Author's response to reviews

Title: Dual regulation of cell death by Akt kinase inhibitor MK-2206 in colorectal cancer

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Author's response to reviews: see over
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To,
The Editor-in-Chief
BMC Cancer

Subject: Revised manuscript submission (Ms. 2799715021026821)

Dear Editor,

Thank you for your response. We appreciate both the enthusiasm for the manuscript and the constructive nature of the reviewers’ comments. We have revised the manuscript in response to each of the reviewers’ comments and have made a point-by-point list addressing the reviewers’ concerns as indicated below:

Reviewer #1:

There is a lot of discussion in the results part (e.g. discussion of BAD function page 12, discussion of the role of XIAP and survivin on page 12, discussion of AIF/Akt and Ezrin/Akt interaction on page 14 and 15, respectively). Please rewrite the results/discussion part.

The above comment has been addressed and the discussion in the results section has been deleted and reorganized in the revised manuscript.

Figure 2E (of which results are reported on page 12) is missing.

This has been corrected in the figures section in the revised manuscript.

For in vitro tests, the dose of 350nm and 500nm was used. Please show specificity of obtained results by testing MK-2206 in this dose on IGF1R-independent colon cancer cells and non-colon cancer cells/ non-malignant cells.

This has been addressed and data has been included in the supplementary section as fig.S1. We tested the specificity of MK-2206 (using the two doses 250nm and 500nm) on HCT116 (an IGF1R-independent colon cancer cell line) and MiaPaCa (pancreatic cancer cell line with constitutive activation of IGF-1R). While HCT116 showed marginal loss of pAkt S473 following drug treatment; MiaPaCa cells showed robust loss of pAkt with MK-2206 treatment.

Please comment on the correlation of the in vivo MK-2206 dose (120mg/kg) compared to in vitro testing cells.

The in vitro and in vivo doses were provided by Merck &Co based on their confidential in-
house observations. We have a copy of the confidential report which details the in vitro IC50s in sensitive cell lines are usually below 1uM. For in vivo, standard mono therapy efficacy study in mice xenograft model, the dose of 120 mg/kg, po, 3 times/week or 480 mg/kg weekly was found to be standard and achieved clinically relevant dose. We performed both the 120 mg/kg, po, 3 times/week and 480 mg/kg weekly (data not shown) studies. The 120 mg/kg, po, 3 times/week dose was found to be more effective in anti-tumor activity as has been reported in the manuscript.

Reviewer #2:
Title: “Dual regulation of cell death” can be suitably expanded to allow a glimpse of the content of the paper to the prospective reader.
The title has been changed in the revised manuscript to:

The Akt inhibitor MK-2206 promotes anti-tumor activity and cell death by modulation of AIF and Ezrin in colorectal cancer

Corresponding author: Is it a policy of the journal to mention the ‘co-corresponding author’.

We have changed the “co-corresponding author” to two “corresponding authors” in the revised manuscript (Sanjib Chowdhury and Michael Brattain). We have earlier published with BMC journals (Journal of Molecular Signaling – Hedrick et al., Oct 2013) with 2 corresponding authors.

Reference 4 ‘Cell Signal’ is incomplete.

This has been corrected in the revised manuscript.

DNA fragmentation assay: The classical ladder formation could have been done and shown as a supplementary data.

We have performed DNA Fragmentation assay using the Cell Death Detection ELISA plus kit from Roche. The advantage of this method is its fast performance (one –step ELISA, high sensitivity and has been extensively used as a reliable tool for amplification of apoptosis. We have extensively used the above method for studying apoptosis in several of our studies. However, if the reviewer decides on the “DNA-ladder formation” as a mandatory revision, we could perform the DNA ladder formation experiment.

Cell Death Detection ELISA plus information as obtained from Roche website: This assay is a Photometric enzyme-immunoassay for the qualitative and quantitative in vitro determination of cytoplasmic histone-associated-DNA-fragments (mono- and oligonucleosomes) after induced cell death.

