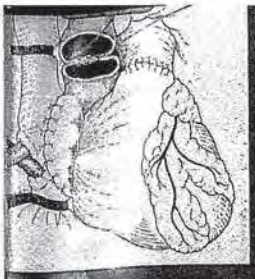
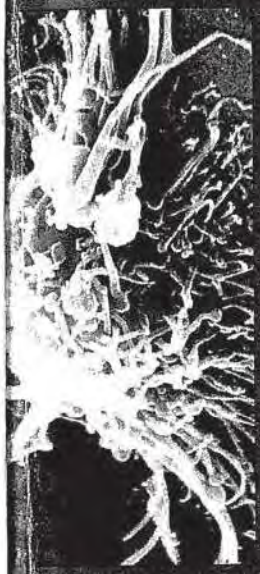
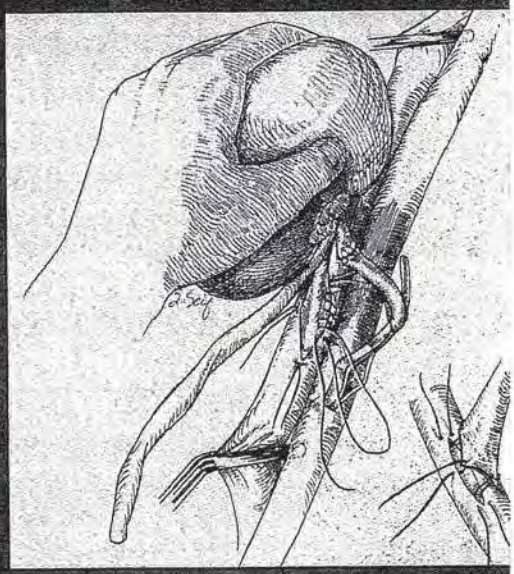
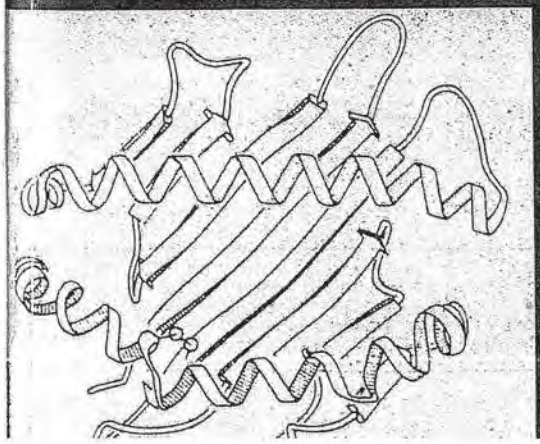


VOLUME 6
NUMBER 1
JANUARY
1992

TRANSPLANTATION REVIEWS







Rapamycins: Antifungal, Antitumor, Antiproliferative, and Immunosuppressive Macrolides

Randall Ellis Morris

What we know is a drop. What we don't know is an ocean.
Isaac Newton

Progress in rapamycin (RPM) research has been rapid and is poised to accelerate even more dramatically. An Investigational New Drug application (IND) for phase I trials of RPM as a treatment for prospective graft recipients was approved less than 2 years after the first published reports[1,2] and public disclosure[3] of the ability of RPM to prolong graft survival in experimental animals. RPM is a macrolide fermentation product that has antifungal and antitumor activity. However, its effects on the immune system have generated the most interest because RPM is structurally similar to another new immunosuppressive macrolide, FK506. RPM is particularly intriguing because it inhibits the activation of immune cells by unique, relatively selective, and extremely potent and highly effective mechanisms. For example, one half microgram of RPM administered daily to mouse recipients of completely mismatched heart allografts prolongs graft survival. When these mice are treated for only 2 weeks with higher doses of RPM, or when a single dose of RPM is administered to rat heart allograft recipients, strain-specific unresponsiveness is induced, and grafts survive indefinitely in both species.

The research on RPM is representative of a significant shift in emphasis in transplantation from the macrocosmic world in which innovative surgical techniques predominated from the 1950s through the 1970s to our current focus on the microcosm of cellular and molecular immunopharmacology. A revolution in the discovery, development, and clinical use of new strategies to control the immune response is clearly upon us: it took more than 35 years to accrue the four imperfect mainstays of immunosuppression for transplantation—steroids, azathioprine, anti–T-cell antibodies, and Cyclosporin A (CsA). In 1992, six new xenobiotic immunosuppressants will be in clinical trials (Fig 1).

This new era in immunosuppression can be traced to the convergence of several lines of research: (1) the discovery and successful clinical use of CsA; (2) an increased understanding of the fundamental biology of immune cells that enables the actions of different immunosuppressants to be better understood and thus lay the foundation for more rational means to discover, develop, and use improved drugs; and (3) organized preclinical research programs designed to identify potentially valuable immunosuppressants and to generate the knowledge needed for these agents to be used intelligently in the clinic. Figure 2 shows the research program used for several years in the Laboratory for Transplantation Immunology at Stanford University that enabled us to identify RPM[3-11] and the morpholinomethyl ester of mycophenolic acid (MPA)[12-16] as immunosuppressants for graft rejection. The mechanisms of action and immunopharmacology of these two compounds, as well as FK506,[17-19] deoxyspergualin (DSG),[20,21] and brequinar sodium (BQR)[22] have also been studied and compared with one another in our laboratory.

Our spectrum of experimental systems begins with in vivo mouse models that are so rapid, quantitative, and inexpensive that we have been able to evaluate hundreds of molecules for suppression of alloimmunity. The vast majority of these drug candidates fail during testing in rodents because they lack efficacy or safety, and they are discarded quickly so that our resources can be concentrated on compounds with the greatest potential. Compounds that show promise are evaluated further in rodent models to identify those with the following ideal characteristics: (1) unique mode of action; (2) high efficacy for the prevention or treatment of acute, accelerated, or chronic rejection; and (3) low toxicity. This Darwinian selection process accomplishes two tasks: first, it insures that only the agents with the greatest poten-

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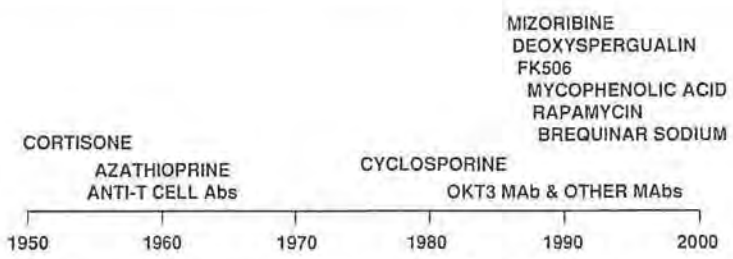

Figure 1. History of the use of drugs used to control graft rejection. All of the following xenobiotics recently discovered to suppress graft rejection in preclinical models have advanced to clinical trials: the antimetabolites such as mizoribine (MZR), MPA in its prodrug form of RS-61443, and BQR; the cyclosporine-like drug FK506, and drugs that define two new classes of immunosuppressants, DSG and RPM.

tial are advanced to the expensive nonhuman primate transplant model; and second, it prepares us to be able to use these compounds intelligently in nonhuman primates. The nonhuman primate model is important because it is highly predictive of the safety and efficacy of a test drug in humans. The sum of all knowledge produced from well-planned preclinical studies is the essential foundation from which successful clinical trials are designed and executed. New drug development is a highly complex, multidisciplinary task, and our contribution to the development and clinical use of new immunosuppressants depends on very close collaboration with scientists and clinicians in the pharmaceutical industry.

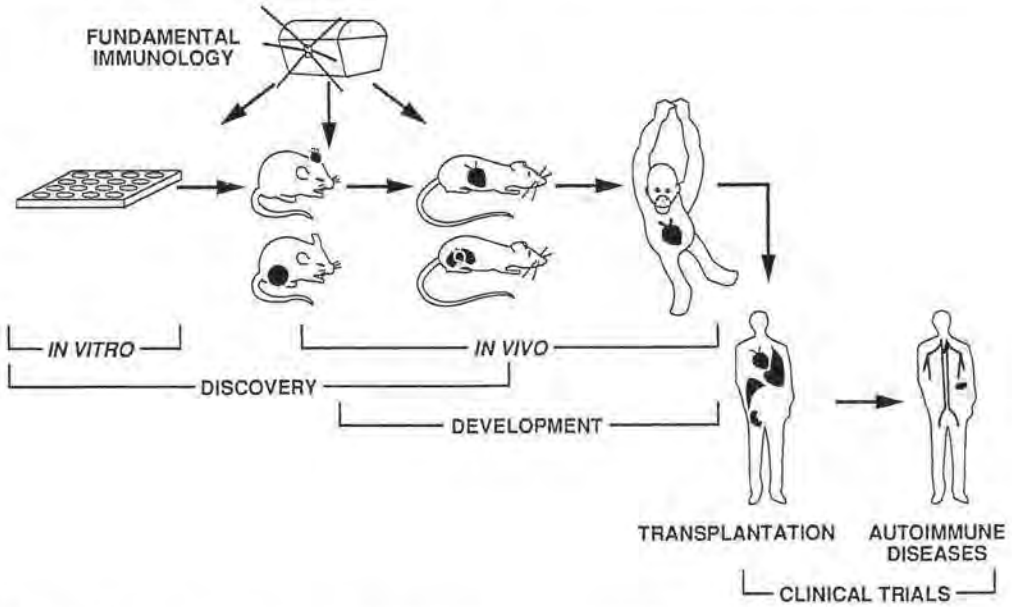

Figure 2. Schematic representation of the program used at the Laboratory of Transplantation Immunology at Stanford University to identify compounds with immunosuppressive activities for transplantation and to develop these compounds for clinical use for the prevention and treatment of rejection. Fundamental knowledge of the immune system coupled with an appreciation of the characteristics of the drug candidate is used to design experiments to profile the activity of the compound and define its mechanisms of action. Heterotopic transplantation of neonatal mouse heart allografts into the ear pinnae of mouse recipients and alloantigenic and mitogenic stimuli of popliteal lymph node hyperplasia are used as rapid and quantitative bioassays before proceeding to the more laborious techniques of primarily vascularized heterotopic (abdominal) and secondarily vascularized heterotopic (subrenal capsule) heart allograft and xenograft transplantion in the rat. Assessment of the efficacy and the safety of the compound in cynomolgus monkey recipients of heterotopic allografts precedes phase I clinical trials in transplant patients and patients with autoimmune diseases.

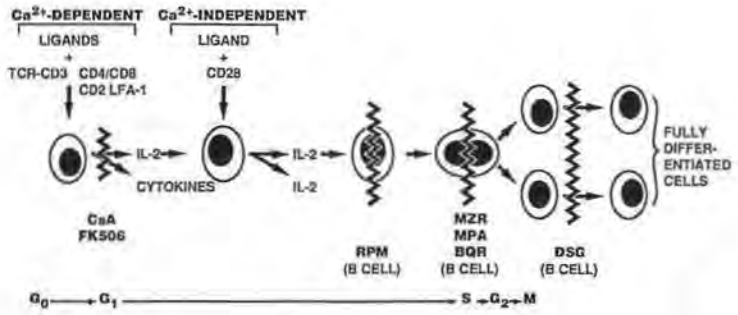

Figure 3. Schematic representation of the possible sites of action of the following immunosuppressants on activated T cells: CsA and FK506 prevent the transcription of early phase cytokine genes; RPM inhibits the signal transduction of IL-2 bound to its receptor and may have other antiproliferative effects unrelated to lymphokine signals; MZR, MPA, and BQR all inhibit purine (MZR, MPA) or pyrimidine (BQR) nucleotide synthesis; DSG seems to inhibit late stages of T-cell maturation. RPM, MZR, MPA, BQR, and DSG also act on activated B cells at the sites shown.

Even more important than the relatively large number of new immunosuppressants that have been discovered is their variety. Each of these new molecules suppresses the immune system by blocking distinctly different biochemical reactions that initiate the activation of immune cells that cause the many forms of graft rejection (Fig 3). Briefly, CsA and FK506 act soon after Ca^{2+}-dependent T-cell activation to prevent the synthesis of cytokines important for the perpetuation and amplification of the immune response.[23-25] RPM acts later to block multiple effects of cytokines on immune cells including the inhibition of interleukin-2–(IL-2–)triggered T-cell proliferation,[26-30] but its antiproliferative effects are not restricted solely to T and B cells. RPM also selectively inhibits the proliferation of growth factor-dependent and growth factor-independent nonimmune cells. Mizoribine (MZR),[32] MPA,[33] and BQR[34] are antimetabolites that inhibit DNA synthesis primarily in lymphocytes. These new antimetabolites are more selective than azathioprine because these compounds block the activity of enzymes restricted only to the de novo purine or pyrimidine biosynthetic pathways. Lymphocytes are more dependent on these pathways for nucleotide synthesis than other cells.

