Author's response to reviews

Title: Mechanistic studies of Gemcitabine-loaded nanoplatforms in resistant pancreatic cancer cells

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Author's response to reviews: see over
RE: Resubmission to BMC Cancer

Dear Drs Manibo and Kominsky,

Thank you very much for your promptness in reviewing our manuscript entitled *Mechanistic studies of Gemcitabine-loaded nanoparticles in pancreatic cancer*. We also wish to thank both reviewers for their time, promptness and insightful comments, which are addressed below:

**Reviewer 1: Dr Tarun Mandal**

- Are the methods appropriate and well described?
  
  Some of the methods described are not well described. The authors described the method of preparation of the nanoparticles but amount of drug in each formulation needs to be described. The drug loading has been reported but it is essential to calculate and report the Efficiency of Encapsulation in details. It is a water soluble drug, so it is expected to have lower efficiency of encapsulation.

- Thank you for these comments, which give better clarity to the manuscript. The amounts of drug and vectors were added on p.6 and 7. Effectively, Gemcitabine, as other water soluble drugs, lead to low encapsulation efficiency in PLGA and liposome nanoparticles, as compared to lipophilic drugs (Tewes *et al*, *European Journal of Pharmaceutics and Biopharmaceutics* 2007). The encapsulation efficiencies were added to the revised version of the manuscript on p.12. The % encapsulation were calculated as follows:

  **GemPo**: 4 mg of gemcitabine were used. After evaporation, hydration, extrusion and Sephadex steps, 1 mL of the GemPo was produced. This sample was submitted to UV-visible to determine the amount of Gemcitabine remaining in the sample. The concentration was found as being 1.4 μg. μL⁻¹. The total volume of sample was 1 mL so the total amount of Gemcitabine in the sample was 1.4 mg. Consequently, the encapsulation efficiency (EE) for GemPo was 35 %.

  **PLGem**: 122 mg of this product was produced after synthesis. The UV-visible characterization of the sample showed that Gemcitabine was remaining as 5.3 μg.mg⁻¹ of sample. So, the total amount of Gemcitabine after synthesis was 0.65
mg (5.3 \(\mu\)g.mg\(^{-1}\) of Gemcitabine x 122 mg of product). Consequently, the encapsulation efficiency for PLGem was 10%.

- It is also important to measure and report the saurface charge of the particles. Both size and charge of the particle dictate the fate of the particle in cellular delivery.

- Effectively, this is a crucial parameter. The zeta potential values for each nanoparticles were reported on p.11.

- The method of measuring the total content of the nanoparticles needs to described in details.

-Although we didn’t measure the total number of nanoparticles for the cellular experiments, we instead used empty nanovectors as internal controls, since they were treated exactly the same as their drug-loaded counterparts. Also, we normalized all nanoparticles to Gemcitabine content. As the size of all nanoparticles are similar, we hypothesize this translates to similar amounts of all nanoparticles.

- Figures 3A and 3B does not necessarily showed a significant burst release. The drug release also does not show any significant release after abot 20 hours. How much drug/nanoparticles were used for the release studies?

-We agree that the release stays constant after about 20hrs, which is characteristic of burst profiles associated with nanoparticle encapsulation (Sunqrot S et al, Bioconjug Chem 2011). For the goal of this paper, it was simply to compare the Gemcitabine release profiles from 2 vector types in PBS versus PANC1 cells, and hence, this methodology was deemed suitable for our purpose. Again, we normalized both nanoparticles amounts with respect to Gemcitabine content, which was 420 \(\mu\)g, and not number of nanoparticles. Taking into account this initial drug content, the release was about 95% and 33% respectively for PLGem and GemPo over the tested period of time. We added these precisions to the manuscript to bring more clarity on p.12. However, we respectfully believe there is significant release after 20hrs, based on the MTS cytotoxic studies, were drugs were kept for 3 days.

- Apparently, the PLGA batch has significantly less drug, i.e., 5 micro gram/mg whereas, the liposome batch has about 50 microgram/mg. However, the PLGA batch showed much more total drug than the liposome batch. Please explain.

-This is a very good point. We believe this is due to the higher degradation rate of PLGA compared with liposome in aqueous media (Hasirci V et al, J Biotechnol 2001, 86:135-150).
Page 8 Please change the title from Physico-chemical release kinetics.... to In vitro drug release.

-This has been done.

- Encapsulating the drug in nanoparticle does not change the half life of the drug, but it only prolong the drug release. Please change the statement throughout the manuscript.

-This has been done.

**Reviewer 2: Dr Davidson D Ateh**

**Major Compulsory Revisions:**

- The authors have used a single pancreatic cell line (PANC1) known to be resistant to Gemcitabine. The authors must therefore limit the scope of their conclusions unless they choose to do further experiments with other pancreatic cell lines including those that are not resistant to Gemcitabine. It has to be noted that the achieved results are significantly influenced by the underlying drug resistance mechanisms inherent to the cell line. So for instance, the poor performance of free form Gemcitabine could be due to cell membrane efflux pump exclusion which are by-passed by internalised nanoparticles. The only way to conclude a generally superior nanoparticle performance in pancreatic cancer would be to test more cell lines including those that are non-resistant.

-This is a very good point: thank you. Effectively, we have preliminary data in PANC1 and other cell types indicating that the sensitivity of cells to Gemcitabine/Gemcitabine nanoparticles relates to the presence of membrane efflux pumps. As such, the cell lines not having high levels of these pumps were significantly more sensitive to the drugs. These results are for another manuscript, and hence, we only focused only on PANC1 for this study. We have taken in account your remarks and have limited the scope of our conclusions.

- If the paper is to be published without further experimentation, this reviewer views the following changes as essential:
  1. Change paper title to: Mechanistic studies of Gemcitabine-loaded nanoplatfroms in a resistant pancreatic cancer cell line

-This has been done.

2. Clarify and discuss the limitations of the use of a single drug resistant cell line (including possible resistant mechanisms in this cell line and reasons why nanoparticles would be advantageous) in the Discussion section.
- As stated above, we have preliminary data in other cell types implicating membrane efflux pumps; we have hence clarified this point in the text (p.16). We also stated that the reason for reporting PANC in this paper is that of all the cell lines, it most closely mimics the PDA phenotype, and hence implies how the choice of nanovector can circumvent resistance.

3. Limit the scope of the conclusions to findings with respect to this cell line.

- This has been done.

We have addressed the reviewers comments to the best of our abilities, and feel that these reviews will significantly enhance the quality of our manuscript for publication in *BMC Cancer*.

We thank you very much in advance for considering us, and look forward to hearing from you.

Sincerely,

[Signature]