**Author's response to reviews**

**Title:** Knockdown of autophagy-related protein 5, ATG5, decreases oxidative stress and has an opposing effect on camptothecin-induced cytotoxicity in osteosarcoma cells.

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**Version:** 2 **Date:** 13 October 2013

**Author’s response to reviews:** see over
October 12, 2013

Dafne Solera, Ph.D.
Editor in Chief
BMC Cancer

Dear Dr. Solera,

We are excited and pleased to submit our revised manuscript entitled: “Knockdown of autophagy-related protein 5, ATG5, decreases oxidative stress and has an opposing effect on camptothecin-induced cytotoxicity in osteosarcoma cells” for consideration of publication in BMC Cancer. As before, all authors have reviewed and approved the revised manuscript.

As a reminder, in this manuscript, we demonstrate that ATG5 protein knockdown-mediated inhibition of autophagy has an opposing effect on camptothecin-induced cytotoxicity in two metastatic murine osteosarcoma cell lines. Our results indicate that the mechanism of protection against camptothecin-induced cytotoxicity involves autophagy inhibition-mediated reduction in oxidative stress. Our results also indicate that autophagy inhibition reduces basal oxidative stress levels. To our knowledge, this is the first report of autophagy protein knockdown-mediated autophagy inhibition reducing basal or drug-induced oxidative stress in osteosarcoma or any other cancer for that matter.

Our responses to the reviewers’ comments are included in the ‘Cover Letter’ stage of the revised manuscript submission step. We have organized our responses to the reviewers’ comments in a format that we hope will help facilitate easy interpretation.

Again, thank you for your consideration.

Sincerely,

Mario Hollomon, Ph.D.
Reviewer's report #1

**Title:** Knockdown of autophagy-related protein 5, ATG5, decreases oxidative stress and has an opposing effect on camptothecin-induced cytotoxicity in osteosarcoma cells.

**Version:** 1  **Date:** 21 June 2013

**Reviewer:** Stefania Meschini

**Reviewer's report:**

**General Comments.**

The paper by Hollomon M.G. et al. reports the results by a wide experimental work aimed to demonstrate that autophagy inhibition (ATG5 knockdown) has an opposite effect on the cytotoxicity induced by Camptothecin in murine metastatic osteosarcoma cell lines (DLM8, K7M3).

The use of two different osteosarcoma cell lines is justified because the K7M3 cells have a high basal autophagy compared to DLM8 cells. This difference results in a different response of the cells to the inhibition of autophagy and a different response to CPT treatments. The autophagy inhibition reduces cell death in the DLM8 cell line with low basal level of autophagy while increases cell death in K7M3 cells with higher basal autophagy. The inhibition of autophagy by ATG5 gene silencing reduces basal and CPT oxidative stress in DLM8 cells. The authors conclude that the inhibition of autophagy by ATG5 gene silencing protects of osteosarcoma DLM8 cells from CPT cell death.

In this work the authors conclude that the autophagy modulation (particularly inhibition) may have an effect on the chemotherapic response in cancer cells. Certainly the data of the effect of CPT on osteosarcoma cell lines is new, but it is not the fact that the CPT by inducing autophagy inhibition increases the effectiveness of chemotherapy ... see the work listed below:

*PLoS One. 2012;7(9)*
The inhibition of autophagy sensitises colon cancer cells with wild-type p53 but not mutant p53 to topotecan treatment.
Li DD, Sun T, Wu XQ, Chen SP, Deng R, Jiang S, Feng GK, Pan JX, Zhang XS, Zeng YX, Zhu XF.

Although the discussion is well balanced the experimental part needs a careful review.

In conclusion, in my opinion, this paper must respond to the major compulsory revisions.

**Specific points:**

The materials and methods are appropriate and adequately described but the figures are missing key parts:

**Figure 1A:**
Insert a table that supports cell viability in which are included the total cells
values, the percentage of live cells (TB negative) and dead cells (positive TB). This request is made to verify whether the treatment induces mortality or slowdown;

We did observe decreased cell growth but this is not an uncommon observation or response of cancer cells to anticancer drugs. We are confident that the data accurately reflects the degree of cell death. We are not able to present a comprehensive table that also includes total number of cells for each treatment group. For a number of the cell viability experiments, only the cell viability value was recorded.

Flow cytometric analysis in this paper is accurate, but changes are necessary to clarify the results:

Figure 3A: Specify the parameters in x-axis and y-axis;

We have changed the x-axis and y-axis to read FL1-H and FL3-H respectively. We have indicated in the figure description that the FL3-H axis indicates degree of AVO formation.

