Author’s response to reviews

Title: Salinomycin increases chemosensitivity to the effects of doxorubicin in soft tissue sarcomas

Authors:

Sven T Liffers (sven-thorsten.liffers@rub.de)
Daniel J Tilkorn (d.tilkorn@web.de)
Ingo Stricker (ingo.stricker@rub.de)
Christoph G Junge (christophjunge@gmx.de)
Sammy Al-Benna (sammyalbenna@gmail.com)
Markus Vogt (markus.vogt@rub.de)
Berlinda Verdoordt (berlinda.verdoordt@rub.de)
Hans U Steinau (hans-ulrich.steinau@uk-essen.de)
Andrea Tannapfel (andrea.tannapfel@rub.de)
Iris Tischoff (iris.tischoff@rub.de)
Alireza Mirmohammadsadegh (alireza.mirmohammadsadegh@rub.de)

Version: 2 Date: 20 August 2013

Author’s response to reviews: see over
Dear Prof. Bruce Baguley

We appreciate the constructive review of our manuscript: MS: 2494687009468224 "Salinomycin increases chemosensitivity to the effects of doxorubicin in soft tissue sarcomas". We have revised the manuscript according to all the reviewers’ suggestions. A series of further experiments were performed (including cytotoxicity assays, isobologram analysis and annexin V staining) to fulfill the criteria raised by the reviewers.

The point-by-point list of changes to the manuscript is given below. We hope that our revised manuscript will find approval by the referees.

Sincerely yours

Sven-Thorsten Liffers, PhD
Point-by-point reply to reviewers` comments

Reviewer I comments (Ugo Testa):

Specific Comments

1. In order to better elucidate the occurrence of a possible synergism between salinomycin and doxorubicin the authors should perform experiments of quantification of living cells and of apoptotic cell number growing the cells in the presence of a dose-response of doxorubicin, either in the absence or in the presence of salinomycin (1uM). After that, they have to express the results in terms of percentage with respect to their respective control (the value of the control is 100%) and plot them on a X, Y graph. This is an intuitive way to directly visualize the occurrence or not of synergism.

   In order to better differentiate between viable and dead cells in response to the different treatment regimes cells were analysed following treatment with the MultiTox-Glo Cytotoxicity Assay (Promega). The new data was added to the material and methods (page 4; line 19-27), results (page 9 line 1-7) and in figure 2A-C.

2. Furthermore, an isobologram analysis is important.

   An isobologram analysis is added to the manuscript and the manuscript was changed accordingly (material & methods page 7; line 22-27), results (page 9 line 7-18) and figure 3A-B.

3. Annexin V binding experiments would be useful in addition to the subG1 fraction analysis reported in Fig.2D.

   Annexin V binding experiments were performed, and the new data are presented in material & methods (page: 7 line 22-27), the results section (page 9 line 24-25; 33-34 and page 10 line 1-5), in the discussion (page 12 line 16-17) and figure 5A.

4. The data reported in Fig.4 provide very preliminary evidence that Salinomycin affect the CD133+ cell population of the 1080 cell line. These results must be considered very preliminary. Given the high positivity of this cell line for CD133, it would be important to sort CD133+ and CD133- cell populations and then evaluate their sensitivity to slaminomycin, doxorubicin and both drugs.

   Regarding the issue of subfractioning the HT1080 cells into CD133- and CD133+ cell populations, we agree with the reviewer that it would be highly interesting to focus only on the CD133+ cell fraction, but their are some concerns regarding to the subfractionation of these cells. According to Torino et al. 2011 subfractionation leads to a homogenous CD133-cell fraction whereas the CD133+ cell population will produce both CD133+ and CD133- cells after a few doublings, which is also consistent with other cell lines. Therefore we decided to present the CD133 data without subfractionation prior treatment, because the intention was to propose a model which explains the higher activation of NFkB signalling and p53 phosphorylation. In conjunction with figure 4 the increased response rate correlates well with an increase number of affected cells in both the CD133+ and CD133- subfractions. Hence the
subdivision in CD133+ and CD133- cells will not lead to new insights in terms of sensitizing soft tissue sarcomas for doxorubicin treatment. The different dose response for each sub-fraction and the fact that Doxorubicin alone is able to target CD133- cells is already demonstrated in figure 4.

5. Furthermore, in addition to vitality and apoptosis study, it would be particularly important to assess the effect of the two drugs at least on tumor sphere-forming capacity.

