Author's response to reviews

Title: A-770041 reverses paclitaxel and doxorubicin resistance in osteosarcoma cells

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The Editorial Board of **BMC Cancer**

Dear Editor,

We appreciate the thoughtful comments and suggestions from both you and the reviewers regarding our manuscript, “*A-770041 Reverses Paclitaxel and Doxorubicin Resistance in Osteosarcoma Cells*” (MS: 3783918591317638). In this revised manuscript, we have addressed each of the editor and reviewer’s concerns point by point with additional experimental data and clarifications. Specific responses to the editor and reviewers’ comments and criticisms are listed below:

Reviewer #1:

Thank you for the opportunity to review this manuscript. This is an interesting, nicely written and well structured study addressing the issue of multidrug resistance (MDR) in osteosarcoma. The figures are in general of good quality, although some clarifications are necessary as indicated below. The authors screened a kinase specific inhibitor compound library in two MDR osteosarcoma cell lines to identify compounds capable of reversing chemoresistance to doxorubicin and paclitaxel. A-770041, a potent Src-family kinase inhibitor, was identified as the most effective kinase inhibitor to overcome MDR. Combination of a Src inhibitor with conventional chemotherapy could therefore be a worthwhile treatment option to further explore in osteosarcoma patients.

*Response:* We thank this reviewer for finding our study to be interesting, nicely written and well structured addressing the issue of multidrug resistance (MDR) in osteosarcoma.

I have a few comments/questions which could aid in improving the manuscript:

**Major Compulsory Revisions:**

1. Results, first paragraph. After screening of 3000 compounds, the authors identified 18 small molecule inhibitors that can increase chemotherapy effectiveness in two MDR osteosarcoma cell lines. Next, they further verified efficacy of those 18 compounds by serially titrating drug combinations with paclitaxel,
of which 8 showed an improved effect in combination with paclitaxel. Further studies validated A-770041 as the most potent MDR reversing agent. I have some comments/questions regarding this part:

a. Although the authors mention some of the identified compounds in the text, it would be interesting to have an overview of all 18 identified small molecule compounds in a (supplemental) table including their target-kinases, also highlighting the 8 compounds identified after the second screen.

**Response:** Per the reviewer’s suggestion, we have included all 18 identified small molecular compounds in a supplementary table (Supplementary Table 1) including their target-kinases in the revised manuscript. We also highlighted the 8 compounds identified after the second screen (highlighted in bold in the supplementary Table 1).

b. Please define ‘further studies’ in regard to A-770041. Why exactly was A-770041 selected for further analysis over the other 7 compounds remaining after the titration studies?

**Response:** A-770041 is the top one of only eight compounds shown to be the most effective MDR reversing agents when used in combination with doxorubicin or paclitaxel, as determined by drug sensitivity assays.

c. Why did the authors use the two doxorubicin-resistant cell lines U-2OSMR and KHOSR2 for the initial screening, and not also the taxol/paclitaxel-resistant cell line U-2OSTR?

**Response:** Two doxorubicin-resistant cell lines U-2OSMR and KHOSR2 were initially established by selection with doxorubicin, while taxol/paclitaxel-resistant cell line U-2OSTR was established by exposure to taxol. As doxorubicin is a frontline chemotherapeutic drug for osteosarcoma, we used doxorubicin-resistant cell lines U-2OSMR and KHOSR2 for the initial screenings.

d. Why did the authors perform the serial titrating combination studies with paclitaxel (and not with doxorubicin) on the two doxorubicin-resistant cell lines?

**Response:** We performed the serially titrating drug combination studies with both doxorubicin and paclitaxel on the two doxorubicin-resistant cell lines. We have made corrections in the revised manuscript (Page 10).

2. General comment: throughout the manuscript, the authors have conducted various experiments to examine effectiveness of certain compounds and combinations. However, I cannot find any statistics or p-values indicating significant differences. Although the data look sound, statistical tests are necessary to confirm the findings. Figures 3 and 5A will also be more convincing when statistics are performed.

**Response:** Statistical analyses were performed using the GraphPad PRISM5 software from GraphPad Software, Inc (San Diego, CA, USA). The student t test was used to analyze the differences between two groups. Results are expressed as mean ± SD and P < 0.05 was considered statistically significant. We have added these descriptions on statistical analyses in the Methods section (Page 9) and the p values in the legends of Figure 3 and Figure 5A.

