanism of action of the two compounds suggests a potential for synergy. In mouse mixed lymphocyte immune response assay RAD and CysA (Neoral) effectively showed improved activity as measured by an *in vitro* cell proliferation assay.

RAD, like rapamycin was also capable of dose dependently inhibiting (1) IL-6 dependent proliferation of a B-cell hybridoma subclone and (2) IL-2 and IL-15 dependent T cell proliferation. IL-2 (produced by CD 4<sup>+</sup> and CD 8<sup>+</sup> T cells) and IL-15 (produced by activated macrophages, muscle and epithelial cells) receptors are thought to be closely related since both are known to utilize  $\beta$  and  $\gamma$  chains of the IL-2 receptor. However, recent studies show that IL-15 is pleiotropically expressed and this expression pattern plays a unique role in both innate and adaptive immune cell homeostasis (Lodolce *et al.*, 2002).

RAD dose dependently inhibits *in vitro* and *in vivo* mouse humoral immune responses against T cell-independent and T cell-dependent antigens. When rapamycin was used as a comparator, RAD demonstrated 5 to 7 fold less activity in responses against DAGG-Ficoll and SRBC but not for responses to TNP-LPS. In comparison to CysA, RAD, like rapamycin, inhibited *in vitro* T-independent immune response against TNP-LPS.

RAD, like rapamycin, was shown to partially inhibit bovine smooth muscle cell proliferation. However, adriamycin was shown to completely block serum-induced bovine smooth muscle cell proliferation.

The metabolites 46-hydroxy-RAD and 24-/25-hydroxy-RAD showed very low activity in the MLR assay (566-fold and approximately 91-fold, respectively). The two opened ring degradation (b) (4) also demonstrated very low activity in mouse MLR experiment.

RAD, when compared with rapamycin, was effective at improving heterotopic heart and orthotopic kidney grafts in rats and primates. In the heart and kidney transplantation models, RAD and rapamycin demonstrated increased activity with CysA (Neoral) in prolonging graft survival.

In the rat lung transplant model, the administration of RAD in combination with CysA showed a slight increase in the ability to suppress severe acute lung rejection. However, neither monotherapy with RAD, nor cyclosporine prevented severe acute rejection in unilateral lung transplant animals.

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RAD was shown to dose dependently inhibit intima thickening (80% inhibition) in rat allogeneic aorta transplantation. However, complete inhibition was not demonstrated. In studies involving CysA, only 28% inhibition was demonstrated. In drug combination studies, CysA and RAD showed increased activity in preventing intima thickening.

RAD, like rapamycin, was effective at inhibiting some of the immune responses *in vivo* such as (1) localized graft-versus-host reaction, (2) antibody responses to T-dependent and T-independent antigens, and (3) antibody responses to hepatitis A and B antigens.

The sponsor has measured the anti-fungal activity of RAD *in vitro* against six *Candida* species including a multi-drug resistant strain of *C. albicans* strain NFI 2023. However, the clinical significance of such an effect is not known.

The immunosuppressive activity of RAD was shown to be mediated by binding to FKBP-12. RAD demonstrated a binding affinity to FKBP-12 that was 2-3-fold weaker than that of rapamycin. In addition, RAD, like rapamycin, was shown to inhibit the activity of p70 S6 kinase activity of a B cell hybridoma subclone. The studies show that RAD binds to FKBP-12 and inhibits p70<sup>s6k</sup> kinase activity. However, there is not enough evidence to show that the FKBP-12-RAD complex affects mTOR activity similar to that of FKBP-12-rapamycin.

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**8. RECOMMENDATIONS:**

This NDA is approvable with respect to the immunopharmacology, pending the accepted version of the label.

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Microbiologist, HFD-590

**CONCURRENCES:**

HFD-590/Deputy Dir \_\_\_\_\_ Signature \_\_\_\_\_ Date \_\_\_\_\_  
HFD-590/Micro TL \_\_\_\_\_ Signature \_\_\_\_\_ Date \_\_\_\_\_

CC:

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