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Everolimus in Combination with Cyclosporin A as Pre- and Posttransplantation Immunosuppressive Therapy in Nonmyeloablative Allogeneic Hematopoietic Stem Cell Transplantation

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Everolimus (RAD001) is an mTOR inhibitor that has been successfully used as an immunosuppressant in solid-organ transplantation. Data in allogeneic hematopoietic stem cell transplantation (HSCT) is limited. This study aimed to investigate pharmacokinetics, safety, and efficacy of RAD001 in a canine allogeneic HSCT model. First, pharmacokinetics of RAD001 were performed in healthy dogs in order to determine the appropriate dosing. Doses of 0.25 mg RAD001 twice daily in combination with 15 mg/kg cyclosporin A (CsA) twice daily were identified as appropriate starting doses to achieve the targeted range of RAD001 (3-8 μg/L) when orally administered. Subsequently, 10 dogs were transplanted using 2 Gy total body irradiation (TBI) for conditioning and 0.25 mg RAD001 twice daily plus 15 mg/kg CsA twice daily for pre- and posttransplantation immunosuppression. Seven of the 10 transplanted dogs were maintained at the starting RAD001 dose throughout the study. For the remaining 3 dogs, dose adjustments were necessary. RAD001 accumulation over time did not occur. All dogs initially engrafted. Five dogs eventually rejected the graft (weeks 10, 10, 13, 27, and 56). Two dogs died of pneumonia (weeks 8 and 72) but were chimeric until then. Total cholesterol rose from median 4.1 mmol/L (3.5-5.7 mmol/L) before HSCT to 6.0 mmol/l (5.0-8.5 mmol/l) at day 21 after HSCT, but remained always within normal range. Changes in creatinine and triglyceride values were not observed. Long-term engraftment rates were inferior to sirolimus/CsA and mycophenolate mofetil (MMF)/CsA regimen, respectively. RAD001/CsA caused a more pronounced reduction of platelet counts to median 2×10^9/L (range: 0-21 $\times 10^9$/L) and longer time to platelet recovery of 21 days (range: 14-24 days) compared with MMF/CsA. CsA c_{2h} levels were significantly enhanced in the RAD001/CsA regimen, but c_{0h} and area under the curve from 0 to 12 hours ($AUC_{0\text{-}12h}$) values did not differ compared with an MMF/CsA immunosuppression. In summary, immunosuppression consisting of RAD001 and CsA is well tolerated but not as efficient as with other established immunosuppressants in a canine nonmyeloablative HSCT regimen. Hence, our study does not support the application of RAD001/CsA as standard practice in this setting.

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INTRODUCTION

Everolimus (RAD001) is an orally active proliferation signal inhibitor of the mammalian target of rapamycin (mTOR) with immunosuppressive activity. It interferes with the mTOR signaling pathway by inhibiting mTOR phosphorylation activities, thereby halting the translation of proteins critical for proliferation and cell survival. It was successfully used alone or in combination with cyclosporin A (CsA) as an immunosuppressant in solid-organ transplantations [1-3]. Combined application of RAD001 and CsA resulted in synergistic immunosuppressive activity that allowed CsA dose reduction and minimized CsA-related nephrotoxicity. Comparative analyses revealed that immunosuppression with RAD001/CsA resulted in equivalent graft survival

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and rejection rates compared with mycophenolate mofetil (MMF)/CsA after renal transplantation and in superior efficacy compared with azathioprine/CsA in cardiac transplant recipients [2,4]. In addition, RAD001 significantly reduced the incidence of viral infections, particularly cytomegalovirus (CMV), in heart and kidney transplantation [4,5]. However, RAD001/CsA administration was associated with increased incidents of renal dysfunction when used with full-dose CsA, hyperlipidemia, and thrombocytopenia compared with MMF/CsA. The exposure-response relationship and the safety profile determined in solid-organ transplantations support a therapeutic range of RAD001 trough level of 3-8 μg/L.

Studies on the application of RAD001 in hematopoietic stem cell transplantation (HSCT) are limited. One study used RAD001 in combination with tracrolimus as graft-versus-host disease (GVHD)-prophylaxis in allogeneic HSCT following busulfan-based intensive conditioning [6]. This study was prematurely terminated because of an increased rate of sinusoidal obstruction syndrome.