3. How did the authors assess drug synergy? Figure 3 shows that the combinations are indeed more effective than the monotherapies, but it would be more appropriate to use a formula to officially assess drug synergy, for instance using the combination index (CI) method (e.g. Zhao 2004 Clin Cancer
Response: The synergistic effect of A-770041 on paclitaxel and doxorubicin in osteosarcoma MDR cell lines was assessed by GraphPad PRISM5 software as described in the Methods section of the revised manuscript (Page 9). The combination index (CI) analysis (Zhao 2004 Clin Cancer Res;10:7994-8004 and van Gaal 2013 Eur J Cancer 49(16):3462-70) needs specific computer software packages and the calculations use SAS language procedures. We have contacted the Massachusetts General Hospital Biostatistics Center and plan to evaluate and validate these synergistic effects in osteosarcoma MDR cell lines in our future investigations. It should be noted that the inability to conduct such combination index (CI) based analysis in this revised manuscript does not undermine the manuscript, which focuses on screening a kinase specific inhibitor compound library in human osteosarcoma MDR cell lines to identify inhibitors that are capable of reversing chemoresistance to doxorubicin and paclitaxel.

4. Results, fifth paragraph (‘effect of... Src, Lck’): ‘Src kinase...osteosarcoma phenotype.’ Please provide references for this statement.

Response: We have added references (Ref 34,35) to this statement in the revised manuscript.

Minor Essential Revisions:
1. Figure 1: Please indicate what the green, blue and red boxes represent, and also what the red and blue line represent.

Response: We have indicated these descriptions in the legend of Figure 1. Red: no effect on drug sensitivities; green: minor effect on drug sensitivities; blue: significant effect on drug sensitivities.

2. Figure 2B, 2C and 5A: Please indicate what type of error bars were used. Standard deviations?

Response: We have added: “Results are expressed as mean ± standard deviations (SD)” in both legends of Figure 2 and Figure 5.

3. The experiments in figure 3 were performed multiple times as indicated in the figure legend, please give error bars. Did the authors also measure the absorbance of untreated cells?

Response: We have added error bars in the bars of Figure 3. We have also updated the Figure with the absorbance of untreated cells.

4. Figure 5: Do the authors also have data about cytokeratin 18, PARP and Pgp levels in untreated U-2OSMR, KHOSR2 and U-2OSTR cells? It also would have strengthened the results if these assays were performed upon A-770041 monotherapy, since A-770041 itself might already exert effects on for instance apoptosis.

Response: Expressions of cytokeratin 18 (by ELISA), PARP and Pgp (by Western blot) in untreated U-2OSMR, KHOSR2 and U-2OSTR cells are shown in our previous publications (Ref 6, 7, and 20). A-770041 monotherapy (at the dose of reverse doxorubicin resistance) resulted in undetectable apoptosis activity (by ELISA) in these osteosarcoma drug resistant cell lines.

Discretionary Revisions:
1. Discussion, final sentence “these preclinical... treat osteosarcoma”. Although I agree with the authors that the combination of Src inhibitors with chemotherapy could definitely be a promising approach to
treat osteosarcoma patients in the future, I would not state that clinical studies would be the next step. It would in my opinion be appropriate to include a sentence like ‘further (in vivo) research is warranted’.

Response: We have removed and changed the final sentence to: Further in vivo research is warranted to understand the implication of A-770041 in overcoming drug resistance.

Reviewer #2:

The manuscript entitled “A-770041 reverses paclitaxel and doxorubicin resistance in osteosarcoma cells” by Zhenfeng Duan et al, reports on the identification of the Src family kinase inhibitor A-770041, acting as one of the most effective multidrug resistance (MDR) reversing agents when combined with doxorubicin or paclitaxel in human osteosarcoma MDR cell lines. The paper is well written, pretty straightforward and is containing new information. In addition the manuscript is of appropriate length and its design is clear. The experimental approach is well performed and results support enough the conclusions of the Authors. Nevertheless, this reviewer feels some changes and additional data would greatly increase the reliability of this study and asks some “easy to perform” amendments which will improve the overall quality of the paper. Finally, the reviewer considers the manuscript worth of publication, once the questions apart mentioned will be addressed.

Response: We thank this reviewer for finding our study to be well written, straightforward and containing new information; of appropriate length and clear design; and the experimental approach to be well performed and results supporting our conclusions.

