mTOR Inhibitor RAD001 Promotes Metastasis in a Rat Model of Pancreatic Neuroendocrine Cancer

Stefan E. Pool, Sander Bison, Stuart J. Koelewijn, Linda M. van der Graaf, Marleen Melis, Eric P. Krenning, and Marion de Jong

Abstract

Inhibition of mTOR is commonly considered a valid target in cancer treatment, but this assertion does not address effects on the immune microenvironment that may be detrimental to cancer treatment. Here we show how administration of the mTOR inhibitor RAD001 (everolimus) results in the occurrence of distant metastasis in a rat model of pancreatic cancer. RAD001 was administered twice weekly for 4.5 weeks as a single treatment or combined with [177Lu-DOTA,Tyr3]octreotate (177Lu-DOTATATE), where the latter targets the somatostatin receptor-2. The hypothesized synergistic therapeutic effect of RAD001 combined with 177Lu-DOTATATE was, however, not observed in our experiments. The combination was shown to be less effective than 177Lu-DOTATATE alone. Unexpectedly, tumor metastasis was observed in 77% of the subjects treated with RAD001, either alone or as part of the combination treatment. This was a striking effect, because metastasis did not occur in control or 177Lu-DOTATATE-treated animals, including those where the primary tumor was surgically removed. These findings may be important clinically among noncompliant patients or patients that discontinue RAD001 therapy because of adverse effects.

Introduction

Gastroenteropancreatic neuroendocrine tumors (GEPNET) are usually slow growing and most patients present with already metastasized disease at time of diagnosis. As published by Missiaglia and colleagues (1) in many pancreatic neuroendocrine tumors (PNET), the mTOR signal transduction pathway is upregulated. This pathway plays a key role in regulating cell growth, metabolism, proliferation, and angiogenesis. Inhibition of the mTOR signal transduction pathway in PNETs by RAD001 has shown promising results with a clinical benefit of 76%, when given as a single treatment (n = 115), and of 82%, when given in combination with octreotide-LAR (n = 45; ref. 2). The RADIANT-3 study, a randomized, double-blind, placebo-controlled, multicenter phase III trial of 10 mg RAD001 daily in PNET-patients (n = 410) has been completed recently. Median progression-free survival for RAD001 plus best supportive care was 11 months compared with 4.6 months in the placebo group (P < 0.001) with acceptable toxicity (3). Most recently, the U.S. Food and Drug Administration approved RAD001 for the treatment of PNET patients.

From the late 1990s, clinical peptide receptor radionuclide therapy (PRRT) studies have been carried out with radiolabeled somatostatin analogs, such as 177Lu-DOTATATE and [111In-DTPA-DOTA,Tyr3]octreotide (111In-DOTATOC; Onalta), targeting sst2 overexpressed on most GEPNETs. These studies have shown very promising results with regard to tumor response, overall survival, and quality of life (4, 5).

Considering the promising results of both RAD001 and PRRT for the treatment of GEPNETs, we combined these 2 therapies in the CA20948 syngeneic pancreatic tumor model in the rat. This radiosensitive model is commonly used for preclinical PRRT experiments and also shown to be susceptible to RAD001 treatment (6). For comparison, studies were also carried out in the xenograft H69 tumor model in nude mice. The aim of the current study was to investigate the therapeutic effects of combined treatment with RAD001 and 177Lu-DOTATATE compared with those of the 2 monotherapies.

Materials and Methods

Cell lines

The CA20948 rat pancreatic tumor cell line (derived from a rat pancreas at our institution) is of acinar origin (7), has high sst2 expression, and was cultured and reported previously (8, 11). The cells were passaged for a maximum of 20 times and checked for mycoplasma infection and sst2 expression every 3 months.

The H69 tumor cell line is a human small-cell lung carcinoma (the American Tissue Culture Collection, Wesel, Germany), has high sst2 expression, was passaged for a maximum of 10 times, and was cultured according to the supplier’s protocol.

