In Vitro and In Vivo Activity of the Low-Immunogenic Antimesothelin Immunotoxin RG7787 in Pancreatic Cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) has a dismal prognosis, and new therapies are needed. RG7787 is a novel low-immunogenic antimesothelin recombinant immunotoxin (RIT), engineered to overcome the limitations of SSIP, a RIT now in clinical trials. In vitro activity was evaluated on five established PDAC cell lines (KLM-1, AsPC-1, BxPC-3, Panc 3.014, and PK-1) and on PDAC cells directly established from a patient tumor (GUMC108). RG7787 had subnanomolar IC50s in most cell lines, and was significantly more active than SSIP in GUMC108, KLM-1, and Panc 3.014 cells. GUMC108 was most sensitive, with RG7787 killing >99% of the cells. In a subcutaneous KLM-1 xenograft mouse model, two cycles of 3 × 2.5 mg/kg RG7787 QOD combined with two cycles of 1 × 50 mg/kg paclitaxel induced near-complete responses, with all tumors regressing below 5 mm3 within 30 days after therapy was initiated (>95% decrease) and no significant growth increase for at least another 3 weeks. RG7787 alone gave limited but significant regressions and paclitaxel by itself arrested tumor growth. Quantifying the uptake of Alexa Fluor 647–labeled RG7787 in tumors showed that the RIT reached only 45% of KLM-1 cells, accounting in part for the limited responses. Paclitaxel did not improve RG7787 uptake, which thus cannot explain the beneficial effect of the combination therapy. In conclusion, RG7787 has high cytotoxic activity on PDAC cell lines as well as on primary patient cells. In vivo, this novel RIT gives durable near-complete tumor responses when combined with paclitaxel. RG7787 merits further evaluation for the treatment of PDAC.

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Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the United States (1). The most common subtype, pancreatic ductal adenocarcinoma (PDAC), is notoriously resistant to most chemotherapeutic agents, and alternative treatment options are urgently needed.

Mesothelin is a 40-kDa cell surface glycoprotein (2) that is uniformly and highly expressed in virtually all PDAC and several other malignancies, including mesothelioma (3, 4). Because mesothelin is not expressed on normal essential organs, it is an established target for direct therapies against (5, 6). Our laboratory produces and evaluates recombinant immunotoxins (RIT) for cancer treatment. Current RITs in clinical trials are composed of an antigen-binding Fv fused to a 38-kDa portion of Pseudomonas exotoxin A (PE; ref. 7). The Fv binds to the cancer cells, after which the RIT is internalized via receptor-mediated endocytosis, and traffics via the endocytic compartment and Golgi to the endoplasmic reticulum. During this process, the toxin gets separated from the Fv by the action of furin. PE is subsequently transferred to the cytosol, where it ADP-ribosylates and inactivates elongation factor 2. This halts protein synthesis and leads to programmed cell death (8).

We have been evaluating the activity of the anti-mesothelin SSIP and anti-CD22 Moxetumomab pasudotox (MP) RITs in the clinic. In a phase I trial, MP produced durable complete remissions in 46% of patients with refractory hairy cell leukemia (9) and a phase III trial is now open (ClinicalTrials.gov Identifier: NCT01829711). In phase I clinical trials in patients with solid tumors, SSIP was well tolerated but the high immunogenicity of the PE portion typically induced neutralizing antidrug antibodies after one treatment cycle, resulting in limited antitumor activity (10, 11).

Our laboratory has focused on reducing this dose-limiting immunogenicity. One approach aims at suppressing the host immune system, by combining SSIP with immune-depleting chemotherapeutic agents. In a recent phase I trial (ClinicalTrials.gov identifier: NCT01362790),...
this allowed for multiple SS1P cycles that resulted in striking and unprecedented responses in patients with advanced refractory mesothelioma (12). These findings clearly illustrate that RITs can have high antitumor efficacy in malignancies with a poor prognosis. A second approach aims at minimizing PE immunogenicity via re-engineering RITs. By removing B-cell epitopes and protease-sensitive regions of PE38, a truncated de-immunized 24-kDa PE fragment (PE24) has been developed. PE24 variants have less reactivity with human anti-sera, are resistant to lysosomal degradation, and display a decreased nonspecific toxicity in rodent models in vivo (13–15). In collaboration with Roche Innovation Center Penzberg, Germany, this low-immunogenic PE24 backbone has been integrated into a novel antimesothelin RIT by linking it to a humanized antimesothelin Fab (huSS1), thereby increasing size and circulatory half-life. This clinically optimized RIT is named RG7787 (Fig. 1) and is being rapidly developed for evaluation in patients.

