**In vitro and in vivo activity of the low-immunogenic anti-mesothelin immunotoxin RG7787 in pancreatic cancer**

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**Running title:** Activity of RG7787 in pancreatic cancer cells

**Keywords:** immunotoxin; KLM-1; mesothelin; pancreatic cancer

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Conflict of interest: I.P. is an inventor on several patents on immunotoxins that have all been assigned to NIH (including US 8357783, US 20120263674, US 20140094417 and E-771-2013/1-US-01) and has a Cooperative Research and Development Agreement (#2791) with Roche Pharmaceuticals. G.N. and S.I are inventors on patent application E-771-2013/1-US-01. The other authors disclose no potential conflicts of interest.

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) has a dismal prognosis, and new therapies are needed. RG7787 is a novel low-immunogenic anti-mesothelin recombinant immunotoxin (RIT), engineered to overcome the limitations of SS1P, 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 SS1P 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 x 2.5 mg/kg RG7787 QOD combined with two cycles of 1 x 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 Alexa647-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.
INTRODUCTION

Pancreatic cancer is the fourth leading cause of cancer death in the US (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 to direct therapies against (5,6). Our laboratory produces and evaluates recombinant immunotoxins (RITs) 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) (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 SS1P 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 3 trial is now open (ClinicalTrials.gov Identifier: NCT01829711). In phase I clinical trials in patients with solid tumors, SS1P was well-tolerated but the high immunogenicity of the PE portion typically induced neutralizing anti-drug antibodies after one treatment cycle, resulting in limited anti-tumor activity (10,11).
Our laboratory has focused on reducing this dose-limiting immunogenicity. One approach aims at suppressing the host immune system, by combining SS1P with immune-depleting chemotherapeutic agents. In a recent phase I trial (ClinicalTrials.gov Identifier: NCT01362790), this allowed for multiple SS1P cycles which resulted in striking and unprecedented responses in patients with advanced refractory mesothelioma (12). These findings clearly illustrate that RITs can have high anti-tumor 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 non-specific 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 anti-mesothelin RIT by linking it to a humanized anti-mesothelin Fab (huSS1), thereby increasing size and circulatory half-life. This clinically-optimized RIT is named RG7787 (Figure 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, anti-mesothelin RITs were not yet evaluated in PDAC xenograft models. The aims of the current study were a) to evaluate the cytotoxicity of RG7787 in established and primary PDAC cell lines, b) to evaluate the anti-tumor activity of RG7787 in a PDAC mouse model, both alone and in combination with paclitaxel, and c) 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. (Kensington, MD) and Roche Innovation Center Penzberg, Germany, respectively. RG7787 is a re-engineered version of SS1P (Figure 1 shows a comparison of their structures) consisting of a humanized anti-mesothelin 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 (US7081518 B1) were grafted onto human V_H and V_L 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 min vs. 20 min 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:

PTGAEFLGDDGVDSFSTRGTQNWTVERRLLQAHAQLEERGFVFVGYHGTLEAAQSIVF
GGVAARSLADAIWAGFYIAQDGPAAYGAQDQEPDAAGRIRNGALLRHYVPAASLPFG
FYRTSTLAAPEAAGEVERLIGHPLPLALDAITGPEEEGGRLETILGWPAERTVVIPSAP
TDPRNVGGDLPSSIPDKEQAISALPDYASQPGKPPREDLK. 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, seven 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. Udo 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. Elizabeth Jaffee (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, Bethesda, MD). RPMI-1640 was supplemented with 2 mM L-glutamine, 1 mM 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. Richard Schlegel in July 2012 (Georgetown University Medical Center, DC). GUMC108 cells were cultivated on irradiated (3000 rad) Swiss 3T3 fibroblasts (J2 strain) in F medium [3:1 (v/v) F-12 Nutrient Mixture (Ham)-DMEM (Invitrogen), 5% fetal bovine serum, 0.4 μg/mL hydrocortisone (Sigma-Aldrich), 5 μg/mL insulin (Sigma-Aldrich), 8.4 ng/mL cholera toxin (Sigma-Aldrich), 10 ng/mL epidermal growth factor (Invitrogen), and 24 μg/mL adenine (Sigma-Aldrich)] with addition of 5 μmol/L ROCK inhibitor Y-27632 (Enzo Life Sciences). This approach has previously been shown to support indefinite proliferation of primary cells while maintaining the phenotype of the primary tumor (19).

