Everolimus in Combination with Mycophenolate Mofetil as Pre- and Post-Transplantation Immunosuppression after Nonmyeloablative Hematopoietic Stem Cell Transplantation in Canine Littermates

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A B S T R A C T
The mammalian target of rapamycin inhibitor everolimus (RAD001) is a successfully used immunosuppressant in solid-organ transplantation. Several studies have already used RAD001 in combination with calcineurin inhibitors after hematopoietic stem cell transplantation (HSCT). We investigated calcineurin inhibitor free pre- and post-transplantation immunosuppression of RAD001 combined with mycophenolate mofetil (MMF) in a nonmyeloablative HSCT setting. After nonmyeloablative conditioning with 2 Gy total body irradiation, 8 dogs received HSCT from dog leukocyte antigen identical siblings. Immunosuppressives were given at doses of 1.5 mg RAD001 twice daily from day 1 to +49, then tapered until day +56, and 20 mg/kg MMF from day 0 to +28, then tapered until day +42. An historical cyclosporin A (CsA)/MMF regimen was used in the control group. All dogs engrafted. Median platelet nadir amounted in all dogs to 0/2×10⁹/L (median, day +10; duration <50/2×10⁹/L, 22 days) and median leukocyte nadir was 1.0/2×10⁹/L (range, 1 to 2.5 × 10⁹/L; median, day +13). Eventually, 5 of 8 (63%) animals rejected their grafts. Two dogs died of infections on day +19 and +25. Pharmacokinetics of RAD001 and MMF showed median trough levels of 19.1 (range, 10.5 to 43.2) mg/L and .3 (.1 to 1.3) mg/L, respectively. The median area under the curve was 325 (range, 178 to 593) mg/L × hour for RAD001 and 29.6 (range, 7.9 to 40.5) ng/L × hour for MMF. All dogs developed clinically mucosal viral infections during the clinical course. Compared with the control group, the level of toxicities for RAD001/MMF increased in all qualities. Combined immunosuppression of RAD001 and MMF after nonmyeloablative HSCT is associated with significant toxicities, including a prolonged platelet recovery time as well as increased infections compared to the CsA/MMF regimen.

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INTRODUCTION
The mammalian target of rapamycin (mTOR) kinase is a central cell cycle regulator and its inhibition leads to G1 arrest in lymphocytes as well as in various cancer cells [1]. Therefore, mTOR inhibition has been used as an immunosuppressive as well as an anti-cancer drug. Sirolimus (SRL) was the first investigated mTOR inhibitor. It has a long half-life of approximately 70 hours and, therefore, it may be difficult to adjust and dose. In a 2 Gy canine hematopoietic stem cell transplantation (HSCT) model, Hogan et al. demonstrated a similar engraftment by using cyclosporin A (CsA)/SRL compared with CsA/mycophenolate mofetil (MMF) [2]. A triple combination of SRL, CsA, and MMF only allowed a stable engraftment in 1 of 8 dogs after using suboptimal conditioning with 1 Gy total body irradiation (TBI) [3].

Everolimus (RAD001) is a derivative of SRL. It is used in solid-organ transplantation for prevention of graft rejection [4,5]. In renal transplantation, RAD001 combined with CsA treatment was associated with significantly improved renal function [6,7]. RAD001 in combination with MMF showed promising renal outcome after liver and kidney transplantation [8].

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transplantation [8,9]. In heart transplantation, cytomegalovirus infections occurrence was significantly reduced by combining CsA/RAD001 compared with CsA/azathioprin [10].

Former studies for HSCT used RAD001 in combination with calcineurin inhibitors (CNIs). A clinical phase II study with tacrolimus was canceled at an early stage because of the high incidence of sinusoidal obstruction syndrome [11]. An engraftment study after HSCT in canine litters using RAD001 in combination with CsA showed no disadvantage, compared with treatment with CsA and MMF [12].

In addition, SRL in combination with MMF and antithymocyte globulin was shown to be a feasible combination for CNI-free graft-versus-host disease prophylaxis after an intensive combined conditioning of 4-day chemotherapy and 4 Gy TBI [13].