Recent reviews[35-38] discuss these and other immunosuppressants. RPM has recently been the subject of four brief reviews,[7,39-41] a long review,[42] and has been included in reviews that have primarily focused on FK506.[43-45] This review provides a complete profile of RPM from work published through the end of August 1991. Despite the progress made in understanding RPM since the first publication on this compound in 1975,[46] the description of its ability to suppress graft rejection has stimulated renewed interest by a wide variety of investigators whose work has not yet been published. As a result, research on macrolide immunosuppressants has become fluid and extremely fast-paced. Because unpublished data generally are not available for evaluation, I have not referred to unpublished work or personal communications. However, I have relied on many studies of RPM from the Laboratory of Transplantation Immunology at Stanford University that have yet to be published in full. In most of these cases, I have supplied the data from which conclusions in the text are drawn.

Because this review is being written relatively early in the research life of RPM, and because the majority of the work on this complex molecule has yet to be published, the material subsequently presented should be regarded more as a preview rather than as a review. At the very least, this article will provide a logical framework that other investigators can use to organize and to evaluate new information on RPM as it is published. For many investigators with highly specialized interests, only selected sections will be of use. For others, it is essential to understand all that is known about a new and unique molecule such as RPM. Without an understanding of RPM that is both deep and broad, it will be difficult to meet the challenging tasks of using RPM as a tool to learn more about the immune system, maximizing its therapeutic potential, and discovering new and improved members of this class of immunosuppressant. If we strive to understand thoroughly the little that is now known about RPM, we will make more efficient and rapid progress toward our goal of understanding all of the important biological effects of this molecule.

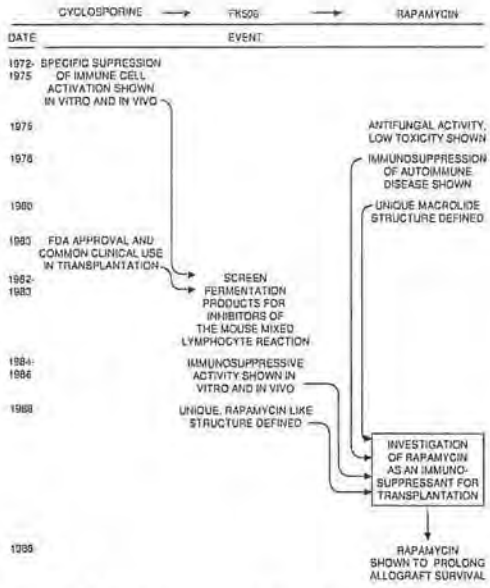

Figure 4. Evolutionary path of RPM as an immunosuppressant for transplantation.

In addition to reviewing the information on RPM, this article warns of the danger of inductive reasoning in which, in an adolescent field like immunology, arguing from highly specific cases to general laws often promotes the illusion of knowledge rather than its true acquisition. However, by interrelating information concerning the structure, the molecular mechanisms, and the actions of RPM on defined cell types in vitro, its effects in vivo, as well as its disposition in the body and its toxicity, new and important insights into the actions of RPM can be gained. In general, the conceptual tools used in this review to analyze the data from experiments on RPM can be applied to the study of many other immunosuppressants, especially other xenobiotics.

Before dissecting and examining every aspect of RPM in detail, it is worth reviewing the events that led to the attention RPM is now receiving. Figure 4 shows the relationship of the evolution of RPM as an immunosuppressant to the development of CsA and FK506 as immunosuppressants. Table 1 provides a more detailed outline of the sequence of the main events that have defined progress in RPM research in its first 15 years.[1-4,27-29,46-52] The ancestors of RPM are CsA and FK506. As shown in Fig 4, CsA stimulated the organization of a rational screening program designed to discover other fermentation products with mechanisms of immunosuppressive action identical to CsA. The discovery of FK506 was the product of this program,[31] and when the structure of FK506 was defined, its similarity to the structure of RPM was immediately recognized.[31] Years before, the structure of RPM had been determined as a conse-

Table 1. History of RPM Drug Development: The First 15 Years

Discovery	*Year*	*References*
Isolation from Easter Island (Rapa Nui) soil sample and characterization of antimicrobial activity	1975	Vezina, Kudelski, and Sehgal[46] Sehgal, Baker, and Vezina[47]
In vivo use: Toxicity Pharmacokinetics Bioavailability Antifungal activity	1978	Baker, Sidorowicz, Sehgal, et al[48]
Immunosuppression of autoimmune disease	1977	Martel, Klicius, and Galet[49]
Elucidation of structure	1980	Findlay and Radics[50]
Antitumor activity described	1981	Douros and Suffness[51]
Immunosuppression of allograft rejection RPM alone	1989	Morris and Meiser[1] Calne, Collier, Lim, et al[2]
RPM in combination with CsA	1990	Meiser, Wang, and Morris[3]
Differentiation of effects of RPM and FK506 on immune cells in vitro	1989 1990	Tocci, Matkovich, Collier, et al[27] Metcalfe and Richards[28] Dumont, Staruch, Koprak, et al[29]
Differentiation of effects of RPM and FK506 on immune system in vivo	1990	Morris, Wu, and Shorthouse[4]
Demonstration of binding of RPM to FK506 binding protein	1989	Harding, Galat, Uehling, et al[52]

quence of the identification of RPM as an antifungal antibiotic (Table 1). Shortly after the antibiotic activities of RPM were described, it was found to have immunosuppressive activity. This was only a few years after the immunosuppressive activity of CsA was discovered, but ironically, RPM was not developed as an immunosuppressant at that time. In a review[36] of immunosuppressive agents published in 1988, Devlin and Hargrave encouraged "a detailed comparison of the biological profile of these macrolides [FK506 and RPM]." These investigators suggestion was based on the structural similarity of both compounds and their known immunosuppressive activity.

Sehgal was aware that investigators at the Laboratory for Transplantation Immunology at Stanford University had developed a quantal bioassay for the evaluation of immunosuppressant potency and efficacy, had validated the assay with CsA,[35] and had used it to study FK506.[17] In 1988, he offered to provide us with enough RPM to enable us to determine whether its activity differed from FK506 in mouse as well as rat heart transplant recipients. As subsequently discussed, the activity of RPM is extremely dependent on the vehicle in which it is suspended and the route by which it is administered. Had our first experiment used suboptimal conditions for the administration of RPM, we would have found no difference in potency or efficacy between RPM and FK506 and might not have pursued our study of RPM. In retrospect, the mode of administration used at the outset was optimal and, under those conditions, RPM was clearly more potent and effective than FK506. This clear difference in pharmacological effect between these two structurally related macrolides prompted our continued investigations of the activity of RPM. At the same time as these studies were being conducted, investigators at the University of Cambridge, England, were testing the immunosuppressive activity of RPM in rodents, dogs, and pigs.[7] Simultaneous studies[27-29] performed at Cambridge and by various groups of investigators at Merck Sharp and Dohme Research Laboratories, United States showed that RPM and FK506 affect immune cells quite differently in vitro.

Origin and Characterization of the Bacterium Producing RPMs

RPM (AY-22,989 [Fig 5]) is made by a filamentous bacterium from the streptomycete group that was isolated from an Easter Island soil sample by Vezina et al and Sehgal et al at Ayerst Research Laboratories in the middle 1970s.[16,17] The aerial mycelium of this bacterium is monopodally branched (Fig 6), contains sporophores terminated by short, coiled spore chains, and absorbs water. It was ultimately identified as belonging to the species *Streptomyces hygroscopicus*, designated by Ayerst Research as strain AY B-994, and deposited in both the ARS culture collection of the United States Department of Agriculture (assigned number NRRL 5491) and the American Type Culture Collection (ATCC 29253). A structurally related compound,[56] 29-demethoxyrapamycin (AY-24,668 [Fig 5]) is coproduced with RPM. Another culture isolated from the same soil sample and designated AY B-1206 produces higher levels of RPM than AY B-994 and little or no 29-demethoxyrapamycin.[57]

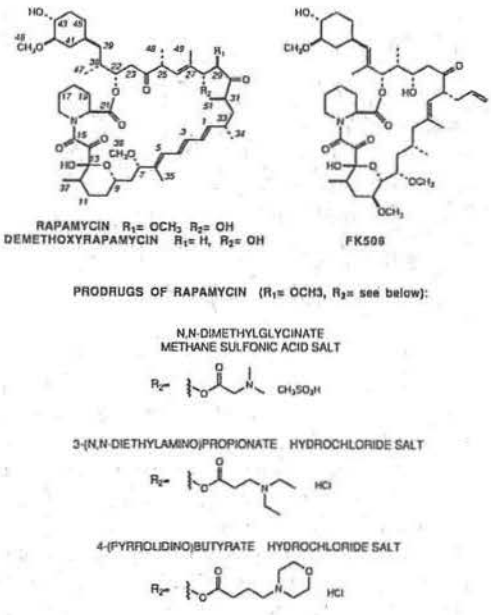

Figure 5. Chemical structures of RPM, 29-demethoxyrapamycin, FK506, and the prodrugs of RPM.

Fermentation, Purification, and In Vitro Antimicrobial Activity of RPMs

Fermentation of RPM

Soon after the availability of a pure strain of *S hygroscopicus*, fermentation conditions (type of media, media pH, and temperature) were varied to define its cultural characteristics.[16,48] Although this microbe grows and sporulates in a wide range of culture

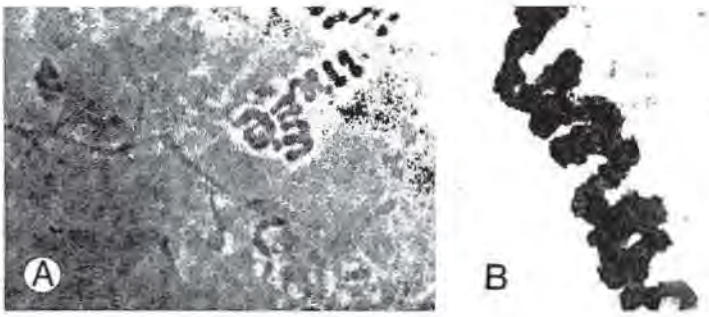

Figure 6. (A) Photomicrograph of the filamentous bacterium, *S hygroscopicus*, that produces RPM (magnification ×455). (B) Electron micrograph of *S hygroscopicus* (magnification ×2,500). (Reprinted with permission.[36])

conditions, more narrowly defined conditions are necessary for the optimum production of RPM. RPM has been produced by aerobic submerged fermentation similar to that used for most antibiotics. Inoculum is prepared in two stages in a medium containing soybean meal, glucose, $(NH_4)_2SO_4$, and $CaCO_3$ and used at 2%. For the fermentation in stirred vessels, the starting medium was soybean meal, glucose, $(NH_4)_2SO_4$, and KH_2PO_4. Glucose is fed continuously after the 2nd day and the pH was controlled at 6.0 with NH_4OH. Maximum titers of RPM are reached in 96 hours. Paper disc-agar diffusion assays with *Candida albicans* are used to determine the antibiotic titer.

The fermentation methods required to produce 29-demethoxyrapamycin are the same as those described for RPM.[56]

Purification of RPMs

The purification scheme (Fig 7) adopted for the production of RPM was developed shortly after the identification of the antifungal activity of RPM and is subsequently summarized.[37] After fermentation, the pH of the beer is adjusted to 4.0. The mycelium, extracted with trichloroethane, is filtered off and the extract is dried with anhydrous sodium sulfate to produce about 500 gm of oily residue from a 160-liter fermentation run. After extracting the residue with methanol, the extracts are evaporated to yield approximately 50 gm of residue that is then dissolved in 15% acetone in hexane and mixed with silica gel. The dissolved RPM is adsorbed to the silica gel and remains bound to the gel after the mixture has been filtered and washed onto a column from which RPM is eluted with an acetone:hexane mixture. After evaporating the column eluate to dryness, the residue is dissolved in ether from which pure crystals of RPM are obtained. In this initial purification process, recoveries of RPM are on the order of 40%; 10 L of broth produce 300 mg pure RPM. A more recent method of purification has been reported.[57]

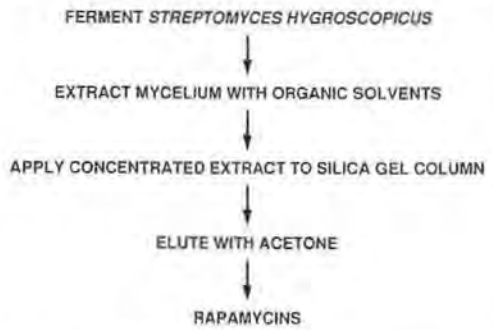

Figure 7. Fermentation and isolation of RPMs.

