Author's response to reviews

Title: Increase of Gap Junction Activities in SW480 Human Colorectal Cancer Cells

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Re: Manuscript #1631422451239827, entitled “Increase of Gap Junction Activities in SW480 Human Colorectal Cancer Cells”

Dear Reviewers,

On behalf of all the authors, I would like to thank for the opportunity to address the reviewer’s concerns and resubmit a revision of manuscript #1631422451239827.

Reviewers’ comments:

Reviewer: Marisa Ionta

1. Cell culture conditions should be added.
   
   The authors have added the conditions on page 5 lines 93 and 94. Cells were grown with 0% CO₂ in Leibovitz’s L-15 Medium with 10% Gibco Fetal Bovine Serum.

2. In subtopic “Western Blot” it is necessary to include: 1) cell density used in plating; 2) cell growth area; 3) how long cells were cultured before the treatment.
   
   The authors have added more information on page 5 lines 95 and 96. Cells were seeded to 50% density in a T-25 cm² flask for 24 hours and allow the density to reach 90% prior to treatment.

3. Figure 4A is not mentioned in the text.
   
   This has been corrected. Figure 4A is mentioned and discussed starting on page 9 lines 190.

4. The authors affirm “Isoform expression of Cx43 has shifted from P0 form to P1 form in the Cx43 transfected cells”. However, immunoreactive bands for Cx43 are not observed in control sample (figure 1). Therefore, it is necessary to revise this point.
   
   Thank you for pointing this out. The figure has been updated to show the results that correlate with the graph. In order to visualize the control samples on the sample blot, the intensity of the transfected sample had to be decreased as indicated above each band. The label, “0.1” above
the transfected band, indicates the signal was decreased to 10% relative to previous image. This approach allows the analysis of the control sample to detect the Cx43.

Reviewer: Jerome Gilleron

1. The authors should add the citations referring to the intracellular localization of Cx43 phosphorylated isoforms since this information is important.

   Thank you for the suggestion. Citation was added on page 4 line 67.

2. The scrape loading assay is not the more sensitive assay used today to determine the functionality of gap junction, but it should be sensitive enough to support or invalidate the hypothesis of the authors. However, essential control using AGA, oleamide or Carbenoxolone seems required to determine the transfer distance after fully blocking GJIC. Indeed, if TPA is disturbing Gap junction, it is not per se a GJIC functional inhibitor.

   Thank you for your input and suggestion. Additional experiment of scrape loading assay was performed using Carbenoxolone (CBX). In the presence of CBX, PQ1 does not lead to an increase in GJIC. The data is shown in figure 3D and is analyzed on page 9 lines 179 through 185.

3. In discussing the results of the figure 4, the authors concluded that the findings suggest that PQ1 act on existing Cx43 and not on expression. The authors should show by imaging an increase of Cx43 staining at plasma membrane. This result is important and easy to obtain.

   Immunofluorescence of Cx43 after treatment with PQ1 has been added as Figure 5. It shows a shift from cytoplasmic Cx43 in controls to plaque formation after PQ1 treatment. Analysis has been added to page 10 lines 203 to 205.

4. Based on the lack of stars, it seems that the results plotted on the figure 5 is not significant, why the authors conclude that increase of gap junction functionality by PQ1 may act on Akt activation? First this could be correlative and not causal, second the data don’t support the hypothesis since not significant.

   An apology is owed to the reviewer. The asterisk was missing on the graph. The active Akt expression is significant compared to DMSO control and has now been added to the graph in figure 6B (a new figure number due to the addition of immunofluorescence data).

5. Same question for the figure 6. Indeed if here the effect is significant, how the author could exclude that PQ1 acts on MAPK activation independently to GJ?

   Thank you for your insight in the author’s interest to address this question. Inhibiting GJIC could address this concern and demonstrate causality.

   To test PQ1’s ability to increase GJIC via kinases, staurosporin and calphostin C (kinase inhibitors) were used. Scrape loading assay showed that in the presence of kinase inhibitors PQ1 did not lead to an increase in GJIC. This data is shown in figure 8 and analyzed on page 11 lines 219 to 224.
6. Also to address Dr. Gilleron’s statement that due to no decrease in proliferation or viability the data is diminished.

Thank you for pointing out the author’s failed to describe the reasoning behind not capturing the effective dose on proliferation and viability. In this study, the effect of PQ1 is examined at not as a direct chemotherapeutic drug but as an enhancer of other chemotherapeutic drugs. If overexpressing Cx43 or PQ1 caused a decrease in proliferation and viability then gap junctions would not be able to form due to no connections with adjacent cells (cell-to-cell contact). This study does prove PQ1’s ability to enhance gap junctions and therefore its ability to be a potential chemotherapeutic enhancer by way of gap junctions. This is now stated on page 13 lines 253 to 255.

Additional changes

1. Figures 2A, 2B, 4B, 6 and 7 were all normalized to DMSO as it is the more accurate control than the no treatment controls.

2. Discussion using figures 5 and 8 was added to page 12 on lines 239 and 240 as well as 249 to 251.

3. Figure numbers were corrected now that there are 8 figures instead of 6 figures.

4. Figure legends were also change to reflect the new data. Figure 3 changes are on page 19 lines 379 to 380. Legend for figure 5 was added on page 20 lines 392 to 394. What used be figures 5 and 6 were changed to figures 6 and 7 on line 395 and 402 respectively. Legend for figure 8 was added on page 21 lines 410 to 416.