SS1P is highly cytotoxic to cells obtained from patients with ovarian cancer and mesothelioma (6), but has limited activity in mesothelin-expressing PDAC cell lines (16, 17). Consequently, antimesothelin RITs were not yet evaluated in PDAC xenograft models. The aims of this study were (i) to evaluate the cytotoxicity of RG7787 in established and primary PDAC cell lines, (ii) to evaluate the antitumor activity of RG7787 in a PDAC mouse model, both alone and in combination with paclitaxel, and (iii) to quantify the percentage of PDAC cells in vivo that are reached by RG7787 and link this uptake to response.

Materials and Methods

**Recombinant immunotoxins**

Clinical-grade SS1P [SS1(dsFv)-PE38] and RG7787 [huSS1(Fab)-LR-GGS-LO10-PE24] were manufactured by Advanced BioScience Laboratories, Inc. and Roche Innovation Center Penzberg, Germany, respectively. RG7787 is a re-engineered version of SS1P (Fig. 1 shows a comparison of their structures) consisting of a humanized antimesothelin Fab linked to a truncated and de-immunized PE24 moiety. Its detailed development is as follows: the CDR sequences of the light and heavy chain of the murine SS1 antibody (US70/8158 B1) were grafted onto human V_{L1} and V_{H1} domains chosen by structural similarity. To compensate for the reduced size of PE24 (24 kDa instead of 38 kDa in SS1P), C_{L1} and C_{H1} domains were added. As a result, RG7787 (73 kDa) is somewhat larger than SS1P (62 kDa) and has a similar half-life in the circulation of mice (30 minutes vs. 20 minutes for SS1P). To enhance cellular potency, the end of the human C_{H1} domain was fused to a GGS-based linker sequence that encompasses the furin cleavage site of PE (RHRQPR\#GWEQL; see PCT/US2012/036456). The linker sequence in turn was fused to the following modified PE24 sequence: PTGAEFLGDGGDVSFSTR-GTQNWTLVERL1QHAQLEERGYVVFVYGHTFLEA-AQSIFVGVAARSQDLAIAWAGFY1AGDPA1AYGYA-QDQEPDAAGRIRNGALLRVYYPASSLPGFYRTSL-TAAPEAAGEVERLHGPLLALDAITPGEESGRLE-TILGWPLAERTVIVPSAIPFTVRNVGGDLPSSIPDKEQAISALPDYASQPGKPPREDLK. PE24 was designed by removing the bulk of the original PE domain II (residues 251–273 and 284–394), which includes protease-sensitive regions and B-cell epitopes (13, 15). In addition, 7 mutations in domain III (R505A, R427A, R490A, R467A, D463A, R458A, and R538A) were introduced to silence previously identified B-cell epitopes (14).

**Cell culture**

PDAC cell lines AsPC-1, BxPC-3, KLM-1, and PK-1 were provided by Dr. U. Rudloff (NCI, Bethesda, MD).
in September 2011 and maintained in RPMI-1640 with 10% FBS. KLM-1 is originally derived from PK-1, a cell line established from a PDAC liver metastasis, by repeated passaging in mice (18). Panc 3.014 was obtained from Dr. E. Jaffe (Department of Oncology, Johns Hopkins University, Baltimore, MD) in December 2010 and maintained in RPMI-1640 with 20% FBS and 0.2 unit/mL humulin R U-100 (Eli Lilly and Company, NIH Pharmacy). RPMI-1640 was supplemented with 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). Cell line identities were verified using short tandem repeat analysis in August 2012 (NCI, Frederick, MD). GUMC108 is a primary PDAC cell line, directly derived from a patient tumor as previously described (19), and was a gift from Prof. R. Schlegel in July 2012 (Georgetown University, Baltimore, MD) in December 2010 and maintained at 37°C in a humidified incubator, with 5% CO₂. The collagenase-based dissociation process established method (21). In brief, animals were sacrificed, tumor volume was measured regularly using 0.2 Wunch U/mL Liberase Research Grade TM (Roche Diagnostics) and 0.1 mg/mL DNAse I (Sigma-Aldrich) treated controls.