Except for minor modifications, the methods described for the isolation of 29-demethoxyrapamycin are the same as those used for RPM.[56]

In Vitro Antimicrobial Activity of RPMs and Mechanisms of Their Antimicrobial Actions

The antimicrobial screening program at Ayerst Research Laboratories identified RPM for its antifungal activity. RPM inhibits the growth of yeasts and filamentous fungi including the dermatophytes *Microsporum gypseum* and *Trichophyton granulosum*.[46,47] The minimum inhibitory concentrations (MIC) of RPM against ten strains of *C albicans* were in the range of less than 0.02 to 0.2 μg/ml, representing greater potency than that of amphotericin B, nystatin, or candicidin in this assay. RPM has no antibacterial activity. The spectrum of antimicrobial activity of 29-demethoxyrapamycin is similar to RPM, but its potency is only about 25% that of RPM although nearly as potent as amphotericin B.[56]

One study has investigated the mechanisms by which RPM mediates its antifungal effects,[58] and the results of this study are summarized in Table 2. Approximately 90 minutes after adding RPM to *C albicans* cultures, growth is inhibited and subse-

Table 2. Mechanisms of Antifungal Actions of RPM

Test System	*Effect of RPM Treatment*
Sorbose retention by *C albicans*.	Not inhibited
Increased hemolysis of rat red blood cells, efflux of K^+, Pi, UV-absorbing material from *C albicans*.	Not increased
C albicans anerobic glycolysis, aerobic respiration.	Not inhibited
Protein synthesis by cell-free preparations of *C albicans*, *E coli*, rat liver, and mitochondrial preparations of *C albicans*.	Not inhibited
Amino acid metabolism by glutamic-oxaloacetic transaminase, glutamic-pyruvate transaminase in *C albicans*.	Not inhibited
Glucosamine and N-acetyl-glucosamine incorporation into whole *C albicans*.	Not inhibited
Oxidative deamination of glutamic and aspartic acids in *C albicans*.	Inhibited
Incorporation of glucose into mannan in *C albicans*.	Inhibited
Incorporation of Na acetate and methionine into total lipid of *C albicans*.	Inhibited
Incorporation of adenine and phosphate into RNA and DNA of *C albicans*.	Inhibited more for RNA than DNA
Degradation of ^{32}P-labelled intracellular macromolecules and leakage through *C albicans* membrane.	Increased

quently yeast cells lose viability and begin to lyse. The candicidal actions of RPM differ from polyene antibiotics that increase yeast cell permeability by binding to sterols in the cytoplasmic membrane, thus causing leakage of cellular components. Not only do sterols not reverse the actions of RPM, but RPM does not increase the leakage of sorbose or the efflux of potassium, phosphate, or UV-absorbing materials from yeast cells.

The effects of RPM on other metabolic systems of *C albicans* have also been investigated.[58] For example, RPM does not inhibit anaerobic glycolysis or aerobic respiration, nor does it inhibit the incorporation of glucosamine or N-acetyl-glucosamine. RPM does not inhibit protein synthesis in cell-free preparations of *C albicans*, rat liver, or mitochondria from *C albicans*.

Although RPM inhibits the incorporation of glucose into mannan and acetate into lipids, the synthesis of glucan is minimally affected, indicating that inhibition of cell wall synthesis is not the primary site of the antifungal action of RPM.[58]

The most profound effects of RPM on *C albicans* may also provide clues to its actions on mammalian cells. For example, very low concentrations (.02 ~ .1 μg/mL) of RPM inhibit the incorporation of adenine and phosphate into RNA and DNA. At the MIC for RPM, phosphate-containing molecules leak out of the yeast cell membrane. The degradation of these molecules, presumably including nucleic acids, seems to be promoted in some way by RPM.[58]

Physico-Chemical Properties of RPMs

Structure of RPMs

Although the initial analysis of the structure of RPM by infrared and nuclear magnetic resonance (NMR) spectroscopy did not provide the complete picture of its structure,[47] these techniques indicated that RPM was a completely new type of macrolide antibiotic. Ultimately, x-ray crystallographic data clarified the structure of RPM.[59] RPM is a 31-membered macrocycle lactone containing an amide with a C15 carbonyl and a lactone with a C21 carbonyl (Fig 5). Additional analyses of the ^{13}C and ^{1}H NMR spectra of RPM confirmed the x-ray crystal structure of RPM.[59] X-ray studies showed that RPM in its solid crystal form is conformationally homogeneous; in solution however, RPM exists as a mixture of two conformational isomers caused by *trans* to *cis* amide isomerization via hindered rotation about the pipecolic acid N-CO bond. The ratio of *trans* to *cis* rotamers in chloroform solutions is 3 to 4:1.[59,60]

Illustrations of the structure of RPM were initially inconsistent: different enantiomers were drawn,[59] a novel numbering system was used,[59] and incorrect stereochemistry at C28 was represented.[50] Ultimately, the correct structure was published,[61] and the coordinates are deposited in the crystal data bank. Using advanced 2-dimensional NMR spectroscopic methods, new assignments of the proton and carbon spectra for the major rotamer of RPM have been made and a new numbering system suggested.[60]

The closest structural relative to RPM is the antifungal and immunosuppressive macrolide FK506, which is also produced by a streptomycete.[53] FK506 is a 23-membered macrocycle lactone that shares a unique hemiketal masked α,β-diketopipecolic acid amide substructure with RPM,[51] but lacks the C1-C6 triene segment of RPM.

The results of ^{13}C-labelled acetate and propionate

and ^{14}C-labelled methionine incorporation studies of the biosynthesis of RPM were consistent with the proposed polyketide pathway in which the carbons of the lactone ring of RPM are derived from condensation of acetate and propionate units in a manner similar to that responsible for fatty acid synthesis. The methyl group of methionine is an efficient source for the three methoxy carbons of RPM. Because none of the labelled precursors was incorporated into either the cyclohexane or heterocyclic ring, these moieties probably orginate from the shikimate pathway and lysine, respectively.[62]

When ^{1}H and ^{13}C NMR, infrared, UV, mass spectroscopy, and optical rotary dispersion/circular dichroism (ORD/CD) analyses were used to compare the structures of RPM and 29-demethoxyrapamycin, these molecules were shown to be configurationally identical at all chiral centers and to have identical structural features at all but C29. Like RPM, approximately 20% of 29-demethoxyrapamycin in solution exists as the *cis* rotamer form.[63]

In addition to the naturally occurring 29-demethoxyrapamycin, amino acid ester analogues of RPM have been synthesized to produce three water soluble prodrugs of RPM[64] (Fig 5). The amine functions of the appended esters can be converted to water soluble salts that are enzymatically hydrolyzed in the plasma to produce RPM. Although RPM forms both monoester and diester adducts depending on the reaction conditions, only monoester salts are discussed because these are sufficiently water soluble to obviate the need for the disubstituted forms. The 28-hydroxyl group of RPM has been proposed as the site of esterification for each of these prodrugs, but this remains to be confirmed.

Physical Properties of RPMs

Table 3 lists the physical properties of RPM.[47,50,64] Although 29-demethoxyrapamycin is also a white crystalline solid, it has a lower melting point (107° to 108°C) than RPM.[36] Both RPM and its 29-demethoxy form are lipophilic and only minimally soluble in water. The water solubilities of both the mono-N,N-dimethylglycinate methanesulfonic acid salt and the mono-N,N-diethylpropionate hydrochloride salt prodrugs of RPM are more than 50 mg/mL. The water solubility of the mono-4-(pyrrolidino)butyrate hydrochloride salt prodrug is 15 mg/mL.[64]

Because MICs for the antifungal activity of RPM in vitro vary depending on the medium used and the length of the assay, it was suggested that RPM is unstable.[46] Subsequent studies showed that 5 μg/mL of RPM in uninoculated broth loses 80% of its antimicrobial activity after 7 days of incubation at 37°C.[47] Later analysis showed that 50% of the antimicrobial activity of 1 or 5 μg/mL concentrations of RPM are lost after only 24 hours of incubation in culture medium.[38]

High-pressure liquid chromatography (HPLC) has also been used to examine the stability of RPM in

Table 3. Physical and Chemical Properties of RPM

31-Membered macrocyclic lactone $C_{51}H_{79}NO_{13}$ FW = 914.2

3-4:1 ratio of *cis-trans* rotamers about the pipecolic acid N-CO bond

White, crystalline solid MP 183-185 C

Solubility:
- 20 μg/mL in water
- sparingly soluble in ether
- soluble in methanol, ethanol, acetone, chloroform, methylene dichloride, trichloroethane, dimethyl formamide, dimethyl acetamide, dimethyl sulfoxide

Stability (degradation by hydrolysis):

Temperature	Vehicle	pH	T½ (hrs) by HPLC
25°C	acetate buffer	3.3	35.8
	phosphate buffer	7.1	47.6
37.5°C	acetate buffer	3.3	9.9
	phosphate buffer	7.4	10.2
	human plasma	—	3
	rat plasma	—	2.83

different diluents subjected to different temperatures[61] (Table 3). RPM is particularly susceptible to degradation in plasma and in low and neutral pH buffers at 37°C. The degradation products of RPM have less antifungal activity than RPM and are identifiable by HPLC, but their structures are unknown. It is known that base-catalyzed hydrolysis of RPM at reflux temperature produces well-characterized neutral and poorly characterized acidic fragments.[50]

HPLC has also been used to determine the in vitro half lives of the RPM prodrugs in human and rat plasma at 37.5°C before their conversion to RPM. These half lives varied in length between 1 and 5 hours.[64] There are no published stability data on 29-demethoxyrapamycin.

Absorption, Distribution, Metabolism, and Excretion of RPMs

Measurement of Serum Levels of Antifungal Activity

Soon after the discovery of the potent antifungal activity of RPM in vitro, studies were conducted in mice and dogs to determine whether treatment with RPM could produce blood levels that would be sufficient for the compound to be an effective antifungal drug in vivo.[48] The levels of RPM antifungal activity in the sera of dogs and mice were determined by an agar diffusion method that measures the zone of inhibition of *C albicans* growth caused by the antifungal activity of RPM (or RPM metabolites) in a given serum sample. The concentration of this antifungal activity in the serum sample was determined from a standard curve created from zones of inhibition produced by increasing concentrations of pure RPM. Because it is not known whether mammals convert RPM into metabolites that are also highly effective antifungal compounds, the anti-*Candida* activity measured in this assay may not necessarily be caused solely by the RPM parent. Thus, this method of determining RPM serum levels actually defines the pharmacodynamics of antifungal activity after RPM treatment and may not necessarily represent the pharmacokinetics of RPM itself.

Absorption, Distribution, and Elimination of the Antifungal Activity of RPMs

Regardless of whether a 15 mg/kg dose of RPM (micronized in 5% acacia) is administered to mice by the subcutaneous (SC) or oral (PO) route, near maximal concentrations of anti-*Candida* activity are reached within 1 hour. For both routes of administration, relatively constant serum levels of anti-*Candida* activity are maintained for the first 3 hours after dose administration.[48]

Either 250 or 500 mg of RPM in gelatin capsules was administered PO as a single dose to dogs weighing 10 kg and anti-*Candida* serum levels measured. Compared with the mouse, it took longer to reach maximum inhibitory concentrations in the dog: 2 hours for the 250 mg dose and 5 hours for the 500 mg dose.[48]

The bioavailability of the antifungal activity of RPM in the mouse was estimated by comparing the blood levels of anti-*Candida* activity 4 hours after administration of RPM by the SC or PO route. Administration of RPM by the SC route produces a peak level of anti-*Candida* activity in the serum that is equivalent to 4.3 μg/mL of RPM, and a peak serum level equivalent to 1.85 μg/mL of RPM is achieved after PO administration of RPM.[48] These data suggest that RPM (defined as its serum level of antifungal activity) is more bioavailable after systemic injection than by oral administration. The lower bioavailability of RPM administered PO could be caused by degradation of the RPM before absorption, poor absorption, or a significant first-pass effect leading to efficient conversion of RPM by the liver into metabolites that are either incapable of inhibiting *C albicans* growth or are rapidly excreted.