We hypothesized that RAD001 might be a potential drug to replace CNIs and minimize side effects in HSCT regimens. Thus, we investigated the pre- and post-transplantation combination of the mTOR inhibitor RAD001 and MMF after 2 Gy TBI in a canine littermate model, evaluating engraftment as well as pharmacokinetic and toxicity properties of this treatment.

MATERIAL AND METHODS

Animals
Litters of random-bred beagles were purchased from commercial kennels possessing a license for animal breeding and husbandry. All dogs were dewormed and vaccinated against rabies, distemper, parvovirus, leptospirosis, hepatitis, and parainfluenza virus. The selection of dog leukocyte antigen–identical donor/sibling pairs was based on matching for highly polymorphic MHC class I and II microsatellite markers [14].

At the beginning of this study, the median age of the dogs was 24 months (range, 12 to 30 months), and the median weight was 16.6 kg (range, 9.8 to 20.0 kg). This study was approved by the review board of the State Institute for Agriculture, Food Safety, and Fishery Mecklenburg-West Pomerania, Germany. (LALLF M-V/TSD/7221.3-11-013/09)

Pharmacokinetics
It was intended to keep RAD001 trough levels not lower than 8 μg/L [15]. Initial pretransplantation pharmacokinetics were performed in healthy dogs, as previously described [12]. Briefly, animals received either 1.5 mg RAD001 alone (group 1), 15 mg RAD in combination with 20 mg/kg MMF (group 2), or 20 mg/kg MMF alone (group 3) twice daily orally for 5 consecutive days. On the fifth day, blood samples were taken at different time points, and RAD001 concentrations were quantified using a fluorescence polarization immunoassay. Plasma levels of mycophenolic acid (MPA), which is the active metabolite of MMF, were measured by the CEDIA® mycophenolic acid assay (Thermo Fischer Scientific Inc.; Waltham, MA) on a Roche COBAS® Mira Plus analyzer (Roche; Basel, Switzerland). The study included a calculation of pharmacokinetic parameters such as maximum concentration, time to reach maximum concentration, area under the curve over 12 hours (AUC0–12h), and half-life. Pharmacokinetics of the dogs that underwent transplantation were measured similarly at day +5 and +21 after HSCT.

HSCT
All dogs (n = 8) received 2 Gy TBI at a dose rate of 1 Gy/minute by using a high linear accelerator (Siemens Primus®, 10 MV X-ray; Siemens, Munich, Germany). Donor bone marrow was collected from the femur, humerus, and iliac crest by aspiration under general anesthesia. Within 24 hours after TBI, bone marrow was infused intravenously (day 0).

Recipients were treated with pre- and post-transplantation immunosuppression. Therefore, the animals received RAD001 (Cortecia®, Novartis; Basel, Switzerland) from day −1 to +49 at a dose of 1.5 mg twice daily orally and subsequently a half dose (0.75 mg) until day +56. Twenty milligrams per kilogram MMF (CellCept®, Roche) was given twice daily orally from day 0 to +28 and continued with 10 mg/kg until day +42. Endpoints of this study were initial and long-term engraftment, as well as the toxicity evaluation. Long-term engraftment was defined as chimerism of donor granulocytes and peripheral blood mononuclear cells (PBMCs) after 26 weeks.

Chimerism
To evaluate the chimerism, blood samples were collected weekly until day +77 and at larger intervals afterwards. Samples were analyzed as previously described [16].

Toxicities
Toxicities were monitored daily during the drug administration period (day −1 until +56) by surveying activity, defecation, ingestion, body weight, and temperature. Grades of toxicity, supportive care, and criteria of euthanasia are specified in the supplement (Supplementary Items S1, S2). Leucopenia was defined as ≤1.0 × 10^9 leukocytes/L, and thrombocytopenia as ≤50 × 10^9/L. Blood cell counts were monitored daily until cell count recovery and then afterwards in weekly intervals. The need for blood transfusion was defined by either clinical signs of hemorrhage or platelet counts <5 × 10^10/L. The biochemical parameters creatinine, urea, cholesterol, triglycerides, transaminases, alkaline phosphatase, and gamma-glutamyltransferase were assessed weekly to biweekly.