**Mesothelin surface expression**

To quantify surface mesothelin expression, harvested cells were stained with 5 μg/mL of mouse antihuman mesothelin MN antibody (ref. 20; Rockland Immunoclonicals, Inc.) and processed as previously reported (17). Fluorescence intensity was analyzed by flow cytometry on a FACSCalibur. QuantibRITE R-PE beads (BD Pharmingen) were used to quantitate the number of mesothelin sites per cell.

**Cell proliferation, cell death, and protein synthesis inhibition assays**

For each assay, RITs were added approximately 16 hours after seeding of the cells in a 6- or 96-well plate. Growth inhibition was evaluated by measuring ATP levels with the Cell Titer-Glo Luminiscence Cell Viability assay (Promega). Values were normalized between controls of Dulbecco’s phosphate-buffered saline without Ca and Mg (D-PBS; Quality Biological, Inc.) containing 0.2% human serum albumin (HSA; Division of Veterinary Resources, NIH, Bethesda, MD) and 1 μmol/L staurosponine (Sigma-Aldrich). Cell death was visually verified with bright-field microscopy, and quantified using a cell counting assay. Pictures were taken on a Zeiss microscope with a ×10 EC Plan-NeoFluar objective using the AxioCam MRc camera and the AxioVision 4.7.2 acquisition software. Cells were counted in triplicate using a Cellometer Vision (Nexcelom), and dead cells were excluded using Trypan blue staining. Protein synthesis inhibition was quantified by measuring [³H]leucine (Perkin Elmer) incorporation as previously reported (17). Values are presented relative to controls of D-PBS HSA 0.2% and 100 μg/mL cycloheximide- (Sigma-Aldrich) treated controls.

**In vitro cellular RIT uptake**

RITs were labeled with the Alexa Fluor 647 Labeling Kit (Invitrogen) for 3.5 hours and purified according to manufacturer’s instructions. Harvested cells were incubated for 30, 60, and 120 minutes at 37°C with 2 μg/mL of SS1P-Alexa Fluor 647 or RG7787-Alexa Fluor 647 and processed as previously described (17). Fluorescence intensity was analyzed on a FACSCalibur.

**Mice experiments**

KLM-1 cells form tumors when implanted into immune-deficient mice and can therefore be used for antitumor studies. Five-week-old female athymic nude mice (NCR-Nu/Nu 01B74; NCI Frederic, MD) were injected subcutaneously in the flank with 4 × 10⁶ KLM-1 cells in 200 μL RPMI with 4 mg/mL matrigel (BD Biosciences). Tumor volume was measured regularly with electronic caliper, by taking a length and width measurements perpendicular to each other. Tumor volume was calculated as (length × width²) × 0.4. Mice were handled according to the NIH guidelines, and studies were approved by the Animal Care and Use Committee of the NCI.

