Author's response to reviews

Title: Lovastatin induces apoptosis of ovarian cancer cells and synergizes with doxorubicin: immediate therapeutic relevance

Authors:

Anna Martirosan (amartiro@uhnres.utoronto.ca)
James W Clendening (jclenden@uhnres.utoronto.ca)
Carolyn A Goard (cgoard@uhnres.utoronto.ca)
Linda Z Penn (lpenn@uhnres.utoronto.ca)

Version: 2 Date: 21 December 2009

Author's response to reviews:

Dear Drs. Alexandersson and Marshall,

RE: MS 1760521282714808

We wish to thank you for providing editorial guidance and managing the review of our manuscript, “Lovastatin induces apoptosis of ovarian cancer cells and synergizes with doxorubicin: immediate therapeutic relevance”. We are very pleased that you and the reviewers felt our research was of merit and that it may have the potential to be acceptable for publication in BMC Cancer after a few revisions. To this end, please find below our point-by-point responses to each request or comment.

We hope this letter addresses the concerns of the reviewers and that you will find this re-submission to be acceptable for publication.

Sincerely,

Dr. Linda Z. Penn

Reviewers' Comments:

Reviewer 1: Dr. Jung-Hwan Yoon

“1. The authors demonstrated that lovastatin augmented doxorubicin-induced apoptosis by measuring olive tail moment and TUNEL-positive cells. It would be more convincing if the authors show additional biochemical evidences, such as more enhanced activation of caspase 9 in A2780ADR cells following lovastatin+doxorubicin as compared to single drug-treated cells.”

The reviewer is correct in pointing out that more than one assay is helpful in demonstrating the induction of apoptosis. To this end, we have added new data in the form of fixed PI experiments (Figure 6) to complement our TUNEL results. This additional information further strengthens the conclusions of our manuscript and we thank the reviewer for his suggestion.
“2. The authors claimed that lovastatin increased the accumulation of intracellular doxorubicin, which was not reversed by MVA. Additional GGPP & FPP data are also needed in Fig.5.”

We understand and share Dr. Yoon’s interest in our observation that MVA was unable to reverse lovastatin-induced increase in doxorubicin accumulation. In our experience, MVA is the more potent “reversing” agent with respect to the effects of statin exposure:


While GGPP and FPP are also usually able to reverse these statin-induced effects, it is rare that they do so as robustly as MVA, as evident in Figure 1B. As such, MVA reversibility was our criterion for determining if a lovastatin-induced increase in doxorubicin accumulation is MVA-dependent.

“3. Doxorubicin induces p53-dependent apoptosis. Then it is interesting to see if A2780ADR cells expressing p53DD undergoes less apoptosis following combined treatment compared to A2780ADR cells.”

Although we agree with Dr. Yoon that further work to fully flesh out the mechanism of doxorubicin-induced death as potentiated by lovastatin treatment will be of great interest, we believe it falls beyond the scope of this manuscript. Doxorubicin-induced cell death is, of course, partly dependent on p53, but many other factors play important roles as well, including Myc and Rb:


While this would potentially be of great interest, our main focus was to explore the role statins can play in the eradication of ovarian carcinoma and to provide important pre-clinical data to suggest how to maximize their utility in the clinical setting.
“4. A2780ADR and A2780CIS cells show higher lovastatin IC50 as compared to A2780 cells. Then this may suggest that P-gp and/or MRP2 may be responsible for lovastatin resistance. Interpretation and discussion of this data is also necessary.”

Dr. Yoon is correct in suggesting that our data supports the notion that the MDR phenotype may play a role in resistance to statins. This is particularly interesting in light of previously published reports that demonstrated P-gp expressing cells were more sensitive to statin-induced apoptosis:


We had chosen not to discuss this topic in the manuscript for the sake of space and clarity of message, but have included it in the discussion of the current re-submission as requested. It is an important point and something that will require further investigation in the future.

“5. In Fig-1B, why 20µM was used, since this concentration is much higher as compared to IC50 values of A2780, HOC7 and OVACA432.”

A fixed dose was used to be able to compare across cell lines and obtain a relative level of sensitivity. We further used a dose of 20µM in order to induce a robust level of apoptosis. The primary goal of the experiment was to determine if lovastatin-induced apoptosis was MVA-dependent. The secondary purpose was to examine whether GGPP or FPP played a more substantial role in mediating the MVA-dependent reversal of lovastatin-induced apoptosis. We believe that rescuing cells from a stronger induction of cell death provides a better test to compare the relative reversals of MVA, GGPP and FPP. Minor text changes in the Results section have been made to clarify this rationale.