Tumor models

The animal studies were in agreement with the Animal Welfare Committee requirements of our institution and...
conducted in accordance with generally accepted guidelines. For the first 2 experiments, male Lewis rats (Harlan; Horst, the Netherlands) with a mean body weight of 275 g were used. For tumor induction, 10^7 CA20948 tumor cells in 0.5 mL ice-cold PBS per animal were injected subcutaneously in the lower flank. Monitoring of body weight and tumor size by caliper measurements was carried out by a technician blinded for the treatment groups. Tumor volume was calculated according to \( \frac{0.4 \times \text{length} \times \text{width} \times \text{height}}{3} \). In the first study (Fig. 1), animals were euthanized when tumor size had reached a volume of more than 4 cm^3 or when a tumor was bleeding due to skin penetration. In the second study, the primary subcutaneous tumors were surgically removed when tumor volume exceeded 4 cm^3 or when the tumor was bleeding due to skin penetration, thus saving the animals for follow-up. Animals were euthanized when more than 10% loss of body weight was detected. For the third experiment, 24 male NMRI Nu/Nu mice with a mean body weight of 35 g xenotransplanted sst2-expressing H69 cells were used. Animals were euthanized when tumor size reached a volume of more than 2 cm^3, when a tumor was bleeding due to skin penetration, or when more than 10% loss of body weight was detected.

**RAD001**

In the first experiment, RAD001 (kind gift from Novartis, Basel, Switzerland) and placebo was prepared according to the manufacturer’s protocol. In the second and third experiment, RAD001 powder (LC laboratories, Woburn, USA) was dissolved in 2 mL ethanol and further diluted to the appropriate concentration in 5% (w/v) glucose solution. RAD001 was administered orally by gavage with a blunt needle.

**Radionuclides and peptides**

DOTA,Tyr^3^-octreotate was obtained from Mallinckrodt, St Louis, Missouri. ^177^LuCl\textsubscript{3} was obtained from NRG, Petten and was distributed by IDB-Holland, Baarle-Nassau, the Netherlands. ^177^Lu-DOTA,Tyr^3^-octreotate was locally prepared as described previously (9) in a specific activity of 125 MBq/3.4 μg peptide. Labeling of ^111^In-DTPA-octreotide (Octreoscan; Tyco Health Care, Petten, the Netherlands) in a specific activity...
of 30 MBq $^{111}$InCl$_3$/0.5 µg DTPA-octreotide was carried out as previously described (10). For the mouse experiments, a specific activity of 30 MBq $^{111}$InCl$_3$/0.1 µg DTPA-octreotide was used.

In vitro autoradiography and hematoxylin and eosin staining

These procedures were carried out as previously described in detail (11).

Experimental setup

In experiment 1, RAD001 treatment was started 10 days after tumor inoculation, followed by $^{177}$Lu-DOTATATE injection. Animals were randomized into matching treatment groups with regard to tumor size at the start of treatment. Six treatment groups were created using 1 dose of RAD001 (5 mg/kg body weight twice a week) and 2 doses of $^{177}$Lu-DOTATATE, 125 or 278 MBq, either as monotherapy or in combination (Table 1). Experiment 1 ended at day 60. To keep animal discomfort as low as possible and for practical reasons, RAD001 was administered twice weekly.

Experiment 2 was essentially similar as experiment 1; variations were 2.5 next to 5 mg/kg body weight dose of RAD001, alone or combined with 125 MBq $^{177}$Lu-DOTATATE (Table 1). The most important difference in comparison with experiment 1 was the resection of the subcutaneous tumor when tumor size increased to more than 4 cm$^3$. Experiment 2 was terminated at day 150. All animals still alive at the end of the experiment were checked for distant metastases by $^{111}$In-DTPA-octreotide single-photon emission computed tomography (SPECT) or computed tomography (CT) and autopsy.

Surgical procedure

During all surgical procedures, isoflurane/O$_2$ anesthesia was applied and animals were kept warm using a heating pad. After shaving and disinfection, an incision was made just adjacent to the tumor. The capsule surrounding the tumor was carefully dissected from the surrounding tissue. The wound was closed by using absorbable sutures.

SPECT/CT scanning

After 24 (experiment 1) or 48 hours (experiment 2) of intravenous (i.v.) injection of $^{177}$Lu-DOTATATE, a helical SPECT scan was acquired covering the tumor region using the 4-headed NanoSPECT/CT system (Bioscan) using Nuclide software (v2.01, Mediso) for the quantification of $^{177}$Lu-DOTATATE uptake.