**In vivo RG7787 uptake in tumors**

The cellular uptake of RG7787 within subcutaneous KLM-1 tumors was quantified by modifying a previously established method (21). In brief, animals were sacrificed, tumors were harvested and dissociated, and the percentage of human tumor cells that internalized the Alexa Fluor 647-labeled RG7787 was identified. When tumors reached a volume of approximately 120 to 130 mm³, approximately 1 week after cell engraftment. 0.4 mg/kg SS1P or 2.5 mg/kg RG7787 were injected intravenously in a volume of 200 μL D-PBS-HSA 0.2%. Paclitaxel (Hospira, Inc.) was purchased from the NIH Pharmacy, and injected intraperitoneally at 50 mg/kg in a volume of 800 μL D-PBS HSA 0.2% 1 day before the first dose of RG7787. All animals were handled according to the NIH guidelines, and studies were approved by the Animal Care and Use Committee of the NCI.
strips surface-bound RITs from the cell surface (21). Dissociated cells were filtered through a 40-μm cell strainer (BD Biosciences) and washed. To prevent nonspecific binding to the Fc receptor, cells were preincubated with 10 μg/mL rat anti-mouse CD16/32 (BD Pharmigen). To distinguish human tumor cells from murine cells, filtered cells were stained with R-PE anti-human CD71 (transferrin receptor; Biolegend). Rather than relying on flow cytometry forward and side scatter to identify viable cells as done previously (21), we standardized the selection of viable cells by staining cells with SYTOX Dead Cell Stains (Life Sciences). Doublet cells were excluded from the analysis. The fraction of viable single cells was further gated for CD71+ cells, which represent the human KLM-1 tumor cells. By comparing RG7787-treated tumors with a control tumor (D-PBS HSA 0.2%), the percentage of Alexa Fluor 647-positive tumor cells could be gated and quantified. This population represents the KLM-1 cells that internalized RG7787. Representative flow cytometry scatter dot plots and the required gating steps to obtain the presented uptake data are shown in Supplementary Fig. S1. Data were collected on an LSRII (BD Biosciences) and analyzed in FlowJo 10 software (Tree Star, Inc.).

**Tumor immunohistochemistry**

Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) on KLM-1 tumors were performed by Histoserv, Inc. with the following primary antibodies: anti-cleaved caspase-3 (1:200; Cell Signaling, #9661L) and antimesothelin MN (1:100; Rockland Immunochemicals, Inc.). Detailed procedures are presented in the Supplementary Methods. Pictures of the slides were taken on a Zeiss microscope, as described above.

**Statistical analysis**

Each experiment was performed independently at least twice, and representative or average data are displayed. Data are presented as mean ± SEM of duplicate or replicate experiments. Applied statistics include (paired) Student’s tests and one-way ANOVA with Tukey multiple comparison tests. Statistical analysis and figure drafting was done with GraphPad Prism 6 software (GraphPad Software, Inc.). A P value of less than 0.05 was considered statistically significant.

**Results**

**Mesothelin surface expression of PDAC cell lines**

Mesothelin surface expression was evaluated by flow cytometry in 5 established PDAC cell lines and one cell line (GUMC108) directly derived from a patient PDAC tumor. Panc 3.014, KLM-1, GUMC108, and AsPC-1 had high mesothelin expression (30,000 to 90,000 sites per cell), whereas PK-1 and BxPC-3 had low expression (3,000–5,000 sites per cell; Table 1).

**In vitro cytotoxic activity of RG7787 versus SS1P**

**Cell growth inhibition.** PDAC cell lines were treated for 72 hours with SS1P or RG7787 and cell viability was evaluated by ATP measurements. To account for the differences in molecular weight between SS1P (62 kDa) and RG7787 (73 kDa), IC50s are presented in ng/mL and pmol/L (Table 1). RG7787 had subnanomolar IC50s in most cell lines, and was significantly more active than SS1P in 3 of 6 cell lines: Panc 3.014 (P = 0.008), KLM-1 (P < 0.0001), and GUMC108 (P = 0.0005). SS1P and RG7787 did not significantly differ in AsPC-1 (P = 0.15) or BxPC-3 (P = 0.53). In PK-1, RG7787 did not reach an IC50 below 1 μg/mL (Table 1). SS1P and RG7787 showed the highest activity in the primary cell line GUMC108 and the established cell line KLM-1 (Fig. 2A).