“6. Since cis-platin resistance is related to MRP2 (Cancer Res 1997; 57(16):3537-47), it is more appropriate to use A2780CIS cells expressing MRP2 for cis-platin experiments than A2780ADR cells.”

Dr. Yoon is correct in stating that A2780CIS cells would be more appropriate for studying the effect of lovastatin on MRP2 activity. However, because the focus of the combination experiments within this manuscript is on P-gp, we chose not to
include the suggested experiments. In light of both Dr. Yoon and Dr. Cuello’s comments, we have performed additional experiments addressing the interaction of lovastatin and cisplatin in A2780CIS cells. Interestingly, in A2780CIS cells, lovastatin and cisplatin do not synergize. If anything, there is a trend towards antagonism at lower doses, but this is not statistically significant. When lovastatin and doxorubicin are combined in A2780CIS cells, however, there is some degree of synergy. While this suggests that elements other than P-gp are involved in the interaction between these two drugs, the degree of synergy observed in A2780ADR cells is much higher, indicating that inhibition of P-gp is likely an important mechanism of how lovastatin synergizes with doxorubicin. These results are included now in Supplementary Figure 1.

“7. Since statin is known to induce apoptosis by activating mitochondrial apoptotic signals, it is not clear why statin-induced apoptosis is inhibited by bcl-2 over-expression.”

We are a little confused by Dr. Yoon’s last comment. Bcl-2 is a mitochondrial protein that has a very well-defined role in inhibiting apoptosis mediated by mitochondrial apoptotic signals. Our results on lovastatin-induced apoptosis of ovarian cancer cells are consistent with the mechanisms of statin-induced apoptosis demonstrated in other cell systems in that it can be inhibited by Bcl-2:


“Level of interest: An article whose findings are important to those with closely related research interests”

We thank Dr. Yoon for his thorough and insightful review. We are pleased that the significance of our work to the field is appreciated. His questions and comments have been very helpful in ensuring that the quality of our manuscript will be high and we are grateful for his work and input into this review.

Reviewer 2: Dr. Paul W. Sylvester

“This manuscript examines the antiproliferative and apoptotic effects of lovastatin on ovarian cancer cells in culture and demonstrates synergistic and additive anticancer effects when lovastatin in used in combination with doxorubicin and cisplatin, respectively. Additional studies showed that these anticancer effects are mediated by lovastatin inhibition of HMGCoA reductase and increasing cellular retention of doxorubicin believed to be due to an inhibition of P-glycoprotein. In general, the studies described in this manuscript are a logical
extension of previous work conducted by this laboratory. Studies are well designed and the manuscript is well written. The experimental data provides convincing data regarding the potential use of statins for the treatment of ovarian cancer. Much of this information is new and may be useful in providing insights for further investigations into the use of statins either alone or in combination with other traditional chemotherapies. Although the experimental evidence described in this manuscript is based in vitro studies and there is no direct evidence to suggest that these results can be duplicated in vivo, these data are interesting and suggest that this class of compounds may have some therapeutic value in the treatment of ovarian cancer in women. Therefore, it is the opinion of this reviewer that this manuscript is acceptable for publication with minor revision in BioMedicalCentral Cancer.”

We would like to sincerely thank Dr. Sylvester for his judicious review of our manuscript. We are pleased that he felt our studies were well designed and that he found the paper to be similarly well written.

“Minor Essential Revisions:
1. Because the data presented was exclusively from in vitro studies and not validated in subsequent in vivo animal tumor studies, it is an extremely large leap of faith to declare that these findings have “immediate therapeutic relevance” or “clinicians (are now provided) with a rational basis upon which decisions can be made regarding the addition of statins to their patients’ chemotherapy regimens.” Although the results presented in this manuscript are interesting, it is clearly evident these data need to be followed up in relevant animal tumor models before clinical trials can be justified. Therefore, the title of this manuscript, as well as parts of the Abstract, Introduction and Discussion sections need to be “toned down” in order to avoid overstepping or sensationalizing the basic findings of this study.”

We agree that the overall tone of the paper can be altered accordingly without underselling the importance of our results. To this end, we have modified the tone and language in the sections identified in Dr. Sylvester’s comments.

