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OVERVIEW

Balancing the immune system for tolerance: a case for regulatory CD4 cells

E.H. Field, Q. Gao, N. Chen, and T.M. Rouse

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SDZ RAD, A NEW RAPAMYCIN DERIVATIVE

PHARMACOLOGICAL PROPERTIES IN VITRO AND IN VIVO

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Background. **This report describes the preclinical pharmacological profile of the new rapamycin analog, SDZ RAD, i.e., 40-O-(2-hydroxyethyl)-rapamycin.**

Methods. **The pharmacological effects of SDZ RAD were assessed in a variety of in vitro and in vivo models, which included an autoimmune disease model as well as kidney and heart allotransplantation models using different rat strain combinations.**

Results. **SDZ RAD has a mode of action that is different from that of cyclosporine or FK506. In contrast to the latter, SDZ RAD inhibits growth factor-driven cell proliferation in general, as demonstrated for the in vitro cell proliferation of a lymphoid cell line and of vascular smooth muscle cells. SDZ RAD is immunosuppressive in vitro as demonstrated by the inhibition of mouse and human mixed lymphocyte reactions and the inhibition of antigen-driven proliferation of human T-cell clones. The concentrations needed to achieve 50% inhibition in all of these assays fall into the subnanomolar range. SDZ RAD is effective in the in vivo models when given by the oral route in doses ranging between 1 mg/kg/day and 5 mg/kg/day. When compared with rapamycin, the in vitro activity of SDZ RAD is generally about two to three times lower; however, when administered orally, SDZ RAD is at least as active in vivo as rapamycin.**

Conclusions. **In conclusion, SDZ RAD is a new, orally active rapamycin-derivative that is immunosuppressive and that efficiently prevents graft rejection in rat models of allotransplantation. SDZ RAD has therefore been selected for development for use in combination with cyclosporine A to prevent acute and chronic rejection after solid organ allotransplantation.**

It was first reported in 1989 that the macrolide rapamycin (RPM*), a secondary metabolite of *Streptomyces hygroscopicus*, effectively suppresses the rejection of transplanted allogeneic solid organs in experimental animals (*1*, *2*). RPM is of particular interest as a new immunosuppressant because its mode of action is different from that of both cyclosporine (CsA) and FK506. The latter drugs prevent T-cell proliferation by blocking transcriptional activation of early T cell-specific genes, thus inhibiting the production of T-cell growth factors, like interleukin (IL)-2. RPM, in contrast, acts at a later stage of the cell cycle, blocking not the production of growth factors but rather the proliferative signal that is provided by these factors; RPM arrests the cells at the late G1 stage of the cell cycle, preventing them from entering the S phase. (For a review on RPM and its mechanism of action see *3*, *4*). It is of note that this effect of RPM is not restricted to IL-2-driven proliferation of T cells; RPM inhibits growth factor-dependent proliferation in general of any hematopoietic as well as nonhematopoietic cells tested so far (*5–7*), including vascular smooth muscle cells (VSMC) (*8*). The different modes of action of RPM and CsA provide a rationale for synergistic interaction of the two compounds, and this synergism has indeed been demonstrated (*9–11*). Further, the ability to inhibit growth factor-driven cell proliferation makes RPM a potential compound for the prevention of late graft loss due to graft vessel disease (GVD); growth factor-driven proliferation of VSMC leading to intimal thickening and eventually vessel obstruction seems to play a crucial role in the development of GVD (for a review see *12*). RPM has indeed been shown to inhibit arterial intimal thickening in rat recipients of orthotopic femoral artery allografts (*13*) as well as such thickening produced by mechanical injury where no immunological mechanism is involved (*14*).

These features make RPM and RPM analogs very interesting compounds for clinical transplantation. However, development of a proper oral RPM formulation with acceptable stability, bioavailability, and predictability has proven difficult and has impeded successful clinical development. So far, the majority of published preclinical work demonstrating the potent immunosuppressive effect of RPM deals with parenteral administration of the compound (for references see *15*); efficacy of an oral RPM formulation was shown only very recently in a pig and a rat model of allotransplantation (*15*, *16*). However, wide interindividual variation in the pharmacokinetic parameters was noted in the pig study as well as in a recent report on first clinical experience with an oral RPM formulation (*17*).

The formulation of a compound can have a marked effect on clinical outcomes in transplantation, as seen with the introduction of the microemulsion preconcentrate of CsA (Neoral; Sandoz, Basel, Switzerland) (*18–20*). The 40-O-(2-hydroxyethyl)-RPM, SDZ RAD, is a new RPM analog that resulted from our efforts to overcome the formulation problems by chemical derivation, while maintaining the pharmacolog-

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* Abbreviations: CsA, cyclosporine; FCS, fetal calf serum; FKBP12, FK506 binding protein; GVD, graft vessel disease; IC_{50}, concentration of compound needed to reach 50% inhibition; IL, interleukin; MLR, mixed lymphocyte reaction; PBMC, peripheral blood mononuclear cells; RPM, rapamycin; VSMC, vascular smooth muscle cells.

