Author's response to reviews

Title: Novel treatment option for MUC16-positive malignancies with the targeted TRAIL-based fusion protein Meso-TR3

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Version: 3 Date: 2 January 2014

Author's response to reviews: see over
Re: Resubmission of MS: 1993410459108578 – “Novel treatment option for MUC16-positive malignancies with the targeted TRAIL-based fusion protein Meso-TR3” in revised form.

Dear Editors,

We are pleased to learn that our manuscript was favorably received by the Reviewers and the Editorial board of *BMC Cancer*. We appreciate the Reviewers’ comments and the opportunity to address these critiques in a revised form of our current manuscript. Changes within the manuscript have been highlighted throughout in yellow to facilitate easy identification of the modifications.

Reply to the Reviewers’ comments:

**Reviewer's report #1 (Adnan M Nagrial)**

The authors have shown that mesothelin linked TR3 (a TRAIL-based drug platform) causes cell-death in the OVCAR3 cell-line and an OVCAR3 xenograft more effectively than either TR3 or TRAIL alone. Interest in novel linked targeted-drugs is high with the recent approval of T-DM1 in breast cancer.

**Minor Essential Revisions**

1. *The cell death figures (Figures 3 and 4) are somewhat unconventional in that drug volume rather than drug concentration has been provided. It would make interpretation of the benefit of meso-TR3 easier if simple concentrations of TR3 and meso-TR3 were used.*

We agree with the comment made by the Reviewer. We originally attempted to determine our drug concentrations using conventional sandwich ELISAs. However, the data we obtained were unreliable and we began to suspect that the mesothelin domain of our Meso-TR3 fusion protein could have conformational properties which would interfere with an ELISA-based assay system.
In order to overcome this limitation, functional drug titrations based on killing activities of Meso-TR3 and TR3 (drug concentration unknown) and commercially available recombinant TRAIL (drug concentration known) were performed on the TRAIL-sensitive and MUC16-negative cell line Jurkat (compare Fig. 3A). Based on this information, the relative abundance of Meso-TR3 and TR3 was then determined employing semi-quantitative Western blotting, a detection method that is not sensitive to potentially epitope-masking tertiary and/or quaternary structures. The results show that Meso-TR3 was \approx 6 to 8-fold more abundant than TR3. This was unexpected and led to our hypothesis that Meso-TR3 was exhibiting prodrug properties. This also explained the inconsistencies we obtained from our ELISA-based initial detection system. As a result, we believe the method we selected to present our data is currently the most accurate that we have available.

2. **The effect on HELA cells (Figure S3) is stated in the results section as showing a greater benefit of meso-TR3 vs TR3. Although this looks numerically true, it is not actually statistically significant and thus somewhat misleading.**

In the revised manuscript, we repeated these experiments and observed a substantially more rapid cell death induction by Meso-TR3. For this reason, we assayed the HeLa cells at 6 h post-treatment and included this modification into the legend of Additional Figure S3 (page 34). Our new data confirm that HeLa cells are indeed more sensitive to Meso-TR3 compared to TR3 alone \((P < 0.016)\). We have now replaced the original data and provide a new supplementary Figure S3.

**Discretionary Revisions**

3. **The effect of meso-TR3 has only been shown in a single cell line and a xenograft using the same cell-line. The benefit of a MUC16 targeted drug would be more convincing if more than 1 cell line was used. The entire concept would also be more convincing if a non-ovarian cancer cell line was also used.**

In the present manuscript, we included additional data on the MUC16-expressing cervical cancer cell line HeLa. Our binding data (OVCAR3, 100% MUC16+ [Fig. 2D] and HeLa, 80% MUC16+ [Fig. 5A]) nicely demonstrate the ability of Meso-TR3 to attach to MUC16+ cells while it is hardly taken up from MUC16-negative cells (Fig. 5A, arrow). We could further demonstrate selective elimination of MUC16-positive cells from the mixed HeLa cell pool, studies which would not have been possible in a cell line with 100% MUC16-positive population such as OVCAR3. These data suggest that the nature and the source of MUC16 present on two unrelated types of cancer cell membranes are equally suited as a receptor for recombinant cancer therapeutics such as Meso-TR3.
Figure R2. MUC16 as a putative, drug targetable biomarker in pancreatic cancer.

