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Targeting angiogenesis for radioimmunotherapy with a ^{177}Lu -labeled antibody

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Abstract

Purpose—Increased angiogenesis is a marker of aggressiveness in many cancers. Targeted radionuclide therapy of these cancers with angiogenesis-targeting agents may curtail this increased blood vessel formation and slow the growth of tumors, both primary and metastatic. CD105, or endoglin, has a primary role in angiogenesis in a number of cancers, making this a widely-applicable target for targeted radioimmunotherapy.

Methods—The anti-CD105 antibody, TRC105 (TRACON Pharmaceuticals), was conjugated with diethylenetriaminepentaacetic acid (DTPA) for radiolabeling with ^{177}Lu ($t_{1/2}$: 6.65 days). Balb/c mice were implanted with 4T1 mammary carcinoma cells, and five study groups were employed: ^{177}Lu -only, TRC105 only, ^{177}Lu -DTPA-IgG (a nonspecific antibody), ^{177}Lu -DTPA-TRC105 low dose, and ^{177}Lu -DTPA-TRC105 high dose. Toxicity of the agent was monitored through body weight measurements and analysis of blood markers. Biodistribution studies of ^{177}Lu -DTPA-TRC105 were also performed at 1 and 7 days post-injection. *Ex vivo* histology studies of various tissues were conducted at 1, 7, and 30 days post-injection of high dose ^{177}Lu -DTPA-TRC105.

Results—Biodistribution studies indicated steady uptake of ^{177}Lu -DTPA-TRC105 in 4T1 tumors between 1 and 7 days post-injection (14.3 ± 2.3 %ID/g and 11.6 ± 6.1 %ID/g, respectively; $n = 3$) and gradual clearance from other organs. Significant inhibition of tumor growth was observed in the high dose group, with a corresponding significant increase in survival ($p < 0.001$, all groups). In

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Compliance with ethical standards

Conflict of interest: C.P. Theuer is the CEO of TRACON Pharmaceuticals. No potential conflicts of interest were disclosed by the other authors.

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most study groups (all except the nonspecific IgG), mouse body weight did not decrease by more than 10%, indicating the safety of the injected agents. Serum ALT quantification indicated steady levels and no damage to the liver (a primary clearance organ of the agent), confirmed with *ex vivo* histological analyses.

Conclusion— ^{177}Lu -DTPA-TRC105, when administered at a sufficient dose, is able to curtail tumor growth and provide a significant survival benefit without off-target toxicity. Thus, this targeted agent holds promise to be combined with other treatment options in order to slow tumor growth and allow for greater therapeutic indices.

Keywords

angiogenesis; radioimmunotherapy; lutetium-177 (^{177}Lu); CD105; endoglin; cancer

Introduction

Angiogenesis, or the formation of new blood vessels, is a characteristic trait of many cancers, involving a wide variety of pathways [1, 2]. The vascular endothelial growth factor (VEGF) and its receptor (VEGFR) have been the target of many clinical attempts to curtail angiogenesis, with varying levels of success in nearly every type of cancer [3–5]. These treatments, often utilizing bevacizumab, induce responses in a subpopulation of patients; however, even these responders often progress [6, 7].

CD105, or endoglin, is an endothelial cell marker that has been implicated to be a suitable biomarker for angiogenesis, as well as a possible therapeutic target [8–10]. As CD105 has a very limited normal tissue biodistribution [11], it has become an attractive target for both treatment and imaging of angiogenic tumors [12]. TRC105, a human/murine chimeric antibody targeting CD105, has been clinically studied in a wide variety of cancers in phase I and II trials [13–17]. While limited toxicity has been observed in patients enrolled in these studies, clinical activity of TRC105 alone has also been rather limited, with minimal benefit being reported from this drug as monotherapy. TRC105 is thus being explored as a combination therapy with other anti-cancer treatments.