**Cell death.** ATP assays cannot differentiate between cell proliferation arrest and cell death (22). We evaluated cell death in the 2 most sensitive cell lines, KLM-1 and GUMC108, by bright-field microscopy and counting viable cells. Cells were incubated for 72 hours with 100 ng/mL SS1P or 117 ng/mL RG7787 (the molar equivalent of 100 ng/mL SS1P). Photomicrographs of KLM-1 and GUMC108 cells at day 1 (just before adding the RITs),

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Mesothelin binding sites/cell (× 10⁶)</th>
<th>IC₅₀ ± SEM (ng/mL)</th>
<th>IC₅₀ ± SEM (pmol/L)</th>
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<tr>
<td></td>
<td></td>
<td>SS1P</td>
<td>RG7787</td>
</tr>
<tr>
<td>GUMC108</td>
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<td>3.98 ± 0.49</td>
<td>1.38 ± 0.15</td>
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<td>KLM-1</td>
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<tr>
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<td>3</td>
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<td>33.28 ± 6.47</td>
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<tr>
<td>Panc 3.014</td>
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<td>108.5 ± 2.4</td>
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<tr>
<td>PK-1</td>
<td>5</td>
<td>638.6 ± 11.9</td>
<td>&gt;1000</td>
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NOTE: Average data from at least three independent experiments are displayed. Mesothelin expression is evaluated by flow cytometry, and IC₅₀ are derived from 72-hour ATP cell viability assays. Cell lines are ranked from most to least sensitive to RG7787.
day 2, and day 4 (72-hour incubation) confirmed that RG7787 was more effective in killing cells than SS1P (Fig. 2C and D). With KLM-1, there were 2.5$\times$10^5 viable cells after a 72-hour incubation with SS1P (85% decrease compared with untreated cells day 4), whereas after RG7787 treatment there were very few cells detected (0.5$\times$10^5 cells, 97% decrease compared with untreated cells day 4). With GUMC108, a 72-hour SS1P treatment gave 2.7$\times$10^4 cells (82% decrease compared with untreated day 4), whereas RG7787 left only 1.6$\times$10^4 viable cells (99.9% decrease compared with untreated cells day 4; Fig. 2B). RG7787 in KLM-1 and both treatments in GUMC108 gave fewer cells remaining on day 4 than were counted on day 1. These data confirm that the RITs kill cells, and not just inhibit growth.

In vitro uptake of RG7787 and SS1P

An early step in RIT action is its binding and internalization. To evaluate whether the difference in activity between SS1P and RG7787 was because of higher cellular uptake of RG7787, we measured the in vitro internalization of both Alexa Fluor 647-labeled RITs in KLM-1 cells at 30, 60, and 120 minutes. Flow analysis showed that the uptake of SS1P and RG7787 was similar at the 3 time points (Fig. 3A). The enhanced activity of RG7787 is thus not because of increased uptake, but some other event.

Inhibition of protein synthesis by RG7787 and SS1P

A later step in RIT action that is essential for cell killing is protein synthesis arrest because of the inactivation of elongation factor 2. To evaluate whether the enhanced RG7787 activity could be detected at the level of protein synthesis inhibition, KLM-1 and GUMC108 cells were incubated with 100 ng/mL SS1P and 117 ng/mL RG7787 for 16 hours (Fig. 3B), after which [3H]leucine incorporation was measured. RG7787 was more potent than SS1P in KLM-1 (P < 0.0001) and GUMC108, although the latter difference did not reach significance (P = 0.26). In accordance with the higher sensitivity of GUMC108,
In vitro activity of RG7787 in a KLM-1 xenograft