Differential trypsinization was used to separate feeder and epithelial cells during passaging. In short-term cell-based assays, GUMC108 cells were cultured without the irradiated fibroblasts, to avoid interference from the feeder layer. All cells were maintained at 37°C in a humidified incubator, with 5% CO₂.
Mesothelin surface expression

To quantify surface mesothelin expression, harvested cells were stained with 5 μg/ml of mouse anti-human mesothelin MN antibody (20) (Rockland Immunochemicals, 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 hrs 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 Luminescent 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 μM staurosporine (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 10X 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 hrs and purified according to manufacturer’s instructions. Harvested cells were incubated for 30, 60 and 120 min at 37°C with 2 μg/ml of SS1P-Alexa647 or RG7787-Alexa647 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 anti-tumor studies. Five-week old female athymic nude mice (NCR-Nu/Nu 01B74; NCI Frederic, MD) were injected subcutaneously in the flank with 4 x 10^6 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 x width^2) x 0.4. Mice were grouped and treatment started when tumors reached approximately 120-130 mm^3, approximately one week after cell engraftment. 0.4 mg/kg SS1P or 2.5 mg/kg RG7787 were injected iv in a volume of 200 μl D-PBS-HSA 0.2%. Paclitaxel (Hospira, Inc.) was purchased from the NIH Pharmacy (Bethesda, MD), and injected ip at 50 mg/kg in a volume of 800 μl D-PBS HSA 0.2%, one day prior to the first dose of RG7787. All animals were handled according to the National Institutes of Health guidelines, and studies were approved by the Animal Care and Use Committee of the National Cancer Institute.

**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 Alexa647-labeled RG7787 was identified. When tumors reached a volume of approximately 120-30 mm$^3$, Alexa647-labeled RG7787 was injected iv. At the indicated time points, animals were sacrificed, and tumors were excised and dissociated into single cells using 0.2 Wunch U/ml Liberase Research Grade TM (Roche Diagnostics) and 0.1 mg/ml DNase I (Sigma-Aldrich). The collagenase-based dissociation process 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 non-specific binding to the Fc receptor, cells were pre-incubated 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 Alexa647-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 Figure S1. Data was collected on an LSRII (BD Biosciences) and analyzed in FlowJo 10 software (Tree Star, Inc.).
Tumor immunohistochemistry

H&E staining and immunohistochemistry (IHC) on KLM-1 tumors was performed by Histoserv, Inc. with the following primary antibodies: anti-cleaved caspase 3 (1:200, Cell Signaling, #9661L) and anti-mesothelin 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.

Statistics

Each experiment was performed independently at least twice, and representative or average data are displayed. Data are presented as mean ± standard error of measurement (SEM) of duplicate or replicate experiments. Applied statistics include (paired) Student t-tests and one-way ANOVA with Tukey’s 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 five 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 (3000 to 5000 sites per cell) (Table 1).

In vitro cytotoxic activity of RG7787 versus SS1P

Cell growth inhibition: PDAC cell lines were treated for 72 hrs 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), IC_{50}s are presented in ng/ml and pM (Table 1). RG7787 had subnanomolar IC_{50}s in most cell lines, and was significantly more active than SS1P in three out of six 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 IC_{50} 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 (Figure 2A).

Cell death: ATP assays cannot differentiate between cell proliferation arrest and cell death (22). We evaluated cell death in the two most sensitive cell lines, KLM-1 and GUMC108, by bright-field microscopy and counting viable cells. Cells were incubated for 72 hrs 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), day 2 and day 4 (72 hr incubation) confirmed that RG7787 was more effective in killing cells than SS1P (Figure 2C-D). With KLM-1, there were 2.5 x 10^5 viable cells after a 72-hr incubation with SS1P (85% decrease
compared to untreated cells day 4), while after RG7787 treatment there were very few cells detected \((0.5 \times 10^5 \text{ cells}, 97\% \text{ decrease compared to untreated cells day 4})\). With GUMC108, a 72-hr SS1P treatment gave \(2.7 \times 10^4 \text{ cells} \) (82\% decrease compared to untreated day 4), whereas RG7787 left only \(1.6 \times 10^2 \text{ viable cells} \) (99.9\% decrease compared to untreated cells day 4) (Figure 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 due to higher cellular uptake of RG7787, we measured the *in vitro* internalization of both Alexa-647-labeled RITs in KLM-1 cells at 30, 60 and 120 min. Flow analysis showed that the uptake of SS1P and RG7787 was similar at the three time points (Figure 3A). The enhanced activity of RG7787 is thus not due to 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 due to 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 hrs (Figure 3B), after which \([^{3}\text{H}]\)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 \((p = 0.26)\).
with the higher sensitivity of GUMC108, protein synthesis was significantly more inhibited by SS1P ($p < 0.0001$) and RG7787 ($p = 0.01$), compared to KLM-1. This indicates that the improved activity of RG7787 is related to events occurring prior to the protein synthesis inhibition step.

