Author's response to reviews

Title: Benzofuroxan derivatives N-Br and N-I induce intrinsic apoptosis in melanoma cells by regulating AKT/BIM signaling and display anti metastatic activity in vivo.

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Author's response to reviews: see over
Dear Editor

Thank you very much for the revision of our manuscript submitted for publication in BMC Cancer. We now indicate our disposition to both reviews, focusing on all points raised and suggestions made.

Referee 1.

1. Authors should show the B16F10-Nex2 tumor volume data with N-Br/N-I compounds in C57BL/6 mice. This data will complement the lung metastasis data with these compounds.

*Reply.* The metastatic model with melanoma cells was chosen because of the compounds’ poor solubility in the present configuration. At the concentration used the volume used was adequate for intraperitoneal administration but not for subcutaneous injections, using a peritumoral via. Since the results obtained were quite significant with the lung colonization method we did not explore at this stage other models and routes of drug injection.

2. Since N-Br/N-I derivative compounds mediated effects (increased cytotoxicity/apoptosis etc) on B16F10-Nex2 cells are due to the generation of ROS, vitamin C (quencher of ROS) should be used to confirm the effects of derivative compounds in in vitro experiments instead of NAC which works by replenishing depleted glutathione levels in the cells. Since, NAC supplementation prevented N-Br/N-I induced decreased viability of B16F10-Nex2 cells (Figure 4), these data also indicate the possible role of glutathione in this effect.

*Reply.* N-acetylcysteine is a powerful reducing agent and anti-oxidant that protects cells from damage induced by reactive oxygen species (ROS). The thiol (sulfhydryl) group is able to reduce free radicals. As an anti-oxidant and a mucolytic amino acid, N-AcCys contributes to the generation not only of L-glutathione but also of reduced forms of key components such as thioredoxin, glutaredoxin, peroxiredoxin, that build a redox state which can globally interact with and reduce reactive oxygen radicals. We fail to see why vitamin C would give a more efficient result as a quencher of ROS.

3. Were cells pretreated, simultaneously treated or post-treated with NAC to observe NAC effects on derivative compounds mediated effect (cell viability) on B16F10-Nex2 melanoma cells? Please state this in the method section for the clarity.

*Reply.* Melanoma cells were simultaneously treated with NAC. This information was added in the Methods section.

4. What is the rationale of using IC100 dose of N-Br/N-I compounds for apoptosis assay (Figure 3A)? Usually at higher doses agents with potent cytotoxic activity kill cancer cells and thus are not...
considered optimal doses. IC25 and IC50 doses should be considered to test the efficacy of anti-cancer drugs.

Reply. We agree with the referee. When considering the number of cells necessary for the apoptosis experiment, the IC50 concentration as determined for a different number of cells proved insufficient to trigger the apoptotic response. We therefore adjusted the concentration to the number of cells (moles/cell) but used the IC100 indication which was misleading. In the revised manuscript we changed it to 100 µM, the actual concentration used for 5 x 10^5 cells.

Minor Essential Revisions:

1. Table 2, the IC50 concentration of different benzofuroxan compounds ranges between 6.9µM to 25.4µM, instead 9.7µM to 25.4µM. Please change this.

Reply. This was corrected in the text.

2. At various places “Nac” is used instead of “NAC”. Please fix this.

Reply. A single form (NAC) is now used in the text as indicated.

3. Please state the level of significance for figure 2C and figures 4.

Reply. P values were included in the figures and legends

Referee 2

1. The emphasis of this paper is on metastatic melanoma as stated in the abstract and the rest of the manuscript. Yet the authors only used one mouse melanoma cell line to draw several conclusions. To confirm the antitumor effects of their 2 benzofuroxan derivatives, additional melanoma lines (preferably of human origin) should be evaluated as well. Furthermore, melan-a cells were mentioned in the methods section but not thereafter. Were they tested and if so how did they respond to the compounds?

Reply. The B16F10-Nex2 cell line was used because it is syngeneic in C57Bl/6 mice, the selected model of our laboratory. The advantage over xenogeneic and allogeneic systems is that although in presence of an active imune system the tumor is recognized as self, much like an autoctonous human cancer. Therefore, the compounds are tested in the “real” condition of an immunocompetent animal. In a syngeneic system any effect observed against a highly invasive melanoma tumor is quite relevant.

The melan-a cell line was tested, but this result, among others were included in another article to be published, therefore mention to it was removed from Methods, but kept in Results.

2. The toxicity of their 23 compounds were evaluated also in non-tumorigenic cell lines (Fibro T75 and GM637) and the IC50 was found to be within the range of tumor cell lines (10-30uM) that
responded to their 2 selected drugs. No comment on toxicity was made in the discussion, however.

*Reply.* Although there was little difference in the IC50 values in tumor cells and non-tumorigenic cells, further experiments should be carried out with primary normal cells for a better appraisal of drug toxicity. Cultured cells in the laboratory differ from cells in a living organism in many aspects. Both compounds did not exhibit toxicity in vivo at the concentrations used and yet showed definite antitumor effects in syngeneic mice. A comment on toxicity was added in Discussion.

3. There is no explanation as to why a clone from B16-F10 (B16F10-Nex2) had to be generated. The parental line is well documented to be highly metastatic.

*Reply.* An in vitro cultured tumor cell line tends to form heterogeneous populations with time giving rise to detectable different phenotypes, molecular, enzymatic or even related to metastatic ability. For instance, the original B16F10 tumor cell line, and sublines recognized at the Experiment Oncology Unit (UNONEX) such as Nex2, when sub-cloned in our laboratory generated new sets of melanoma cells that were studied in detail (see Freitas et al. Melanoma heterogeneity: differential, invasive, metastatic properties and profiles of cathepsin B, D and L activities in subclones of the B16F10-Nex2 cell line. Melanoma research, 2004;14(5):333-44). A strongly melanotic and invasive strain, B16F10-Nex2 was deposited at the Rio de Janeiro Banco de Células (BCRJ), number 0342, as referred to in Methods, and has been routinely used in our laboratory.

4. Figures 1B and 1C are two animal studies performed in two different strains of mice (B6 and Scid-NOD). Yet the corresponding tumor images presented for each figure panel are identical.

*Reply.* The lung images had been misplaced and are now in the proper place in Figs 1B and 1C.

5. A subcutaneous tumor model was described in the materials and methods section but no data were presented or mentioned. Did the compounds reduce tumor size?

*Reply.* Due to solubility problems and the maximal volume (50 µl) allowed for compound administration via the subcutaneous (peritumoral) route, this experiment was not possible and was not included. The description in Materials and Methods was a mistake and was removed.

Minor Comments:

1. A few grammatical errors/typos were found throughout (i.e. Line 142; medium were add)

*Reply.* Grammatical errors have been corrected

2. Figure 5 has a title but is missing a legend.

*Reply.* The legend has been added.

3. Line 116; the proper title should read “Cell lines” and not Cell lineages.
Reply. “Cell lines” is now being used in the mentioned title.

We hope that the revised version is now acceptable for publication in BMC Cancer.

Sincerely yours,

Luiz R Travassos

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