In both the mouse and dog studies previously described, it seemed that a second peak of anti-*Candida* activity followed the first peak, suggesting a role for enterohepatic circulation of RPM or its anti-*Candida* metabolites.[48]

Because the MIC of RPM in the presence of 5% horse serum was found to be greater than 10 μg/mL compared with less than .02 μg/mL in growth medium without serum, it was concluded that the reduced antifungal potency of RPM is caused by its binding to serum constituents in vitro.[48] Despite the inhibition of the antifungal activity of RPM by serum in vitro, antifungal activity is present in the serum after administration of RPM in vivo (discussed previously) and RPM treatment also protects mice against systemic candidiasis.[48] These in vitro and in vivo findings can be reconciled if the binding of RPM to serum is reversible or if RPM is converted in vivo to metabolites with antifungal activity that are not serum bound.

After these studies were completed, HPLC analyses showed that RPM is unstable in plasma (dis-

cussed previously). Therefore, degradation of RPM in serum may be another explanation for the low in vitro anti-*Candida* activity of RPM in the presence of serum and may also contribute to the metabolism of RPM in vivo. Because the stability of RPM in whole blood may differ from the stability of RPM in plasma or serum, it is not possible to extrapolate with certainty from the currently available stability studies. Studies of the stability of RPM in the blood of different species need to be conducted to understand more precisely the fate of RPM in vivo.

Pharmacokinetics of RPMs Measured by HPLC

A reversed phase HPLC technique has been used to monitor the pharmacokinetics of the mono-N,N-dimethylglycinate methanesulfonic salt of RPM and its RPM parent after intravenous (IV) injection in mice.[65] These studies showed that 20 minutes after administration of 100 mg/kg of the prodrug, plasma levels of RPM exceed those of the prodrug. This is followed by steadily decreasing levels of RPM during the first 48 hours after injection. In addition, the area under the curve is not linear with prodrug dose.

Using the HPLC method, a pharmacokinetic analysis of the prodrug in mice[65] showed that the concentration decay of the prodrug in the plasma is triexponential when a dose of 100 mg/kg is administered, but biexponential when lower doses are used. Total body clearance and volume of distribution of the prodrug increases with drug dose. The volume of distribution of the prodrug is 1.74 L/kg and 8.76 L/kg for doses of 10 and 50 mg/kg, respectively. These data indicate that plasma binding of the prodrug is saturable and that excess prodrug is distributed in the tissues.

Although another HPLC method has recently been developed, it has not been used to detect RPM in biological fluids.[91] Clearly, more sensitive assays for RPM and its metabolites are needed. Until additional methods that detect picogram levels of RPM in the blood and tissues become available, the pharmacokinetics, metabolism, distribution, and elimination of RPM and its metabolites will remain largely unknown. The doses of RPM in experimental animal graft recipients that are associated with the highest therapeutic indexes for RPM fail to produce blood levels detectable by currently available analytic techniques. Consequently, we have been unable to correlate dose and blood level with either immunosuppressive drug efficacy or toxicity. Even though a suitably sensitive blood level monitoring technique is not available, the structural similarity between RPM and FK506 suggests that RPM, like FK506,[67] will be distributed widely throughout the body (including red blood cells), principally and completely metabolized by the liver, and excreted in the bile.

Toxicity of RPMs

Limitations of Preclinical Models

Fortunately, there is remarkably good agreement in results among rodent and large animal models of allograft rejection concerning the relative efficacy and indications for use of an immunosuppressant. If the appropriate boundary conditions are set, the results from these experiments are usually highly predictive of the efficacy of a compound in human graft recipients. However, the ultimate value of a new drug is determined not only by its superior efficacy, but by whether the ratio of its toxic dose to its immunosuppressive dose (therapeutic index) is substantially greater than conventional therapies.

Unfortunately, preclinical toxicology data have been poor predictors of the toxicity of immunosuppressive xenobiotics in humans. Animal studies have either underestimated toxic effects ultimately found to be significant in humans (CA and FK506 nephrotoxicity) or certain species of experimental animals have grossly over estimated drug toxicity never noted in humans. For example, now that some of the newer immunosuppressive xenobiotics have completed phase I trials, it is clear that dogs are far more sensitive to the toxic effects of this class of drugs than are humans. Humans have tolerated FK506, MPA, and DSG better than dogs, and the dose-limiting toxicities of these drugs in humans are different from those in dogs.

There is no question that the extreme sensitivity of the dog to the toxicity of these xenobiotics has slowed the progress of the development of several of these agents. In addition, the increasing interest in immunosuppressive monoclonal antibodies and cytokines that are only effective in nonhuman primates places additional limitations on the value of dog models for the evaluation of these new classes of immunosuppressive agents. The determination of a "no toxic effect" dose of a new chemical entity may be the primary value of dogs for immunosuppressive drug development. In view of the sensitivity of the dog to drug toxicity, it is likely that this "no effect" dose in the dog will also be a safe level for the humans to whom the drug is being administered for the first time.

Toxicity of RPMs in Rodents

The toxicity of RPM in humans is not known. Furthermore, none of the published toxic effects of RPM in animals has been generated by formal, Good Laboratory Practices studies. On the contrary, most of the available information on the toxic effects of RPM is a byproduct of studies of the efficacy of the drug, thus limiting the interpretability of the data. Because an IND application for phase I trials of RPM has been submitted to the FDA and because it was accompanied by the necessary and complete toxicology results from rodent and large animal experiments, the extensive information on the toxicity of RPM in animals has been gathered, but has yet to be published.

The data that have been reported[46,48] indicate that mice and rats are very resistant to the acute (single dose) toxic effects of RPM (Table 4). The mouse intraperitoneal median lethal dose (IP LD_{50}) value is considerably higher than the 15 mg/kg IP dose of RPM that produces high serum levels of anti-*Candida* activity in this species. Furthermore, the LD_{50} values for RPM administered IP and by other routes in the mouse and rat are also much greater than the doses of RPM needed to suppress graft rejection and autoimmune diseases in these animals (discussed subsequently: refer to sections headed Effects of RPM on Autoimmune Diseases and Effects of RPM on Graft and Tissue Rejection). Because RPM is administered as multiple doses in most rodent models designed to evaluate efficacy, the subchronic LD_{50} values for mice and rats that have been treated daily for 14 days with RPM would be a more relevant estimate of the therapeutic index of RPM than the acute LD_{50} values shown in Table 4.

Unfortunately, no LD_{50} values from subchronic toxicity studies have been published. However we have administered 24 mg/kg of RPM in suspension in carboxymethyl cellulose IP daily to mice for 2 weeks and then performed necropsies, complete blood counts, and serum chemistries on day 14 or day 28. None of the mice died during treatment or during the 2 week recovery period. The dose level and schedule of RPM administered by this route causes thymic involution, lymphoid cell depletion in the lymph nodes and spleen, and lowers the white blood cell (WBC) count (Zheng B, Morris RE: unpublished observation, 1989). In another study (Zheng B, Morris RE: unpublished observation, 1990), we treated mice IP with 6 mg/kg of RPM for a maximum of 14 days and then necropsied the mice 7, 14, 40, or 102 days after the start of treatment. There was no evidence of renal, cardiac, or liver damage in any of the animals, and the marrow cellularity was normal. Testicular atrophy is a delayed drug effect, because it is observed only in mice necropsied on days 42 and 102. The effects of RPM on the thymus and spleen of these animals are discussed in the section headed Effects on the Morphology and Function of Central Lymphoid Tissues.

Table 4. Acute Toxicity of RPM in Rodents

Animal	*Route*	*Formulation*	*Acute LD_{50} (mg/kg)*
Mouse	IP	Suspension in acacia	597
	PO	Suspension in acacia	>2,500
Rat	IP	Suspension in acacia	>1,600
	PO	Suspension in acacia	>1,600

When 2.5 to 10 mg/kg of RPM was administered PO daily to rats for 7 to 14 days to evaluate the effect of the drug on experimental allergic encephalitis, the only drug-related adverse effect noted was a depression of the growth curve.[49] We have also noted that the rate of weight gain in rat heart allograft recipients treated with RPM is lower than normal, but that weight gain accelerates after cessation of RPM treatment (Morris RE, Wang J: unpublished observation, 1990). Others have also found that rats treated with 50 mg/kg of RPM in oil administered intramuscularly (IM) daily causes a 10% weight loss.[2] The cause for the weight loss could be a direct or indirect effect of RPM on the central nervous system or a direct or indirect effect on the absorption or metabolism of nutrients.

We have noticed that rats treated with RPM have high blood glucose levels that return to normal after cessation of treatment (Morris RE, Wang J: unpublished observation, 1991). In addition, rats treated with RPM once weekly for 50 days often died more than a month after the last dose of RPM of what seemed to be pneumonia that was probably secondary to prolonged and irreversible nonspecific immunosuppression (Morris RE, Wang J: unpublished observation, 1990).

The most complete study[48] of the toxicity of a two-week course of an immunosuppressive dose (1.5 mg/kg as suspension, IP daily) of RPM in rats showed that RPM had no effect on: (1) the marrow; (2) WBC count or its differential, (3) percentage of T cells, T-cell subsets, or B cells; or (4) liver function. RPM treatment does cause elevations in plasma glucose, atrophy of the thymus medulla, and necrosis of the myocardium. When this same immunosuppres-

sive dose of RPM is combined with a high daily dose of CsA (15 mg/kg PO), plasma glucoses are higher.

In mice, the LD_{50} of a single IP dose of 29-demethoxyrapamycin formulated as a suspension in acacia is greater than 900 mg/kg,[36] which is much higher than the LD_{50} for identically formulated and administered RPM in this species (Table 4). The lower toxicity and lower antifungal efficacy in vivo (previously discussed) of 29-demethoxyrapamycin compared with RPM could be caused by poor absorption or increased elimination, or because of its metabolism to less toxic or more rapidly excreted metabolites. However, these reasons alone cannot account for the lower in vitro antifungal activity of 29-demethoxyrapamycin compared with RPM (previously discussed). Therefore, perhaps the methoxy group at C29 contributes directly to the as yet unknown molecular events that are ultimately responsible for the antifungal effects and toxicity in the whole animal (discussed in section headed Molecular Mechanisms of the Antifungal and Immunosuppressive Activities of RPM). There are no other published data on the toxicity of 29-demethoxyrapamycin in rats or larger animals; the LD_{50}s for RPM prodrugs also are not available from the literature.

Toxicity of RPM in Large Animals

Long-term toxicity studies of RPM in dogs show that RPM causes hypoplasia in lymph nodes, spleen, and thymus.[19] The dose, route, and schedule of RPM treatment used in these studies were not stated. In separate studies,[2,69] 0.25 to 5 mg/kg of RPM administered PO daily to dogs also depleted central lymphoid tissues (particularly of B cells) and caused vomiting, diarrhea, and thrombocytopenia. Ulceration occurring from the mouth to the colon secondary to necrotizing fibrinoid vasculitis was also seen.

Pigs treated with 2 mg/kg of RPM PO daily gained weight normally but exhibited microscopic evidence of colitis without vasculitis. After 50 days of treatment with RPM, interstitial pneumonitis occurred in 50% of the animals; this was ascribed to nonspecific immunosuppression.[69]

During the last 2 years we have gained experience treating cynomolgus (*Maccaca fasicularis*) recipients of heart allografts with RPM administered IM and formulated in suspension in carboxymethyl cellulose.[8,9] Other animals were treated with RPM plus IM CsA. No animal was treated for more than 100 days posttransplantation. RPM doses ranged from 0.5 mg/kg every other day (QOD) to 7 mg/kg once per week, and doses of either 4 mg/kg/d or 2 mg/kg QOD of CsA were used. Lethargy and a loss of appetite occurs in animals treated with high-dose RPM. Although animals treated with RPM or CsA lose similar amounts of weight, these weight losses are less severe than in monkey heart graft recipients treated with FK506.[19] In fact, 1 mg/kg of FK506 in suspension administered IM daily causes significant mortality in cynomolgus monkeys.[70,71]

In our recipients, we have also found that high-dose RPM produces hypoplasia of central lymphoid tissues in monkeys. Testicular atrophy with thinning of the seminiferous tubules occurs in all animals necropsied regardless of RPM dose or whether RPM is used alone or combined with CsA. RPM alone or in combination with CsA is not diabetogenic in cynomolgus monkeys and does not cause myocardial necrosis. Necrotizing vasculitis was present in only one animal, but several monkeys showed some microscopic evidence of enterocolitis manifested by lymphoplasmacytic infiltrates in the small and large intestines. The cellularity of the bone marrow in all RPM-treated monkeys was normal. It is not known whether the lethargy observed in animals treated with high-dose RPM alone is a direct or indirect effect of RPM treatment. Despite the administration of RPM doses that are effective for the prolongation of allograft survival, all monkeys have remained free of malignancy during treatment and for as long as 100 days after the last treatment dose. The incidence of severe infection was low in animals treated with only RPM and nonexistent in animals treated with low-dose RPM plus CsA.