**In vivo 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 x 0.4 mg/kg SS1P QOD iv (n=3) showed no anti-tumor response compared to control mice (n=3) (Figure 4A). In contrast, animals treated iv with 3 x 2.5 mg/kg RG7787 QOD (n=6) showed a significant decrease in tumor size on day 5, from two days after the second dose on (-13%, $p = 0.008$) (Figure 4B). A second cycle of 3 x 2.5 mg/kg RG7787 QOD was initiated 5 days after the third RG7787 dose, when the tumors started to regrow. These three additional doses, however, did not induce additional tumor response. To try to improve the anti-tumor response, we combined RG7787 with paclitaxel. The combination of RITs with chemotherapeutics has previously shown to benefit tumor response (21,23-25). One day prior to the start of a first 3 x RG7787 cycle, 50 mg/kg paclitaxel was administrated ip. The combination of paclitaxel and RG7787 gave a significant tumor regression, which increased with a second treatment cycle of paclitaxel and 3 x RG7787. On day 26, 12 days after the last dose of RG7787, all six tumors had regressed below 5 mm$^3$ (average 3 mm$^3$) and were almost impalpable. Tumors only showed a significant regrowth after another 3 weeks (day 26 versus day 46; $p = 0.04$), after which most tumors slowly regrew (Figure 4B). Nine months post-therapy, one mouse still has a tumor below 100 mm$^3$ that does not grow, and another mouse has an ongoing complete regression with no palpable tumor. Mice that received the two 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 four weeks after the last dose. Follow-up of tumor growth was plotted on Figure 4B as long as the combination group was complete (day 88), i.e. no mice were euthanized. When considering a threshold of 300 \( \text{mm}^3 \), average tumor growth was delayed for approximately 4 weeks by RG7787, for 5 weeks by paclitaxel, and for >14 weeks by the combination, compared to 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 x 4 mg/kg RG7787 QOD iv, demonstrating that the currently applied dose was below MTD. LD\(_{50}\) was 1 x 10 mg/kg, and 1 x 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 (Figure 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 (Figure 5A). The effect of paclitaxel was evaluated by harvesting a tumor 1 day after a 50 mg/kg injection (1d+), the standard time point at which RG7787 is administrated in the combination group. Paclitaxel disrupted tumor organization and induced apoptosis (5A). We also evaluated the effect of two treatment cycles of 50 mg/kg paclitaxel and/or 3 x 2.5 mg/kg RG7787 QOD (Figure 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 two 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 (Figure 5B). Of note, the KLM-1 tumors stained strongly for mesothelin, and expression was not affected by RG7787 treatment (Supplementary Figure 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-647-labeled RG7787, the tumor is dissociated and the percentage of Alexa-647 positive tumor cells is quantified with flow cytometry. To determine maximal uptake, mice were injected iv with 2.5 mg/kg RG7787-Alexa647, and tumors were harvested at times ranging from 5 min to 14 hrs after injection. The number of KLM-1 cells that internalized RG7787 increased over time, with a highest uptake of 45% at 6 hrs (Figure 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-Alexa647, alone or preceded by 50 mg/kg paclitaxel one or three days earlier. Paclitaxel had no effect on RG7787 uptake (Figure 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 advanced mesothelioma patients (12).

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

RG7787 was evaluated in a panel of five established PDAC cell lines. Since 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, e.g., 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

RG778 had a significantly enhanced activity compared to 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 x 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 x 4 mg/kg RG7787 QOD iv can be safely administered. We chose to give 3 x 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 due to 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 RITs 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 (20). 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 regards to cytotoxicity.