Here we asked whether RAD001 can be combined with CsA as pre- and posttransplantation immunosuppression in a nonmyeloablative HSCT setting. Therefore, we investigated the pharmacokinetics as well as the efficacy and safety of a combined immunosuppression consisting of RAD001 and CsA after a 2 Gy total body irradiation (TBI) conditioning in a canine allogeneic HSCT model.

MATERIALS AND METHODS

Laboratory Animals

Litters of random-bred beagles were purchased from commercial kennels licensed by the district administration of the county of Paderborn. All dogs were routinely dewormed and obtained standard immunization against rabies, parainfluenza, leptospirosis, distemper, hepatitis, and parvovirus. Furthermore, custom-prepared canine papilloma virus vaccine was given. This study was approved by the review board of the State Institute for Agriculture, Food Safety and Fishery Mecklenburg-Vorpommern, Germany.

Dog leukocyte antigen (DLA)-identical donor/recipient sibling pairs were selected on the basis of matching for highly polymorphic DLA class I and class II microsatellite markers and DLA-DRB1 single-strand conformation polymorphism analysis [7,8]. At study entry, dogs weighed a median of 12.5 kg (range: 9.8-14.6 kg) and were a median of 16 months (range: 11-29 months) old.

Pharmacokinetic Assessment

Pharmacokinetic studies targeted at RAD001 trough levels in the range of 3-8 μg/L as recommended in solid-organ transplantations. Therefore, healthy dogs were given doses of 1.5, 0.5, and 0.25 mg of RAD001 twice daily orally alone and in combination with 15 mg/kg CsA twice daily orally for 5 consecutive days. At day 5, serial EDTA-blood samples were collected for full (9-point) pharmacokinetic studies before and 0.5, 1, 1.5, 2, 3, 4, 6, and 12 hours after the morning dose and stored at −20°C. To exclude an effect of food on RAD001 absorption, dogs had an overnight fast before pharmacokinetic analyses and continued to fast for 4 hours after RAD001 morning dose application. Whole-blood concentrations of RAD001 were determined by fluorescence-polarization-immunoassay with a TDx/FLx analyzer (Abbott Laboratories, Abbott Park, IL) using a RAD001-specific monoclonal antibody (Innofluor® Certican® Assay System; Seradyn Inc., Indianapolis, IN). A noncompartmental analysis (KINETICA 4.4; Thermo Fisher Scientific Inc., Waltham, MA) was used to calculate the maximum concentration (C_{max}), time to reach maximum concentration (T_{max}), and half-life as well as the area under the curve over the 12-hour dosage interval ($AUC_{0\text{-}12h}$).

Pharmacokinetic profiles of transplanted dogs were similarly assessed on days 5 and 21 after HSCT. In addition, whole-blood concentrations of CsA were analyzed in parallel with the RAD001 pharmacokinetics in healthy dogs and after HSCT by a fluorescence-polarization-immunoassay (TDx®/TDxFLx® Cyclosporin monoclonal [whole-blood] assay, Abbott Laboratories) with a TDx/FLx analyzer. CsA AUC were estimated using the following equation developed by Novartis for renal transplant patients: $AUC = 990 + [10.74 \times c_{0h}] + [2.28 \times c_{2h}]$. Several full pharmacokinetics of CSA were recorded as well to prove the applicability of this formula in the canine HSCT model.

HSCT

Dogs (n = 10) were administered 2 Gy of TBI delivered at a dose rate of 0.25 Gy/min from a high-energy linear accelerator (Siemens Primus; 10 MV X-ray). Bone marrow of DLA-identical littermates was collected under general anesthesia from the humeri, femora, and iliac crest. Taking into account animal protection aspects, we reduced the number of required animals by using 5 donors that were sensitized to the recipient by a previous HSCT in which rejection occurred (Table 1). Marrow grafts contained a median of 3.6×10^8 total nucleated cells per kg (range: 1.9-11.8 $\times 10^8$ total nucleated cells/kg) and a median $CD34^+$ cell count of 6.0×10^6 cells/kg (range: 2.6-18.2 $\times 10^6$ cells/kg). Marrow was infused intravenously within 24 hours after TBI. The day of marrow infusion was designated as day 0. Pre- and posttransplantation-immunosuppression consisted of 15 mg/kg CsA (Sandimmun Optoral®, Novartis, Nürnberg, Germany) twice a day orally