Figure 4B: Specify the parameters in x-axis and y-axis;

The changes that were made for figure 3A have been made for figure 4B

Figure 4C and 4D Specify the cell lines which are discussed;

These figures have been revised to specify the cell lines.

Figure 6A: Eliminate the written + TMRE, seen that this dye is used for the determination of all samples;

Caption of Figure 6:

This figure has been revised as suggested.

1. CPT induces cell arrest and .... where are the data of the cell cycle??

Figure 6 was mistitled. The correct title is “CPT decreases mitochondrial membrane potential and induces caspase-9 activation”.

2. Because the mitochondrial membrane potential was made to 24h when all other experiments in 48h, specify?

The mitochondrial membrane potential disturbance is an early event and precedes cell death. We wanted to ensure that we made our measurement of mitochondrial membrane potential (MMP) at a time point that would not miss any CPT-induced MMP changes compared to control group.
Experimental part of western blotting:
1. Specify the molecular weights beside the proteins and determine the bands quantization relate to the control

Molecular weights for LC3I and LC3II have been added.

2. Specify the exact concentrations of the drugs used and no low ... medium...

The descriptions low, medium and high have been removed and replaced with the actual treatment doses.

3. Figure 3C: is necessary to put LC3I protein expression to demonstrate the conversion of the molecule from the form 1 (indicating basal level of autophagy) to 2 and specify the difference in molecular weight (18 kDa, 16kDa)

Films have been rescanned to indicate both LC3I and LC3II bands.

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

Quality of written English: Acceptable

Statistical review: Yes, and I have assessed the statistics in my report.

Declaration of competing interests:
I DECLARE THAT I HAVE NO COMPETING INTERESTS
Reviewer's report #2

**Title:** Knockdown of autophagy-related protein 5, ATG5, decreases oxidative stress and has an opposing effect on camptothecin-induced cytotoxicity in osteosarcoma cells.

**Version:** 1  **Date:** 26 August 2013

**Reviewer:** Vincent C O Njar

**Reviewer's report:**

This manuscript attempts to investigate the impact of autophagy inhibition on camptothecin–induced cytotoxicity in osteosarcoma cell lines DLM8 and K7M3. Although the manuscript reports some interesting findings there are innumerable shortcomings which make the paper unacceptable for publication in the present form. Following is a detailed critique of the manuscript:

Major comments:

1. The introduction and discussion sections are lengthy, vague and confusing. The authors certainly need to revise both these sections.

   We have shortened the background section by 15% and the discussion and conclusion by 17%. We also attempted to make clearer these sections which was primarily achieved by reducing their length. However, it was difficult to eliminate a large portion without being able to adequately tell our story.

2. Although the authors clearly delineate the reason for decreased cell death in DLM8 cells upon autophagy inhibition, solid and clear cut reasons for increase in cell death upon autophagy inhibition in K7M3 cells is not demonstrated clearly.

   Considering that the results observed in DLM8 cells are in the minority, we focused our attention on this cell line with respect to investigating the mechanism responsible for the results observed in DLM8. Still, we have added a figure indicating that autophagy knockdown in K7M3 cells increases 9NC-induced caspase-3 activation. While this is not an extensive investigation into K7M3 it does suggest that the mechanism is related to increased caspase-3 activation in K7M3.

3. The reasons for the opposing effects of autophagy inhibition and its subsequent effects on cell death in the different OS cell lines need to be explained in detail.

   This comment is somewhat related to the comment above (#2). Our finding of reduced autophagy inhibition being associated with deceased anticancer drug efficacy is not common and we felt that investigating this lesser observed impact of autophagy inhibition on the response of osteosarcoma cancer cells to anticancer drug treatment would be of more interest to the scientific community. Additionally, this is the first report of autophagy protein knockdown–mediated autophagy inhibition reducing basal or drug-induced oxidative stress in osteosarcoma or any other cancer.
4. CPT induced autophagy is demonstrated only by LC3 immunoblot analysis. Other reliable autophagy associated proteins such as Beclin 1, Atg proteins and Ulk1 also need to be examined. The morphological changes associated with autophagy may also be evaluated by TEM or fluorescene microscopy to confirm CPT- autophagy induction.

We have confirmed autophagy induction using an additional autophagy marker, p62 (supplementary figure).