Table 1. Characteristics of treatment groups in studies 1 and 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>n</th>
<th>Tumor volume &gt;4 cm$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Control</td>
<td>Vehicle only</td>
<td>7</td>
<td>†</td>
</tr>
<tr>
<td>2 RAD001</td>
<td>RAD001 (2×/week, 5 mg/kg bw)</td>
<td>6</td>
<td>†</td>
</tr>
<tr>
<td>3 Combination of low-dose $^{177}$Lu-DOTATATE with RAD001</td>
<td>RAD001 (2×/week, 5 mg/kg bw) and 125 MBq $^{177}$Lu-DOTATATE</td>
<td>7</td>
<td>†</td>
</tr>
<tr>
<td>4 Combination of high-dose $^{177}$Lu-DOTATATE with RAD001</td>
<td>RAD001 (2×/week, 5 mg/kg bw) and 275 MBq $^{177}$Lu-DOTATATE</td>
<td>7</td>
<td>†</td>
</tr>
<tr>
<td>5 Low-dose $^{177}$Lu-DOTATATE</td>
<td>125 MBq $^{177}$Lu-DOTATATE</td>
<td>6</td>
<td>†</td>
</tr>
<tr>
<td>6 High-dose $^{177}$Lu-DOTATATE</td>
<td>275 MBq $^{177}$Lu-DOTATATE</td>
<td>7</td>
<td>†</td>
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<tr>
<td>Study 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 Control</td>
<td>Vehicle only</td>
<td>8</td>
<td>Surgery</td>
</tr>
<tr>
<td>8 Low-dose RAD001</td>
<td>RAD001 (2×/week, 2.5 mg/kg bw)</td>
<td>8</td>
<td>Surgery</td>
</tr>
<tr>
<td>9 High-dose RAD001</td>
<td>RAD001 (2×/week, 5 mg/kg bw)</td>
<td>8</td>
<td>Surgery</td>
</tr>
<tr>
<td>10 Combination of low-dose RAD001 with $^{177}$Lu-DOTATATE</td>
<td>RAD001 (2×/week, 2.5 mg/kg bw) + 125 MBq $^{177}$Lu-DOTATATE</td>
<td>7</td>
<td>Surgery</td>
</tr>
<tr>
<td>11 Combination of high-dose RAD001 with $^{177}$Lu-DOTATATE</td>
<td>RAD001 (2×/week, 5 mg/kg bw) + 125 MBq $^{177}$Lu-DOTATATE</td>
<td>8</td>
<td>Surgery</td>
</tr>
<tr>
<td>12 $^{177}$Lu-DOTATATE</td>
<td>125 MBq $^{177}$Lu-DOTATATE</td>
<td>8</td>
<td>Surgery</td>
</tr>
<tr>
<td>13 RAD001, no tumor</td>
<td>RAD001 (2×/week, 5 mg/kg bw)</td>
<td>7</td>
<td>n.a.</td>
</tr>
</tbody>
</table>

NOTE: All animals were bearing CA20948 tumors, except for animals in group 13. Abbreviations: BW, body weight; n.a., not applicable. †, euthanasia.
tumor uptake. Just before euthanizing an animal, a whole-body SPECT/CT scan was carried out 4 to 24 hours after i.v. injection of $^{111}$In-DTPA-octreotide for detection of distant metastasis.

