Author's response to reviews

Title: Enhancing chemosensitivity to gemcitabine via RNA interference targeting the catalytic subunits of protein kinase CK2 in human pancreatic cancer cells

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Author's response to reviews: see over
The authors investigated whether the efficacy of gemcitabine could be enhanced by modulation of various subunits of CK2. CK2 plays a role in cell division and proliferation. Although there was an earlier report on gemcitabine and CK2 it was not particularly clear to me why the authors chose to investigate this combination specifically in pancreatic cancer.

Protein kinase CK2 plays an important role in cell division, proliferation but also in cell death and its expression is often elevated in cancer cells making it a potential molecular target for anti-cancer treatment. Hamacher et al., (ref. 36 in the manuscript) conducted a study testing the viability of Mia PaCa-2 cancer cells following cell incubation with two inhibitors of protein kinase CK2, i.e. DRB and apigenin, which have a rather broad specificity. Giroux et al., conducted a siRNA-based large screening (ref. 14 in the manuscript) identifying, among other proteins, CK2 as a potential target for decreasing resistance of pancreatic cancer cells towards gemcitabine. Further investigations have not been conducted.

In our study, we aimed to:
1. verify whether the specific down-regulation of CK2 by RNAi technology would sensitize pancreatic cancer cells resistant to gemcitabine
2. identify the specific role of the individual CK2 subunits
3. explore the molecular mechanisms underlying the observed effects

It is not clear to me whether there is an interaction (in case of cytotoxicity, the effects should be more than additive) or whether the effects are additive, which probably means no interaction. We believe that the effects are additive as the reviewer has also commented below.

p. 9-10: an extensive description (almost each concentration of each cell line) of the sensitivity of cells to gemcitabine is given. This is redundant; Fig 1 and Fig 2 should be exchanged; first give growth inhibition and subsequently the associated disturbance in cell cycle.

As suggested by the reviewer, we have exchanged Figures 1 and 2 and reduced the description of the results which refer to the same figures. The figure legends have been modified accordingly.

Fig. 2: it is unclear to me why no normal growth inhibition curves are found in Fig. 2a, although a concentration effect was seen in Fig. 2B. E.g. PANC-1 shows no growth inhibition (Fig. 2A), but shows inhibition of BrdU labelling and disturbance of cell cycle. Possibly the WST-1 is not the most suitable assay for this purpose or the cells did not grow sufficiently during the assay. What is the doubling time? I prefer to get a list of IC50 values.

What is shown in Fig. 2A (now Fig. 1A) is the results of a viability assay which provides information on the metabolic status of the cells. The test indicates that gemcitabine induces cytotoxicity in all cell lines except PANC-1. The effect on growth (i.e. proliferation) is instead demonstrated by the BrdU assay (now Fig. 1B). Here, Mia PaCa-2, BxPC-3 and Capan-1 cell lines are severely affected by the treatment. The two assays demonstrate that incubation of cells with 50 nM gemcitabine is partially cytostatic but not cytotoxic for PANC-1 cells while for the remaining cell lines is cytostatic and cytotoxic.

The doubling time of PANC-1 is about 50 hrs.
The IC50 are as follows: Mia PaCa-2 50 nM, PANC-1 >> 1µM, BxPC-3 40.4 nM, Capan-1 40.4 nM.

An additional experiment (i.e. WST-1 test) has been performed with PANC-1 cells. Results show that gemcitabine up to 100 µM is slightly cytotoxic for the cells.
Fig. 1A: it is strange that there are no cells in the S-phase of MiaPaCa cells. What is the purpose of Fig 2B; it only shows that there are fewer cells when treated with gemcitabine.

In Fig. 1A (now Fig. 2), the border of the graphs is now thinner so that the percentage of Mia PaCa-2 cells in S phase is more visible.

We agree with the reviewer that Fig. 2B (now Fig. 1C) shows that after treatment cells are fewer. We believe that this piece of information (i.e. the morphological evaluation) complements results obtained with the viability and proliferation assays. Therefore, we would like to include the cell pictures in Fig. 1.

In Fig 3C cell death of 7% is reported but this is not in agreement with the number in Fig 1A, which is just 2-3%.

The experiment shown in Fig. 3, which reports the percentage of cell death following the indicated treatments, includes the effect of the transfection reagent (i.e. Lipofectamine) which has been used for transfecting the CK2-siRNAs. In Fig. 1A (now Fig. 2), transfection reagent has not been used.

p. 11: do not give number of lanes in the text; the effects are difficult to follow. Clearly indicate treatment in the figure. I do not agree that cells are efficiently killed in the combination, actually cell kill in the combination is less than what can theoretically be expected from the effect of gemcitabine alone or anti-CK alone.