Major Compulsory Revisions

As far as “Effects on drug sensitivities from inhibiting Src expression by shRNA” concerned (Fig 2), Authors claim that the Src protein expression in U-2OSMR or KHOSR2 cells was down-regulated using lentiviral Src kinase shRNA. However, Src protein levels are not shown. In addition, results would be more convincing and conclusions by the Authors would be highly supported if they would have provided data that include also the evaluation of drug-induced cytotoxicity after transduction with control shRNA.

Response: We have made changes in the revised manuscript by stating: the Src expression in U-2OSMR or KHOSR2 cells was inhibited by using lentiviral Src kinase shRNA. We have also added Src protein levels in the revised Figure 4. Regarding the drug-induced cytotoxicity after transduction with control shRNA, we have tested negative controls (empty lentiviral shRNA vector and non-target lentiviral shRNA controls) both in our previous publications (ref 30, and Duan, Z et al. Mol Cancer Ther. 2008 Aug;7(8):2377-85 ) and in the current study; no drug-induced cytotoxicity after trasduction with control lentiviral shRNA was noted.

As far as “Synergistic effect of A-770041 with paclitaxel or doxorubicin in drug resistant cell line” concerned (Fig 3), in the relative legend is indicated that “The results are shown as the mean value of triplicate samples and are representative of 3 independent experiments”. However, statistical data (mean value, standard deviation…) are not shown in such figure.

Response: Statistical analyses were performed using the GraphPad PRISM5 software from GraphPad Software, Inc (San Diego, CA, USA). The student t test was used to analyze the differences between two groups. Results are expressed as mean ± SD and P < 0.05 was considered statistically significant. We
have added these descriptions on statistical analyses in the Methods section (Page 9) and the p values in the legends of Figure 3 and Figure 5.

As far as “Effect of A-770041 on the expression and activation of Src, Lck” concerned (Fig. 4), it is claimed that “A-770041 inhibits Src and Lck expression in drug-resistant osteosarcoma cells in a dose-dependent manner” and that “Western blot analysis revealed that A-770041 inhibits both Src and Lck activation and expression in osteosarcoma MDR cells, but has less or no effect on other kinases such as pAKT, pmTOR or CDK11”. It’s not completely true. I mean, in Fig. 4 it is shown only p-Src and p-Lck protein levels and not also total Src and Lck protein levels (even in “Methods Section, anti-Src and anti-Lck antibodies are not mentioned). In my opinion, statistical and densitometric analysis of pSrc/Src and pLck/Lck is critically needed to support the above claim, and it should be provided. In addition, pAKT, pmTOR protein levels (at least in U-2OSMR cells) also appear down regulated in response to A-770041 treatment.

Response: We have added total Src and Lck protein levels in the revised Figure 4. Densitometric analysis of Western blot results confirmed that A-770041 inhibits both Src and Lck activation and expression in osteosarcoma MDR cells as highlighted in the revised Figure 4. We did notice that pAKT and pmTOR protein levels also appear down regulated in response to A-770041 treatment in U-2OSMR cells, however, these effects are not as significant as the effects of A-770041 on pSrc and pLck expression, especially in multiple cell lines.

As far as “A-770041 enhances apoptosis induced by doxorubicin in drug resistant osteosarcoma cells” concerned (Fig. 5), it is claimed that “PARP cleavage was detected after the treatment of U-2OSMR or KHOSR2 cells with A-770041 in combination with doxorubicin (Figure 5B)”. In my opinion, the data reported in such figure are not so much obvious and PARP cleavage is not clearly evident. In addition, my feeling is that results would be more convincing and conclusions by the Authors would be highly supported if they would have provided data that include the monitoring of caspases activation also by Western blot and/or Flow cytometric analysis of apoptosis.

Response: In addition to the PARP cleavage assay, quantification of apoptosis was also evaluated using the Apo-ONE Homogeneous Caspase-3/7 Assay kit from Promega according to manufacturer’s instructions. Compared with cells treated with doxorubicin alone, the combination of A-770041 resulted in greater levels of apoptosis in these osteosarcoma MDR cell lines. We have added these descriptions in the Results section (Page 13).

Discretionary Revisions
Bibliography is not completely adequate and updated. For instance, recent evidence on sensitization to doxorubicin in osteosarcoma cells by different agents is not cited.

Response: We have added several novel publications (Ref 37-39) on sensitization to doxorubicin in osteosarcoma in the revised manuscript.

Some misreadings throughout the text

Response: We have edited and proofread the whole manuscript.
We hope these changes will now make this manuscript acceptable for publication in BMC Cancer.

Respectively submitted,

Zhenfeng Duan, MD, PhD