Round 1

Reviewer 1 Report

The current manuscript by Massaro et al. describes the results of a series of in vitro studies examining the effects of a combination of inhibitors of the various biosynthetic enzymes responsible for polyamine biosynthesis alone and in combination with a polyamine transport inhibitor in a human pancreatic ductal adenocarcinoma line. The overall goal of the study was to determine if the addition of a PTI could effectively improve the overall in vitro response to the combinations of polyamine biosynthesis inhibitors. Although similar experiments have been previously published combining DFMO and inhibitors of both spermidine and spermine synthases, the addition of a PTI does add some novelty to the experiments presented here. Additional relevance is provided by recent studies demonstrating a reduction in polyamines by the combination of DFMO with a PTI can enhance the antitumor immune response. Although not formally tested here, the authors make the argument that their combinations that result in significant decreases in tumor spermine may result in an enhanced immune response in pancreatic cancers, a cancer that has thus far not been amenable to immune therapy.

Although the results are of interest, there are some issues that need to be addressed prior to publication.

Major

1) The authors state that they examined both the human L3.6p1 and mouse PanO2 cells for their response, but only results for the L3.6p1 cells appear to be included.

2) The authors use the terms toxicity and % viability throughout the manuscript. However, they appear to only be referring to MTS assay results. The MTS assay is not well-suited to determine the difference between cytostasis and cytotoxicity. DFMO treatment of most cancer types leads only to cytostasis rather than overt toxicity and cells will continue to grow after DFMO is removed from culture. Consequently, rather than using the terms toxicity and % viability, the authors should refer to their results as an indication of growth inhibition rather than overt toxicity unless they do the proper experiments do demonstrate toxicity. (Trypan blue exclusion, colony formation, measures of cell death/apoptosis, DNA fragmentation, etc).

Minor

1) In the abstract a comma should be used before the pronoun/determiner “which”.

2) Throughout the manuscript the bracketed reference numbers are sometimes preceded by a space and sometimes they are not. (eg. Reference [1], reference[2]).

3) Page 2, lines 50 & 51 should read, “The normal pancreas has the highest level of the native polyamine spermidine (Spd) of any mammalian tissue.”

As the sentence is currently worded it is difficult to understand.

Author Response

We first thank this reviewer for their insightful comments. We removed the PanO2 references in the paper (lines 23, 91 and 95) as this cell line was not the subject of the paper. We instead focused the paper on the human pancreatic cancer cell line L3.6pl as it is more relevant to the clinic, where patients present with metastatic disease. The other major concern was the use of the word 'toxicity'. We changed all references to toxicity to 'growth inhibition' as suggested by this reviewer. We also added commas in Lines 21 and 25 (before 'which') as suggested. In addition, we made all the bracketed reference indications consistent throughout the manuscript and restated the sentence on lines 50-51 regarding the normal pancreas having the highest level of spermidine. In sum we have addressed the concerns of reviewer 1 and hope that the paper is now suitable for publication in the Journal.

Reviewer 2 Report

The authors Massaro et al. studied the effects of polyamine biosynthesis inhibitors against pancreatic cancer using human and mouse cancer cell lines. Importantly, the concept of dual inhibition of intracellular polyamine synthesis and transport of polyamines into the cell by using different inhibitors is appealing. Experiments are well planned and executed. The present studies showed that combined targeting of ODC, SMS and polyamine import were most effective in reducing PDAC viability. There are some minor comments that need authors attention.

All experiments are performed in invitro cultures. Although out of the scope of this manuscript, use of invivo models would validate the present results.

Only cell viability is measured. Why did the authors not measure other relevant biomarkers using western blotting or histochemistry or PCR.

Please provide details on the polyamine transport mechanism (channel etc) and how the PTI will act on it to inhibit polyamine transport.

Author Response

We first thank reviewer #2 for their insightful comments.

Although out of the scope of this manuscript, use of in vivo models would validate the present results.

We agree and the in vivo studies will be the subject of future work. Note: we have already shown that the combination therapy of DFMO+PTI works in other cancers like melanoma (ref 28) and augments the immune response.

Only cell viability is measured. Why did the authors not measure other relevant biomarkers using western blotting or histochemistry or PCR?

We have measured other markers in other manuscripts by histology and Western and have shown that these L3.6pl cells shunt to apoptosis upon polyamine starvation. Here we sought to understand the plasticity of the metabolic system itself and used the MTS reagent (which measures cell viability) to assess how these cells respond to inhibitors and combinations of inhibitors. Since these inhibitors affect polyamine biosynthesis and intracellular polyamine levels and relative polyamine distributions (e.g., relative spermidine and spermine levels), we felt the best tool to assess the metabolic affect of the combination therapies was the direct HPLC determination of intracellular polyamine distributions/levels. the MTS measurements provided a convenient method to assess how these changes in polyamine levels affected cell viability.

Please provide details on the polyamine transport mechanism (channel etc) and how the PTI will act on it to inhibit polyamine transport.

The nature of the specific target of the PTI is unknown at this time. there is evidence for both a plasma membrane transporter and a caveolin dependent endosomal uptake route and this area has been recently reviewed by Poulin et al. Amino Acids. 2012 Feb;42(2-3):711-23. (which we have now included as ref 29.

To address this concern we added line 370: While the precise genes and proteins involved in polyamine transport are only now coming to light [29], this area is ripe for future drug discovery.

We hope these changes are sufficient and allow us to publish these interesting findings to the scientific community.

Round 2

Academic Editor

In the revision of the manuscript by Massaro et al., the majority of concerns raised by the reviewers have been addressed. However, the use of the terms "viable" and "% viability" remains throughout the manuscript and is not an appropriate description of the results presented. The data in Figures 2-5 should be either reformatted as % growth inhibition, or the axis labels should be changed (maybe to "viable cell number (% of untreated)".

Line 132: The IC50 should be described as the point at which cell growth was inhibited by 50% compared to untreated cells.

Lines 136, 138 and others: "high viability" could be more appropriately replaced with "high proliferative ability" or "high growth rate".

Throughout the manuscript, "viability" can be replaced with "growth" or "proliferation".

Tables 1 and 2: The legends refer to viability percentages, but only polyamine data are included.

Author Response

All the comments have been addressed.