Author's response to reviews

Title: Parallel Screening of FDA-Approved Antineoplastic Drugs for Identifying Sensitizers of TRAIL-Induced Apoptosis in Cancer Cells

Authors:

  David J Taylor (dtaylor70@gmail.com)
  Christine E Parsons (ceparson@asu.edu)
  Arul Jayaraman (aruli@mail.che.tamu.edu)
  Kaushal Rege (kaushal.rege@asu.edu)

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Author's response to reviews: see over
We sincerely thank the reviewers for their very helpful comments about our manuscript. We believe that addressing these comments has significantly strengthened our manuscript. We have substantially revised the manuscript in accordance with these comments and have carried out several additional experiments for these. We now have eleven figures and one table in the manuscript in addition to six figures in the supplemental information section. Specific responses to reviewer comments are listed below.

**Reviewer 1**

**Comment 1:**
“There are missing controls with normal pancreatic or prostate human epithelial cells or with human hepatocytes”

**Response:**
We addressed this comment by testing mitoxantrone and mithramycin in human epithelial cells (HPDE6) as shown in Figure 8 in the revised manuscript. We found that the mitoxantrone-TRAIL combination is selective towards malignant pancreatic cells compared to normal pancreatic cells at lower concentrations. The mithramycin-TRAIL did not show this selectivity in vitro, but it is possible that a window of operation exists for this combination in vivo.

**Comment 2:**
“Only MTT assay was used for the analysis of cell proliferation; additional cell proliferation (e.g CellTiter Blue, x-Celligence or clonogenic assay) should be used at least in selected experimental setup; also apoptosis array as AnnexinV-FITC/PI would be helpful.”

**Response:**
We addressed this comment by using a Live/Dead® stain to determine cell viability. In this assay, cells with compromised membranes (dead cells) are red fluorescent, while living cells are green fluorescent. The assay was used to investigate cell death with PC3-TR (TR: TRAIL resistant) prostate cancer cells following single agent treatments with mitoxantrone and mithramycin and combination treatments with each of the drugs and TRAIL (Figure 6 in the revised manuscript).

Following suggestions from the reviewer, the AnnexinV-PI assay was used to investigate if these drug treatments induced apoptosis in PC3-TR cells. The assay was used to determine the
effects of single agent mitoxantrone and mithramycin and combination treatments with TRAIL in PC3-TR (Figure 11 and Supplementary Figure 6 in the revised manuscript). We have shown that the combination treatment induced significant apoptosis in these cells, although additional experiments using Western blots, etc. will be required to further demonstrate this.

Comment 3:
“It might be worthy to titrate TRAIL at fixed e.g. LD50 concentration of the sensitizing agent – 10 ng/ml is relatively low concentration and thus some effects with less enhancing drugs might be missed.”

Response:
We addressed this comment by running the suggested experiments with two leads, mitoxantrone and mithramycin, at their LD50 values in combination with TRAIL in a concentration from 0-100 ng/mL in PC3-TR (Figure 9 in the revised manuscript). We found that increasing TRAIL concentrations did not result in significantly increase loss of cancer cell viability with these two drugs. Thus, lower concentrations of TRAIL possess sufficient activity in combination with the chemotherapeutic leads tested obviating the need for higher concentrations.

Comment 4:
“In addition to cancer cells pre-treatment with the sensitizing drugs it would be interesting to analyze an effect of co-treatment/co-application.”

Response:
We have addressed this comment by comparing loss in cancer cell viability following sequential treatment (drug for 24 h followed by TRAIL for 24 h) with that following simultaneous treatment (cells co-treated with drug and TRAIL for 24 h) for mitoxantrone and mithramycin at the concentrations previously studied and the single 10 ng/mL TRAIL concentration in PC3-TR (Figure 10 in the revised manuscript). Treatment modality influenced the efficacy of the mitoxantrone-TRAIL treatment, but did not influence that of mithramycin-TRAIL treatment.

Comment 5:
“Pg. 8 - 20% of less proliferating (presumably apoptotic) PC3-TR cells is not a negligible susceptibility – it would be if sensitivity to TRAIL at 1 #g/ml drops below 5%.”

Response:
We agree that 100 ng/mL of TRAIL does demonstrate approximately 20% decrease in cell viability which is not negligible. However, the single agent effect of TRAIL is low relative to the single agent drug treatments especially considering our use of 10 ng/mL for most combination
experiments. Following this comment by the reviewer, the phrase, "negligible susceptibility," was reworded in the revised version of the manuscript.

**Comment 6:**
“Why PC3 and PC3-TR so differ in their sensitivity to sole doxorubicine and mithramycin”

**Response:**
We have not looked into the phenotypic differences between the PC3 and PC3-TR cells, but some of the characteristics of the PC3-TR cells have been previously described in the literature [1]. However, the following passage was added in the manuscript for clarification: "It is important to note that while PC3-TR cells are derived from PC3 cells, the two lines are inherently different and therefore, it can be expected that the two cell lines respond differently to drug treatments. For example, we have previously shown that closely related prostate cancer cell lines, PC3 and PC3-PSMA cells, demonstrate markedly different behavior in response to nanoparticle treatment [2]."

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**Reviewer 2**

**Comment 1:**
“The major problem in this manuscript is that the authors actually emphasized apoptotic cell death in their manuscript... However the authors did not use any standard method to assess apoptosis induction/progression.”

**Response:**
Following this concern, we have modified the text throughout the manuscript to reflect ‘loss of cancer cell viability. However, we used the AnnexinV-PI staining, in concert with fluorescence microscopy, to demonstrate the presence of a significant number of apoptotic cells following the mitoxantrone-TRAIL and mithramycin-TRAIL combination treatments (Figure 11 and Supplementary Figure 6 in the revised manuscript). Detailed analyses of the sensitization mechanisms are currently in progress in our laboratory.

**Comment 2:**
“Figure 1 shows results of treatment of PC3-TR cells with different concentrations of TRAIL for 24 h assessed by the MTT assay, Therefore the Y axis of the graph in Figure 1 should be the percentage of cell viability, not cell death. The same problem applies to other figures (6-10).”
Response:
We have now changed the y-axes in all relevant figures to ‘Reduction in Cell Viability (%)’ following the recommendation of the reviewer.

Comment 3:
“Figure 2 shows the experimental design of the drug testing, which is also explained in the text of the manuscript. Thus, Figure is redundant and should be deleted.”

Response:
We had included this figure to provide the reader with a visual of the screening process. Following this comment, we have moved this figure to the supplementary information section (Figure 2 in the Supplementary information section).

Comment 4:
“The authors first pre-screened the selected drug candidates using a 20 uM concentration for each chemical, then 10 uM as a lower concentration for sensitizing TRAIL-induced apoptosis. There must be a reason for the author to do so. However this strategy is unclear and should explain.

Response:
The above procedure was followed in order to limit the toxicity of drugs so that meaningful comparisons between the single-agent treatments and the combination treatments with TRAIL could be made. The manuscript has been revised to make this point clearer.

Comment 5:
“In pages 11, 12 and 13, the authors document the best combination treatment points of doxorubicin, mithramycin and mitoxantrone. In the related Figures (6, 7 and 8), an arrow should indicate the right point to make it clear.”

Response:
The figures were altered as suggested.

Comment 6:
“There are too many figures in this manuscript showing the same type of testing using different drugs. The author should combine some of them, as they were generated using same testing method”

Response:
Although no figures were combined, we did move some figures to the supplementary information section to help clean up the manuscript. However, the figure count is still somewhat high since additional experimental work was requested by the reviewers.

References