KLM-1 cells were grown as a subcutaneous xenograft in athymic nude mice. We first evaluated SS1P at MTD; 3 × 0.4 mg/kg SS1P QOD i.v. (n = 3) showed no antitumor response compared with control mice (n = 3; Fig. 4A). In contrast, animals treated intravenously with 3 × 2.5 mg/kg RG7787 QOD (n = 6) showed a significant decrease in tumor size on day 5, from 2 days after the second dose on (–13%, P = 0.008; Fig. 4B). A second cycle of 3 × 2.5 mg/kg RG7787 QOD was initiated 5 days after the third RG7787 dose, when the tumors started to regrow. These 3 additional doses, however, did not induce additional tumor response. To try to improve the antitumor response, we combined RG7787 with paclitaxel. The combination of RITs with chemotherapeutics has previously shown to benefit tumor response (21, 23–25). One day before the start of a first 3 × RG7787 cycle, 50 mg/kg paclitaxel was administrated intraperitoneally. The combination of paclitaxel and RG7787 gave a significant tumor regression, which increased with a second treatment cycle of paclitaxel and 3 × RG7787. On day 26, 12 days after the last dose of RG7787, all 6 tumors had regressed below 5 mm³ (average 3 mm³) and were almost impalpable. Tumors only showed a significant regrowth after another 3 weeks (day 26 vs. day 46; P = 0.04), after which most tumors slowly regrew (Fig. 4B). Nine months posttherapy, two out of six mice have ongoing complete regressions with no palpable tumor. Mice that received the 2 single doses of 50 mg/kg paclitaxel alone (n = 6) had stable disease without significant tumor regression (baseline versus smallest volume; P = 0.46), and the tumors started to regrow approximately 4 weeks after the last dose. Follow-up of tumor growth was plotted on Fig. 4B as long as the combination group was complete (day 88), that is no mice were euthanized. When considering a threshold of 300 mm³, average tumor growth was delayed for approximately 4 weeks by RG7787, for 5 weeks by paclitaxel, and for >14 weeks by the combination, compared with the control tumors. These experiments were independently repeated and reproducible results were obtained (data not shown). Throughout the study, no toxicity or weight loss were observed. An exploratory dose-escalating toxicity study of RG7787 in athymic nude mice showed no weight loss with doses up to 4 × 4 mg/kg RG7787 i.v., demonstrating that the currently applied dose was below MTD. LD₅₀ was 1 × 10 mg/kg, and 1 × 20 mg/kg killed all animals (Supplementary Table S1).

IHC evaluation of KLM-1 tumors

To evaluate the effect of treatment on tumor histology, untreated and treated KLM-1 tumors were harvested at different time points, and stained with H&E and cleaved caspase-3 (Fig. 5). In an untreated tumor, KLM-1 cells were organized in clusters surrounded by connective tissue and limited apoptosis was detected, as indicated by the lack of cleaved caspase-3 staining (Fig. 5A). The effect of paclitaxel was evaluated by harvesting a tumor 1 day after a 50 mg/kg injection (1 d+), the standard time point at which RG7787 is administrated in the combination group. Paclitaxel disrupted tumor organization and induced apoptosis (Fig. 5A). We also evaluated the effect of 2 treatment cycles of 50 mg/kg paclitaxel and/or 3 × 2.5 mg/kg RG7787 QOD (Fig. 5B). Tumors were harvested at 17 and 24 days after the last dose of RG7787 and paclitaxel, respectively. In tumors treated with RG7787 or
paclitaxel alone, the effect of treatment was obvious with a disrupted organization and empty spaces present within the tumor. In the tumor treated with 2 cycles of the combination, the effect was striking. There was an abundance of connective tissue and few nests of tumor cells left, consistent with the near-complete regressions we observed in this treatment group (Fig. 4B). Of note, the KLM-1 tumors stained strongly for mesothelin, and expression was not affected by RG7787 treatment (Supplementary Fig. S2).

### RG7787 uptake in KLM-1 tumors

To evaluate how many of the tumor cells take up RG7787 in vivo, we optimized a previously developed method (21) for use in KLM-1 tumors. Briefly, after injection of Alexa Fluor 647–labeled RG7787, the tumor is dissociated and the percentage of Alexa Fluor 647–positive tumor cells is quantified with flow cytometry. To determine maximal uptake, mice were injected intravenously with 2.5 mg/kg RG7787-Alexa Fluor 647, and tumors were harvested at times ranging from 5 minutes to 14 hours after injection.
The number of KLM-1 cells that internalized RG7787 increased over time, with a highest uptake of 45% at 6 hours (Fig. 4C). This indicates that the RG7787 dose we treat mice with reaches only about half of the tumor cells, which in part can explain the limited tumor response of RG7787 as a single therapy. We previously found that paclitaxel treatment of mice with A431/H9 tumors increased the uptake of SS1P into the cells within the tumor (21). To determine if paclitaxel treatment did the same for RG7787 in KLM-1 tumors, we evaluated uptake after administration of 2.5 mg/kg RG7787-Alexa Fluor 647, alone or preceded by 50 mg/kg paclitaxel 1 or 3 days earlier. Paclitaxel had no effect on RG7787 uptake (Fig. 4D).

