Author's response to reviews

Title: The 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, simvastatin, lovastatin and mevastatin inhibit proliferation and invasion of melanoma cells.

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Author's response to reviews: see over
Dear Dr. M. Jazayeri,
Assistant Editor
BMC-Series Journals

We are submitting our revised manuscript (MS-1614590434152778) entitled “The 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, simvastatin, lovastatin and mevastatin inhibit proliferation and invasion of melanoma cells.” to be considered for publication in the *BMC Cancer*.

The editorial office requested revisions to our previous manuscript to address the points raised by the reviewers. We appreciate the reviewers’ comments and have revised the manuscript according to their suggestions. Attached to the cover letter is our response to each point raised by the reviewer with an explanation of the changes that we made to the manuscript. We feel the revisions have substantially improved the quality of the manuscript and believe that it is now suitable for publication in *BMC Cancer*.

Thank you for your time and consideration.

Sincerely,

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Reviewer’s comments:

Reviewer #1:

**Major Compulsory Revisions**


Response: The recent meta-analysis by Freeman SR et al, described the incidence of melanoma in 20 of 36 qualifying randomized controlled trials (12 statin trials and eight fibrate trials), with a total of 70,820 participants. They reported that, a total of 127 melanomas occurred among the 39,426 participants in the statin trials (59 among the 19,872 statin group participants and 68 among the 19,554 control group participants). The reported odds ratio for melanoma was in the direction of a protective effect, but did not reach significance (OR = 0.87, 95% CI = 0.61 to 1.23). The authors were of the conclusion that statins do not prevent melanoma. However, with such a small number of incident cases of melanoma, this conclusion can not be final. A case-control melanoma study, that includes information on statins may have greater power to answer this question.

We added the new information to our manuscript, under Introduction, on page 3: “A recent meta-analysis described the incidence of melanoma in 12 qualifying randomized controlled statin trials, with a total of 39,426 participants. They reported that, a total of 127 melanomas occurred (59 among the 19,872 statin group participants and 68 among the 19,554 control group participants). The reported odds ratio for melanoma was in the direction of a protective effect, but did not reach significance (OR = 0.87, 95% CI = 0.61 to 1.23). As the number of incident melanoma cases was low, a specific study to address the role of statins in preventing melanoma needs to be designed, with the required power to provide answers [4].”

2. Given the poor prognosis of advanced melanoma patients I agree with the call to investigate the effects of adding high dose statins with current chemotherapy. The authors should discuss whether this further research should start with in vitro work showing synergistic killing of melanoma cell lines by the combination of statins and chemotherapy, proceed to in vitro animal studies showing increased efficacy due to the combination, and be followed by Phase I studies showing no undue toxicity due to the addition of high dose statins to chemotherapy in humans before proceeding to a definitive RCT for efficacy.

Response: As requested by reviewer 1, we have expanded on the description of future work to assess the viability of statin in combination with chemotherapeutics.
We added the new information to our manuscript, under Discussion on page 18: “Further research is required to examine whether statins can act either additively or synergistically in combination with chemotherapeutics to increase cell kill, and whether these effects are reversible with the addition of mevalonic acid. Combinations of statins with chemotherapy should first be tested in vitro and then proceed to in vivo animal studies to examine effects on tumour burden and/or tumour invasion and metastatic spread, prior to initiation of clinical trials examining the addition of high dose statins to chemotherapy in humans.”

**Minor Essential Revisions**

None.

**Discretionary Revisions**

None.

Reviewer #2:

**Major Compulsory Revisions**

1. It is not clear if the statins had to be activated before use or did the authors purchased the active form?

Response: On page 6, in the methods section, we state that pravastatin sodium, mevastatin sodium, lovastatin sodium and simvastatin sodium used in this paper, which are the activated forms. For better clarification we have added to the script on page 6, that they are indeed activated.

2. The effects observed should be reversed by treatment with mevalonic acid. Such experiments are missing.

Response: We respectfully agree with the reviewer that reverse treatments with mevalonic acid would add to this research. We have addressed this in the conclusion (page 18), were we have expanded on future work as requested by reviewer 1.

**Minor Essential Revisions**

1. Fig. 4 is not cited in the text.

Response: This oversight has been corrected on page 13 of the manuscript.
**Discretionary Revisions**

1. It would be useful to investigate the effect of low doses of statins in combination with some chemotherapeutics used in treatment of melanoma or other type of cancer investigated in this study.

Response: This has been addressed in our response to reviewer 1, revision no. 2 as described above.

**Reviewer #3:**

**Major Compulsory Revisions**

1. Introduction section should present relevant data describing key findings of previous studies evaluating effects of statins on melanoma cells.