Table 1. Marrow Grafts from DLA-Identical Donors, RAD001, and CsA Dose Adjustment and Outcome after 2 Gy TBI HSCT

	Marrow Cells		Dose Adjustment		Max. Donor Chimerism			
Dog	TNC (10^8/kg)	CD34 (10^6/kg)	RAD001 (mg bid)	CsA (mg/kg bid)	Granulocytes (%, [day])	PBMC (%, [day])	Rejection (week)	No Rejection (week)
H6E-5395	11.8	10.3	0.25	15	100 [287]	81 [385]	—	>65
H6E-5868	7.6	6.7	0.25/0.5‡	16.5	100 [28]	33 [378]	—	>64
H6E-5399	6.4	13.7	0.25	15	95 [21]	52 [35]	—	8†
H5L-9233	6.2	18.2	0.125	7.5	82 [28]	62 [21]	56	—
H5F-8690*	3.7	8.2	0.25	15	100 [91]	100 [77]	—	>82
H5F-8686*	3.5	5.3	0.25	15	29 [35]	19 [29]	10	—
H5L-9235*	3.1	3.0	0.25	10	48 [20]	16 [14]	13	—
H6E-5872	2.7	4.0	0.5	7.5	54 [29]	26 [34]	10	—
H6E-5876*	2.2	2.6	0.25	7.5	24 [28]	15 [28]	27	—
H5L-9630*	1.9	3.6	0.25	3.75	100 [70]	100 [77]	—	>71†

DLA indicates dog leukocyte antigen; TNC, total nucleated cell.
*Dogs received grafts from donors that were sensitized to the recipient by a previous HSCT in which rejection occurred.
†Dogs died at days 60 and 503 with mixed and full donor chimerism at death, respectively.
‡Evening dose only was raised to 0.5 mg.

from day −1 to +35 and 0.25 mg RAD001 (Certican®, Novartis) twice a day orally from day 0 to +27. Doses were adjusted to reach RAD001 trough levels of 3-8 μg/L. The endpoints of the study were engraftment, level of chimerism, graft loss, death, and the incidence of GVHD.

Assessment of Hematopoietic Chimerism

Before and after HSCT peripheral blood of the recipients was obtained weekly up to day 77 posttransplantation and in larger intervals thereafter. Granulocyte and peripheral blood mononuclear cell (PBMC) fractions were separated by standard Ficoll-Hypaque density gradient centrifugation (density 1.074 g/mL). Donor/recipient chimerism was also assessed from bone marrow samples aspirated at days 0, 14, 28, 56, and 140 after HSCT. Genomic DNA of peripheral blood cell fractions and unfractionated marrow were isolated (Nucleobond CB 100; Macherey-Nagel, Düren, Germany). Tetranucleotide repeats that were polymorphic between donors and recipients were amplified and quantified as described previously [9].

Toxicity

Toxicity was evaluated from day 0 to day 35 (time of drug administration) after HSCT by assessment of activity, defecation, ingestion, and weight loss. Grade of activity and ingestion was determined according to the following score: grade 0 = normal, grade 1 = reduced, grade 2 = no activity or ingestion, respectively. Defecation was graded as follows: grade 0 = normal stool, grade 1 = loose stool, grade 2 = diarrhea, grade 3 = bloody stool. The grading of weight loss was established according to the following criteria: grade 0 = <0.5 kg loss of body weight, grade 1 = 0.5-1.0 kg weight loss, grade 2 = >1.0 kg weight loss always in regard to starting weight. Hematologic toxicities were evaluated by blood cell counts. Leukopenia and thrombocytopenia were defined as leukocyte counts $<1.0 \times 10^9$/L and platelet counts $<20 \times 10^9$/L. Furthermore, safety assessment included determination of laboratory parameters such as blood lipids and creatinine levels.

Statistics

The distribution of data was described using medians and ranges. Statistical analyses between treatment groups were performed by using the Mann-Whitney *U* test. Within the treatment groups, data of different days were analyzed by the Wilcoxon matched-pairs signed rank test. Probability of $P < .05$ was considered significant.