“2. The Results section is excessively large due to unnecessary discussion or redundant descriptions of methodology. The Results section can be greatly shorten by restricting lengthy discussion or repeated methodology to the Discussion or Materials and Methods sections, respectively.”

We also agree with Dr. Sylvester that the results section is longer than necessary. We have made several modifications to implement this suggestion and have shorted the Results section by approximately 300 words.

“Level of interest: An article of importance in its field.”

Again, we thank Dr. Sylvester for his comments and are pleased that the significance of our work to the field is appreciated.

Reviewer 3: Dr. Mauricio A. Cuello
“After reading the Article from Martirosyan et als entitled ‘Lovastatin induces apoptosis of ovarian cancer cells and synergizes with doxorubicin: immediate therapeutic relevant’, major objections arose that make the manuscript non acceptable for publication in its current version. A major compulsory revision through all the manuscript must be done before considering a new revision and potential publication.

a) First at all, the authors state in abstract section: ‘this is the first evidence that lovastatin triggers apoptosis in ovarian cancer cells as a single agent by a mevalonate-dependent mechanism’, statement which unfortunately is not true. At least three papers non-cited by the authors have already addressed several of the questions explored in the manuscript. The authors might eliminate this type of asseverations (present in the abstract and discussion) and necessarily cited those papers already available online (including one already published in your own journal):


While the first of these papers (from the lab of Dr. Cuello, in fact) does indeed show that ovarian cells undergo apoptosis in response to lovastatin exposure, it was not available either in print or online at the time of our own submission. Unfortunately, timing simply did not allow us to reference this paper in the original manuscript. The other three papers actually do not show induction of apoptosis in response to lovastatin. Paper #3 exclusively uses an ATP-based tumour chemosensitivity assay and Paper #4 measures invasiveness. Paper #2 is a review article which claims lovastatin-induced apoptosis of an ovarian cancer cell line but actually does not present any such data. We have therefore added Dr. Cuello’s recent and interesting manuscript (Paper #1) to the references and the text has been modified accordingly.

“2) Despite a well design, conclusive results with a generally good interpretation, no new or relevant information is given in this manuscript considering the evidence provided in the papers above mentioned where already have been demonstrated not only in cell lines but also in primary tissue cultures that lipophilic but not hydrophilic statins are able to induces apoptosis in ovarian
cancer cells as well as in other gynecological cancers. Moreover, evidence of synergism has also shown for some chemotherapeutic reagents, including doxorubicin. In one of the paper cited by the authors (Rozados et al., ref.41), they only mentioned the citation in terms of the lack of toxicity on normal cells but they did not mention the previous finding of synergistic antitumoral activity observed with this combination by this group in the murine model. The authors wrongly cited the paper from Liu et al. (ref.14) saying this work was done in Chinese hamster ovary cells when in fact it was done in Hey-1b and Ovcar-3 human ovarian cancer cells. In that paper, evidence of statin-induced cell death was also provided. They might better discuss previous findings from other papers and rectify those wrongly cited.

We thank Dr. Cuello for bringing this to our attention – as described above, his group’s paper was not available at the time of our original submission but has now been added to our references and text. The Liu et al. paper was, in fact, mis-referenced in our original submission and this has been corrected in the most recent version of the manuscript. We thank Dr. Cuello for pointing out this error.

Dr. Cuello is also correct in his statement that evidence of synergism has also been shown with doxorubicin and lovastatin. However, we were expanding this body of literature by addressing the synergy in ovarian cancer, a disease in desperate need of novel treatment options. The paper by Rozados et al. made use of a rat B-cell lymphoma and mouse mammary tumor model – we wanted to highlight the importance of the lack of toxicity observed when combining lovastatin and doxorubicin (not a cancer-type-specific issue) and did not discuss the synergy because it was not observed in a cell system relevant to our study.

“3) The only new piece of evidence is related with the effects of statins on P-glycoprotein functionality and as a possible explanatory mechanism for synergism between lovastatin and doxorubicin in resistant ovarian cancer cells. They consider this finding relevant for rescuing patient who became resistant after a primary treatment. Here there are some vaguenesses should be addressed in the text (particularly in the background section). For example, the first line chemo-treatment after achieving optimal surgical cytoreduction is the use of a combination of carboplatin plus paclitaxel (with about 70 to 80% response rate). After a remission period (about 18-24 months) about half to two thirds of the patients experience a recurrence. Not all of them will be resistant to the primary scheme (particularly if the interval is major than 12 months). They should be more specific in terms of information provided to the reader to really estimate the importance of the problem.”