One such option for enhancing the efficacy of TRC105 is the addition of a beta-emitting radionuclide such as ^{177}Lu ($t_{1/2}$: 6.65 days, E_{max} : 0.497 MeV) for targeted radionuclide therapy. Unlike external beam radiation treatments, in which the exact location of a tumor needs to be known, radioimmunotherapy depends on knowledge of the biology of a cancer, regardless of its location within the body [18, 19]. This means that such targeted strategies are feasible for metastatic disease, or for tumors in radiation-sensitive areas. Ideal targets for radioimmunotherapy are preferentially expressed on cancerous tissue with minimal normal tissue expression, as in the case of CD105. Potential therapeutic targets on cancer cells are easily identified through biopsy, which is nearly always performed for primary tumors. Anti-angiogenic agents have been combined with both external and targeted radiotherapy with success as a dual treatment preclinically [20–22], and VEGF-targeting has been studied as well for potential radioimmunotherapy [23, 24]. CD105-targeted agents hold promise to be applied in a number of cancers, as its expression has been noted throughout the literature in many tumor types [11]. We therefore herein present a widely-applicable targeted

radionuclide therapy agent based upon TRC105 for targeting of angiogenesis for therapy with ^{177}Lu .

Methods and Materials

Antibody chelation and determination of radiolabeling conditions

The anti-CD105 antibody, TRC105 (TRACON Pharmaceuticals) was obtained commercially. The antibody was prepared for radiolabeling through conjugation with the monofunctional versions of either DOTA or DTPA (Macrocyclics), using standard methods [12]. $^{177}\text{LuCl}_3$ was obtained from Perkin Elmer. As both DOTA and DTPA may be employed for chelation of ^{177}Lu ($t_{1/2}$: 6.65 days), radiolabeling efficiencies were compared. Various ratios of antibody to isotope (from 0.05 $\mu\text{g}/\mu\text{Ci}$ to 0.5 $\mu\text{g}/\mu\text{Ci}$) were incubated for varying lengths of time from 5 to 60 minutes, and labeled and unlabeled fractions were quantified using iTLC. Similar procedures were utilized for radiolabeling the nonspecific isotype-matched antibody, ^{177}Lu -DTPA-IgG (Invitrogen).

Flow cytometry

Flow cytometry was utilized to evaluate the binding of TRC105 and DTPA-TRC105 to 4T1 cells (CD105 negative) and human umbilical vein endothelial cells (HUVECs, CD105 positive). Cells were prepared and stained according to previously reported procedures [25]. Control groups of cells alone, secondary (goat anti-human AlexaFluor488 antibody, Invitrogen) alone, and a nonspecific IgG isotype control antibody (Invitrogen) were employed. Samples were tested using a BD FACSAria cell sorter, and data was analyzed using FlowJo V10 software.

Murine breast cancer models

4T1 murine breast cancer cells were purchased from the American Type Culture Collection and maintained in RPMI 1640 medium (Invitrogen) with 10% fetal bovine serum supplementation. Cells were incubated in a 37°C environment with 5% CO_2 and used for *in vivo* experiments when they reached nearly 75% confluence.

All animal studies were conducted with approval from the University of Wisconsin – Madison Institutional Animal Care and Use Committee. To generate the breast cancer model, 4 to 5 week old female Balb/c mice (Harlan) were implanted with 2×10^6 4T1 cells in a 1:1 mixture of Matrigel (BD Biosciences) in the lower flank. Mice were monitored and used for therapeutic studies 10 days after implantation, when the tumors had reached 5–10 mm in diameter.

Therapy preparation and administration

DTPA-TRC105 and DTPA-IgG were radiolabeled with ^{177}Lu using procedures similar to those described previously [26]. In summary, $^{177}\text{LuCl}_3$ was diluted in sodium acetate buffer (pH 6.5) and 0.15 $\mu\text{g}/\mu\text{Ci}$ DTPA-TRC105 (or DTPA-IgG) was added. The reactants were incubated for 1 h at 37°C with constant shaking and purified via a PD-10 column (GE Healthcare) prior to preparation for injection.

Five study groups were employed for the *in vivo* therapy study, as outlined in Table 1. Mice were injected with the proper agent in a total volume of 100 μ L in PBS via tail vein injection, for a one-time administration of the therapeutic agent.

Monitoring of treatment safety and efficacy

Every other day, the body weight, tumor volume (through caliper measurement), and general appearance of the mice in all study groups were evaluated. Mice were euthanized by CO₂ asphyxiation if the tumor volume increased to >500% of its initial size, body weight dropped by more than 15%, or hindlimb movement was significantly impaired by the tumor. Complete blood count analyses and liver function tests were performed on untreated mice as well as those in the high dose treatment group a month after therapy administration.