Table 2. Steady-State Pharmacokinetic Parameters of RAD001 ± CSA in Healthy Dogs

						Platelet Counts	
Doses*	c_{0h} (μg/L)	c_{max} (μg/L)	t_{max} (h)	$AUC_{0\text{-}12h}$ (μg/L × h)	Half-life (h)	day 0	day 8
1.5 mg RAD001 alone (n = 4)	6.2 (4.8-13.1)	16.2 (12.6-27.1)	1.5 (1.0-1.5)	117 (96-197)	10.5 (6.2-19.1)	331 (298-352)	242 (195-277)
1.5 mg RAD001 + CsA (n = 2)	28.7 (24.1-33.4)	77.2 (76.7-77.6)	2.3 (1.5-3.0)	643 (579-707)	11.1 (8.2-14.0)	296 (285-307)	149 (103-195)
0.5 mg RAD001 + CsA (n = 2)	14.5 (14.1-14.9)	22.1 (16.8-27.5)	3.0 (2.0-4.0)	190 (159-220)	12.5 (10.0-15.1)	328 (308-347)	173 (155-190)
0.25 mg RAD001 + CsA (n = 2)	6.6 (5.5-7.7)	11.7 (9.4-14.0)	2.8 (1.5-4.0)	97 (85-110)	10.7 (10.0-11.4)	364 (311-416)	223 (179-267)

Half-life indicates elimination half-life.
Data are presented as medians (ranges).
*CsA was given at a constant dose of 15 mg/kg. Indicated doses of RAD001 and CsA were administered twice daily.

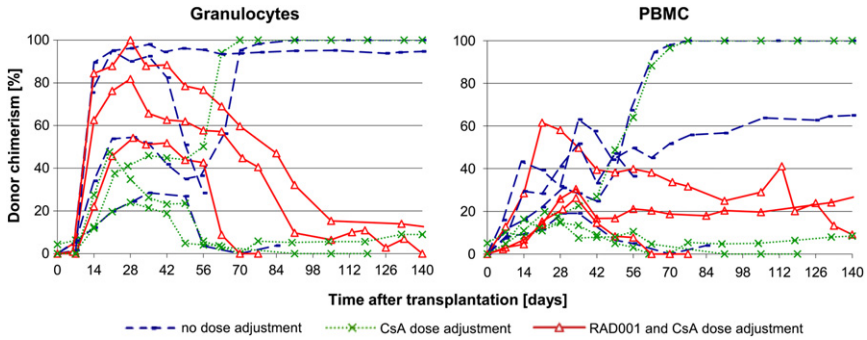

Figure 1. Development of hematopoietic donor chimerism following HSCT (n = 10). Chimerism was determined within the granulocyte as well as within the PBMC compartment. - - - no dose adjustment; ••••x••• CsA dose adjustment; –Δ– RAD001 and CsA dose adjustment.

RESULTS

RAD001 Pharmacokinetics in Healthy Dogs

RAD001 pharmacokinetics alone or in combination with CsA were determined in the blood of healthy dogs in order to establish a suitable dosing scheme for RAD001 in dogs. The steady-state pharmacokinetic parameters at different dose levels are summarized in Table 2.

Initially, 4 dogs received 1.5 mg RAD001 twice daily for 5 consecutive days, resulting in a median trough level of 6.2 ng/mL. Following, dogs received 1.5 mg RAD001 in combination with CSA to investigate potential pharmacokinetic interactions. Under this arrangement, a strong increase in median RAD001 trough levels to 28.7 μg/L was observed. Consequently, RAD001 dose was titrated down to a final dose of 0.25 mg to achieve the targeted range of 3-8 μg/L.

Monitoring of the dogs' blood counts and biochemical parameter on days 0, 5, and 8 did not reveal any evidence of clinically relevant toxicity. However, considering all cases regardless of RAD001 dose, a significant decrease in platelet counts ($P = .005$) from 316 $\times$ 10^9/L (285-416 $\times$ 10^9/L) at day 0 to 195 $\times$ 10^9/L (103-277 $\times$ 10^9/L) at day 8, as well as a significant increase in cholesterol levels ($P = .008$) from 4.5 mmol/L at day 0 to 6.7 mmol/L at day 8 could be observed. Both effects seemed to be RAD001 dose dependent.

Engraftment after HSCT

All 10 dogs showed rapid initial engraftment (Figure 1, Table 1). Five dogs eventually rejected their grafts and survived with complete autologous recovery. One engrafted dog (H6E-5399) died on day 60 as a result of pneumonia but was mixed chimeric until then. The 4 remaining evaluable dogs showed sustained chimerism for >64 weeks of follow-up. One of them (H5L-9630) died because of multiple organ failure following signs of GVHD (skin change, hair loss) and pneumonia 72 weeks posttransplantation, but was full chimeric at that time.