In the text of the results section, lanes number has been eliminated. At page 11, “they indicate that PANC-1 cells are efficiently killed by a combination treatment of gemcitabine and siRNA-mediated depletion of the CK2α subunit” We have rephrased the sentence as follows: “they indicate that PANC-1 cells are sensitized to gemcitabine treatment following cellular depletion of the CK2α subunit by RNA interference”.

p. 13: the effect on JNK and of the JNK inhibitor SP600125 is interesting, however, it does not provide a sufficient explanation; the authors claim that gemcitabine-induced cytotoxicity is mediated by CK2α mediated JNK signalling. However, the authors did not investigate cell death in the presence of SP60012, only an effect on signalling was investigated.

The experiment shown in Fig. 5B refers to an in vitro, non-radioactive assay where cell extract containing endogenous JNK, was incubated with GST-cJun fusion protein linked to glutathione agarose beads. The JNK inhibitor SP600125 has been included in the assay as a control exclusively for the purpose of verifying that indeed the kinase phosphorylating cJun was the precipitated JNK.

We observed enhanced JNK phosphorylation in cells depleted of CK2α and incubated with gemcitabine. Beside the intention to use the aforementioned inhibitor in a control experiment, we did incubate cells with SP600125 and performed a FACS analysis. We did not observe a decreased percentage of cells in sub-G1 as we predicted from our results. Bain et al. (Biochem. J. 371, 199-204, 2003) showed that although SP600125 was developed as an inhibitor of JNK, 13 other protein kinases are inhibited with similar or greater potency suggesting a limitation in the usage of this compound for specifically inhibiting JNK in cultured cells.

p. 14: specify what was done in vivo, clarify in vivo as well?

It refers to measurement of PDK1 activity in whole extract from control and treated cells. We have rephrased the sentence as follows: “results indicated that the observed lack of phosphorylation of AKT at T308 in cells depleted of CK2α’ was not a direct consequence of decreased activity of endogenous PDK1 (data not shown)”.

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p. 15: cell death is postulated to be increased, but as indicated above, I believe it is only additive or even less then additive. The JNK pathway may be of interest, but essential experiments have not been done.

We have modified the sentence as “Cellular depletion of the individual CK2 catalytic subunits in combination with gemcitabine resulted in enhanced cell death with respect to the sole gemcitabine treatment”.

Last paragraph: the authors mention that “the gemcitabine resistance mechanism” is with Akt; although there is an interaction, this is only one out of many possibilities. Pancreatic cells may have a decreased uptake of gemcitabine, a decreased phosphorylation or better repair of DNA damage, which may be more important.

We agree with the reviewer that the modulation of AKT activity is not the only factor affecting cell death under the mentioned treatments. Therefore, the sentence has been modified as: “the reported difference in the phosphorylation levels of AKT suggests that the modulation of AKT activity contributes to the different amounts of cell death observed following the aforementioned treatments”.

Figure 1: why are the bars not the same; they do not all reach 100%, especially in the BxPC3
As mentioned above, Fig. 1 is now Fig. 2. The percentage of cells in the various cell cycle phases has been corrected considering as 100% the gated population of cells (i.e. with the exclusion of duplets, triplets etc…). We have overlooked it.

Figures 2, 4 and 6 are unreadable. Remove the horizontal grid, because this makes the letters unreadable; next use a better readable font and increase size of the legend.

The font size has been increased to 10 pt in all legends. We believe that some legends are not clearly readable because the figures were originally made in pptx and converted into TIFF before submission. We will now submit the figures as Pdf format. In Fig. 4, we have eliminated the scheme below the bar graph and wrote instead a text under each bar.

Fig. 2: growth inhibition should not be given in bars but as curves similar to presentation of the NCI of growth inhibition; the figure can then be simplified, with 4 lines in one figure.
Fig. 2 (now Fig. 1), bar graphs have been converted in line graphs.

Figures 3, 4, 5 and 6: the explanation of the numbers is given in one figure, but when one looks at the subsequent figures, one has to refer to the previous legends or even the text in the paper. Each figure should have an adequate description of each lane in the western blots, not based on numbers; each figure should be self-explaining without reference to another figure or text.
Following the reviewer’s suggestion, we have described more extensively the figures and avoid referring to previous figures. Lane numbers have been eliminated and text has been added for explanation.

Check citation of ref 14: Y de CW, this seems to be incorrect.
We have checked citation 14 and there are no mistakes in the text. Yde CW stands for Christina Westmose Yde.
Reviewer: Wainer Zoli

1. Add some references to the fifth sentence of the “Background” section first paragraph
   In agreement with the reviewer’s remark, we have added two new references (now indicated as [4] and [5]).

2. Some additional data/suggestions on CK2 functions should be inserted in the “Background” section
   Additional data on CK2 have been added in the aforementioned section.

3. Cell cycle data should be re-evaluated utilizing Modfit software or other software able to use the correct mathematical model or algorithm
   We believe that the reviewer refers to the fact that the bars do not all reach 100% in Fig. 1A (now Fig. 2). The percentage of cells in the various cell cycle phases has been corrected considering as 100% the gated population of cells (i.e. with the exclusion of duplets, triplets etc…). We have overlooked it.