Discussion

Our goal is to develop a new treatment for pancreatic cancer. We report here that RG7787 has high cytotoxic activity against mesothelin-expressing PDAC cells in vitro, and in combination with paclitaxel causes profound and durable tumor regressions in mice. RG7787 is a novel RIT designed to have low immunogenicity and fewer side-effects than SS1P, with the latter recently reported to have high activity in patients with advanced mesothelioma (12).

**RG7787 has significant in vitro activity in PDAC cell lines and primary cells**

RG7787 was evaluated in a panel of 5 established PDAC cell lines. Because their characteristics can differ greatly from the original primary tumors (26), we also studied PDAC cells directly derived from a patient tumor, GUMC108. These cells were grown using an approach that maintains the phenotype of the primary tumor, allowing for, for example, in vitro chemo-sensitivity testing of patient cells (19, 27). We found that RG7787 had subnanomolar IC50s in most cell lines, and was most active on GUMC108 cells.

**Enhanced activity of RG7787 versus SS1P is cell-line specific**

RG7787 had a significantly enhanced activity compared with SS1P in half of the PDAC cell lines. The ability to inhibit protein synthesis correlated with the cytotoxic activity of SS1P and RG7787, suggesting that the mechanism for the improved activity of RG7787 lies upstream of the protein synthesis inhibition. We found no difference in in vitro uptake between SS1P and RG7787 in KLM-1 cells. The higher activity of RG7787 may be attributable to the deletion of protease-sensitive regions in PE domain II, which makes it resistant to degradation in the endolysosomal system, a potential barrier to effective RIT treatment (28, 29). It remains unclear, however, why the improvement in activity was not observed in all probed cell lines. Further research is needed to elucidate these cell-specific differences.

**RG7787 has significant tumor response and moderate uptake in vivo**

We evaluated the in vivo activity of SS1P and RG7787 in a subcutaneous KLM-1 xenograft model. At the maximum dose that can be given safely QOD (3 × 0.4 mg/kg), SS1P had no effect on tumor growth. RG7787 can be administered at much higher doses because of the lower off-target toxicity associated with the PE24 moiety (13, 15). An exploratory toxicity study in mice indicated that 4 × 4 mg/kg RG7787 QOD i.v. can be safely administered. We chose to give 3 × 2.5
mg/kg. The first RG7787 cycle of 3 doses induced a small but statistically significant decrease in tumor size. This is in contrast with the high sensitivity to RG7787 of KLM-1 in vitro. To determine if the small response was because of low uptake of RG7787, we quantified RG7787 uptake in KLM-1 tumors by optimizing a previously developed flow cytometry method of dissociated tumor cells (21). This dissociation process removes bound Rit from the cell surface and allows us to quantify the cells that internalized RG7787. Other methods measure both bound and internalized molecules as well as molecules trapped in the extracellular fluid or by other cell types (21). We found that a dose of 2.5 mg/kg RG7787 reached only about half of the tumor, suggesting that the small tumor regression is linked to tumor accessibility, rather than RG7787 activity. Further research will focus on the amount of Rit that accumulates in the tumor cells, which is also critical with regard to cytotoxicity.