Median maximum PBMC chimerism after RAD001/CsA treatment amounted to 42% (range: 15-100%) at day 35 (range: 14-385 days). Maximum granulocyte donor chimerism of median 88% (range: 24-100%) was obtained at day 29 (range: 20-287 days) after HSCT.

Hematologic Recovery after HSCT

Hematologic toxicities after HSCT were reflected by a moderate decrease in leukocytes and a severe diminution of platelets counts (Figure 2). Median leukocyte

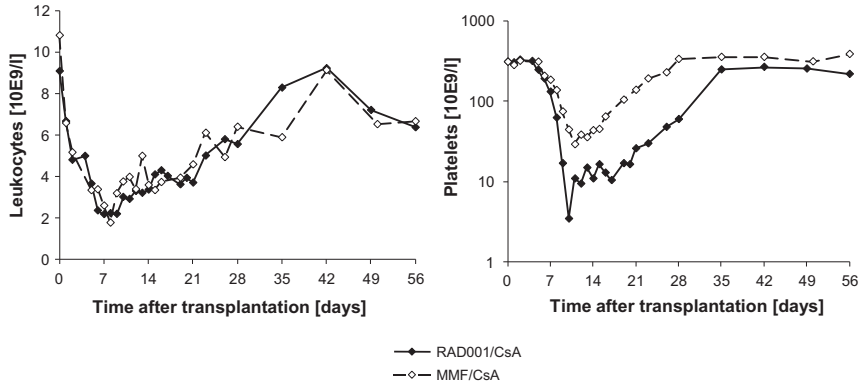

Figure 2. Hematologic recovery in dogs after application of a starting dose of 0.25 mg and 15 mg/kg CsA twice a day after HSCT (n = 10). Median leukocyte and platelet counts after RAD001 treatment (solid line) were compared with the MMF-related toxicity (dotted line, historic data [31]).

Table 3. Pharmacokinetic Parameters of RAD001 in Dogs after HSCT

	Day	c_{0h} (μg/L)	c_{max} (μg/L)	t_{max} (h)	$AUC_{0\text{-}12h}$ (μg/L × h)	Half-life (h)
No RAD001 or CsA adjustment (n = 4)	5	6.3 (4.0-9.8)	11.6 (7.1-15.0)	2.0 (2.0-2.0)	100 (60-129)	11.6 (11.5-13.9)
	21	5.2 (4.0-7.8)	10.4 (9.5-13.0)	1.5 (1.0-4.0)	89 (80-115)	13.3 (12.0-14.3)
CsA adjustment after day 5 (n = 3)	5	7.9 (6.9-12.0)	15.4 (13.5-15.4)	3.0 (1.0-4.0)	138 (124-154)	13.0 (10.9-14.0)
	21	5.3 (4.9-7.4)	10.8 (9.3-13.6)	1.5 (1.5-1.5)	92 (85-113)	11.7 (10.2-22.5)
RAD001 and CsA adjustment after day 5 (n = 3)	5	2.3 (2.0-11.7)	6.3 (5.9-17.3)	2.0 (1.0-3.0)	46 (40-165)	6.2 (5.3-16.8)
	21	5.7 (4.6-8.6)	7.5 (7.0-13.4)	3.0 (1.5-6.0)	72 (67-129)	7.5 (6.7-25.5)
All cases (n = 10)	5	7.2 (2.0-12.0)	13.6 (5.9-17.3)	2.0 (1.0-4.0)	123 (40-165)	11.6 (5.3-16.8)
	21	5.5 (4.0-8.6)	10.4 (7.0-13.6)	1.5 (1.0-6.0)	89 (67-129)	12.5 (6.7-25.5)

Half-life indicates elimination half-life.
Data are presented as medians (ranges).

counts decreased from 9.1 × 10^9/L (range: 7.0-12.1 × 10^9/L) to 1.6 × 10^9/L (range: 0.7-3.2 × 10^9/L) at day 8 (range: 6-11 days). A total of 8 of 10 dogs never became leukopenic. The remaining 2 dogs had a median duration of leukopenia of 1.5 days (range: 1-2 days) and a median time to leukocyte recovery of 9 days (range: 9-9 days). Median platelet levels were 310 × 10^9/L (range: 264-437 × 10^9/L) before HSCT and platelet nadirs amounted to 2 × 10^9/L (range: 0-21 × 10^9/L) at day 11 (range: 10-14 days) posttransplantation. Thrombocytopenia was detected in 9 of 10 dogs with a median duration of 11 days (range: 2-15 days). Time to platelet recovery was 21 days (range: 14-24 days). Six animals received prophylactic transfusions of whole blood or platelet enriched plasma.