Biodistribution studies were performed to determine the organs of localization of ¹⁷⁷Lu-DTPA-TRC105 at both 1 d and 7 d post-injection. Mice were euthanized after the appropriate number of days, and various organs were excised, wet-weighed, and gamma counting was performed utilizing a WIZARD2 automatic gamma counter (PerkinElmer). Uptake was presented as percent injected dose per gram (%ID/g). Biodistribution studies were also performed on radionuclide-injected mice in all groups as they were euthanized throughout the study.

Ex vivo tissue sample analysis

Tumor, liver, and kidney tissue samples were collected from the high-dose ¹⁷⁷Lu-DTPA-TRC105 group mice at various time points for histological evaluation as described previously [25]. Tissues were embedded and frozen in Optimal Cutting Temperature compound (Sakura Fintek) and sectioned in 7 μ m slices. Immunofluorescent staining was performed on tumor tissues to look for the presence of angiogenesis and existing blood vessel structure, using AlexaFluor488-conjugated anti-CD31 and DyLight550-conjugated anti-CD105 antibodies (Novus Biologicals). H&E staining was also performed on the same tissues to look for morphological evidence of damage from the treatment.

Dosimetric extrapolation

The biodistributions of ⁸⁹Zr-labeled and ⁶⁴Cu-labeled TRC105 have previously been reported by our group [27, 12]. Using the longitudinal PET tracer uptake from these studies in conjunction with biodistribution data of ¹⁷⁷Lu-DTPA-TRC105, a conservative estimate of dose to a tumor following injection of ¹⁷⁷Lu-DTPA-TRC105 was calculated using the dose-to-sphere modeling capabilities of OLINDA/EXM [28].

Statistical analysis

Direct comparisons between groups were made using a two-sided Student's t-test. Data is presented as mean $\pm$ standard deviation. A P-value of less than 0.05 was considered statistically significant.

Results

Radiolabeling conditions and choice of chelator for ^{177}Lu

Radiolabeling efficiencies were compared with both DOTA-TRC105 and DTPA-TRC105. As outlined in Figure 1a, higher labeling yields were achieved through the use of DTPA over DOTA at all antibody-to-isotope ratios and at all reaction time points. After 1 h incubation, labeling yields of >90% were achieved with the use of all DTPA-TRC105 antibody ratios over 0.1 $\mu\text{g}/\mu\text{Ci}$ as determined through iTLC, while DOTA-TRC105 yields were only near 90% for 0.5 $\mu\text{g}/\mu\text{Ci}$ (DTPA, Figure 1b; DOTA not shown). Thus, moving forward, a ratio of 0.15 μg of DTPA-TRC105 for every 1 μCi of ^{177}Lu was employed. For therapy and biodistribution studies, similar labeling yields were consistently achieved. Flow cytometry studies indicated no sacrifice in binding of TRC105 to HUVECs with the addition of the DTPA chelator (Figure 1c–d). Additionally, these FACS studies demonstrated that TRC105 specifically binds only to endothelial cells, and not 4T1 cancer cells themselves. A small amount of binding to HUVECs was noted for the nonspecific IgG.

Biodistribution studies

Gamma counting biodistribution studies were performed at 1 and 7 days post-injection of ^{177}Lu -DTPA-TRC105 in 4T1 tumor-bearing mice. As presented in Figure 2, the radioactive content of various organs was analyzed to determine the distribution of the agent. Persistent uptake between 1 and 7 days of ^{177}Lu -DTPA-TRC105 was observed in tumor tissue, at 14.3 ± 2.3 %ID/g and 11.6 ± 6.1 %ID/g, respectively ($n = 3$). The agent cleared from the blood pool over the course of the week, with values of 18.5 ± 3.1 %ID/g at 1 day post-injection and 10.5 ± 3.9 %ID/g at 7 days. Similar decreases in uptake were seen in other clearance organs such as the liver and kidneys over the course of the week. In mice injected with the nonspecific antibody, ^{177}Lu -DTPA-IgG, tumor uptake of 9.9 ± 4.1 %ID/g was observed at 7 days p.i. Sizable accumulations were also observed in the spleen (14.7 ± 3.4 %ID/g) and bone (10.7 ± 3.9 %ID/g), which may have contributed to the notable toxicity in this group. In other control group mice, biodistribution studies were performed as mice were euthanized (average 13–14 days post-injection) as shown in Figure 2b. $^{177}\text{LuCl}_3$ was found to mainly localize in the liver and spleen, with very minimal tumor uptake (0.1 ± 0.03 %ID/g). Biodistribution of low-dose ^{177}Lu -DTPA-TRC105 was found to match that found for the high-dose group, with similar organ clearance patterns.