Paclitaxel dramatically improves RG7787 tumor response, but not uptake

The entry of antibodies and antibody-derived therapeutics like RITs into solid tumors is known to be limited by the close packing of tumor cells, high interstitial pressure within tumors, and a lack of functional lymphatics (30). One approach to overcome this barrier is to combine RITs with chemotherapeutics. Combining SS1P with paclitaxel, for example, resulted in an enhanced response in A431/H9 xenografts (21). Similar observations were made with our KLM-1 tumor model. We found that 2 cycles of paclitaxel and RG7787 resulted in durable near-complete responses, including 2 out of 6 mice with no detectable tumor 9 months posttherapy. IHC analysis showed that tumors treated with both paclitaxel and RG7787 consisted primarily of connective tissue with very few tumor cells left. Unexpectedly and in contrast to previous findings with SS1P in A431/H9 tumors (21), treatment with paclitaxel did not lead to an improved uptake of RG7787 in KLM-1 tumors. We are now investigating the mechanism by which paclitaxel and RG7787 induce such profound antitumor responses.

Clinical prospects and challenges for RG7787 in PDAC

PDAC is well known for its dense stroma, which results in poor drug uptake. Compounds that target the stroma have been shown to improve PDAC accessibility for cytotoxic drugs (31). Several examples are currently in clinical trials. The combination of gemcitabine with nab-paclitaxel, an albumin-bound formulation of paclitaxel particles, for example, has recently shown to be an active regimen in PDAC (32). The evaluation of stroma-targeting compounds in combination with RG7787 will require in vivo models that closely mimic the PDAC microenvironment (31, 33).

In conclusion, RG7787 is a novel antimesothelin RIT optimized for clinical use with a significant activity in vitro in established and primary PDAC cell lines. RG7787 can be administered safely at high doses in mice, and induces significant tumor responses in a PDAC mouse model. This response is dramatically enhanced when combined with paclitaxel, resulting in durable near-complete regressions. Our preclinical findings demonstrate that RG7787 merits further evaluation for the treatment of PDAC.

Disclosure of Potential Conflicts of Interest

S. Imhof-Jung has ownership interest in patent E-771-2013/1-US-01. C. Niederfellner has ownership interest as a coinventor in patent E-771-2013/1-US-01. I. Pastan has ownership interest in patents US 8357783 (owned by NIH), US 20120263674 (owned by NIH), and US 20140094417 (owned by NIH). No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: K. Hollevoet, S. Imhof-Jung, G. Niederfellner, I. Pastan
Development of methodology: K. Hollevoet, E. Mason-Osann
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Hollevoet, E. Mason-Osann, X.-f. Liu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Hollevoet, E. Mason-Osann, G. Niederfellner, I. Pastan
Writing, review, and/or revision of the manuscript: K. Hollevoet, E. Mason-Osann, S. Imhof-Jung
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Hollevoet, S. Imhof-Jung
Study supervision: I. Pastan

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References

Correction: In Vitro and In Vivo Activity of the Low-Immunogenic Antimesothelin Immunotoxin RG7787 in Pancreatic Cancer

In this article (Mol Cancer Ther 2014;13:2040–49), which appeared in the August 2014 issue of Molecular Cancer Therapeutics (1), the authors request a revision of the following text on page 2041 of the article:

In addition, 7 mutations in domain III (R505A, R427A, R490A, R467A, D463A, R458A, and R538A) were introduced to silence previously identified B-cell epitopes (14).

The revised text is as follows:

In addition, 7 mutations in domain III (R505A, R427A, R490A, R467A, D463A, R538A, and R456A) were introduced to silence identified B-cell epitopes. Six of these mutations are identical to those included in the previously reported LR-LO10 PE moiety: R505A, R427A, R490A, R467A, D463A, and R538A (14). The seventh mutation originally introduced in LR-LO10, R458A, was later found to lead to loss of activity and therefore not included in RG7787. As an alternative and to destroy the targeted B-cell epitope, we focused on changing nearby residues of R458. In the structure of domain III, R456 is close to R458 and was therefore mutated to A. We found that R456A was more active than R458A and also destroyed the B-cell epitope.

Additionally, mutation 456A is incorrectly labeled as 458A in Fig. 1 and in the Fig. 1 legend.

Reference

In Vitro and In Vivo Activity of the Low-Immunogenic Antimesothelin Immunotoxin RG7787 in Pancreatic Cancer

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