Pharmacokinetics after HSCT

Based on the pharmacokinetics in healthy dogs, a RAD001 dose of 0.25 mg twice a day (days 0 to +27) was administered in combination with 15 mg/kg CsA twice a day (days −1 to +35) as pre- and posttransplantation-immunosuppression in HSCT.

Seven of the 10 transplanted dogs were maintained at the starting RAD001 dose of 0.25 mg twice daily throughout the study period. For 1 dog, the dose had to be reduced. The remaining 2 dogs needed a dose increase to achieve the targeted range (Table 1). Data of pharmacokinetic analyses after HSCT are summarized in Table 3 and Figure 3. After dose adjustment, RAD001 trough concentrations reached the targeted range in all dogs, shown at day 21 posttransplantation. In 3 of the 7 dogs in which the RAD001 dose was not changed, CsA dose was diminished leading to a concomitant reduction in RAD001 exposure. Comparison of pharmacokinetic parameters at days 5 and 21 at constant RAD001 and CsA doses (n = 4) revealed no evidence of drug accumulation over time.

In parallel to RAD001 pharmacokinetics, CsA whole-blood concentrations were determined in nonmyeloablative transplanted dogs at days 5 and 21 after HSCT. CsA AUC were generally calculated from abbreviated kinetics (c_{0h}, c_{2h}). To prove the applicability of this method in our canine HSCT model, comparisons with AUC values determined from full pharmacokinetics (n = 9) were conducted. Median AUC levels of 12,310 μg/L × h (range: 8736-24,683 μg/L × h) estimated from abbreviated kinetics and 12,439 μg/L × h (range: 9485-25,540 μg/L × h) determined from full kinetics showed a good comparability of both methods (P = .314).

If healthy dogs (n = 6) were compared with transplanted animals (n = 10), no significant differences in

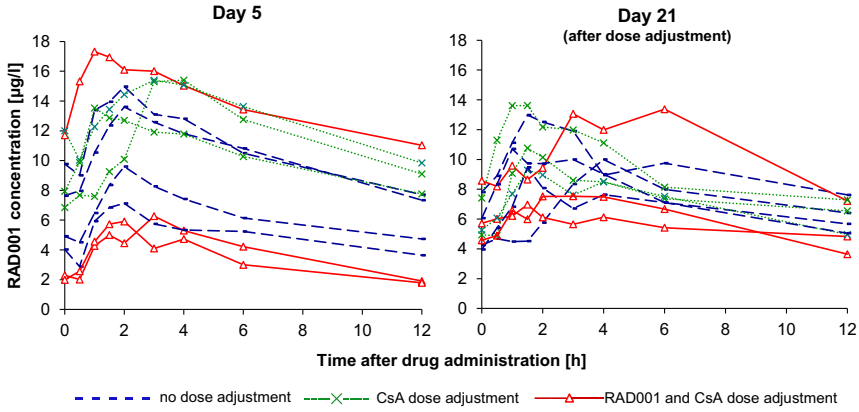

Figure 3. Whole-blood concentration-time profiles of RAD001 in dogs starting with 0.25 mg RAD001 and 15 mg/kg CSA twice a day after HSCT are displayed. Full pharmacokinetic analyses were done at days 5 and 21 after HSCT. In dependence of blood trough levels at day 5 dose adjustment was partially necessary to achieve the targeted range of 3-8 μg/L. --- no dose adjustment; •••••x•••• CsA dose adjustment; –Δ–RAD001 and CsA dose adjustment.