Stage Description
1. The sample (cell-lysate, serum, culture-supernatant etc.) is placed into a streptavidin-coated MP.
2. A mixture of Anti-histone-biotin and Anti-DNA-POD are added and incubated. During the incubation period, the Anti-histone antibody binds to the histone-component of the nucleosomes and simultaneously captures the immunocomplex to the streptavidin-coated MP via its biotinylation. Additionally, the Anti-DNA-POD antibody reacts with the DNA-component of the nucleosomes.
4. Quantitative determination of the amount of nucleosomes by the POD retained in the
immunocomplex. POD is determined photometrically with ABTS as substrate.

**Specificity**

1. The Anti-histone-biotin-antibody binds to histones H1, H2A, H2B, H3 and H4 from various species, e.g. man, mouse, rat, hamster, cow, opossum and xenopus.
2. The Anti-DNA-POD-antibody reacts with single and double stranded DNA.

**Western blot:** Electrophoretic transfer must have been checked on a nitrocellulose membrane by Ponceau S, and that may be mentioned.

We routinely perform Ponceau S staining on every nitrocellulose membrane following electrophoretic transfer. This information has been addressed in the Methods section.

3. **Control:** In cell culture experiments, the control must have received the vehicle of the drug DMSO for MK-2206.

The controls were treated with DMSO (vehicle of the drug).

4. **Make for the confocal microscope must be given.**

LSM 510 microscope (Carl Zeiss GmbH, Oberkochen, Germany) was used to perform laser confocal microscopy. This information has been included in Methods section.

5. **Xenograft studies:** How the dose level of MK-2206 at 120mg/kg body weight had been arrived at? Previous references should be given. Also the duration of 3 weeks in alternate days should be clarified.

Please see Reviewer #1 response above. Briefly, standard mono therapy efficacy studies in mice xenograft model was performed by Merck & Co. The dose of 120 mg/kg, po, 3 times/week or 480 mg/kg weekly was found to be standard and achieved clinically relevant dose. We performed the 120 mg/kg, po, 3 times/week and 480 mg/kg weekly (data not shown) studies. The 120 mg/kg, po, 3 times/week dose was found to be more effective in anti-tumor activity as has been reported in the manuscript.

6. **Animal number:** What does it mean by ‘at least 3 independent control....’

Mention the exact number of animals and repetition to validate the results. Animal pictures, before and after treatment of MK-2206, at least at the final stage could be shown as a supplementary figure and legend.

The experiment was performed in a total of 16 animals. Following euthanization and tumor processing, we used 3 animals each from control and treated groups for further protein, RNA and IHC analysis. The images of the control and treated animals used for further analysis have been included in supplementary figures (fig.S8).

7. **Tumor micro imaging system should be a little bit explained and properly referenced.**

We have used the Near-IR enhanced Macro Imaging System Plus Cooled with the LT-99D2 with the Dual Tool dual excitation upgrade for viewing 2-D and 3-D EGFP and RFP fluorescent samples.

8. **How many cells were counted for TUNEL assay?**

4000 cells each for control and treated group were counted for TUNEL assay. This information has been updated in the methods section of the revised manuscript.
9. **Fig 2B: Is the cell death with MK-2206 strictly in a concentration dependent manner.**

We have performed both time- and concentration-dependent assays for MK-2206. Maximal cell death response has been observed at 48 hours post treatment with 500 nm MK-2206.

10. **Page 12: Bcl-xl caption for Fig 2E is missing in the figure (IP for Bcl-xl).**

This has been corrected in the revised manuscript.

11. **It should be ‘g’ (tumor wt) and not ‘gms’ as written in the text and fig 3D.**

This has been corrected in the revised manuscript.

12. **Fig S4 - Was the densitometric data of tAkt normalized with GAPDH.**

The tAkt densitometric data in fig S6 (fig S4 in the original submission) is the normalization of treated against control IHC data shown in fig. S5. The difference in staining intensity for tAkt was determined by selecting 10 different but histologically similar fields per sample and the fields were analyzed using NIH Image J software. The staining intensity measured by the software was plotted using Graph pad 5.0.