We will gladly add the requested detail to help the reader better estimate the magnitude of the potential clinical impact. It should also be noted, however, that we are not trying to claim that all patients with ovarian cancer stand to benefit from lovastatin and doxorubicin combination therapy. Rather, we are aiming to expand upon the notion of individualized therapy – each individual patient’s cancer is unique and better care will be provided if the specific features of any given patient’s tumor can be targeted. This has been highlighted on p. 4.
“Moreover, the authors do not provide information on the major mechanisms that explain the acquisition of resistance in ovarian cancer. One of them is the overexpression of Bcl2, what is the effect of Bcl2 overexpression in cell death mediated by this combination? They also indicate (but not shown) the lack of effect of cisplatin plus lovastatin in A2780 cancer cells. But apparently they did not test this combination in cisplatin resistant A2780 cells. According to the design, the synergism is observed with the combination only in doxo-resistant cells. To be relevant for the clinician and to rescue patients, the authors might test the combination of dox/lovastatin in cisplatin-resistant and taxane-resistant A2780 cancer cells.”

As discussed previously, in light of both Dr. Yoon and Dr. Cuello’s comments, we have performed additional experiments addressing the interaction of lovastatin and cisplatin in A2780CIS cells. These are results are presented in Supplementary Figure 1.

We have also performed experiments attempting to address the effects of Bcl-2 overexpression on the combination of lovastatin and doxorubicin. Surprisingly, Bcl-2 did not inhibit either doxorubicin-induced cell death or cell death induced by the combination of lovastatin and doxorubicin. These results suggest that the cells have likely become drug-resistant through means other than the MDR machinery, such as upregulation of one or more anti-apoptotic proteins, thus rendering forced expression of Bcl-2 incapable of rescuing cells further. This data is now presented in Supplementary Figure 2, but further elucidation of the likely many anti-apoptotic mechanisms engaged in A2780ADR cells is beyond the focus of this manuscript.

“To better support their findings, the authors also should mention the evidence of P-glycoprotein induction (after taxane and cislatin treatment) observed in recurrent ovarian cancers (Anticancer drugs induce mdr1 gene expression in recurrent ovarian cancer. Hille S, Rein DT, Riffelmann M, Neumann R, Sartorius J, Pfützner A, Kurbacher CM, Schöndorf T, Breidenbach M. Anticancer Drugs. 2006 Oct;17(9):1041-4).”

We thank Dr. Cuello for his suggestion and have added the reference to this revised manuscript.

“In terms of exploring the P-glycoprotein functionality, the evidence provided is limited only to FACS analysis. What about the quantitative expression of P-gp at the membrane upon statin incubation?, localization, traslocation. It is already known that statins are potent inhibitors of p-gp activity (Wang E. et als, ref. 38). The authors also should consider the relationship between P-gp and HMG-CoA levels as described previously by Maksumova et als. (In Increased sensitivity of multidrug-resistant myeloid leukemia cell lines to lovastatin. Maksumova L, Ohnishi K, Murakhodjaev F, Zhang W, Pan L, Takeshita A, Ohno R. Leukemia. 2000 Aug;14(8):1444-50).”

Again, we thank Dr. Cuello for his suggestions. We have referred to Maksumova
et al. in our revised Discussion section. However, we limited our study of P-gp to it’s ability to function as a MDR pump capable of expelling doxorubicin from the cell (Figure 5) because this function is ultimately what impacts tumor sensitivity to chemotherapy. Differences in localization, translocations, expression, etc. are not necessarily of primary importance to the MDR phenotype. Therefore, we focused on the active pumping of doxorubicin (or other substrates) from the tumor cell itself over the course of this study.

“4) Recently it has been published retrospective evidence supporting the role of statins in improving survival outcome of patients with advanced ovarian cancer treated with chemotherapy and concurrently with statins. The authors should comment on this paper in the discussion section (Elmore RG et als, GYn Oncol 111:102-105, 2008).”

This reference has been added to the text of our manuscript. Indeed, these types of retrospective analyses provide excellent rationale for further molecular and clinical studies. We thank Dr. Cuello for his suggestion.

“Level of interest: An article whose findings are important to those with closely related research interests.”

We thank Dr Cuello for his exceptionally thorough review of our manuscript and are pleased that the significance of our work to the field is appreciated. His comments and suggestions have undoubtedly improved the quality and depth of our re-submission.