The toxicity of RPM in baboon renal allograft recipients is significantly worse than in monkeys treated with RPM. These baboons suffered from vomiting and diarrhea that was probably related to the vasculitis present in their intestines.[72]

Nephrotoxicity of RPM

Although not studied systematically, we have not found any blood chemistry or histopathologic evidence that RPM causes impairment of renal function or damage in rodents treated with doses of RPM that are highly effective for the prolongation of heart allograft survival (Morris RE, Wang J: unpublished observation, 1990).

Also, no indication was found from repeated blood chemistry analyses that treatment with RPM alone or in combination with CsA impairs renal function in cynomolgus monkeys; mild renal tubular atrophy was observed in animals treated with RPM alone.[8,9]

Two studies have investigated the effect of RPM

treatment on renal function. In the first study,[73,74] rats were treated with either 10 mg/kg RPM or 25 mg/kg CsA PO daily for 2 weeks at which time the effect of these drugs on plasma creatinine, creatinine clearance, and renal histology was evaluated. In contrast to treatment with CsA, RPM caused no abnormalities. In the second study,[68] rats were treated daily IP with 1.5 mg/kg of RPM in suspension for 2 weeks. This treatment does not change the urinary flow rate but the plasma creatinine is increased. The kidneys in these animals are histologically normal. Renal impairment is exacerbated when this RPM treatment is combined with PO treatment with 15 mg/kg of CsA.

It seems from these preliminary reports that the order of decreasing susceptibility of different species to RPM toxicity is: (1) dog; (2) cynomolgus monkey and pig; (3) rat; and (4) mouse. Although immunosuppressive doses of RPM seem to be minimally nephrotoxic or nonnephrotoxic in rodents and large animals, this important adverse effect needs to be evaluated more thoroughly before it can be concluded that RPM is less nephrotoxic than CsA or FK506 when equi-effective immunosuppressive doses of each drug are used. In addition to signs of nephrotoxicity, phase I trials of RPM will have to monitor the effects of RPM on the central nervous and immune systems, islet cells, and gastrointestinal tract. Because RPM is a nonspecific immunosuppressant, patients treated with RPM combined with other powerful immunosuppressants will be at risk for infection and malignancy. Once the phase I trials of RPM have defined the toxicity of RPM, we will know which experimental animal best predicts the dose-limiting toxicity of RPM in humans. Hopefully, we will also have a blood level assay for RPM that will enable us to determine the important relationships among RPM doses, immunosuppressive efficacy, toxicity, and RPM blood levels in humans and experimental animals. This information can then be used to understand the mechanisms of RPM toxicity and to devise methods of minimizing its toxicity by implementing improved methods of RPM administration, by combination drug therapy, or by developing immunosuppressive, but less toxic, RPM analogues.

Effects of RPM on *Candida* Infections in Vivo

The first therapeutic use of RPM in experimental animals was as treatment of systemic fungal infections.[18] To evaluate the protective effect of RPM treatment, different doses of RPM or 29-demethoxyrapamycin[36] suspended in 5% acacia were administered PO, SC, or IP to mice 1, 4, and 24 hours after IV injection of *C albicans* cells. Data from this experiment were used to calculate the drug dose that protected 50% of the animals from death (PD_{50}) within the 7 days following the injection of *C albicans* (Table 5). Though RPM and 29-demethoxyrapamycin were administered by slightly different routes, RPM is more potent than 29-demethoxyrapamycin; this is consistent with the difference in the anti-*Candida* potency of these two RPMs in vitro (previously discussed). Reasons for the difference in activity between RPM and its 29-demethoxy derivative are discussed in the sections headed Toxicity of RPM and Molecular Mechanisms of the Antifungal and Immunosuppressive Activities of RPM. Because the PD_{50}s for both RPMs are substantially less than their single dose LD_{50}s (previously discussed), the therapeutic indexes for these compounds for this specific test system are very wide.

Despite the differences in origin, structure, and effects on the immune system among RPM, CsA, FK506, MPA, and DSG, each is both an immunosuppressant and an antibiotic. However, except for RPM, the antibiotic activity of these compounds either is restricted to nonhuman pathogens or is too weak for these agents to be seriously considered as clinically useful. Only RPM has the potential to play dual and complementary roles as an immunosuppressant and antifungal in transplant patients. However, this potential may not be realized because the doses of RPM that provide effective immunosuppression in rodents are much lower than the doses needed for protection against yeast infection in this species. If immunosuppressive doses of RPM in patients are lower than its antifungal doses, the availability of other safe and effective antifungals would obviate the need to risk increasing the dose of RPM simply to exploit its antifungal effects. In the final analysis, the therapeutic profile of RPM in humans will depend on the relative therapeutic indexes for its immunosuppressive and antibiotic effects.

Table 5. Doses of RPM that protect 50% (PD_{50}) of Mice from Death by Systemic Candidiasis

Drug	*Route*	*Formulation*	*PD_{50} (mg/kg)*
RPM	PO	Suspension in acacia	11
RPM	SC	Suspended in acacia	9.5
29-Demethoxy-rapamycin	IP	Suspended in acacia	38

Effects of RPM on Tumor Growth in Vivo

The National Cancer Institute's (NCI) Developmental Therapeutics Program has screened hundreds of thousands of molecules for antineoplastic activity through its Natural Products Program. Because it was well known that *Streptomyces* organisms are prolific producers of antimicrobial molecules, the screening of *Streptomyces* broths initially dominated the search for new antitumor antibiotics. Because RPM (NCI designation, NSC 226080) is a product of *Streptomyces* and is structurally unique, it was evaluated for its ability to inhibit the growth of tumor cells in mice.[31]

Initially, RPM was found to be active against the CDF1 mammary tumor, Colon 38 tumor, and ependymoblastoma at doses of 200, 400, and 25 mg/kg, respectively.[31] The activity of RPMs against mouse tumors was further investigated by evaluating the response of the P388 lymphocytic leukemia, B16 melanocarcinoma, and Colon 38 tumors to treatment with RPM or 29-demethoxyrapamycin[36,75] administered IP daily for 9 days. RPM treatment shows antitumor activity against P388 leukemia and B16 melanoma at a dose range of 12.5 to 100 mg/kg. Doses of 200 to 400 mg/kg of RPM are required to increase the survival of mice with Colon 38 tumors. Interestingly, 29-demethoxyrapamycin has no activity against the B16 melanoma or the Colon 38 tumors; slight activity is observed against the P388 leukemia.

The antitumor efficacy of RPM is route-dependent.[75] A 400 mg/kg dose of RPM is equally effective if injected IP or IM; these two routes are superior to the SC route that is, in turn, more effective than the PO route. Other experiments using the mouse tumor model show that RPM treatment is active against Colon 38 tumors that have become established in the host before the start of RPM treatment and that this same tumor is more susceptible to the antineoplastic activity of RPM + 5-fluorouracil + cyclophosphamide than to 5-fluorouracil + adriamycin + cyclophosphamide.[75]

In a separate study, RPM was used to treat human medulloblastoma TE-671 and glioblastoma multiforme U-251 implanted intracranially in nude mice.[76] Intraperitoneal injections of 100 to 800 mg/kg doses of RPM dissolved in ethanol and diluted with saline to give a final concentration of 10% ethanol increase the length of survival of mice implanted with the U-251 tumor but have no effect on TE-671.

RPM is the most recent addition to the following list of parent compounds that were identified as antitumor agents before their potential or the potential of their analogues as immunosuppressants were appreciated: 6-mercaptopurine (and its analogue azathioprine), cyclophosphamide, MPA (and its analogue RS-61443), spergualin (and its analogue DSG), and BQR. On the other hand, CsA was recognized as a member of a new class of immunosuppressants (to which FK506 also belongs) when initial studies showed it to be immunosuppressive but not to inhibit tumor growth in vivo.[77] Subsequently, it was found that high concentrations of CsA are cytostatic to specific malignant cell lines mainly of T-cell origin.[78] We have shown that low concentrations of RPM are potent and effective inhibitors of the proliferation of both the Jurkat T-cell line and the Daudi B-cell line; even when CsA or FK506 are used at relatively high concentrations, the proliferation of these cell lines is not inhibited.[79] It is interesting to consider that RPM might have been rejected from screens for new immunosuppressants limited solely to the identification of lead compounds that specifically inhibit only lymphocyte activation. RPM seems to be in a class by itself: it is a less specific antiproliferative than CsA and FK506, yet like CsA and FK506 and unlike other more general antiproliferative immunosuppressants, RPM seems to allow bone marrow and intestinal epithelial cells to proliferate normally.

The antineoplastic activity of RPM certainly shows that this molecule has a broad spectrum of pharmacological activity. The antineoplastic actions of RPM in preclinical models seem to be less potent and effective than its antifungal and immunosuppressive activities, but RPM may still have application for the treatment of malignant diseases. Perhaps certain human tumors will be found that are more sensitive than tumors in mice, or maybe the antitumor efficacyof RPM can be increased by optimizing its route and schedule of administration and by combining it with other antineoplastic drugs.

Effects of RPM on Autoimmune Diseases

The recent discovery of the ability of RPM to control allograft rejection in experimental animals,[1-3] has renewed interest in the use of RPM to treat autoimmune diseases (Table 6). Although the current re-

Table 6. Effect of Treatment with RPM in Models of Autoimmune Diseases in Rodents

Model	*Strain/Species*	*Treatment*	*Route*	*Schedule (days)*	*Dose (mg/kg)*	*Effect*
						% Protection from paralysis
EAE	Rat	none	(unspecified)	(unspecified)	–	20
		RPM	PO	0 to 13	5	50
		RPM	PO	0 to 13	10	100
		RPM	PO	7 to 13	10	42
	Rat	RPM	PO	?	9	67
						% Survival (day 192)
SLE	MRL/lpr J mouse	Vehicle	PO	3x/wk	–	33
		RPM	PO	3x/wk	12	80
						% Inhibition of disease
Collagen-induced arthritis	Mouse	RPM	PO	18 to 35 (immunization, day 0)	25	44
						ED_{50}(mg/kg) for
	Rat	RPM	PO	3x/d or 2x/wk	–	Inhibition of development arthritis = 2.3 Inhibition of established arthritis = 3.7 Rebound disease inhibited
						Onset of diabetes inhibited as measured by:
Diabetes	Nonobese diabetic mice	RPM	(unspecified)	(unspecified)	6 or 12	Blood glucose Water consumption

ports describing the potency and efficacy of RPM treatment in murine models of autoimmune diseases are brief,[40,41,80-82] the results of these studies confirm the original report[40] of the activity of RPM for this indication.

Treatment of rats with RPM limits the incidence of complete Freund's adjuvant-induced experimental allergic encephalomyelitis (EAE) more effectively when RPM treatment is begun on the day of adjuvant injection than when treatment with RPM is delayed until day 7.[40] Prolonged treatment with RPM also increases the survival of MRL/lpr J mice in which premature death occurs from systemic lupus erythematosis (SLE).[82] RPM treatment also prevents elevated anti-DNA antibody levels, halts progressive glomerulonephritis-like symptoms, and improves the overall appearance of these mice.[82,40]

Arthritis develops in mice injected in the tail with bovine collagen in complete Freund's adjuvant challenged with lipopolysaccharide and in rats injected in the hindpaw with *Mycobacterium butyricum* in complete Freund's adjuvant. RPM not only inhibits the development of arthritis in mice and rats but also is an effective treatment of established disease in rats and prevents the histopathologic signs of joint destruction in these rodents. The normal rebound disease that occurs after cessation of CsA treatment is prevented by RPM.[40,80,41,83]

Finally, there are indications that treatment of nonobese diabetic mice with RPM inhibits the onset of spontaneous diabetes as measured by blood glucose levels and water consumption.[40]

The successful treatment with RPM of autoimmune diseases in rodents with RPM is provocative for several reasons. The doses of RPM that are effective are well below the doses that cause acute toxicity in rodents and in many cases RPM is more potent and more effective than treatment with CsA. Because the number of patients with autoimmune diseases far exceeds any other patient population that could benefit from treatment with the known immunosuppressive actions of RPM, the investigation of the therapeutic potential of RPM for this indication will continue to be very important. Additional preclinical

and clinical studies will be needed to determine whether the therapeutic index for RPM (alone or combined with other drugs) is sufficiently high to warrant its long-term use in chronic autoimmune diseases that are not immediately life-threatening.