CsA concentrations were detected (data not shown). Data obtained at different times after HSCT at a constant CsA dose revealed no evidence for CsA accumulation at day 21 compared with day 5 (n = 4). Median c_{0h} and c_{2h} values were determined to be 550 μg/L (range: 236-2237 μg/L) and 3264 μg/L (range: 1955-5486 μg/L). The respective $AUC_{0\text{-}12h}$ was median 13,725 μg/L × h and ranged from 8736-36,642 μg/L × h.

Toxicity

Biochemical analyses revealed no changes in creatinine and triglyceride values during the time of drug administration. Total cholesterol significantly rose from a median baseline of 4.1 mmol/L (3.5-5.7 mmol/L) before HSCT to 6.0 mmol/L (5.0-8.5 mmol/L) at day 21 after HSCT. However, these changes always remained within normal range and were reversible, because cholesterol values reached base values (as before HSCT) 15 days after RAD001 was withdrawn.

Evaluation of regimen toxicity was further done for the parameters activity, defecation, ingestion, and weight loss. In every dog, gastrointestinal toxicities were temporarily observed that were characterized mainly by loose stools and less frequently by diarrhea and bloody stools. While on treatment, dogs experienced mild effects on activity and ingestion that resulted in weight loss >0.5 kg in 3 dogs (range: 0.7-1.8 kg).

DISCUSSION

In recent years, RAD001 has been successfully used as an immunosuppressant mainly in kidney and heart transplantation. Because of these positive experiences, further studies were initiated to investigate RAD001 in other areas of transplantation. This study aimed to determine pharmacokinetics, safety, and efficacy of RAD001 in a preclinical HSCT model.

The pharmacokinetic analyses of oral RAD001 monotherapy in healthy dogs revealed comparable c_{max} and AUC values to healthy human volunteers. In several single-dose studies, RAD c_{max} values in the range of 15-21 μg/L and AUC values in the range of 90-115 μg/L × h have been reported [10,11]. In contrast, RAD001 seems to have a considerably shortened half-life of 12 hours in dogs compared with 24-32 hours in humans [10-12]. However, in dogs given 1.5 mg RAD001 twice daily, a trough level of 3-8 μg/L can be easily achieved. Interactions of RAD001 and CsA were investigated in healthy dogs, because both drugs are metabolized primarily by CYP3A isoenzymes and are substrates for the P-glycoprotein efflux transporter. The concomitant administration of 15 mg/kg CsA twice daily increased the steady-state parameter of RAD001 c_{min}, c_{max}, and AUC 5- to 6-fold in our studies. An enhancement of RAD001 exposure was also demonstrated in clinical studies in healthy volunteers [13] and transplant patients [14,15] when dosed with CsA. In these studies, CsA caused a 2- to 3-fold increase in RAD001 exposure. The more pronounced effect in our dogs might be related to the high CsA levels observed, which could intensify the influence of CsA on RAD001. A 6-fold reduction of RAD001 dose in healthy dogs that received concomitantly CSA allowed the achievement of targeted RAD001 levels that were otherwise achieved using 1.5 mg RAD001 twice daily alone. This confirms the dose-proportionality of RAD001 described in previous clinical studies [16].

For the first time, RAD001 pharmacokinetic analyses were also conducted after HSCT. Irradiation containing conditioning is known to damage oral and intestinal mucosa and may affect drug absorption. However, the pharmacokinetic profiles determined 5 and 21 days after HSCT did not differ from parameters identified in healthy dogs, emphasizing the low toxicity of 2 Gy TBI. Successful dose adjustment in dogs was easily feasible as shown by the narrower range of c_{min}, c_{max}, and $AUC_{0\text{-}12h}$ at day 21 compared with day 5 after HSCT, respectively. In 3 dogs, CsA dose reduction resulted in a decreased RAD001 exposure at day 21 compared with day 5, respectively. This reflects the strong influence of CsA on RAD001 pharmacokinetics after HSCT as we already described in healthy dogs. No drug accumulation over time was observed, suggesting that the steady state of RAD001 was reached within 5 days. These results are in accordance with data after solid-organ transplantation [17,18].