Results and Discussion

Our first study, described in Table 1 (groups 1 – 6), showed inhibition of CA20948 tumor growth by RAD001 monotherapy as was previously found by Boulay and colleagues as well (6). In addition, in clinical studies mTOR inhibition generally seems to elicit a cytostatic, rather than a cytotoxic, response (12, 13). Boulay and colleagues also showed twice weekly administration of RAD001 to be as effective as daily administration in this tumor model (6). All animals in the control and the RAD001-only group had to be euthanized within 36 days after start of treatment because of tumor growth beyond 4 cm$^3$ or a ruptured tumor. As expected, all animals receiving $^{177}$Lu-DOTATATE with or without RAD001 exhibited significant antitumor response compared with controls ($P < 0.05$). However, $^{177}$Lu-DOTATATE in combination with RAD001 did not show evidence of being more effective than...
Figure 3. RAD001 treatment resulted in the occurrence of distant metastasis, shown by $^{111}$In-DTPA-octreotide SPECT/CT and in vitro autoradiography. A, SPECT/CT of animals representing all treatment groups. The SPECT/CT of control and $^{177}$Lu-DOTATATE-treated animals were carried out at the end of the experiment at day 150 and only showed uptake in the kidneys. The SPECT/CTs of the other animals were made just before euthanasia because of tumor growth and showed clear uptake in tumor tissue and kidneys. Kd, kidney; LuM, lung metastasis; LiM, liver metastasis; LnM, lymph node metastasis; and PST, primary subcutaneous tumor. B, autopsy images. Healthy livers (Liv) in control and $^{177}$Lu-DOTATATE-treated animals. Distant metastasis in the liver (LvM) in RAD001-treated animals. C, hematoxylin and eosin-stained sections of primary subcutaneous tumors (PST). D, corresponding $^{111}$In-DTPA-octreotide in vitro autoradiography images. E, corresponding $^{111}$In-DTPA-octreotide in vitro autoradiography images with addition of a 1000× excess of unlabeled DTPA-octreotide. F, hematoxylin and eosin-stained sections of distant metastasis in RAD001-treated animals. G, corresponding $^{111}$In-DTPA-octreotide in vitro autoradiography images. H, corresponding $^{111}$In-DTPA-octreotide in vitro autoradiography images with block.
had taken place. We, therefore, hypothesize that RAD001, alone or in combination with PRRT, might be responsible for the appearance of spontaneous metastases in the subcutaneous CA20948 tumor model. The suppression of the VEGF pathway by RAD001 could have stimulated glucose uptake, glycolysis, and de novo lipid biosynthesis, which are considered hallmarks of cancer and cancer metastasis. A possible explanation for the unexpected metastases in lung, liver, and lymph nodes in the rat model cannot be based on the basis of these studies. Understanding the pathways involved and how they are interconnected is needed to explain the current findings. As recently discussed by Ebos and colleagues (15), sustained suppression of the VEGF pathway may lead to a rebound in tumor growth, after it is discontinued. Comparable with our findings with RAD001, acceleration of metastasis was found in preclinical models after short-term treatment with the vascular endothelial/platelet derived growth factor receptor (VEGFR/PDGFR) kinase inhibitor sunitinib (16). The fact that, in our studies, RAD001 treatment was discontinued after 4.5 weeks may have resulted in such rebound effect via VEGFR as well. The twice-weekly administration of RAD001 could also have resulted in an incomplete inhibition resulting in an (twice-weekly repeated) upregulation of growth pathways. Discontinued mTOR inhibition could also have stimulated glucose uptake, glycolysis, and de novo lipid biosynthesis, which are considered hallmarks of cancer and cancer metastasis. A possible explanation for the higher therapeutic effects found for 177Lu-DOTATATE-only treatment compared with the combination treatment of RAD001 and 177Lu-DOTATATE could be the fact that, in the combination treatment, the tumor cell proliferation rate is decreased by RAD001, resulting in decreased radiosensitivity. The acceleration of metastasis could be caused by an effect on the immune system that could be unique to this particular preclinical CA20948 tumor model. Therefore, comparable experiments with other preclinical tumor models, such as the H69 mice model with surgical removal of the primary subcutaneous tumor, will have to be conducted. If the results of our studies can be translated to humans, mTOR inhibition treatment should be closely watched, especially after discontinuation of this therapy, because of adverse effects or in noncompliant patients, despite the positive therapeutic results of mTOR inhibition in different types of tumors.

177Lu-DOTATATE alone (Fig. 1A). A dose–effect relationship for the low- and high-dose 177Lu-DOTATATE was not found, which is in agreement with similar levels of 177Lu-DOTATATE tumor uptake in these 2 groups 24 hours after injection, as quantified on the basis of SPECT (Fig. 1E). Partial saturation of the sst2 receptors on the tumor cells in the high-dose group could play a role in this regard. Strikingly, from day 40 after start of treatment, most animals in the combination therapy groups showed unexpected decrease in body weight (Fig. 1B). Tumor metastasis to the liver and occasionally to the lung was found in these animals on autopsy (Fig. 1D). In the combination treatment groups, 11 out of 14 animals had to be euthanized because of loss of body weight and apparent metastasis (Fig. 1C). Metastases could be visualized after injection of 111In-DTPA-octreotide by SPECT/CT, indicating sst2 expression. The 177Lu-DOTATATE-only-treated animals did not show loss of body weight and were all free of metastasis, as confirmed by negative 111In-DTPA-octreotide SPECT/CT scans and negative autopsies (Fig. 1D). The occurrence of spontaneous metastasis in the subcutaneous CA20948 tumor model has not been described previously, despite the fact that this model has often been used in PRRT studies with long follow-up periods (14). We, therefore, hypothesize that RAD001, alone or in combination with 177Lu-DOTATATE, or the discontinuation of RAD001 treatment might be the cause of the metastasis. Unfortunately, the RAD001 monotherapy treatment group in this study did not survive long enough to develop or to be tested for metastases by SPECT.