Determining treatment efficacy of ^{177}Lu -DTPA-TRC105

The treatment efficacy of ^{177}Lu -DTPA-TRC105 was determined using 4T1 murine breast cancer models. Mice were administered a one-time dose of the given agent via tail vein injection, and tumor volume was monitored every other day. As outlined in Table 1, mice in group 1 were administered only $^{177}\text{LuCl}_3$ and group 2 received 45 μg of TRC105 (which has, as a single agent, been indicated to have therapeutic effects at a higher dose [14–17, 13]), while groups 3 and 5 were given varying doses of ^{177}Lu -DTPA-TRC105. The amount of TRC105 was held constant at 45 μg per mouse in all groups receiving this antibody. Group 4 mice received a similar dose of radiolabeled nonspecific antibody.

Significantly decreased ($p < 0.05$) tumor volumes were measured in mice in group 5 receiving high dose ^{177}Lu -DTPA-TRC105 as compared to those in groups 1 and 2 at 10 days post-injection (or 20 days post tumor inoculation), when the first mouse in these groups reached the euthanasia criteria. Decreased tumor volume was also seen in mice in the low-dose group 3 as compared to ^{177}Lu -only group 1 at this time point ($p = 0.02$). This inhibition of tumor growth in the high dose group led to a corresponding increase in survival, which was significantly longer than the lifespan for mice in any other group ($p < 0.001$) as seen in Figure 3. Mice in group 5 had an average survival of 32.4 ± 4.8 days, while groups 1, 2, 3, and 4 were found to have decreased survival of 13 ± 2.2 , 13.3 ± 1.9 , 14 ± 1.8 , and 8 ± 3.7 days, respectively. Most mice in groups 1, 2, 3, and 5 were terminated from the study on the basis of tumor growth, as none displayed significant weight loss; however, one mouse each in groups 2 and 5 were euthanized due to significant hindlimb impairment prior to tumor volume increasing by 500%. On the other hand, the majority of mice in group 4 (^{177}Lu -DTPA-IgG) were euthanized due to significant weight loss and corresponding toxicity of the nonspecific tracer.

Toxicity and safety determination

The safety of ^{177}Lu -DTPA-TRC105 was monitored via various experiments over the course of the study. Body weights were monitored to check for systemic toxicity every other day, and the weight of any individual mouse did not decrease by more than 10% over the course of the study in groups 1, 2, 3, or 5. Most mice in these groups were near their original pre-therapy weight at the time of euthanasia. However, in group 4, mice injected with the nonspecific antibody demonstrated noticeable toxicity due to off-target accumulation as three (of five) mice were terminated due to body weight decreasing to $<85\%$ of the original value.

Complete blood count analyses and determination of ALT levels were performed pre- and 1 month post-treatment in the high-dose ^{177}Lu -DTPA-TRC105 group (Figure S1). Notably, a sizable increase was noted in monocyte levels, resulting from inflammation during the course of treatment. ALT levels remained near constant, indicating no liver toxicity.

Immunohistochemistry

Tumor tissues were collected at 1, 7, and 30 days post-injection of high dose ^{177}Lu -DTPA-TRC105 from 4T1 mice. Fluorescent immunochemical staining was performed on these tissues to examine CD105 and CD31 expression (Figure 4). No CD105 staining was observed at both 1 and 7 days post-injection, suggesting the destruction of this receptor through targeting with ^{177}Lu -DTPA-TRC105. At one month post-injection, when tumors had recommenced growth, angiogenesis was evident at the periphery of the tumor, visualized through CD105 co-staining with blood vessels in this region. Morphological differences were also noted over the course of the study through H&E staining (Figure S3).