Response:

The following background information has been added to the introduction on page 4:

*A number of studies have investigated the effects of statins on melanoma cells. Lovastatin induces apoptosis in A375 melanoma cells (11) and also enhances response to chemotherapy drugs in the B16 mouse model of melanoma (12, 13). Collisson et al (14) showed that atorvastatin inhibited in vitro invasion and in vivo metastasis of A375M melanoma cells. Depasquale and Wheatley (15) recently showed that lovastatin reduced both melanoma cell growth and angiogenesis in an in vitro co-culture model angiogenesis system. These studies support the hypothesis that statins may play a role in melanoma prevention or treatment.*

2. Significant portion of the presented results is confirmatory in nature. Authors should clearly specify which aspect of their work is novel.

Response:

The following text has been added to the introduction on page 5:

*However, the conflicting evidence from the chemoprevention studies suggests that further investigation is required to determine if standard cholesterol-lowering doses of statins will inhibit the growth of melanoma cells. In this study we compared sensitivity to simvastatin, lovastatin and mevastatin across a panel of melanoma, lung and breast cancer cell lines and systematically examined the effects of the statins on apoptosis, adhesion, motility and invasion in melanoma cells.*
Minor Essential Revisions

1. Methods section, cytotoxicity assays subsection, second sentence: “Toxicity assays were carried out at least twice in triplicate… and each assay contained eight replica...”. The sentence is unclear and should be revised.

Response: We have amended the sentence as follows on page 7 to read, “Toxicity assays were performed on at least two separate occasions, in triplicate (3 individual 96 well plates) for each cell line for each drug. Eight replicate wells on the 96 well plate were used per drug concentration.”

2. Methods section, apoptosis assay subsection. More details should be provided, especially to clarify to the reader the function of 7-AAD as an indicator of cellular structural integrity.

Response: The following text has been added to the methods section to clarify the apoptosis assay methodology.

The Annexin V-PE detects phosphatidyl-serine on the external membrane of apoptotic cells and 7-AAD, a cell impermeant dye, is an indicator of membrane structural integrity. 7-AAD is excluded from live, healthy cells and early apoptotic cells, but permeates late-stage apoptotic and dead cells.

3. Methods section, ECM protein binding subsection. Please clarify abbreviation ECM. Also, a clarification of wavelengths used (405 nm vs. “reference” wavelength of 620 nm) is needed.

Response: We have amended the manuscript accordingly on page 9.

4. Results section: relevance of studying lung carcinoma and breast carcinoma cell lines should be explained.

Response: For clarification the following text has been added on page 11.

“As statins have been proposed as chemoprevention agents for a variety of cancer types including lung cancer, breast cancer and melanoma, we tested a range of cancer cell lines for sensitivity to four statin drugs, simvastatin, lovastatin, mevastatin and pravastatin.”

5. Results section: concentration-dependent effects of individual statins on melanoma cell lines should be presented in detail. Table 1 is not sufficient. For example, it is important to present data on minimal concentrations of statins at which significant inhibition of proliferation was observed.
Response: The purpose of table 1 is to allow us to compare the sensitivity of range of cell lines to statin drugs. Representation of data in the format of an IC50 a standard method, and allows for quick visual comparison of the results. In future work, we plan to assess the effects of statins in combination with chemotherapeutics, at varying concentrations and will determine the minimal concentrations of statins required to inhibit proliferation both as single therapy and in combination, as addressed in our response to reviewer 1, revision no.2.

6. Results section, presentation of regression analysis correlating late apoptosis and statin concentrations would benefit from including the r-values.

Response: We have included the r-values for the regression analysis as requested on page 12.

7. Results section, subsection presenting effects of statins on cell migration and invasion. Second sentence states that individual cell lines were “invasive and motile to varying degrees”. Presentation of statistical analysis is recommended

Response: See response to request no. 9.

8. Results section, last subsection presenting effects on adhesions: reference to Figure 4 should be provided.

Response: This was addressed in response to reviewer 2’s request.

9. Figures 1, 2 and 3 should present statistical differences between treatments and/or controls.

Response: In addition to the regression analysis for figures 1 and 3 as described previously in the results section, we have added supplemental tables 1, 2, 3 (in a separate file) corresponding to figure 1, 2, 3 which show the results of student t-test analysis to test for significant differences in apoptosis, migration and invasion between control and treatment in the case of figures 1 & 3, and differences between migration and invasion in the individual cell lines in figure 2. The supplementary tables are referred to in the figure legends.

10. Figures 4 and 5 should have appropriate clarification of Y-axes. O.D. 560 is not sufficient.

Response: We have amended figures 4 and 5 accordingly.

Discretionary Revisions

None