Author’s response to reviews

Title: Variable NF-kappa B pathway responses in colon cancer cells treated with chemotherapeutic drugs

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Author’s response to reviews: see over
To: Editor, BMC Cancer

RE: Submitting our revised manuscript

Dear Editor,

Thank you for your communication regarding our manuscript (ID #1822424546125758) entitled “Variable NF-kB pathway responses in colon cancer cells treated with chemotherapeutic drugs”. Thanks also for extending the time for re-submission to accommodate my international travel.

We also thank the reviewers for their time, efforts, and useful comments and suggestions, which helped us make our manuscript better.

I am addressing below the comments from the reviewers point by point.

Reviewer 1.
1: Figure 2 and 3: Different response of NF-kB to different drug stimuli and in different cell types is not a novel finding and is described several times in the literature.

We agree the response of NF-kB to different stimuli is not novel. However, the novel aspects of our work include the independence of the response on p53 presence or absence, uncoupling of downstream anti-apoptotic pathway from cytokine response, and the up-regulation of cytokine receptors in cells not responding by NF-kB activation. Therefore, taken together, our reports indeed contain novel results and we have raised important clinically relevant questions (see also #5 below).

2: Figure 4: Why was only CPT influence on NF-kB nuclear translocation evaluated? All cell lines and all drugs used in the study should have been investigated.

Our data from Fig. 3 onwards are focused on the responses of SW480 and HCT116 cells to CPT only. We acknowledge that we failed to indicate this transition in the manuscript. Therefore we have introduced a transition statement in the revised manuscript (Lines 190-195): “Although the drugs used in these experiments are clinically relevant, we decided to further examine the activation of NF-kB by CPT only, because this drug is widely clinically used and it gave consistently higher NF-kB response at sub-micromolar concentrations. Moreover, phleomycin is only radiomimetic, and unlike EGFR inhibition by monoclonal antibodies, EGFR inhibition by tyrosine kinase inhibitors (TKI) such as erlotinib in colon cancer has not achieved wide clinical utility (Alyssa M. Krasinskas, 2011)”.

3: Figure 5 and 6: Why are only results for CPT treatment shown here? Results for all evaluated drugs should be shown.

Not to miss a concentration dependent effect of NF-kB activation by other drugs, we have also performed experiments using various concentrations of 5-FU and Oxaliplatin (two clinically widely used drugs besides CPT) on NF-kB response in reporter HCT116 or SW480 cells. These results (now submitted as Supplemental Figure S1) show the consistency of CPT response, while the lack of or weak NF-kB
activation by 5-FU and Oxaliplatin in concentration ranges that are clinically relevant. Please also see the response to comment #2 above, why CPT was further investigated.

4: Figure 7: Cells NCL-60 and SW620 are mentioned now for the first time. Why are they now evaluated? Please add to Materials and Methods and give good reasons for performing these experiments now with different cells then the ones used in the preceding experiments.

We apologize for the omission in Materials and Methods of the description of CellMiner database, which was used to generate these data sets. We have amended the manuscript to correct this, and a paragraph has been added under Materials and Methods section (Lines 134-140) to describe the method.

5: The Discussion is full of unasked questions which could very nicely have been evaluated for this paper to elucidate the mechanistics of the NF-kB signaling pathway in response to various chemotherapeutics in different colon cancer cells.

In this first manuscript, we have attempted to introduce the complexity of NF-kB response, the uncoupling of this drug response from the classical NF-kB responsive anti-apoptotic signaling, and the involvement of cytokines and receptors in this response. Besides our previous data that show NF-kB activation in response to CPT, in our revised Figure 6 (E-F), for example, we show that the inhibition of Chk1 and Chk2, two kinases activated in response to DNA damage partially abrogates the activation of NF-kB in our system, suggesting nuclear-initiated events in this mechanism.

We acknowledge that we cannot answer all the possible mechanistic questions relevant to the data in this manuscript and in such short time. For example, we have recently generated additional reporter colon cancer cell lines (SW620, RKO parental and RKO subsets) to explore the common mechanics of NF-kB activation in colon cancer cell subsets. The response of these colon cancer cells to the same drugs we used were again variable, making the finding of a common determinant a challenge. We are planning further studies, including comprehensive knockdown studies to find key regulators of the response, at least in a subset of the cancer cells.

Therefore, we believe that given the relevance of our data to clinical applications (as also mentioned by Reviewer #2), publishing the manuscript in an open access forum would enable the research community to immediately start addressing these questions.

**Reviewer 2.**
1. Fig. 1B-D: Please indicate the concentration and treatment time used for TNFalpha.

TNFα was used at 100ng/ml in these experiments. We have revised the legend to indicate this.

2. Fig. 2: Lines 143 and 150 indicate that Fig. 2 contains A-C, which is not consistent with the description in the figure legend which only contains A and B. In addition, the description of Y axis is not consistent with that in other figures.

Thanks, and we regret this and other errors introduced while rearranging the figures for the final presentation. We have labelled the panels as A, B, and C, and also revised the Y-axis description to be consistent with that of the other figures.

3. Fig. 3: The figure and the description in lines 166 and 170 (Fig. 3A-C) are not consistent with the description in figure legends (the left, middle, and right panels).

We have corrected the legend for Figure 3 accordingly.
4. Fig. 6: C and D are missing in the figure legend. In addition, Fig. 6 B-C should be changed to Fig. C-D (lines 208-209).

**Figure 6** and its legend have been fully revised to correct this error, to better present the X-axis, and also to include data from our recent experiment with Chk1/Chk2 inhibitor AZD-7762.

**In addition** to the above point by point responses and revisions accompanying the responses, the following changes have been made and included in the revised version:

1. **Methods:**
   a. *Lines 96-97:* The source of a new compound used in the experiments has been added, “The Chk1/Chk2 specific inhibitor AZD-7762 was purchased from Sigma Aldrich (St. Louis, MO).”
   b. *Lines 141-147:* A short paragraph on immunofluorescent staining has been added.

2. **Results:**
   a. *Lines 172-174:* The phrase “5-FU and oxaliplatin did not induce remarkable activity in these cell lines and therefore were not utilized further (supplemental Fig. S1)” has been revised to accommodate supplemental information.
   b. *Lines 238-244:* We have generated 2 new Figures (6 E and F), and a section in the revised manuscript has been added to accompany the added Figures. Legend for Figure 6 has also been modified accordingly.
   c. Figure 6 has been re-formatted and re-arranged (see also responses to reviewer #2)

3. **References:**
   a. Two new references have been added (#34 and 36).

4. **Legends:**
   a. A legend for Supplemental Figure S1 has been added.

5. We have added one grant number under “acknowledgements”.

Please let me know if you have any questions.

Thank you

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