Dosimetry

Utilizing biodistribution data from previous studies [27, 12] of radiolabeled TRC105 in conjunction with the biodistribution study herein, approximate doses to tumors following administration of ^{177}Lu -DTPA-TRC105 were calculated (Figure S2). As biodistributions of

the positron-emitter and beta-emitter labeled TRC105 were similar, all available data was combined to obtain a larger data set for more accurate dosimetry estimation. For example, in this study, tumors at the start of therapy in group 5 had an average volume of 0.4 cm³, corresponding to 0.4 g mass (assuming 1 g/cm³). Calculated doses to spherical tumors of this size were found to be approximately 145 ± 21 mGy/MBq (n = 5). The mice in this group received 11 MBq of ¹⁷⁷Lu-DTPA-TRC105, resulting in approximate doses of 1.6 Gy to the tumors. Low-dose group 3 mice would have received an accordingly lower dose of 0.54 Gy.

Discussion

Targeted radiotherapy has a number of advantages over other cancer treatment options, including its specificity for diseased tissue and very localized dose deposition [18]. Such strategies are gaining traction in the clinic, notably through peptide receptor targeting in difficult neuroendocrine tumors [29]. Most radioimmunotherapy treatments utilize targets that are very specific for a given cancer, rather than a general approach that may be more widely applicable. CD105, or endoglin, represents one such general target which has been identified in a wide variety of cancers, and may be correlated to overall outcomes [11, 9, 8, 10]. The CD105-targeting monoclonal antibody employed herein, TRC105, has been explored as a single-agent therapeutic, with limited success. The addition of a dose-depositing nuclide was therefore hypothesized to increase the therapeutic efficacy.

The use of murine mammary cancer models allowed the exploration of the therapeutic efficacy of ¹⁷⁷Lu-DTPA-TRC105. Of course, as with any model system, it should be noted that this tracer will need to be thoroughly investigated in any other tumor model or organism. It was determined that a one-time injection of a sufficient dose of this agent significantly slowed tumor growth, as compared to control groups. Mice in the high-dose group displayed an average survival of 32.4 ± 4.8 days, compared to the low dose (three-fold less ¹⁷⁷Lu) group at 14 ± 1.8 days. Control groups of ¹⁷⁷Lu alone, TRC105 alone, and a radiolabeled nonspecific IgG antibody similarly were found to have shorter survival, at 13 ± 2.2, 13.3 ± 1.9, and 8 ± 3.7 days, respectively. Taken together, the increase in survival of the high-dose group indicates the efficacy of this treatment.

In all groups except the nonspecific IgG group, no evidence of significant toxicity was observed. Mouse body weights did not significantly decrease, and blood count analyses indicated minimal differences in toxicity markers. In the nonspecific group, the agent notably accumulated in both the spleen and bone marrow, both of which may contribute to the significant toxicity (indicated by extreme weight loss) observed in this group. Off-target accumulation of radioimmunotherapy agents is a considerable concern; however, the limited normal tissue distribution of CD105 minimizes this risk with this agent. Even though ¹⁷⁷Lu-DTPA-TRC105 is unable to completely eradicate solid tumors with a single dose in this study, significant tumor growth inhibition was observed. This agent may thus find applications in combination with other agents, slowing tumor growth to allow other agents to be more effective. Possible additional therapies to combine with radioimmunotherapy may include checkpoint immunotherapy [30] and chemotherapeutic approaches [31–33], which have shown benefit both preclinically and in human studies. Of note, the absorbed

doses estimated for this tracer are lower than those for other ^{177}Lu -labeled antibodies [34, 35]; however, the efficacy that was observed may be due in part to the combined effects of radiotherapy and the biological effect of TRC105 itself, a phenomenon which is further supported through the lack of tumor inhibition in the nonspecific IgG group.