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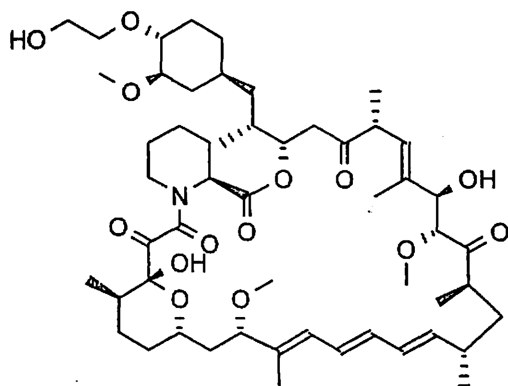

FIGURE 1. Chemical structure of SDZ RAD, 40-O-(2-hydroxyethyl)-RPM.

ical benefits of RPM. In this study we report on the in vitro and in vivo pharmacological characteristics of SDZ RAD. We show that despite a slightly reduced in vitro activity, SDZ RAD has an efficacy after oral dosing that is at least equivalent to that of RPM.

MATERIALS AND METHODS

Reagents

RPM was obtained by fermentation of the actinomycetes strain A91–259211. SDZ RAD, 40-O-(2-hydroxyethyl)-RPM (Fig. 1), was derived by chemical derivation of RPM; the molecular formula is $C_{53}H_{83}NO_{14}$, and it has a molecular weight of 958.25. For the in vitro experiments, 10^{-3} M stock solutions of the compounds in ethanol were used. Stock solutions were stored at −20°C; samples to be tested were diluted on the day of the experiment in phosphate-buffered saline or culture medium. For the in vivo experiments SDZ RAD and RPM were formulated as liquid formulations, which kept the compounds dissolved even after dilution with aqueous vehicles. These formulations were adapted for both compounds with respect to their physicochemical properties. Stored at 4°C, the formulations are stable for >3 months.

In Vitro Assays

The in vitro activity of RPM analogs was assessed by determining in the various assays the concentration of the compounds that results in 50% inhibition (IC_{50}). Serial dilutions of the test compounds, done in duplicate, were tested, and a four-parameter logistic function was applied to calculate the IC_{50} values. RPM was included in each individual experiment as a standard, and the inhibitory activity was expressed as relative IC_{50} compared with RPM (i.e., given as the ratio IC_{50} of test compound/IC_{50} of RPM). To measure in vitro cell proliferation, [^{3}H]thymidine incorporation into DNA was determined following standard procedures.

FKBP12 binding assay. Binding to the FK506 binding protein (FKBP12) was indirectly assessed by means of an ELISA-type competition assay. Microtiter plate wells were coated with FK506 that was covalently coupled to bovine serum albumin. Coupling of FK506 to bovine serum albumin was performed by reacting bovine serum albumin with N-succinimidyl-oxycarbonyl-3′-propionyloxy-33-FK506. Biotinylated recombinant human FKBP12 was allowed to bind to the immobilized FK506 in the absence (as a control) and the presence of the serially diluted test compound or standard. (For technical reasons we used FK506 as the standard, as an exception only in this assay). Bound biotinylated FKBP12 was assessed by incubation with a streptavidin-alkaline phosphatase conjugate, followed by incubation with p-nitrophenol phosphate as a substrate. Readout was the optical density at 405 nm. Binding of a compound to the biotinylated FKBP12 resulted in a decrease in the amount of FKBP12 available for binding to the immobilized FK506, i.e., the magnitude of this inhibition (IC_{50}) reflects the affinity of a compound for FKBP12.

IL-6-driven proliferation of a B-cell hybridoma. The hybridoma B13–29-15 is a subclone of the hybridoma B13–29, which was kindly provided by L. Aarden (Central Laboratory of the Netherlands Red Cross-Blood Transfusion Service, Amsterdam, The Netherlands); this clone is strictly dependent on IL-6. To determine the IC_{50} of a compound in this assay, 10^4 cells per microtiter well (supplemented to contain 0.3 ng of IL-6 per ml) were incubated for 72 hr with serial dilutions of the compounds. [^{3}H]thymidine was added at the end of the incubation period, 5 hr before harvesting the cells for measuring the [^{3}H]thymidine incorporation into DNA.