The effect of chemotherapeutic treatment regimes on stem cell like cells is a highly interesting aspect in the treatment of solid tumors. This topic seems closely related to cell surface antigens which are associated with stem cell characteristics; however this was not the focus of the article and will be beyond the scope of our manuscript. The focus lays on the improvement of anthracyclin based treatment of sarcoma cells through the combination with a second antibiotic. To assess tumor sphere forming capacity following treatment would show the impact of the treatment on tumor stem cell like cells. In our view more experimental data would be required to proof the impact on stem cell like tumor cells than a tumor formation assay alone. The old figure 4 aims to reflect that sarcomas are composed of a heterogeneous cell population which widely differ in their response to chemotherapy and to show that a more resistant subfraction of cells can be targeted, which was not affected by doxorubicin alone. In our opinin this is a very important observation to better understand the direct response rate of soft tissue sarcoma cells. Nevertheless, according to the recommendations from Reviewer I (Ugo Testa) and Reviewer II (Jinhui Wu) we decided to exclude the presented data from the manuscript because the additional experiments will not lead to substantial new insights in terms of sensitizing sarcoma cells to doxorubicin treatment.
Reviewer II comments (Jinhui Wu):

Major Compulsory Revisions:

1. In figure 1, it is weird that the author used different concentration of DOX (fig 1B, ng/nM, 1C ng/ul, 1C, ng/ml). If it were true, the author should clarify this, since the concentration was totally different.

   We apologize for erroneously up-loading a pre-production version of the figure which was mislabelled. Labeling of figure 1B and 1C was corrected accordingly.

2. In figure 1C and D, 1µM salinomycin had already caused about 40% cell death, why author did not use a lower concentration?

   Our aim was to improve the response of soft tissue sarcomas to doxorubicin by salinomycin. 1 µM Salinomycin was used because HT-1080 and SW872 cells displayed the strongest difference in response to the single treatment at this concentration. Further the combination index and isobologram analysis show that the synergy between salinomycin and doxorubicin was detectable at 1 µM salinomycin for HT-1080 and A204 cells.

3. In addition, figure 1c, the author showed that salinomycin caused 40% cell death and 30 ng/ml dox caused 30% cell death. Why the combination only caused 40% cell death?

   The data presented in figure 1C is in accordance with the data presented in figure 1D. In both graphs the main effect on relative cell viability is determined by Salinomycin (i.e. there is no significant difference between the combined treatment and Salinomycin alone). In addition there is a significant effect on the cell number in the new cytotoxicity data (figure 2B) based on the activity of live- and dead-cell proteases. Therefore we propose that at a doxorubicin concentration of 30 ng/mL cell proliferation affecting mechanisms occur (e.g. cell cycle arrest) rather than the initiation of apoptosis. This leads to no detectable synergism between Salinomycin and Doxorubicin by MTT assay.

4. The CD133 assay was totally confused. P11, line26 the author claimed that “These findings indicate that the combination of 1µM salinomycin sensitized the CD133+ cell fraction for doxorubicin mediated apoptosis.” How did author conclude the conclusion? We even could not find how author calculated the fraction CD133 negative cells. How did author define the control cells? How did they calculate the CD133 positive fraction? It is difficult to get any conclusion from the current data. Also, the author should explain more about the relationship between CD133 and apoptosis.

   Please refer to the response to reviewer 1 point 4 and 5.

Minor Essential Revisions:

1. “IC50” # 50 should be subscript
   The IC₅₀ were corrected as mentioned

2. Line 17, can author redefine “synergistic effects”? The formula is not clear.
The manuscript was changed accordingly (page 7 line 22-27)

3. Figure 2ABC, can author use a larger marker? It is difficult to differentiate different groups.
   To distinguish the different treatment regimes the graphs are now colour coded (see Figure 4 A-C).

4. Line32, “in the presence of salinomycin doxorubicin”, missed “,”
   This was corrected as mentioned (page 11, line 15)

5. Page 21, figure1C, DOX concentration
   The doxorubicin concentrations are now uniformly labelled for the graphs 1B, 1C and 1D.

6. Page 21, figure1A, A205 should be A204
   We corrected figure 1A accordingly

7. Should uniform the unit, such that all uM should be ng/ml
   Now it is stated in the material and method section that 1µM is equal to 751 ng/mL (page 4 line 24).