Statistics
For statistical analyses within the treatment group, the Wilcoxon matched-pairs signed rank test was used. Differences between groups were evaluated by the Mann-Whitney U-test. To describe the distribution of data, medians and ranges were stated. Probabilities of P <.05 were considered to be significant.

RESULTS

Pharmacokinetics and Toxicities in Healthy Dogs
Results of pharmacokinetics are displayed in Figure 1 for the combined RAD001/MMF application (group 2) and in the supplement (Supplementary Table S1) for all groups. After receiving RAD001 alone (group 1), median trough level was 15.6 μg/L (range, 11.5 to 21.3 μg/L). The combination with MMF (group 2) resulted in a comparable RAD001 median

![Figure 1. Pharmacokinetics of RAD001 (left) and MPA (right) in healthy dogs. Dogs received RAD001 (1.5 mg twice daily) and MMF (20 mg/kg twice daily) for 5 consecutive days. At day 5, pharmacokinetic analyses were performed and results are displayed for each dog. Median RAD001 (left) and MPA (right) levels are illustrated as solid lines.](image-url)
trough level of 22.0 μg/L (range, 10.2 to 36.5 μg/L). When given in combination (group 2), the median MPA AUC(0-12h) increased significantly from 24.1 mg/L/C2 hour (range, 11.8 to 36.5 mg/L/C2 hour) alone (group 3) to a median of 45.2 mg/L/C2 hour (range, 36.9 to 72.3 mg/L/C2 hour) (P = .029).

Assessment of hematotoxicity showed a mild decrease in platelet counts in all groups. The most apparent reduction starting at 290/C2 109/L at day 0 to 161/C2 109/L at day 8 was observed in group 2 (data not shown). No further clinically relevant toxicities were observed.

Engraftment and Hematologic Recovery after HSCT

Transplantation and chimerism data are shown in Table 1 and Figure 2. All dogs engrafted. Median maximal donor chimerism for granulocytes was 84% (range, 33% to 96%) at median day +18. Median maximal donor PBMC chimerism was 58% (range, 3% to 85%) at median day +25. Dog I developed a stable high donor chimerism until the end of the observation period. Five of 8 animals rejected their grafts and recovered with autologous marrow at median day +91 (range, 63 to 239). Dogs III and VIII died unexpectedly from infections, despite daily check of health status, on days +25 and +19, respectively. In spite of supportive therapy, both of them developed mucosal viral infections with ulcerations and fever up to 41.5°C. Dog III died at day +25 with a high donor chimerism of 90% granulocytes and 52% PBMCs. Dog VIII showed a rising donor percentage of 33% for granulocytes and 3% for PBMCs, before dying at day +19.

The hematological recovery after HSCT is displayed for the leukocyte and platelet counts in Figure 3. The median leukocyte nadir was 1.0 × 10⁹/L (range, 1 to 2.5 × 10⁹/L) at the median day +13 (range, 7 to 24). Dogs III and VIII showed a prolonged leucopenia of 8 and 15 days, respectively, before dying, compared with the leucopenia duration of the rest, with median 0 days (range, 0 to 5 days). The platelet nadir of all dogs was 0 × 10⁹/L. The duration of thrombocytopenia was a median of 22 days (range, 11 to 29 days). The median time to platelet recovery was 36 days (range, 29 to 36 days). Seven of 8 recipients required whole blood transfusions to prevent hemorrhage (median, 2.5 transfusions/dog). Median hemoglobin nadir was 5.3 mmol/L (range, 3.6 to 9.9 mmol/L) at median day +35 (data not shown).