Mixed lymphocyte reaction (MLR). To determine the IC_{50} values of the compounds in a two-way MLR, 10^5 spleen cells per well each of BALB/c and CBA mice were incubated in flat-bottom microtiter plates, either in the absence or the presence of the serially diluted compounds. Serum-free tissue culture medium supplemented with serum replacement factors (CG medium, Camon GmbH, Wiesbaden, Germany) was used. After 4 days of incubation [^{3}H]thymidine was added, and the cells were harvested after another 16-hr incubation period.

Proliferative response of antigen-specific human T-cell clones. CD4-positive (helper type) T-cell clones specific for the hemagglutinin peptide 307–319 were derived from peripheral blood mononuclear cells (PBMC) of a normal healthy volunteer as described (*21*). To determine the IC_{50} of the compounds in this antigen-specific T-cell proliferation assay, cloned T cells (2×10^4) were cultured in a total volume of 200 μl of RPMI medium (supplemented to contain 5% human AB serum) in 96-well round-bottom microtiter plates with 10^5 irradiated PBMC from normal HLA-DR matched donors, together with the peptide antigen (hemagglutinin) and the serially diluted test compounds. As a control, T cells plus PBMC in the absence of peptide antigen or T cells in medium alone were included. Cultures were set up in duplicate. After 48 hr of incubation, [^{3}H]thymidine was added, and the cells were harvested after another 16 hr.

Proliferation of VSMC. Bovine VSMC were derived by the explant technique from small pieces of media (dissected free of adventitia and intima) from fresh bovine aortae. Explants of about 1 mm^3 were placed in culture dishes, covered with medium, and after about 10 days the cells grew out of the explants. The cells were characterized as VSMC by morphology in culture and by immunostaining with an anti-VSMC actin antibody (clone 1A4; Sigma, St. Louis, MO). They were used at passages 2 through 10. The cells were grown in DF10 medium consisting of equal volumes of Dulbecco's modified Eagle's medium and Ham's F12 (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS) and glutamine. For the experiments, the cells were seeded in 96-well plates (1×10^4 or 2×10^5 per well) and allowed to grow to confluence (3 days). They were then growth arrested by serum deprivation for 48 hr in serum-free medium (DF10 without FCS) supplemented with insulin (0.5 mM; Boehringer Mannheim, Mannheim, Germany), transferrin (5 μg/ml; Sigma), and ascorbate (0.2 mM; Sigma). After 3 days the medium was replaced with fresh medium containing 10% FCS and [^{3}H]thymidine (1 mCi/ml), together with serial dilutions of the compounds to be tested (three to four replicate wells for each concentration). The cells were harvested after a 24-hr incubation period and the [^{3}H]thymidine incorporation into the DNA was measured.

In Vivo Experiments

Throughout all of the in vivo experiments the compounds were given once daily, for the indicated period of time. Freshly prepared

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dilutions of the SDZ RAD and RPM formulations with water were given orally by gavage. Control animals received only the administration vehicle as placebo.

Localized graft-versus-host reaction. Spleen cells (2×10^7) from Wistar/F rats were injected subcutaneously into the right hind footpad of (Wistar/F × Fisher 344)F_1 hybrid rats. The left footpad was left untreated. The animals were treated with SDZ RAD or RPM on 4 consecutive days (days 0–3). The popliteal lymph nodes were removed on day 7, and the weight differences between two corresponding lymph nodes were determined. The results were expressed as the inhibition of lymph node enlargement (given in percent) comparing the lymph node weight differences in the experimental groups to the weight difference between the corresponding lymph nodes from a group of animals left untreated with a test compound.

Mercuric chloride-induced glomerulonephritis. Autoimmune glomerulonephritis was induced by treatment with $HgCl_2$ (*22*). Female Brown Norway rats, 9 weeks of age, were injected subcutaneously during a 3-week period, three times per week, with 1 mg of $HgCl_2$ per kg body weight (10 injections in total). SDZ RAD and RPM were given on 5 consecutive days per week. On days 0, 7, 14, and 21, urine was taken and the protein concentration was determined by means of bromophenol blue staining and colorimetric detection using a TCL scanner. The detection limit of this method is 1 mg protein/ml; the upper threshold of this method is 16 mg protein/ml. The experiment is normally terminated between day 21 and day 24 because the control animals, treated with $HgCl_2$ only, start to succumb to the disease at this point.

Orthotopic kidney allotransplantation. Donor kidneys were transplanted orthotopically into recipient rats. The left kidney of the recipient animal was removed and replaced with the donor kidney, with end-to-end anastomoses of blood vessels and ureter. After 1 week, contralateral nephrectomy was performed, leaving the animal fully dependent on the grafted kidney. Recipients were treated with the immunosuppressive compounds or the placebo for the initial 2 weeks after transplantation.