Effects of RPM on Inflammation, the Delayed-Type Hypersensitivity Response, and the Primary Humoral Response in Passive Cutaneous Anaphylaxis

The very first study that showed RPM suppresses EAE in the rat also showed that RPM does not affect the early inflammatory reaction to the adjuvant used to induce EAE.[9] This finding was confirmed in later experiments that indicated that RPM is unable to inhibit the inflammation caused by cargeenan injections into rat paws.[10,11] Studies in vitro have shown that RPM is a poor inhibitor of histamine release from basophils[84,85] (discussed in section headed Effects of RPM on Immune Cells in Vitro).

Oral RPM treatment inhibits the bovine serum-induced delayed-type hypersensitivity response in mice more potently than CsA (median effective dose [ED_{50}]: CsA = 50 mg/kg, RPM = 4 mg/kg).[81]

Treatment of rats with 10 mg/kg of RPM PO inhibits passive cutaneous anaphylaxis caused by IgE-like antibodies stimulated by immunization with egg albumin.[19] Because RPM is only effective if treatment begins on the day of immunization, it was concluded that RPM suppresses the formation of the antibodies causing passive cutaneous anaphylaxis rather than suppressing mediators of the response.

Effects of RPM on Graft and Tissue Rejection

Suppression of Acute Allograft Rejection and Induction of Donor-Specific Unresponsiveness

We reported, for the first time, that RPM administered PO prolongs the survival of secondarily vascularized heterotopic ear-heart allografts in mice and primarily vascularized abdominal heart grafts in rats.[1,86] Publication of this report and in its corrected form[86] was followed shortly by an article describing work performed independently and simultaneously in Cambridge, England.[2] These investigators showed that RPM prolongs abdominal rat heart allograft survival and the survival of pig recipients of kidney allografts. Additional publications from this institution[3-11,79,87,88] and from Cambridge[69,72] have extended these original findings. Subsequently, investigators at the Laboratory for Transplantation Immunology at Stanford University (Morris, RE, Shorthouse R: unpublished observation, 1990) and Eng et al[89] have shown that RPM very effectively and potently prolongs the survival of fully MHC-incompatible skin allografts in mice, and we have found that a brief course of RPM will enable even these highly histoincompatible grafts to survive indefinitely (Morris RE, Shorthouse R: unpublished observations, 1990). Recently, RPM has been used to prolong the survival of rat lung, heart, kidney, pancreas, small bowel, and pancreaticoduodenal allografts.[90-94] Finally, we have shown that RPM suppresses the rejection of abdominal heart allografts in cynomolgus monkeys.[8,9]

Immunosuppressive potency in mouse heart graft recipients. We have now enlarged on our initial evaluation[1] of the effects of the route of administration and formulation of RPM on its potency (Table 7) measured by the quantal BALB/c to C3H/km mouse ear-heart bioassay.[33] A logistic regression analysis of the percent graft survival on posttransplant day 14 on log dose enables the dose producing 50% (ED_{50}) graft survival on day 14 to be estimated (refer to Appendix). Thus, this bioassay provides a sensitive and highly quantitative way to analyze the effect of different variables on immunosuppressive drug potency and to compare the relative potencies among

Table 7. Effect of Route of Administration and Vehicle on Immunosuppressive Potency of RPM, FK506, or CsA by the Quantal Mouse Ear-Heart Assay (BALB/c → C3H)

Drug	*Route*	*Formulation*	*ED_{50} (mg/kg)*
RPM	IP	Suspension in carboxymethyl cellulose	0.23
FK506	IP	Suspension in carboxymethyl cellulose	0.7
RPM	IP	Solution in Cremophor EL/ethanol	~.075
CsA	IP	Solution in Cremophor EL/ethanol	10
RPM	PO	Suspension in carboxymethyl cellulose	7
RPM	PO	Dispersion in oral formulation	1.37
FK506	PO	Suspension in oral formulation	4
RPM	PO	Solution in Cremophor EL/ethanol	0.81
CsA	PO	Solution in Cremophor EL/ethanol in olive oil	21

drugs. This model also presents a particularly stringent test of the potency and efficacy of new immunosuppressants, because rodent recipients of nonprimarily vascularized heart grafts are more resistant to the antirejection effects of immunosuppressants than are recipients of primarily vascularized grafts transplanted into the abdomen.[93]

The immunosuppressive potency data in Table 7 suggest the following conclusions:

1. RPM formulated in Cremophor EL/ethanol is such a potent immunosuppressive agent that it represents, to our knowledge, the most potent xenobiotic immunosuppressant described.
2. RPM is more potent when administered systemically (IP) compared with administration by the PO route.
3. Regardless of the route of administration, RPM formulated in solution (especially in Cremophor EL/ethanol) is more potent than RPM formulated in suspension.
4. Lacking a blood level assay for RPM, oral bioavailability can be approximated by a comparison of the ED_{50}IP and PO of the routes; RPM has higher oral bioavailability when formulated in solution in Cremophor EL/ethanol compared with its bioavailability in suspension, thus providing at least one explanation for the relatively high potency of RPM in solution compared with RPM in suspension.
5. Regardless of its formulation or route of administration, the immunosuppressive potency of RPM is greater than FK506 and much greater than CsA.
6. The oral bioavailability of RPM in suspension is less than FK506 specifically formulated[96] for oral use (solid dispersion form in suspension), and RPM in solution is less bioavailable than CsA in solution when both drugs are administered orally.

Improved absorption probably accounts for the improved oral bioavailability of RPM administered in solution compared with its preparation in suspension. The lower oral bioavailability of RPM compared with FK506 and CsA may be caused by the comparatively lower stability of RPM in the gastrointestinal tract, its less efficient absorption from the gut, or a high first-pass effect in the liver. Ideally, the ED_{50} for different drugs should be expressed as μmol/kg rather than mg/kg to account for differences in the formula weights (FW) of the drug, but because RPM (FW = 914), FK506 (FW = 822), and CsA (FW = 1203) have similar FWs it is acceptable to compare their relative ED_{50}s (in units of mg/kg). The high potency of RPM may relate to the sensitivity of the molecular targets of RPM within the immune system to the immunosuppressive effects of this drug. For example, the structure of RPM is particularly suited to its binding to a cytosolic immunophilin (discussed in section headed Molecular Mechanisms of the Antifungal and Immunosuppressive Activity of RPM). In any case, the differences in immunosuppressive potency between RPM and FK506 or CsA suggest that RPM mediates its immunoregulatory effects by mechanisms that differ from the other two drugs. When a blood level assay for RPM becomes available, the relative immunosuppressive potencies of these three drugs can be more accurately compared by deriving the ED_{50} from blood levels of each drug rather than from drug doses.

A multiple dose-response study in mouse skin allograft recipients found that RPM is 151 times more potent than CsA.[89] Although a multiple dose-response study was not performed when CsA or RPM was administered by continuous infusion directly into heterotopic rat heart allografts, the immunosuppressive potency of the dose of RPM that was tested was 10 times greater than CsA.[91]

Finally, the data in Table 7 suggest that, at least for the mouse, the immune system is unusually sensitive to RPM compared with the effects of RPM on other biological systems. For example, administration of RPM to mice produces acute LD_{50}s that are several hundredfold higher than its ED_{50}s from the mouse ear-heart bioassay (Table 4 *v* Table 7). These LD_{50}s were known when we first began evaluating the immunosuppressive activity of RPM in our ear-heart bioassay. The striking difference in the ED_{50} of RPM compared with its LD_{50} was critical to our decision to continue to investigate RPM after the initial use of this compound. Finally, the ED_{50} of RPM for prolongation of graft survival is also substantially lower than the PD_{50} of RPM for protection from death from systemic *C albicans* infection (Table 5 *v* Table 7).

Immunosuppressive efficacy in mouse heart recipients. Selected data from our evaluation of the effect of route of administration, formulation, and dose schedule on the efficacy of RPM on the prolongation of the survival of mouse ear-heart grafts are shown in Table 8. These data extend our previous investigation of these variables[4] and show:

1. A brief course of RPM prolongs heart graft survival indefinitely (Group 7). In this model, graft survival beyond 150 days can be considered indefi-

Table 8. Effect of Route of Administration, Formulation, and Schedule for treatment with RPM, FK506, or CsA in Mouse (BALB/c → C3H) Ear-Heart Transplant Models of Acute, Ongoing, and Accelerated Rejection

Group	*Drug*	*Route*	*Formulation*	*Schedule (days)*	*Dose (mg/kg)*	*Median Graft Survival Time (days)*
1	Saline	IP	—	1 to 13	—	10
2	RPM	PO	Suspension in carboxymethyl cellulose	1 to 13	24	24
3	RPM	PO	Solution in Cremophor EL/ethanol	1 to 13	6	25
4	RPM	PO	Dispersion in oral formulation	1 to 13	6	20
5	FK506	PO	Suspension in oral formulation	1 to 13	24	24
6	CsA	PO	Solution in Cremophor EL/ethanol	1 to 13	48	26
7	RPM	IP	Suspension in carboxymethyl cellulose	1 to 13	12	190
8	RPM	IP	Solution in Cremophor EL/ethanol	1 to 13	48	26
9	FK506	IP	Suspension in carboxymethyl cellulose	1 to 13	12	62
10	CsA	IP	Solution in Cremophor EL/ethanol	1 to 13	25	24
11	RPM	IP	Suspension in carboxymethyl cellulose	1	6	31
12	FK506	IP	Suspension in carboxymethyl cellulose	1	6	10
13	CsA	IP	Solution in Cremophor EL/ethanol	1	6	10
14	RPM	IP	Suspension in carboxymethyl cellulose	6 to 13	24	29
15	RPM	IP	Suspension in carboxymethyl cellulose	−7 to −3	6	26*
16	RPM	IP	Suspension in carboxymethyl cellulose	0 to +13	6	14*
17	RPM	IP	Suspension in carboxymethyl cellulose	−7 to +13	6	113*

*Secondary graft.

nite because even isografts begin to atrophy and stop contracting after this time.

2. IP administration of RPM is more effective than PO-administered RPM (Groups 2 to 4 *v* Groups 7 and 8).
3. When RPM is administered by the PO route, the dose of RPM formulated in suspension must be four times greater than the dose of RPM formulated in solution or dispersion to achieve similar median graft survivals (Group 2 *v* Groups 3 and 4). Although RPM administered IP is more potent when formulated in solution than when formulated in suspension, RPM administered IP is a considerably more effective immunosuppressant when formulated in suspension compared with its formulation in solution (Group 7 *v* 8).
4. To achieve comparable graft survival, less PO RPM formulated in solution or dispersion is needed than either CsA or FK506 formulated specifically for PO use (Groups 3 and 4 *v* 5 and 6).
5. Administration of RPM IP in suspension is far more effective than the same dose of FK506 formulated in suspension or twice the dose of CsA in solution (Group 7 *v* Groups 9 and 10).
6. A single IP dose of RPM in suspension administered on posttransplant day 1 prolongs graft survival; the same dose of FK506 or CsA is completely ineffective (Group 11 *v* Groups 12 and 13).

Immunosuppressive efficacy in rat heart recipients. We have used a fully MHC-mismatched, primarily vascularized heterotopic rat heart allograft model in which Brown Norway hearts are transplanted into high-responder Lewis recipients to learn more about the immunosuppressive effects of RPM (Table 9).[1,3,3-11] Graft contractility was not assessed after 200 days. By administering the same dose of RPM in suspension by different routes, we showed that the route descending order of immunosuppressive efficacy is IP, IM, SC, and PO (Groups 2, 3, 4, and 6). Administration of RPM IP in suspension for 2 weeks causes all heart grafts to be accepted indefinitely (Group 6). A daily IV dose of .025 mg/kg of RPM in solution effectively prolongs graft survival (Group 7). Not all formulations of RPM in suspension are equivalently immunosuppressive: RPM dissolved in ethanol and then precipitated out of solution by dilution of the ethanol stock solution in an aqueous carboxymethyl cellulose vehicle loses immunosuppressive efficacy (Group 4 *v* 5). Formulation of RPM in dispersion improves the immunosuppressive efficacy of RPM administered PO compared with RPM formulated in suspension (Group 8 *v* 9).