**Paclitaxel dramatically improves RG778 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, e.g., resulted in an enhanced response in A431/H9 xenografts (21). Similar observations were made with our KLM-1 tumor model. We
found that two cycles of paclitaxel and RG7787 resulted in durable near-complete responses, including one mouse with an ongoing complete regression and one with a small tumor that shows no sign of regrowth, nine months post-therapy. 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 anti-tumor 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, e.g., 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 anti-mesothelin 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 pre-clinical findings demonstrate that RG7787 merits further evaluation for the treatment of PDAC.
REFERENCES


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Table 1. Mesothelin expression and cytotoxicity of SS1P and RG7787 in PDAC cell lines

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<tr>
<th>Cell lines</th>
<th>Mesothelin binding sites/cell (x 10^3)</th>
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<td>GUMC108</td>
<td>35</td>
<td>3.98 ± 0.49</td>
<td>1.38 ± 0.15</td>
</tr>
<tr>
<td>KLM-1</td>
<td>60</td>
<td>23.90 ± 2.81</td>
<td>4.44 ± 0.41</td>
</tr>
<tr>
<td>AsPC-1</td>
<td>30</td>
<td>14.76 ± 2.24</td>
<td>22.5 ± 1.34</td>
</tr>
<tr>
<td>BxPC-3</td>
<td>3</td>
<td>24.4 ± 2.47</td>
<td>33.28 ± 6.47</td>
</tr>
<tr>
<td>Panc 3.014</td>
<td>90</td>
<td>848.2 ± 151.8</td>
<td>108.5 ± 2.4</td>
</tr>
<tr>
<td>PK-1</td>
<td>5</td>
<td>638.5 ± 11.9</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

Average data from at least three independent experiments is displayed. Mesothelin expression is evaluated by flow cytometry, and IC₅₀s are derived from 72-hr ATP cell viability assays. Cell lines are ranked from most to least sensitive to RG7787.
FIGURE LEGENDS

Figure 1. Structural models of recombinant anti-mesothelin immunotoxins

Structural models of SS1P [SS1(dsFv)-PE38] and RG7787 [huSS1(Fab)-LR-GGS-LO10-PE24] are shown. Cartoons are created using VMD (34), based on the X-ray crystal structure of PE (Protein Data Bank code: 1IKQ). Models are hypothetical only and do not represent actual structural determinations. Native PE consists of three structural domains organized from a single polypeptide sequence. SS1P includes a 38-kDa PE truncation (PE38) that contains deletions in domain Ia and domain Ib. The model of SS1P shows the anti-mesothelin dsFv on the left and P38 on the right. RG7787 is a re-engineered version of SS1P. Modifications include removing the bulk of PE domain II, leaving a furin cleavage site, and adding a GGS peptide linker after the furin cleavage site. RG7787 is further optimized by replacing the mouse anti-mesothelin Fv (SS1) with a humanized anti-mesothelin Fab (huSS1) and by introducing seven mutations (R505A, R427A, R490A, R467A, D463A, R458A, and R538A) in the catalytic domain III of PE to silence B-cell epitopes.

Figure 2. Cytotoxicity of RG7787 versus SS1P in PDAC cell lines

A: Cell viability assays show that RG7787 is more cytotoxic than SS1P in the established cell line KLM-1 and primary GUMC108 cells. Cells were incubated for 72 hrs with SS1P or RG7787, and growth inhibition was evaluated with an ATP viability assay. Data is normalized between D-PBS HSA 0.2%- and staurosporine-treated controls. B: Viable cell counts done in triplicate of SS1P- and RG7787-treated KLM-1 and GUMC108 cells. Day 1 is just before addition of the RITs, and day 4 equals 72 hrs of treatment. C-D: Bright-field microscopy pictures of KLM-1 and GUMC108 confirm that RITs induce cell death. Cells were incubated with 100
ng/ml of SS1P or 117 ng/ml RG7787 for 72 hrs. Pictures (10X) were taken from identical locations within the wells at each time point. Representative series of pictures are shown. At day 4, floating dead cells were washed out prior to taking pictures. The cell count data (B) quantifies the visual observations.

**Figure 3. Cellular uptake and protein synthesis inhibition of RG7787 versus SS1P in PDAC cell lines**

A: *In vitro* cellular uptake of SS1P- and RG7787-Alexa647 is similar in KLM-1. Flow cytometry was used to obtain geomean fluorescence intensities (GFI) at each time point. B: RG7787 inhibits protein synthesis inhibition more than SS1P in KLM-1 and GUMC108. Cells were incubated with 100 ng/ml of SS1P or 117 ng/ml RG7787 for 16 hrs. Protein synthesis was evaluated by measuring [³H]leucine incorporation. Data is normalized between D-PBS HSA 0.2%- and cycloheximide-treated cells.