The low expression of CD105 in normal tissues allows for high-contrast imaging of CD105 as well through the use of molecular imaging techniques, as we have previously demonstrated [27, 12, 36, 37]. Thus, this single antibody may serve as a theranostic agent, with diagnostic capabilities through conjugation with positron-emitting isotopes such as ^{64}Cu ($t_{1/2} = 12.7$ h) or ^{89}Zr ($t_{1/2} = 78.4$ h) and imaging with positron emission tomography, then radioimmunotherapy of the same target with ^{177}Lu as presented herein. Biodistribution of the ^{177}Lu -labeled TRC105 was found to be similar to that of previously reported PET imaging with TRC105, indicating that this image-then-treat approach is feasible. This personalized approach would be widely applicable in multiple solid tumor types, allowing targeted therapy and real-time monitoring of response. Such a versatile platform may also demonstrate expedited clinical translation, as TRC105 itself is already in clinical trials.

Conclusions

We have herein developed a radioimmunotherapy agent, ^{177}Lu -DTPA-TRC105, targeting angiogenic tissues through CD105. Significantly extended survival was observed in the treatment group, with corresponding slowed tumor growth and negligible toxicity. We believe such an agent will show widely applicable clinical utility in combination with other treatments, in many solid cancer types. In addition, the theranostic capabilities of TRC105 merit further investigation through clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Characterization of labeling conditions and binding properties. (A) DOTA and DTPA chelators were compared for radiolabeling of TRC105 with ^{177}Lu at 0.15 μg of protein per 1 μCi of isotope. DTPA exhibited higher labeling yields at all time points. (B) Varying concentrations of conjugated antibody were used for radiolabeling with DTPA, with similar results observed at all concentrations. A final ratio of 0.15 μg of protein per 1 μCi of isotope was chosen for *in vivo* studies. (C) Flow cytometry showed no binding of the as-developed tracers to 4T1 tumor cells. (D) TRC105 and DTPA-TRC105 were found to bind at similar levels to HUVECs.

Figure 2.

(A) Biodistribution studies were performed at 1 and 7 days post-injection of ^{177}Lu -DTPA-TRC105 or ^{177}Lu -DTPA-IgG in 4T1 tumor-bearing mice. Gradual clearance was seen in most organs, with tumor uptake of the targeted tracer remaining high over this time period at approximately 11–14 %ID/g ($n = 3$). Notable differences in uptake for the nonspecific IgG were observed in the spleen and bone at 7 d p.i., as compared to the targeted tracer. (B) As mice were sacrificed, biodistribution studies were performed in other control groups. These data were collected at an average of 13 days post-injection for the ^{177}Lu only group, and 14 days post-injection for the low dose group ($n = 4$ –6 per group).

Figure 3.

Results of the therapeutic study. (A) Average ($\pm$ S.D.) tumor volumes of all study groups over the first twelve days of the study. The high dose group displayed significantly ($p < 0.05$) smaller tumor volume than all other groups at 12 days post-injection. (B) Body weight measurements were performed every other day for all mice. (C) Kaplan-Meier survival curves for therapeutic groups. (D) Mice in the high dose group exhibited significantly longer average survival than those in all other groups ($p < 0.001$).

Figure 4.

Immunofluorescent staining of tumor tissues at 1, 7, and 30 days post-injection of high-dose ^{177}Lu -DTPA-TRC105. Angiogenic activity, evidenced by co-staining of CD31 and CD105, was evident at 1 month, when tumors began regrowth. Inset at 1 month shows an angiogenic vessel. Scale bar: 10 μm .

Table 1

Therapeutic study groups. Mice bearing 4T1 mammary tumors were administered a one-time injection of the given agent.

Study Group	Group Number	Treatment
¹⁷⁷ Lu only	1	11 MBq of ¹⁷⁷ LuCl ₃ in PBS 1X
TRC105 only	2	45 µg of TRC105 in PBS 1X
¹⁷⁷ Lu-DTPA-TRC105 low dose	3	3.7 MBq of ¹⁷⁷ Lu-DTPA-TRC105 + 30 µg of TRC105 in PBS 1X (45 µg of TRC105 total)
¹⁷⁷ Lu-DTPA-IgG	4	11 MBq of ¹⁷⁷ Lu-DTPA-IgG in PBS 1X (45 µg IgG total)
¹⁷⁷ Lu-DTPA-TRC105 high dose	5	11 MBq of ¹⁷⁷ Lu-DTPA-TRC105 in PBS 1X (45 µg of TRC105 total)