Post-Transplantation Pharmacokinetics

During the course of the study, RAD001 and MMF pharmacokinetics were analyzed on day +5 and +21 after HSCT (Figure 4, Supplementary Table S2). All recipients showed RAD001 trough levels above the lower concentration limit of 8.0 mg/L (median, 19.1 mg/L; range, 10.5 to 43.2 mg/L). The RAD001 morning dose was decreased to 1 mg for dog V because of a high AUC value (571 ng/mL/C2 hour) at day +5. Additionally, MMF administration was prematurely terminated at day +29 and RAD001 dose was adjusted to 1.0 mg twice daily in dog VII because of infections. No significant differences of pharmacokinetic parameters between day +5 and +21 were observed.

Toxicities

Cholesterol values increased significantly but stayed within normal limits. Alkaline phosphatase showed values beyond the upper range during the trial period, starting at median 77 units [U]/L at day 0 and maximal level of 139 U/L at day +21 (normal range <108 U/L). No further increase of other blood parameters could be observed. At day +56, all altered parameters recovered to pretransplantation values after tapering the immunosuppressives.

Table 1

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>Graft Cell Counts</th>
<th>Maximal Donor Chimerism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD34+, 10⁹/kg</td>
<td>TNC, 10⁹/kg</td>
</tr>
<tr>
<td>I</td>
<td>6.3</td>
<td>6.5</td>
</tr>
<tr>
<td>II</td>
<td>1.3</td>
<td>2.0</td>
</tr>
<tr>
<td>III</td>
<td>5.0</td>
<td>4.0</td>
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<td>IV</td>
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<td>2.1</td>
</tr>
<tr>
<td>V</td>
<td>4.8</td>
<td>4.1</td>
</tr>
<tr>
<td>VI</td>
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<td>3.7</td>
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<tr>
<td>VII</td>
<td>6.5</td>
<td>4.3</td>
</tr>
<tr>
<td>VIII</td>
<td>2.4</td>
<td>3.6</td>
</tr>
<tr>
<td>Median</td>
<td>4.2</td>
<td>3.8</td>
</tr>
</tbody>
</table>

TNC indicates total nucleated cells.

Figure 2. Granulocytes (left) and PBMC (right) donor chimerism after HSCT. Chimerism analyses were performed after HSCT weekly until day +77 and afterwards in larger intervals. This figure shows the percentage of hematopoietic donor chimerism for granulocytes (left) and PBMCs (right) after HSCT. Deceased during course.
Multiple mucosal lesions likely due to viral infections occurred in all recipients (Supplementary Figure S1). Dogs III and VIII died unexpectedly without reaching criteria for euthanasia, as outlined in the supplement. During the following autopsy, multiple lesions in mouth and sporadic gastrointestinal lesions were found. No further cause of death could be proven.

Additionally, 7 of 8 dogs showed transient temperatures $>40^\circ$C. Four of 8 recipients showed reduced activity ($\geq$ grade 2) for a median of 5 days (range, 2 to 13 days). All dogs demonstrated reduced appetite. In 5 recipients, parenteral nutrition was required. The MMF dose was adjusted repeatedly, according to the actual individual weight.

**DISCUSSION**

Herein, we investigated the pre- and post-transplantation combination of the mTOR inhibitor RAD001 and MMF after 2 Gy TBI in a canine littermate model.

First, we characterized the pharmacokinetic properties of the compounds in healthy dogs. To replace CNI, trough levels of RAD001 between 6 and 10 mg/L or 8 and 12 mg/L were tested in kidney and liver transplantation [8,9]. Thus, we focused in our HSCT model on a RAD001 trough level above 8 mg/L [15]. Our starting dose of 1.5 mg RAD001 twice daily rapidly exceeded this minimal trough level. Therefore, we used it as the starting dose in our transplantation group. The median half-life of RAD001 in this model amounted to 13.6 hours. In contrast to pharmacokinetic studies in humans, these observed values are remarkably short but are comparable to the half-life of former studies in dogs [12,17]. The MPA concentration in healthy dogs increased rapidly to the maximum concentration value in all recipients and showed similar characteristics in dogs, as previously described [18-20]. Of note, a mild decrease in platelet counts, which was also noted previously in RAD001-based CNI-free immunosuppressive regimens, was observed [7].