5. The authors have mentioned that autophagy inhibition also reduced basal oxidative stress. How was this examined?

Hydrogen peroxide and superoxide anion levels were determined by flow cytometric analysis of DCFH-DA and HE, respectively. Control groups (which received no drug) represented basal oxidative stress levels. See figure 5B.

Minor comment:
1. Acronym BSO need to be described at the first instance.

This has been corrected

Overall impression
The manuscript cannot be accepted in the present form and needs extensive revision taking into account the above points.

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

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
I declare that I have no competing interest.
Reviewer's report #3

Title: Knockdown of autophagy-related protein 5, ATG5, decreases oxidative stress and has an opposing effect on camptothecin-induced cytotoxicity in osteosarcoma cells.

Version: 1 Date: 27 August 2013

Reviewer: Stan G Louie

Reviewer's report:

This paper evaluated the impact of knockdown of ATG5 in relation to camptothecin-induced toxicity. To evaluate this, the authors use osteosarcoma cells, where knockdown of ATG5 have variable results. Autophagy inhibition caused a decrease in cellular metabolism and growth in K7M3 cells as compared to the other mutation found on DLM8. Authors found an increase basal autophagy level in the K7M3, when compared to the DLM8 or non-transformed cells.

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

Quality of written English: Not suitable for publication unless extensively edited

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
No conflicts

Overview

This paper evaluated the impact of knockdown of ATG5 in relation to camptothecin-induced toxicity. To evaluate this, the authors use osteosarcoma cells, where knockdown of ATG5 have variable results. Autophagy inhibition caused a decrease in cellular metabolism and growth in K7M3 cells as compared to the other mutation found on DLM8. Authors found an increase basal autophagy level in the K7M3, when compared to the DLM8 or non-transformed cells.

Major Compulsory Revisions

1. This study would improve dramatically if cells were treated with chloroquine as a positive autophagy inhibitor.

We used Bafilomycin A1 as a positive control for autophagy inhibition. Bafilomycin A1 blocks autophagy by inhibiting lysosomal acidification which is similar to the mechanism of action for chloroquine which decreases lysosomal acidity. This figure has been added as supplementary data.

2. Would improve the paper if data from cells are treated with inducer and inhibitor of autophagy was compared with the cells with mutation.

When this study was first started, we investigated the impact of rapamycin (autophagy inducer), bafilomycin (autophagy inhibitor) and chloroquine (autophagy inhibitor) on CPT-induced cytotoxicity. In our hands, pre-treatment with these agents had no effect on CPT-induced cytotoxicity. Results from these experiments led us to the use of ATG5 protein knockdown to block autophagy.
3. The impact of the mutation on p62, which may tell the readers whether this is the mechanism by which these mutations lead to drug resistance. An overview of that should have been discussed.

p62 is a multifunction scaffold protein that among other functions has a role in triggering protein degradation. p62 is emerging as a protein implicated in tumor suppression. However, the role of p62 was not of interest in this study. We are aware that ATG5 knockdown has been reported to increase p62 levels (Zhang et al., 2013). Considering that ATG5 knockdown inhibits autophagy and increased p62 expression is indicative of autophagy inhibition, it is not surprising that ATG5 knockdown increases p62 expression. In addition, work by Galavotti et al. (2013) indicates that ATG5 knockdown does not affect p62 function.

We do not present any p62 data and worry that discussing p62 might be problematic. Again, we do appreciate the role of p62 and have thoughts of investigating the role of p62 in our cell lines in future studies.

4. The two mutations transfected in the OS line should also be verified in a second cell line to see whether this is cellular specific.

We are not clear as to the meaning of this comment. Two different cell lines (DLM8 and K7M3) were used in this study. The autophagy protein, ATG5, was knocked down in both cell lines.

5. Figure 2 A and B would be much improved if the two lines with ATG5 mutations were compared with cells treated with empty vectors.

We have added a figure (figure 6C) that shows the impact of CPT on caspase-3 activation in wildtype K7M3 and autophagy-inhibited K7M3 cells. This data is already included for wildtype DLM8 and autophagy-inhibited DLM8 cells (see fig. 6B).

6. Figure 3 C would benefit if the results were compared to wild type cells undergoing the same treatments.

These are wildtype cells. The aim of this figure is to show that CPT induces autophagy.

Minor
1. In Figure 2C: Why is the K7M3 cells that were controlled only 90% viable. As a control, would it be 100%?

Control values were not normalized to 100%. Cell viability data was collected by using a Vi-Cell machine. It is expected that there will be some dead cells even in the control group.