We have yet to find a dose of RPM formulated in suspension and administered IM that is not effective; a dose of .75 mg/kg is the lowest dose we have used and it prolongs graft survival to a median of 21 days (data not shown).

Of considerable interest was our finding that a single dose of RPM in suspension on posttransplant day one prolongs the survival of heart grafts much longer than treatment with either FK506 or CsA

Table 9. Effect of Route of Administration, Formulation, and Schedule for Treatment with RPM, FK506, or CsA in Rat (Brown Norway → Lewis) Heterotopic Abdominal Heart Transplant Models of Acute and Ongoing Rejection

Group	*Drug*	*Route*	*Formulation*	*Schedule (days)*	*Dose (mg/kg)*	*Median Graft Survival Time (days)*
1	Saline	IV	—	1 to 14	—	7
2	RPM	PO	Suspension in carboxymethyl cellulose	1 to 14	0.25	7
3	RPM	SC	Suspension in carboxymethyl cellulose	1 to 14	0.25	98
4	RPM	IM	Suspension in carboxymethyl cellulose	1 to 14	0.25	120
5	RPM	IM	Suspension in ethanol/carboxymethyl cellulose	1 to 14	0.25	50
6	RPM	IP	Suspension in carboxymethyl cellulose	1 to 14	0.25	200
7	RPM	IV	Solution in dimethylacetamide	1 to 14	0.025	31
8	RPM	PO	Suspension in carboxymethyl cellulose	1 to 14	6	29
9	RPM	PO	Dispersion in oral formulation	1 to 14	6	42
10	RPM	IP	Suspension in carboxymethyl cellulose	1	12	191
11	RPM	IP	Solution in Cremophor EL/ethanol	1	12	45
12	FK506	IP	Suspension in carboxymethyl cellulose	1	12	43
13	CsA	IP	Solution in Cremaphor EL/ethanol	1	12	17
14	RPM	IP	Suspension in carboxymethyl cellulose	4 to 10	6	140
				11 to 50	3	140
15	RPM	IP	Suspension in carboxymethyl cellulose	5 to 10	6	19
				11 to 50	3	19
16	RPM	IV	Solution in dimethylacetamide	5 to 14	0.5	34

(Group 10 *v* 12 and 13). This same dose of RPM administered identically to Lewis recipients of (Lewis x Brown Norway)F_1 heart grafts prolongs all grafts indefinitely (Morris RE, Meiser BM, Wang J: unpublished observations, 1989). However, formulation of RPM in solution dramatically reduces the immunosuppressive efficacy of one-shot RPM treatment (Group 10 *v* 11).

The conclusions from our studies of the efficacy of RPM in both mouse and rat heart graft recipients are remarkably consistent:

1. A brief treatment with low doses of RPM prolongs heart grafts in mice and rats indefinitely.
2. The immunosuppressive efficacy of RPM is highly route-dependent, thus confirming our studies of the immunosuppressive potency of RPM and the work of others investigating the antifungal and antitumor efficacy of RPM in vivo (previously discussed).
3. The immunosuppressive efficacy of RPM is also very dependent on its formulation.
4. The combination of the optimum route of administration (IP) with the optimum formulation (suspension in carboxymethyl cellulose) is required for RPM to reach its full potential as an immunosuppressant.
5. The immunosuppressive efficacy of RPM is remarkably schedule-independent because graft prolongation continues well beyond the period of RPM treatment.
6. For all routes, formulations and schedules for treatment in the mouse and for most conditions in the rat, RPM is a more effective immunosuppressant than FK506 or CsA.

Interestingly, the studies of RPM treatment of mouse heart graft recipients showed that RPM administered IP is most potent when formulated in solution and is most effective when formulated in suspension. These results can be reconciled as follows: potency (ED_{50}) is derived from the percentage of grafts surviving on posttransplant day 14 as a function of RPM dose. Perhaps RPM in solution is more potent than RPM in suspension because a greater proportion of a given dose of soluble RPM reaches targets in the immune system than does the same dose of RPM in particulate form.

In contrast to potency, immunosuppressive efficacy is measured by the time it takes for grafts to reject. Because treatment of graft recipients with RPM has been arbitrarily restricted solely to the first 2 posttransplant weeks, the immunosuppressive efficacy of RPM depends on the perpetuation of its effects on the immune system after the last dose. Therefore, although RPM formulated in solution may be efficiently absorbed immediately after injection, RPM blood levels probably decline rapidly during treatment intervals. In contrast, a "depot-effect" caused by treatment with RPM in suspension may not only insure continued drug release for a period after the last IP dose, but this "depot-effect" may

also contribute to relatively constant RPM blood levels between treatments. A constant or even steadily increasing RPM blood level may be critical to the effects of the drug on the immune system that are required for its unusual ability to prolong graft survival long after the last dose is administered. In fact, high transient RPM blood levels produced by administration RPM in solution may provide effective immunosuppression as long as treatment continues, but might interfere with the complex changes in the immune system required for induction of long-term unresponsiveness. Careful studies of the associations among RPM blood levels, formulations, routes of administration, immunosuppresive efficacy, and effects on immune cell function will be needed to examine the validity of these hypotheses.

Calne et al also found that RPM prolongs abdominal heart graft survival, but the rats in that study required a dose of 50 mg/kg IM to insure long-term (>100 days) graft survival.[2] In addition to a shorter duration of treatment (10 days) and the use of different donor and recipient rat strains, their formulation of RPM in olive oil may have contributed to the lower potency and efficacy of RPM than noted in the Laboratory of Transplantation Immunology at Stanford University. Less easily explained are the results of the thorough studies of Stepkowski et al in which RPM was administered by constant IV infusion for 14 days by an Alzet pump (Alza Corp, Palo Alto, CA) placed in rat heart or renal allograft recipients.[91,91] If, as hypothesized previously, constant blood levels of RPM provide the optimum mode of administration, then results from constant infusion of RPM should be superior to other methods of administration. For example, treatment with .02 mg/kg/d of RPM by infusion did not prolong heart graft survival, but when we treated rats with .025 mg/kg/d by intermittent bolus IV infusion, RPM does prolong graft survival (Table 9, Group 7). Furthermore, treatment of rats with a dose of .8 mg/kg/d of RPM by constant infusion for 14 days produced a median heart graft survival of only 47 days compared with indefinite allograft survival (>200 days) when we treated rats with .75 mg/kg/d of RPM in suspension by daily IM injection for 2 weeks (data not shown). Because we use a donor-recipient combination that is considered to be a strong mismatch, it is difficult to attribute differences in the potency and efficacy of RPM to the differences in the strains of rats used at this institution, and those used by Stepkowski et al. Perhaps because RPM is unstable in solution, especially at 37°C (Table 3), degradation of RPM occurs in the pump and causes rats treated by this method to receive less than the expected dose of active drug.

Immunosuppressive efficacy in cynomolgus monkey heart graft recipients. Although we had gained 2 years experience defining the immunosuppressive profile of thousands of doses of RPM in hundreds of rodent allograft recipients, a daunting number of unknowns remained as we began to design experiments to learn whether RPM could suppress acute allograft rejection of primarily vascularized hearts transplanted into the abdomens of cynomolgus monkeys. Before we began these studies, the only large animal model in which RPM had been shown to be consistently effective was in pig renal allograft recipients[2,69]; the exceptional sensitivity of dogs to the toxic effects of RPM has prevented RPM monotherapy from being an effective immunosuppressant in this species.[2,69] The use of RPM to suppress the rejection of baboon renal allografts also proved very difficult because of the narrow therapeutic index associated with the RPM formulations, routes of administration, and dose schedules used for this study.[72] The initial uneven success of RPM for the control of rejection in large animal graft recipients was of considerable concern to the few investigators working with the drug at that time. Despite the superiority of RPM immunosuppression in rodent graft recipients, a demonstration of the efficacy and safety of RPM in a large animal transplant model that is predictive of the performance of the drug in humans was absolutely necessary before we could even contemplate the design and conduct of clinical trials.

We knew from previous experience that rodent transplant models can be remarkably predictive of the indications in transplantation for which a new immunosuppressant is best suited, but the high therapeutic indexes for immunosuppressive drugs in rodents narrow considerably when these drugs are used in large animal graft recipients. As discussed in the section headed Toxicity of RPMs, if the experience with FK506 is relevant to RPM, the toxicity caused by RPM in the dog is probably not predictive of how RPM will affect humans. Because allograft rejection in the pig is more sensitive to immunosuppression than rejection in the human, and because we had gained experience with the use of FK506 and RS-61443 in cynomolgus monkey heart allograft recipients,[15,19] we chose to evaluate the immunosuppressive efficacy of RPM monotherapy in monkeys. This model provides several advantages: (1) transplantation of the heart does not impair hepatic or renal function; thus hepatic metabolism of drugs is

normal, and impairment of renal function is an accurate indicator of nephrotoxicity caused by drug treatment; (2) because rejection of the graft does not cause the death of the recipient, animals can be followed-up for prolonged periods to evaluate delayed toxicity and recovery of a variety of parameters after drug treatment has ceased; and (3) monoclonal antibodies exist that can be used to quantitate the effect of drug treatment on numbers of peripheral blood lymphoid cells.

Despite these theoretical advantages of using the monkey to test the immunosuppressive efficacy of RPM, we had little appreciation of the toxicity of RPM in this species. Toxicology studies of RPM in the monkey were not only incomplete, but the routes of administration and formulations of RPM differed from those we planned to use. By drawing inferences from our rodent work, the experience of Collier et al with RPM in dogs, pigs, and baboons,[60,72] and our experience plus those of Flavin et al and Todo et al with FK506 in large animals,[19,97] we chose a formulation, dose, and route of administration for RPM that we believed would be close to providing the optimum therapeutic index.

In fact, it seems that our initial choice of conditions for the use of RPM was far from ideal. Our first cynomolgus monkey heart allograft recipient was treated daily with 1 mg/kg of RPM in suspension in an ethanol/carboxymethyl cellulose vehicle. This animal was anorexic throughout the posttransplant period and died secondary to intraabdominal fat necrosis on day 13. At necropsy, the graft was found to be acutely rejected (Morris RE, Wang J, Lolini PD: unpublished observation, 1991). The combination of lethal toxicity and lack of immunosuppression bode poorly for the clinical use of RPM. We had always prepared RPM for use in rodents by suspending nonsterile RPM powder in an aqueous carboxymethyl cellulose vehicle. Because we believed it was necessary to administer sterile RPM to monkeys, we dissolved RPM in ethanol so it could be filter sterilized. The sterile solution was then diluted in sterile aqueous carboxymethyl cellulose vehicle to precipitate the RPM into a suspension. Because this was a new formulation, it was also evaluated in our primarily vascularized rat heart allograft model. Unlike any RPM formulations we had previously used in rats, this formulation not only causes substantial weight loss, but also prolongs graft survival significantly less effectively than RPM formulated without ethanol (Table 9, Group 4 *v* 5). The reasons for the toxicity of this form of RPM in the rat and monkey and for its limited immunosuppressive efficacy are not known. Perhaps the residual ethanol contributes to increased drug absorption leading to high RPM blood levels that cause toxicity; perhaps immunosuppressive efficacy is diminished if high blood levels are followed by rapid conversion of RPM into inactive metabolities.

All subsequent monkey graft recipients were treated IM with RPM formulated from unsterile RPM powder suspended in a sterile solution of carboxymethyl cellulose (Table 10). Although our experience in rodents showed that the immunosuppressive efficacy of RPM is optimum when the drug is formulated in suspension and administered systemically, we did not know what dose or treatment schedule would be effective and safe in the monkey. Information was available on the pharmacokinetics of FK506 in suspension and administered IM to dogs.