**Figure 4. RG7787 efficacy and uptake in a KLM-1 xenograft model**

Athymic nude mice were subcutaneously injected with 4 x 10⁶ KLM-1 cells, and treatment was initiated when tumors reached a volume of 120-130 mm³. Panels A and B present anti-tumor activity, and panels C and D the uptake of RG778 in the KLM-1 tumors quantified by a tumor dissociation flow cytometry method. A: Tumor-bearing mice were treated with D-PBS HSA 0.2% iv (control, n = 3) and one cycle of 3 x 0.4 mg/kg SS1P QOD iv (n=3). Black arrows present the three SS1P injections. SS1P had no effect on tumor growth. B: Mice were treated with D-PBS HSA 0.2% iv (control, n = 5, white diamonds), two cycles of 3 x 2.5 mg/kg RG7787 QOD iv (n=6, black diamonds), two cycles of 1 x 50 mg/kg paclitaxel ip (n=6, grey diamonds),
or two cycles of 3 x 2.5 mg/kg RG7787 QOD iv and 1 x 50 mg/kg paclitaxel ip combined (n=6, grey-black diamonds), with each dose of paclitaxel administered 1 day before the first of three doses of RG7787. Two cycles of the paclitaxel and RG7787 combination induced durable near-complete regressions. One mouse in the combination group has an ongoing complete response 9 months after therapy. Follow-up in the combination group is plotted as long as groups were complete (day 88), i.e. until no mice had to be euthanized in order to comply with humane endpoints. Grey arrows are the time points of the two paclitaxel injections, black arrows present the six RG7787 injections. 

C: RG7787 uptake in vivo is limited to half of the tumor cells. KLM-1 tumors were harvested 5 min, 45 min, 3 hrs, 6 hrs, 9 hrs and 14 hrs after iv injection of 2.5 mg/kg RG778-Alexa647. The number of KLM-1 cells that internalize RG778-Alexa647 increases over time, with a maximal uptake of 45% at 6 hrs. 

D: Mice were injected with 2.5 mg/kg RG778-Alexa647 one day and three days after the injection of a single dose of 50 mg/kg paclitaxel ip, or without prior injection of paclitaxel. KLM-1 tumors were harvested 3 hrs after injection. Paclitaxel had no significant effect on RG7787 uptake. All experiments were performed at least in duplicate.

Figure 5. Immunohistochemical evaluation of KLM-1 tumors

KLM-1 tumors were stained with H&E and cleaved caspase 3, a marker of cell death. A: In an untreated tumor, KLM-1 cells were organized in clusters surrounded by connective tissue, and limited cleaved caspase 3 staining was observed. One day after 50 mg/kg paclitaxel ip (1d+), tumor organization was disrupted and significant cleaved caspase 3 staining was observed. B: In tumors treated with two cycles of 3 x 2.5 mg/kg RG7787 iv or two doses of 50 mg/kg paclitaxel ip alone, the effect of treatment was obvious. Organization was disrupted, and empty spaces
were present within the tumor. In the tumor treated with two cycles of the combination, there was a vast presence of connective tissue with very few nests of tumor cells left. Representative pictures are shown (10X).
SS1(dsFv)-PE38  = RG7787

huSS1(Fab)-LR-GGS-LO10-PE24  = RG7787
Fig. 2

A. Cell viability (%)

KLM-1

SS1P

RG7787

GUMC108

SS1P

RG7787

RIT (ng/ml)

0.01 0.1 1 10 100 1000

0.00 0.25 0.50 0.75 1.00

0.50 0.75 1.00

B. KLM-1

GUMC108

RIT (ng/ml)

0.01 0.1 1 10 100 1000

0.00 0.25 0.50 0.75 1.00

0.50 0.75 1.00

C. Day 1 Day 2 Day 4

Untreated

100 ng/ml SS1P

117 ng/ml RG7787

KLM-1

D. Day 1 Day 2 Day 4

Untreated

100 ng/ml SS1P

117 ng/ml RG7787

GUMC108
Fig. 4

A. Volume (mm$^3$) vs. Days since treatment start

- Control
- 0.4 mg/kg SS1P

B. Volume (mm$^3$) vs. Days since treatment start

- Control
- Paclitaxel 50 mg/kg
- RG7787 2.5 mg/kg
- Paclitaxel + RG7787

C. In vivo uptake (%) vs. Time since RG7787 injection (min)

D. In vivo RG7787 uptake (%)
Fig. 5

A. Untreated  Paclitaxel 1d+  
   Untreated cleaved casp-3  Paclitaxel 1d+ cleaved casp-3

B. RG7787 17d+  Paclitaxel 24d+  
   Paclitaxel 24d+ RG7787 17d+
Molecular Cancer Therapeutics

In vitro and in vivo activity of the low-immunogenic anti-mesothelin immunotoxin RG7787 in pancreatic cancer

Kevin Hollevoet, Emily Mason-Osann, Xiu-Fen Liu, et al.

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