![Figure 3. Hematologic recovery in dogs after HSCT. Median cell counts of leukocytes (A) and platelets (B) were measured after HSCT and immunosuppression by RAD001 (1.5 mg twice daily) and MMF (20 mg/kg twice daily). Data were compared with our historical CsA/MMF control group (CON).](image)

![Figure 4. Pharmacokinetic profiles of RAD001 and MPA after HSCT. RAD001 was given from day −1 to +49 at a starting dose of 1.5 mg twice daily orally and was tapered by half dose until day +56. MMF (20 mg/kg twice daily) administration was performed from day 0 to +28 and continued with 10 mg/kg until day +42. Pharmacokinetic profiles were tested at day +5 and +21 for both drugs. Results are displayed as medians (solid lines), 25 quantile, and 75 quantile (dotted lines).](image)
All 8 dogs in this study initially engrafted, but toxic effects were significant. The initial engraftment levels in our study were comparable to former studies with 2 Gy TBI [2,12,21]. However, only 1 dog demonstrated a long-term engraftment. When compared with other studies administering CNI in combination with MMF, the herein observed long-term engraftment appears lower [22]. Hogan et al. showed an engraftment in 5 of 6 dogs using CsA combined with the mTOR inhibitor SRL after 2 Gy TBI [2]. Additionally, RAD001 used in lower concentrations combined with CsA was reported to lead to a long-term engraftment in 4 of 9 dogs after HSCT [12]. Herein, 4 dogs rejected their grafts in the first 9 weeks after HSCT, 1 dog rejected within 30 weeks, and 2 dogs died during the course, despite intensive supportive care. The time-wise correlation of dose tapering and decrease of donor percentage may indicate a correlation between the end of the immunosuppressive treatment and graft rejection.

The median RAD001 trough level of 19.1 μg/L after HSCT clearly remained in the targeted range; however, it was higher compared with RAD001 concentrations applied in previous studies [7-9]. The resulting higher immunosuppressive pressure might be one reason for the poor engraftment rate observed in the present study.

No drug accumulation of RAD001 and MPA could be detected between day +5 and +21. This data is in accordance with previous results of RAD001 in combination with CsA [23].

Common side effects of mTOR inhibitors on blood parameters are increased levels of creatinine, cholesterol, and triglycerides and decreased leukocyte and platelet counts [7,17,23]. One of the main obstacles in our study was enhanced thrombocytopenia. Compared with other immunosuppressive regimens, our RAD001/MMF group showed a delayed recovery time and an increased requirement for blood transfusions [2,12]. Similar to our findings, the use of RAD001 in CNI-free solid-organ transplantation showed more frequent drug-related toxicity compared with CNI regimens [7-9]. However, no sinusoidal obstruction syndrome was observed in our study [11].

Mucosal lesions occurred in all dogs with a higher incidence in the deceased dogs than in the surviving animals. This adverse event of RAD001 was previously observed in solid-organ transplantation and treatments of cancer patients, as well [7,24]. Ferte et al. described a dose-related effect of RAD001 on mucosal ulcerations [25]. However, in our study, clinical signs suggested a viral genesis. The lesions may have led to a variable drug uptake and, thus, to a higher variability in pharmacokinetics.

In conclusion, the combination of high dose RAD001 and MMF as pre- and post-transplantation immunosuppression in a nonmyeloablative HSCT led to substantially increased toxicity parameters, in particular mucosal lesions and prolonged thrombocytopenia, which are typical for high RAD001 levels. Consequently, this drug combination appears to be inferior compared with established immunosuppressive regimens used in nonmyeloablative HSCT. To prevent graft rejection, immunosuppression may be administered longer and a more individually adjusted dose of RAD001 may eliminate high pharmacotoxicity.

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Authorship contribution: C.M. and S.L. contributed equally to the study.

SUPPLEMENTARY DATA

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.bbmt.2014.06.004.

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