Table 10. Effect of Treatment with RPM or RPM plus CsA on the Survival of Heterotopic Heart Allografts Transplanted into Cynomolgus Monkey Recipients

Drugs	*Dose (mg/kg)*	*Schedule*	*Route*	*Formulation*	*Median Graft Survival Time (days)*
Vehicle	—	1 to 30	PO	Solution in carboxymethyl cellulose	8
RPM	0.5	1 to 25 QOD	IM	Suspension in carboxymethyl cellulose	
	1	27 to 100 QOD	IM	Suspension in carboxymethyl cellulose	45
RPM	2	27 to 100 QOD	IM	Suspension in carboxymethyl cellulose	41
RPM	7	1 to 100 Q7D	IM	Suspension in carboxymethyl cellulose	36
CsA	4	1 to 15 QD	IM	Oral solution	
	2	17 to 100 QOD	IM	Oral solution	26.5
RPM	1	1 to 100 QOD	IM	Suspension in carboxymethyl cellulose	
+ CsA	4	1 to 15 QD	IM	Oral solution	
	2	17 to 100 QOD	IM	Oral solution	100

This study showed that sustained plasma levels of FK506 are maintained for 24 hours following injection.[98] Extrapolating from these results, we decided to administer RPM IM QOD to avoid the possibility of steadily increasing blood levels caused by daily treatment. When we found that an RPM dose of .5 mg/kg was well tolerated in the 1st posttransplant month, we increased the dose to 1 mg/kg QOD. No animals were treated longer than 100 days. The graft in one of the three animals in this group experienced a rejection episode on day 154 that was so severe that graft contractility was not palpable; later, the graft spontaneously began to contract until complete rejection ensued on day 232. Because two other recipients in this group rejected their grafts during treatment, we used a higher cumulative weekly dose of RPM for the recipients in the next two groups. Animals in both groups were treated IM with a total weekly dose of 7 mg/kg: in one group, this dose was administered as a 2 mg/kg dose QOD, and in the other group 7 mg/kg was administered once per week.

Regardless of the RPM dose and treatment schedule, the median graft survivals for all three groups were similar. As we found in our rodent studies, the immunosuppressive effects of RPM are long lasting because once weekly treatment with RPM prolongs graft survival. Four of the recipients in the two high-dose groups, comprising a total of eight monkeys, rejected their grafts during the treatment period. Two monkeys had to be killed during treatment and three after treament had ceased because of complications most likely related to RPM treatment. These results indicated that although these doses failed to prevent rejection in all monkeys, higher doses would likely be more toxic. Taking into account surface area differences, an RPM dose of 2 mg/kg administered to monkeys is approximately equivalent to 8 mg/kg in the mouse and 4 mg/kg in the rat. Thus, even though relatively high RPM doses were used for the treatment of monkey recipients and the duration of treatment was prolonged, the immunosuppressive efficacy and therapeutic index of RPM in monkey recipients of heart allografts is less than for rodent allograft recipients. Differences in the pharmacokinetics and metabolism of RPM, expression of donor histocompatibility antigens, sensitivity to RPM toxicity and the susceptibility to induction of long-term unresponsiveness between rodents and nonhuman primates all may contribute to the greater immunosuppressive efficacy and therapeutic index of RPM in the rodent compared with the nonhuman primate.

Induction of donor-specific unresponsiveness. We have begun to examine the immunologic specificity of the nonresponsiveness to allografts induced in rat and mouse recipients treated IP with a 2-week course of RPM in suspension.[6] To test for donor-specific unresponsiveness, we created a technique for the simultaneous transplantation of third-party and isogeneic heart grafts into Lewis rats that had not rejected their primarily vascularized abdominal Brown Norway grafts.[13] Adult rat atrial tissue transplanted in the subrenal capsular space becomes vascularized and will contract. These grafts can be monitored for viability by visual inspection through a small diameter arthroscope inserted into the peritoneum. Using this technique, we found that Lewis rats that had failed to reject their original abdominal whole heart allografts for more than 200 days are fully capable of rejecting third-party ACI atrial tissue grafts, but do not reject simulataneously transplanted atrial grafts isogeneic to the donor of the primary whole heart graft.

In similar experiments in mice we found that the induction of specific unresponsiveness to alloantigens is preceded by a period of nonspecific unresponsiveness. Alloantigen must be present for this specifically unresponsive state to be induced and perpetuated. Specifically, survival of C57Bl/6 heart tissue is prolonged if these grafts are transplanted on day 35 into the contralateral ears of C3H recipients of primary BALB/c grafts that were treated with RPM for the first 13 posttransplant days (Morris RE: unpublished observations, 1991). In contrast, the survival of secondary BALB/c grafts, but not secondary third-party C57Bl/6 grafts, is prolonged when these grafts are implanted 85 days after transplantation of primary BALB/c grafts into RPM-treated C3H recipients. If C3H mice are treated with RPM, but no BALB/c heart grafts are implanted on day 0, these mice are fully capable of rejecting BALB/c grafts transplanted on day 85. Even if primary BALB/c grafts remain in RPM-treated C3H recipients before their removal on day 42, long-term survival of secondary BALB/c grafts transplanted on day 85 does not occur. Current experiments are designed to determine whether this state of specific unresponsiveness is caused by central or peripheral clonal deletion or anergy, or possibly active suppression by antibodies or suppressor or veto cells, or a combination of some or all of these mechanisms.

Prevention of Graft Vessel Disease Caused by Chronic Allograft Rejection

For reasons that are unclear and are continuing to be investigated at this institution, treatment of Lewis recipients with certain doses of FK506 causes severe graft coronary disease in both Brown Norway heterotopic abdominal heart allografts and Lewis isografts, but not in the native hearts of Lewis recipients.[10,11] This complication of FK506 therapy has not been observed in cynomolgus recipients of heart allografts and an increase in the incidence of this disease has not been noted in human recipients of heart grafts.[19] Nevertheless, the structural similarity between FK506 and RPM and the similarity between some of their toxicities in rats (weight loss, diabetogenic effects) prompted us to investigate the histopathology of Brown Norway hearts that had been transplanted into Lewis recipients treated IP with 1.5 mg/kg of RPM daily until the donor hearts were removed on day 50. There was essentially no evidence of cellular rejection in the graft interstitium. Furthermore, not only did treatment with RPM not accelerate graft vessel disease, but graft vessel disease was completely prevented. The same dose and schedule of CsA treatment does not exacerbate graft vessel disease, but neither does it completely prevent its occurrence.[10,11]

Despite improved early graft survival in the CsA era, graft vessel disease remains a significant cause of graft loss after 1 year. The pathophysiology of graft vessel disease and the even more clinically significant obliterative disease that occurs in native coronary vessels in nontransplanted patients are not well understood. Inflammatory growth factor-dependent cell proliferation and immune processes may play important roles. In addition to CsA, FK506, and RPM, other drugs (MPA, MZR, BQR, DSG), and monoclonal antibodies that have different antiinflammatory, antiproliferative, and immunosuppressive effects are now available. It is hoped that these drugs will provide powerful and specific tools to understand the mechanisms responsible for vessel pathology and, thus, help in the development of effective means for the treatment and prevention of vascular disease in native and graft vessels.

Treatment of Acute Ongoing Allograft Rejection

As part of our systematic study of the effects of RPM on the control of graft rejection, we investigated the ability of this new immunosuppressant to halt or reverse ongoing rejection. Experiments in non-treated or vehicle-treated C3H mouse recipients of BALB/c ear-heart grafts, show clear evidence of cellular infiltration of allografts on posttransplant day 4 and rapid progression of rejection until the completion of this process on day 10.[21] We initially demonstrated that when treatment with RPM is delayed until day 4, a mean graft survival of 107 days occurs.[1] RPM treatment that is delayed until the rejection process is even more advanced on day 6 also prolongs graft survival (Table 8, Group 14), but not as effectively as when treatment is begun on day 4.

Our recent work in the rat abdominal heart model suggests that it may be critical to achieve therapeutic blood levels of RPM immediately when treating advanced rejection. For example, although IP administration of RPM in suspension beginning 4 days after transplantation of Brown Norway Hearts into Lewis recipients produces a median graft survival of 140 days, the same treatment begun on day 5 and continued until rejection prolongs grafts for a median of only 19 days (Table 9, Group 14 *v* 15). When RPM treatment is begun on day 4, there is histological evidence that progression of rejection is not only halted but is also reversed (Morris RE, Wang J: unpublished observations, 1990). Because the injection of particulate RPM IP is unlikely to produce adequate blood levels immediately after injection, and because rejection proceeds rapidly on day 5 to cause cessation of graft contractions as early as day 6, RPM was administered in solution IV on day 5 to insure that therapeutic blood levels of RPM are achieved before further rejection occurred. Even though we stopped treatment on day 14, this route of administration enables grafts to survive for a median of 34 days (Table 9, Group 16) (Morris RE, Wang J: unpublished observations, 1991). Other investigators recently have shown that when treatment of rat heart, kidney, or pancreas graft recipients with RPM is delayed until day 4, prolonged graft survival is achieved.[9]

These data show that RPM is an effective immunosuppressant for advanced rejection. RPM does not have the same antiinflammatory effects as CsA and FK506 (discussed in section headed Effects of RPM on Inflammation), therefore RPM must suppress ongoing rejection by other mechanisms related to its effects on inhibition of T- and B-cell proliferation, and the actions of cytokines on immune and nonimmune cells in the central lymphoid tissues and the graft (discussed in section headed Effects of RPM on Immune Cells in Vitro). These results indicate that RPM should be tested in nonhuman primate graft

recipients to determine whether it safely halts or reverses advanced graft rejection in this species. These preclinical studies will provide the foundation both for a decision on whether clinical trials for this indication are warranted and for the optimum design of these trials. Perhaps we will have the opportunity to use RPM as maintenance immunosuppression and have the flexibility to briefly increase the dose transiently to reverse acute rejection episodes.

Suppression and Treatment of Accelerated Allograft Rejection

As part of the routine evaluation of any new immunosuppressive molecule that has been shown to be effective for the suppression of acute and ongoing allograft rejection, we determine its efficacy in a mouse model of accelerated rejection.[18,20,21] In this ear-heart model, a primary graft of BALB/c heart tissue is transplanted on day −7 (relative to the secondary graft transplanted on day 0). The primary graft is then removed on day −3 and the survival of the secondary graft is determined. Without immunosuppression, secondary grafts rarely survive beyond day 8. Because primary grafts in nonimmunosuppressed, nonsensitized recipients all survive beyond day 8, rejection of secondary grafts is accelerated. The test compound is administered: (1) only during the period of sensitization (days −7 to −3) to determine whether the drug can prevent sensitization; (2) only for 14 days after the secondary graft has been implanted (days 0 to +13) to learn whether the immunosuppressant can suppress a primed immune system; or (3) throughout the period of sensitization and for 13 days after transplantation of the secondary graft (days −7 to +13).

The results of a study of the effect of treatment with RPM in this model show that RPM can prevent accelerated rejection if it is administered only during sensitization (Table 8, Group 15). Even if treatment with RPM (days 0 to +13) is not begun until after sensitization has occurred, accelerated rejection is delayed (Group 16). The secondary grafts in this group have much shorter survival times than primary grafts because the same dose of RPM prolongs primary grafts in nonsensitized recipients to a median survival of 181 days (data not shown). Therefore, RPM treatment cannot completely erase a preexisting immune response. As demonstrated by the studies of RPM for the treatment of advanced rejection, it may be preferable to administer RPM IV rather than IP in suspension for the control of accelerated rejection so that therapeutic RPM blood levels are achieved immediately. By far, the longest median graft survival (Group 17) in this model is produced by treatment with RPM throughout sensitization and challenge (days −7 to +13). Because this prolongation of survival is still less than the median survival of primary grafts in nonsensitized recipients (181 days), RPM used in this way fails to erase all components of sensitization.

This effect of RPM on an accelerated immune response is noteworthy. It has not yet been demonstrated whether the effects of this drug on this response are primarily restricted to suppression of primed allospecific cells, or their recruitment of other cells that participate in accelerated rejection.

Suppression of Concordant Xenograft Rejection

Despite the marked efficacy of RPM for the prolongation of mouse and rat heart allograft survival, we have found that RPM only weakly prolongs the survival of primarily vascularized hamster hearts transplanted into the abdomens of Lewis rats.[87] Compared with a mean survival of 4.1 days for grafts in untreated control rats, a dose of 9 mg/kg RPM in suspension administered daily from day 1 prolongs graft survival to a mean of only 4.3 days ($P > 0.05$). Only a very high RPM dose of 20 mg/kg produces a statistically significant prolongation of graft survival ($P < 0.05$), but because the mean survival time is only 5 days, the immunosuppressive efficacy is minimal compared with the ability of much lower doses of RPM to prolong the survival of allograft indefinitely. The disparity between the almost complete lack of immunosuppressive efficacy of RPM in this system and the activity of RPM as a suppressant of first set and accelerated allograft survival underscores the difference in the immunologic mechanisms responsible for xenograft and allograft rejection.[100] Because a rapid rat antigraft antibody response is a significant component leading to rejection of hamster hearts, RPM may be a poor inhibitor of the memory humoral response. If this is true, and is applicable to humoral responses in humans, it may mean that patients treated with RPM will be able to produce antibodies to antigens to which they have been previously immunized or exposed. Formal studies need to explore the effect of RPM treatment on primary and secondary responses to a variety of antigens.