For further investigation, a second study was carried out (Table 1; groups 7–13) in which a subcutaneous tumor reaching a volume of 4 cm³ was surgically removed to allow long-term follow-up. Furthermore, in this experiment also a 2.5 mg/kg body weight dose of RAD001 was applied next to the earlier-used 5 mg/kg body weight, combined with 125 MBq 177Lu-DOTATATE (Table 1). In an additional group (group 13), 5 mg/kg body weight of RAD001 was given to control rats without tumor.

The therapeutic effects obtained in this second study were in agreement with the first study, without significant difference with the use of the lower RAD001 dose. PRRT resulted in complete response of the CA20948 tumor in 3 out of 7 animals, whereas in all other animals the subcutaneous tumor had to be removed surgically. In 1 animal treated with 177Lu-DOTATATE, clear tumor spill into the wound bed was observed during the surgical procedure, resulting in tumor regrowth in the wound bed and metastasis in an ipsilateral lymph node in the groin. Therefore, this animal was not included in our analysis. The decrease in body weight seen in the RAD001+PRRT animals in study 1 was less explicit in study 2 (Fig. 2B), probably because of earlier intervention. Again, distant metastasis developed in animals receiving RAD001, either in high or low dose, or in combination with 177Lu-DOTATATE. (Fig. 2A), whereas control and 177Lu-DOTATATE-treated animals remained metastasis free. When development of distant metastasis was suspected on the basis of loss of body weight, 111In-DTPA-octreotide was injected and, 4 to 24 hours later, a SPECT/CT scan (Fig. 3A) was acquired. When a distant metastasis could be visualized, the animal was euthanized, followed by autopsy as illustrated by photographic images (Fig. 3B). From the tumor tissue collected, frozen sections were prepared and used for hematoxylin and eosin staining (Fig. 3C and F) and 111In-DTPA-octreotide in vitro autoradiography with (Fig. 3E and H) or without (Fig. 3D and G) a 1000× excess of unlabeled octreotide (block). All metastasis appeared to be sst2-positive, in agreement with the receptor status of the primary subcutaneous tumors. In a third study, RAD001 administered daily/twice weekly combined with PRRT in a suboptimal dose did again not show any significant additional therapeutic effect on subcutaneous human H69 xenografts in nude mice compared with placebo combined with PRRT (Fig. 2C + D). In this experiment, no distant metastasis was found after RAD001 therapy. A possible explanation could be the slow growth rate of the H69 tumor; longer follow-up and surgical removal of the primary subcutaneous tumor as carried out in the rat experiment 2 was preferred, although this was not allowed according to the animal ethical protocol for this study.

An explanation of the mechanism of action leading to the unexpected metastases in lung, liver, and lymph nodes in the rat model cannot be given on the basis of these studies. Understanding the pathways involved and how they are interconnected is needed to explain the current findings. As recently discussed by Ebos and colleagues (15), sustained suppression of the VEGF pathway may lead to a rebound in tumor growth, after it is discontinued. Comparable with our findings with RAD001, acceleration of metastasis was found in preclinical models after short-term treatment with the vascular endothelial/platelet derived growth factor receptor (VEGFR/PDGFR) kinase inhibitor sunitinib (16). The fact that, in our studies, RAD001 treatment was discontinued after 4.5 weeks may have resulted in such rebound effect via VEGFR as well. The twice-weekly administration of RAD001 could also have resulted in an incomplete inhibition resulting in an (twice-weekly repeated) upregulation of growth pathways. Discontinued mTOR inhibition could also have stimulated glucose uptake, glycolysis, and de novo lipid biosynthesis, which are considered hallmarks of cancer and cancer metastasis. A possible explanation for the higher therapeutic effects found for 177Lu-DOTATATE-only treatment compared with the combination treatment of RAD001 and 177Lu-DOTATATE could be the fact that, in the combination treatment, the tumor cell proliferation rate is decreased by RAD001, resulting in decreased radiosensitivity. The acceleration of metastasis could be caused by an effect on the immune system that could be unique to this particular preclinical CA20948 tumor model. Therefore, comparable experiments with other preclinical tumor models, such as the H69 mice model with surgical removal of the primary subcutaneous tumor, will have to be conducted. If the results of our studies can be translated to humans, mTOR inhibition treatment should be closely watched, especially after discontinuation of this therapy, because of adverse effects or in noncompliant patients, despite the positive therapeutic results of mTOR inhibition in different types of tumors.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S.E. Pool, S. Bison, E.P. Krenning, M. de Jong
Development of methodology: S.E. Pool, S. Bison, M. de Jong
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.E. Pool, S. Bison, M. de Jong
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.E. Pool, S. Bison, M. de Jong

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