BxPC3 cells were treated for 24 hours with MUC16-targeted Meso-TR3 (cell killing ~50%, not shown). The following day, the cells were washed and allowed to recover for two days. After this short recovery phase, the cells were prepared for cell surface staining of MUC16 and submitted to flow cytometry. Of note, after only a single treatment with Meso-TR3, the number of MUC16-positive cells within the culture was dramatically reduced to only ~13% of the initial ratio, suggesting selective elimination of MUC16+ cells from the cell pool.

A, Raw data derived from FACS dot plots; B, Bar graph of the data obtained from (A) normalized to the MUC16 frequency of non-treated control cells.

MUC16 expression profile similar to the HeLa cells with regard to the fact that only a fraction of cells were positive for this tumor marker (Fig. R1).

Figure R1. MUC16 detection in pancreatic cancer cell lines.

A panel of human pancreatic cancer cell lines was assessed for the expression of MUC16 (CA125) using flow cytometry. The cells were detached non-enzymatically to prevent loss of cell surface antigens. Cells treated with a fluorophore-conjugated secondary antibody (a-ms-PE) were used to establish the baseline fluorescence (Control). The cell lines under investigation contained a subpopulation of MUC16-positive cells ranging from 13 - 18%. A, BxPC3; B, Capan1; C, CFPAC.

In an effort to demonstrate broader applicability of MUC16-targeted Meso-TR3, we assessed the MUC16 expression profile on pancreatic cancer cell lines employing flow cytometry. All cell lines investigated displayed a
Treatment of BxPC3 pancreatic cancer cells with Meso-TR3 resulted in selective elimination of the MUC16 cell population, similar to what we have described for the HeLa cells (Fig. R2). Treatment with TR3 did not shift the MUC16 ratio due to the non-targeted nature of this drug format (compare original Fig. 5B of the manuscript). We added a comment in our manuscript on pages 16-17 of the Results section and refer to these additional results as “data not shown”. A related comment has been added to the discussion as well on page 20.

4. **It is somewhat concerning that the only other MUC16 expressing cell-line used, HeLa does not show any additional benefit of meso-TR3 over TR3 alone.**

Please see our response to point 2 above and the additional data provided.

5. **Although I have no criticism of the experimental methods and results, I am personally unconvinced of the conclusions made as the authors have only demonstrated that meso-TR3 is better than TR3 in an OVCAR3 cell-line and xenograft model. However, there are no flaws in methodology that would prevent publication.**

Although 80% of epithelial ovarian cancers are known to express MUC16, very few cancer cell lines actually express MUC16. Similarly, TRAIL resistance is common in cancer cell lines derived from different tumor types. Given these limitations, OVCAR3 was the ideal test model for this proof-of-concept study. Accordingly, this cell line was used primarily for the in vitro and in vivo experiments. We have included additional functional data on Hela cells (Figure S3) and, for the Reviewers only, the pancreatic cancer cell line BxPC3 (Figures R1 and R2).

**Reviewer's report #2 (Chris Scarlett)**

**Major Compulsory Revisions**

The authors describe a very interesting study whereby they have generated a novel TRAIL-based drug platform (TR3), to target MUC16 expressing cancers. It is an interesting approach exploiting a mechanism known to drive tumor invasion and metastasis in MUC16 positive cells to use as a modality for the delivery of a novel therapeutic agent. TR3 has been engineered to fuse with mesothelin to assist in the delivery to MUC16 positive cells via the known high affinity that mesothelin has with MUC16. They authors demonstrate impressive binding of meso-TR3 to MUC16 positive cells, accumulation of meso-TR3 on the MUC16 positive cells, as well as increased cell death in vitro and in vivo of MUC16 expressing ovarian cancer cells. MUC16 overexpression is a hallmark of numerous cancer types, especially for the aggressive cancers of the ovary and pancreas. Investigations into new therapeutic strategies are very important to combat these diseases, which have very few effective therapies.
The manuscript is very well written, however I have a few queries that will need to be addressed prior to publication in BMC cancer:

