**Peer Review File**

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**Reviewer A**

Excellent paper. The methodology was rigorous and the genetic complexity of PC was well explained. Although the proposed drug showed promising results in murine models, would it be possible to extrapolate this data for humans eventually?

**Reply**: We are grateful for the reviewer's positive comments. Although our data obtained using Panc-1 cells in culture and in xenograft nude mice suggest the potential of Pro A for treating PC, much more work is needed before extrapolation to PC in human can be considered. One of the key questions to answer would be whether cell line-specific differences in sensitivity to Pro A are derived from and consequently correlate with different responses of different subtypes of PC to Pro A or similar drugs, as suggested by the reviewer. Our preliminary results regarding SMAD4 indicate this would likely be the case, but more in-depth work is required to identify additional traits associated with sensitivity to Pro A, preferably using primary PC tumor cells. Such work would hopefully lead to the establishment of a practical subtyping criteria for screening candidates for initial testing of Pro A in human PC. We have included a brief discussion on this point in the revised manuscript.

**Changes in text**: We have modified our text as advised (see Page 19, line 406-409).

**Reviewer B**

The authors focus on Proscillaridin A cardiac glycoside effects on pancreatic cancer. To study that, they carry out a number of experiments in cells and in mice models relevant to pancreatic cancer biology. Overall, the experimental design, organization of the manuscript is fair and provide novel insights about effects of Pro A. However, I recommend to improve some aspects of the data based on my comments below:

Methods:

**Comment 1:** The preparation of A Pro A solution is concerning in terms of concentration (if insoluble elements are observed and discarded through filtration the theoretical concentration might be underestimated) and short-term stability (if the solution is stored in -20ºC in 75% water).

**Reply 1**: We are sorry for the incorrect reporting of these details in our original manuscript. We have corrected this error during revision. For cell treatment, stock Pro A solution was prepared at 10 mM (5.3 mg/ml) in DMSO and stored at -80ºC. Working solutions were prepared immediately before use using culture media. For mouse treatment, Pro A was first dissolved in DMSO at 50 mg/ml and then diluted with PBS to a final concentration of 0.5 mg/ml before use. Solution was briefly heated at 37ºC and sonicated till clear.

Solubility of Pro A in DMSO is reported to be higher than 2 mg/ml

(https://www.sigmaaldrich.com/US/en/product/sigma/sml2800) and no less than 100 mg/ml with sonication (https://www.medchemexpress.com/proscillaridin-a.html).
**Changes in text**: We have modified our text as advised (see Page 5, 6, line 105-109).

**Comment 2:** The determination of IC50 in cell proliferation by CCK-8 assay should be explained in detail (differences in starting cell numbers, concentrations used, replicates, errors, and regression analysis). For proper analysis, the curves require sufficient points to define maximal, minimal and slope signals. An accurate determination of the IC50 is needed since the rest of the data is generated on this basis.
**Reply 2**: We appreciate the importance of this comment and re-examination of our original manuscript indeed showed that we forgot to include some data points while preparing Fig. 1A, which resulted in incorrect calculation of $IC_{50}$ for Panc-1 and BxPC-3 cells. The re-calculated $IC_{50}$ is 35.25 nM for Panc-1 and 180.3 nM for BxPC-3, which does not change the pattern of the different sensitivities to Pro A. Panc-1 is still the most sensitive of the three, and AsPC-1 is still the least sensitive one. We have modified Fig. 1A to fix this error and used the corrected $IC_{50}$ in the revised manuscript. We have also included the following details regarding the CCK-8 assay and $IC_{50}$ calculation. Briefly, Cells were inoculated in 96-well plates at 6000 cells per well for Panc-1 and BxPC-3, and 7000 cells per well for AsPC-1. Cells were changed into culture media containing increasing concentrations of Pro A with 5 wells for each concentration, and cultured for 72 hours. CCK-8 reagent was added and OD450 values were measured with Epoch Microplate Spectrophotometer (Bio-Tek, USA). Data were plotted and $IC_{50}$ was calculated using graphpad prism 7.
Although $IC_{50}$ in CCK-8 is an important indicator of drug effects, dosage responsiveness might not necessarily be the same in other assays. For this reason, we used both a higher and a lower concentration compared to the respective $IC_{50}$ in most of other assays.
**Changes in text**: We have modified our text as advised (see Page 8, line 156-160, Fig.1A).