13. **Legend fig 3B: Reduction......after different days of drug treatment etc.**

The figure legend has been corrected in the revised manuscript.

14. **Legend fig 6B and C have been interchanged, please check.**

The figure legend has been corrected in the revised manuscript.

15. **Fig 7 C: Western blot of XIAP had not been shown although mentioned in the legends.**

The figure legend has been corrected in the revised manuscript.

16. **Fig 6 B: Is it really a confocal image, that too taken at 60 X or is it a simple fluorescence microscope, please clarify. Also caption B and C have been interchanged in the legend.**

We would like to clarify that it is indeed a confocal image taken at 60X. The captions have been corrected in the revised manuscript.

**Reviewer #3:**

The author claims that MK-2206 inhibits the expression of XIAP, however this is not clearly shown in fig.2C. A quantitation of this experiment has to be shown. In addition, down–regulation of XIAP has to be shown in the xenografts after MK-2206 treatment.

The quantitation of XIAP shown in fig.2C has been done (normalized against GAPDH) and there was approximately 40% reduction in the XIAP expression on treatment with 500 nm MK-2206.

Robust loss of XIAP expression was observed in xenografts treated with MK-2206. This data has been included in supplemental fig S3 of the revised manuscript.
The reduced binding of phosphoBAD to 14-3-3 shown by co-immunoprecipitation is due to the reduced phosphorylation of BAD (S136) as shown in fig.2C. The immunoblot has to be performed with an anti-BAD antibody instead of an anti-phospho–BAD antibody to show a reduced binding of BAD to 14-3-3.

This has been addressed and reduced binding of BAD with 14-3-3 has been shown in fig 2D of the revised manuscript.

The implication of AIF in the MK-2206 mediated effect on caspase –independent cell death is shown by an AIF inhibitor at a high concentration of 50uM/L. This inhibitor may not be specific anymore at this high concentration. To proof the implication of AIF in the MK-2206 mediated cell death, the authors should down regulate AIF by siRNA and measure the changes in cell death after MK-2206 treatment n comparison to an MK-2206 treated scrambled control.

We have now performed DNA fragmentation assay with AIF siRNA +/- MK-2206 and observed similar results compared to AIF inhibitor. The results have been shown in fig. S9 of the revised manuscript.

The authors report in the results (page 15) that knock down of Ezrin resulted in complete loss of XIAP and survivin (data not shown). These data are important to explain the proposed mechanism and have to be shown in the manuscript. Ezrin siRNA knockdown in colon cancer cells showed loss of XIAP and survivin. The figure has been included as supplementary fig. S11 in the revised manuscript.

Discretionary Revisions

The figure legends should describe the experiments performed only, but should not contain the conclusion made by the authors e.g. Fig 2E...“The interaction increases on treatment with MK-2206 thus leading to increased cell death”.

This interpretation belongs in the discussion and has to be removed from the figure legend.

This concern has been addressed and the interpretation has been deleted from the figure legend in the revised manuscript.

The authors have analyzed AKT1 and AKT2, but not AKT3. It would be interesting at least to investigate the expression of AKT3 in the colon cancer cell lines, analyzed. If AKT3 is expressed, it would be interesting if down regulation of AKT3 has the same effect as observed after down regulation of AKT2.

We performed Akt3 siRNA knockdown on GEO cells and observed no change in pEzrin T567 and total ezrin, thus indicating that Akt2 isoform specifically regulates ezrin phosphorylation at T567. This data has been added to the supplemental fig. S10. in the revised manuscript.

Minor Essential Revisions

Page 28, figure legend 6C: ...in treated as compared to control cells.

This has been corrected in the revised manuscript.

There a few typing errors, e.g.
Page 7, at the end of the page: immuoprecipitation (immunoprecipitation)
Page 8, first line, second paragraph: Akt21si (should be Akt1si).

The typing errors have been corrected in the revised manuscript.

This original research article is expected to be of high interest to the broad spectrum of *BMC Cancer* readers as well as scientists who are working in the field of Akt signaling and colorectal cancer. Thank you very much for your consideration of our work.

Sincerely,

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