Vascular heterotopic heart allotransplantation. Donor hearts were transplanted heterotopically into the abdomen of recipient rats by making end-to-side anastomoses of the donor's aorta with the recipient's infrarenal abdominal aorta as well as with the donor's right pulmonary to the recipient's inferior vena cava. Recipients received the immunosuppressive compounds or the placebo once daily for the entire course of the experiment. The heartbeat of the transplanted heart was monitored daily by palpation of the abdomen. The time of rejection was defined as the day on which a heartbeat was no longer palpable.

RESULTS

Binding to FKBP12

Binding to FKBP12, the abundant intracellular binding protein of FK506, is a prerequisite for the biological activity of RPM-type macrolides (*23*). Therefore, we determined the ability of SDZ RAD to bind to FKBP12. As shown in Table 1, binding of SDZ RAD to FKBP12 is about threefold weaker than that of RPM.

Inhibition of Growth Factor-Driven Proliferation

The immunosuppressive activity of RPM is explained by its ability to inhibit growth factor-driven cell proliferation. We assessed SDZ RAD for this effect in two in vitro systems: IL-6-stimulated cell proliferation of the IL-6-dependent hybridoma clone B13–29-15, and FCS-stimulated proliferation of bovine VSMC. Table 2 shows that the ability of SDZ RAD to inhibit the IL-6-driven proliferation of the hybridoma cells is about two- to threefold less compared with that of RPM (i.e., relative IC_{50} of 2.5±0.7). The relative IC_{50} of SDZ RAD

TABLE 1. Binding to FKBP12[a]

Compound	Relative IC_{50}±SD[b] (range, absolute IC_{50})
FK506	1
	(0.8–1.2 nM)
RPM	0.6±0.2* (n=5)
	(0.4–0.9 nM)
SDZ RAD	2.0±0.4*** (n=3)
	(1.8–2.6 nM)

[a] The ability of the compounds to compete with immobilized FK506 for binding to biotinylated FKBP12 was determined in a competitive binding assay.

[b] FK506 was included as a standard in each individual experiment. Results are expressed as means±SD of the relative IC_{50} values (i.e., ratio of IC_{50} test compound to IC_{50} of FK506). The range of absolute IC_{50} values is given in parenthesis; n=number of individual experiments. Statistical analysis, t test: *$P<0.05$; ***$P<0.001$.

TABLE 2. Inhibition of growth factor-stimulated cell proliferation

Compound	Relative IC_{50}±SD[a] (range, absolute IC_{50})	
	Hybridoma B13-29-15/IL-6	Bovine VSMC/FCS
RPM	1	1
	(0.07–0.5 nM)	(0.4–3.5 nM)
SDZ RAD	2.5±0.7** (n=5)	1.9±0.75[ns] (n=3)
	(0.2–1.4 nM)	(0.9–3.6 nM)

[a] RPM was included as a standard in each individual experiment. Results are expressed as means±SD of the relative IC_{50} values (i.e., ratio of IC_{50} test compound to IC_{50} of RPM). The range of absolute IC_{50} values is given in parenthesis; n=number of individual experiments. Statistical analysis, t test: **$P<0.01$; [ns]not significant.

for inhibition of bovine VSMC was 1.9±0.75 (Table 2); however, this was not statistically significant when compared with inhibition by RPM. The absolute IC_{50} values found here for RPM are in agreement with those reported for platelet-derived growth factor or basic fibroblast growth factor-stimulated VSMC proliferation [5 nM and 0.8 nM, respectively (*8*)].

Immunosuppressive Activity In Vitro

The immunosuppressive activity of SDZ RAD was assessed in two-way MLR experiments with lymphocytes of mouse origin as well as in experiments with antigen-specific human helper T-cell clones. The results are shown in Table 3. The data show that, compared with RPM, the in vitro immunosuppressive activity of SDZ RAD is about two- and fivefold lower, respectively, in these assays.

TABLE 3. Immunosuppressive activity in vitro[a]

Compound	Relative IC_{50}±SD[b] (range, absolute IC_{50})	
	MLR	T-cell clone
RPM	1	1
	(0.06–0.9 nM)	(0.014–0.037 nM)
SDZ RAD	2.1±0.4* (n=4)	5.4±3.5* (n=3)
	(0.2–1.6 nM)	(0.05–0.17 nM)

[a] The effect on two-way MLR performed with mouse spleen cells, as well as on the antigen-specific (hemagglutinin peptide 307–319) proliferation of a human T-cell clone, was tested.

[b] As in Table 2. Statistical analysis, t test: *$P<0.05$.

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