1. **The title describes a novel treatment option for MUC-16 positive malignancies, yet you only show data for the ovarian cancer cell line OVCAR-3. Do you have any data on other cancer types that overexpress MUC16 (such as pancreatic cancer cells) to strengthen your argument? Particularly as pancreatic cancer is eluded to throughout the manuscript?**

   Please see our response to 
   Reviewer #1, where we describe enhanced sensitivity and selective elimination of MUC16-positive HeLa cells by Meso-TR3 (point 2) as well as selective elimination of pancreatic cancer cells by Meso-TR3 (point 3, Figures R1 and R2).

2. **Mesothelin is known to interact with MUC16 to drive invasion and metastasis. How efficient (%) is meso-TR3 in delivering TR3 to the cancer cells? By this I mean is there any chance that meso-TR3 can be dissociated resulting in only mesothelin alone being introduced to MUC16 expressing cells and accelerating tumor progression?**

   The Reviewer raises an interesting question regarding the possibility that Meso-TR3 could “dissociate” (via proteolytic degradation) into mesothelin and TR3 fragments. We have not detected any degradation products when our recombinant proteins were assessed after in vitro production by Western blot analysis (Fig. 1B). We would also conclude that such a degradation process might likely be rather limited, based on the efficient reduction in tumor burden in vivo (Fig. 3C). However, if Meso-TR3 continues to prove effective in our xenograft models, we will need to test this hypothesis more fully. In order to do this, we would employ stable isotope dilution mass spectrometry to investigate this important question in future studies. We have included a statement on the protein stability of Meso-TR3 in the Results section on page 10.

3. **How is the meso-TR3 administered to mice? IP? IV? Oral gavage?**

   TR3 and Meso-TR3 were administered via the intraperitoneal route. This information was added to the Methods section (Animals, page 8), the Results section (page 14) and to the legend of Figure 3C (page 31).

4. **Do the authors have further data that may outline the exact cellular death mechanisms initiated by meso-TR3?**

   In order to test if the traditional death pathways are engaged by Meso-TR3, we performed blocking experiments directed at two strategic points of apoptosis induction - the death receptor level (extrinsic pathway) and the initiation/amplification level (intrinsic pathway).
When soluble death receptor 5 (DR5-Fc) was added to standard killing assays using MUC16-positive OVCAR3 cells, we noticed a dose-dependent reduction in cell death (presented in Fig. 4B of the manuscript). This in itself was a strong indication that the initial step, the binding of the TR3-moiety of Meso-TR3 to the cellular death receptors DR4 and/or DR5 was vital to exert Meso-TR3’s strong apoptosis induction. A similar approach was used in our earlier paper in which we completely blocked both TR3 and recombinant human TRAIL (rTRAIL) with DR5-Fc and prevented Jurkat cell killing in a dose-dependent fashion (Spitzer, et al, 2010).

In this current manuscript, we expanded on our previous investigations and included blockade of the intrinsic death pathway using the pan-caspase inhibitor Z-VAD-FMK. We were able to demonstrate complete rescue of OVCAR3 killing following treatment with Meso-TR3 in the presence of the inhibitor (presented in Fig. 4C of the manuscript). These data strongly suggest that, following activation the extrinsic death pathway by Meso-TR3 at the cell membrane (which does take place and can be blocked with DR5-Fc), caspase activity is absolutely required for completion of apoptosis and does not differ mechanistically from other forms of recombinant TRAIL, such as TR3 and rTRAIL. The downstream pathway has been extensively studied and we therefore felt that it would add little to repeat those studies in this manuscript.

In summary, we are very appreciative of the positive review and helpful comments the Reviewers provided. We believe the Reviewers have helped us to create a stronger and clearer message and we are hopeful that the Editors will find our revised manuscript now acceptable for publication in *BMC Cancer*. Please contact us if you require any further clarifications.

Sincerely,

Gunal Garg and Dirk Spitzer

Reference