To consider possible effects of RAD001 on CsA, pharmacokinetics assessment of steady-state CsA parameters were performed. Comparison of CsA levels with its own historic data in which MMF was coadministered to CsA [19] indicate a significant increase in c_{2h} levels when CsA is combined with RAD001 (P = .001), whereas differences between trough levels and AUC did not reach significance. In most clinical studies, pharmacokinetics of CsA appear not to be affected by RAD001 coadministration [18,20,21]. However, Kirchner et al. [21] observed a nonsignificant 10% augmentation of CsA AUC after administration of a single, oral dose of RAD001. In addition, Budde et al. [20] assumed some minor pharmacokinetic interactions between CsA and RAD001. Results of the pharmacokinetic analyses of RAD001 obtained in our canine model indicate a good agreement between the pharmacokinetic characteristics of humans and dogs, and confirm the usefulness of the dog as a model system for pharmacological investigations.

Pre- and posttransplantation immunosuppression consisting of RAD001 and CsA allowed a rapid hematopoietic engraftment in all dogs. However, long-term chimerism was observed in only 4 of 9 evaluable

animals (44%). The rate of long-term chimeras was lower compared with results from other nonmyeloablative studies. Storb et al. [22,23] achieved in series of experiments long-term engraftment with CsA/MMF in 11 of 12 dogs (92%). Subsequently, MMF was substituted by the mTOR inhibitor sirolimus in the same regimen. Durable engraftment in 5 of 6 dogs (83%) was observed [24].

Several studies in humans have documented that RAD001 in combination with CsA is effective in preventing graft rejection in solid-organ transplantation [2,25-27]. Comparative studies demonstrated an equal efficacy of RAD001 and MMF in preventing graft loss and acute rejection in renal transplantation [4,28]. In addition, RAD001 allowed low rejection rates in heart transplant recipients [2,26]. Effects were equal or superior to MMF or azathioprine immunosuppression.

Other clinical studies investigated the use of sirolimus following myeloablative HSCT [29,30]. In all studies, the use of the mTOR inhibitor was effective in regard to engraftment. However, toxicities were significant. In conclusion, RAD001 seemed to be effective in a nonmyeloablative conditioning setting in regard to short-term engraftment. Nevertheless, the long-term engraftment rates observed are lower compared with a MMF/CsA- or sirolimus/CsA-containing regimen.

The most common reported adverse events associated with RAD001 are leukopenia, thrombocytopenia, hypercholesterolemia, hyperlipidemia, and/or increased creatinine levels [2,4,27]. Consistent with these known side effects, RAD001 caused an increase in cholesterol levels and a significant reduction in platelet counts in both healthy dogs and transplanted animals in our study. However, cholesterol generally did not exceed upper normal limits, and levels were reversible after drug discontinuation and were not of clinical relevance. Magnitude and time course of thrombocytopenia and leukopenia as well as the need for blood transfusion were similar after RAD001/CsA and sirolimus/CsA treatment [24]. Compared with dogs that received nonmyeloablative HSCT with MMF/CsA, thrombocytopenia after RAD001 administration was more pronounced ($P = .002$) and associated with a longer time to recovery ($P \leq .036$). This observation seems to be attributed to the platelet-reductive effect of RAD001 already observed in healthy dogs. The course of leukocyte counts was comparable to that determined in the historic MMF/CsA group. Hence, leukocyte reduction was caused by TBI rather than RAD001. Assessment of the parameters diarrhea, activity, ingestion, and weight loss also showed no differences compared with MMF/CsA [19] and sirolimus/CsA [24] treatment.

In line with our data, Platzbecker et al. [6] observed in 2 of 24 HSCT patients a significant decrease in platelet counts after initial engraftment that did not recover until RAD001 was discontinued. In solid-organ transplantation, the incidence and severity of thrombocytopenia was reported to be dose related [3]. Considering the prolonged time to recovery in dogs after HSCT and the known exposure-response relationship, a dose escalation of RAD001 was not intended in the present study.

The trial by Platzbecker et al. [6] had to be terminated prematurely because of the occurrence of severe adverse events, mainly sinusoidal obstruction syndrome of the liver and transplantation-associated microangiopathy. Severe side effects that required premature termination were not apparent in our study, possibly because of the nonmyeloablative conditioning and the shorter time course of drug administration.

In conclusion, our data demonstrated that pre- and posttransplantation immunosuppression consisting of RAD001 and CsA is feasible in a canine 2 Gy TBI nonmyeloablative HSCT setting and allows initial engraftment in all dogs. However, it is not as effective as sirolimus/CsA and MMF/CsA regimens in regard to long-term engraftment induction. The general use of RAD001/CsA as immunosuppressants therefore cannot be recommended following nonmyeloablative HSCT.

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