Results:
**Comment 3:** Fig 1 The sensitivity to Pro A of Panc-1 is the highest, BxPC-3 is high in proliferation, while in AsPC-1 cells is the lowest but higher to BxPC-3 in colony formation. Can the authors provide a potential explanation, for example regarding the expression of the primary target of Pro A, sodium/potassium-transporting ATPase?
**Reply 3**: We appreciate this keen observation by the reviewer. Indeed, if only the extent of inhibition of colony formation is considered, AsPC-1 does appear to be more sensitive to BxPC-3, despite having a higher $IC_{50}$ in CCK-8 assay. We would like to point out that AsPC-1 is intrinsically less capable of forming colonies

compared to the other two cell lines: without Pro A treatment, AsPC-1 seeded at 1000 cells/well produced far less and far smaller colonies compared to Panc-1 and BxPC-3 seeded at 500 cells/well (Fig. 1C, left). It is therefore more likely that such pre-existing deficiency in colony formation makes AsPC-1 more sensitive to cytotoxicity-inducing Pro A in this particular assay.

**Comment 4:** A Table summarizing the data on sensitivity of PC to Pro A for the phenotypes studied would provide a better comprehensive picture of the differences.
**Reply 4**: We appreciate this suggestion and have added Table 1 to the revised manuscript.
**Changes in text**: We added Table 1 in the revised manuscript.

**Comment 5:** Fig 3. Can the authors provide a rationale about the dose used to test the Pro A effect in vivo relative to in vitro concentrations? How is this dose related to therapeutic human doses?
**Reply 5**: In this work, the dosage used in nude mice (6.5 mg/kg) was based on similar previous work on other types of cancer in xenograft mice. For instance, 5 mg/kg Pro A was used in mice xenografted with PC3 prostate cancer cells (Wang *et al*. Cell Cycle, 2020 19: 541-550), and 7 mg/kg Pro A was used in mice xenografted with glioblastoma cells (Denicolaï *et al*., Oncotarget, 2014 5(21): 10934–10948). Pro A used within this dosage range has not been associated with significant toxic effects in mice, and we did not observe such effects in our work either.
Since this experiment was performed only to demonstrate effects, additional work is required to determine optimal effective dosages. In addition, details regarding bioavailability and other pharmacokinetic properties of Pro A in mice are rather scarce. Consequently, it is as yet difficult to extrapolate such data to potential future applications in human.
For treating heart diseases in human, Pro A has been used orally or intravenously at milligram dosages (Gould *et al*. The Journal of Clinical Pharmacology and New Drugs, 1971, 11: 135-145), which means that the mg/kg dosages used in mice are much higher if compared directly. Although higher toxicity might be considered acceptable in cancer treatment, it is also possible and more desirable to reduce required dosage and side effects through structural modification and/or targeted delivery.

**Comment 6:** Fig 3D. Please provide quantitative data of positive cells.
**Reply 6**: We have added such info to revised Fig. 3D.
**Changes in text**: We added quantitative data in Fig. 3D.

**Comment 7:** Fig 6. Pro A seems to have a slight effect on Ca transients in Panc-1 cells as opposed to the others. Could the authors discuss this in the context of different sensitivity to mitochondrial apoptosis? Also, the Na transients might be

important to study since are related to the MOA of Pro A.

**Reply 7**: Indeed, intracellular $Ca^{2+}$ levels in Panc-1 cells treated with cytotoxic concentrations of Pro A increased only moderately compared to the other two cell lines (Fig. 6A). However, since marked structural and functional damage of mitochondria were found in such Panc-1 cells (Fig. 5&6B), we would like to suggest that this apparent discordance is more likely a result of differences between these cells in other mechanisms affecting $Ca^{2+}$ levels, for instance ER sequestration and plasma membrane $Ca^{2+}$ pump(s).

The reviewer's comment regarding effects on $Na^{+}$ levels of treatment by Pro A is also valid. Studies on anti-tumor effects of Pro A, or other cardiac glycosides for that matter, have generally focused on other potential targets. It is indeed possible that different sensitivities to Pro A between the 3 PC cell lines used here might also be related to differences in $Na^{+}/K^{+}$ ATPase expression, its pump activity and downstream signaling. We hope to be able to address such possibilities in future work.

We have added brief discussion on these points in the revised test.

**Changes in text**: We have modified our text as advised (see Page 18, 19, line 384-391).

**Comment 8:** Figs 8 and 9. How is SMAD4 protein stability affected by Pro A?

**Reply 8**: Based on our currently available data, it is only possible to conclude that SMAD4 degradation through the ubiquitin-proteasome system is affected by Pro A treatment. Wild type SMAD4 has been shown to mainly undergo mono- and oligo-ubiquitination, which enhances its interaction with other SMADs and results in higher transcriptional activation, instead of poly-ubiquitination, which leads to degradation through proteasome (Morén *et al*. J Biol Chem 2003;278:33571-82). It is plausible that Pro A treatment, through as yet unknown mechanisms, promotes poly-ubiquitination of SMAD4. Whether such is the case in Panc-1 cells, or other Pro A-sensitive cells expressing SMAD4, warrants additional work.

We have added brief discussion on these points in the revised test.

**Changes in text**: We have modified our text as advised (see Page 18, line 365-370).