Author's response to reviews

Title: Evaluation of bioactive sphingolipids in 4-HPR-resistant leukemia cells

Authors:

Aintzane Apraiz (aintzane.apraiz@ehu.es)
Jolanta K Idkowiak-Baldys (jldowia@musc.edu)
María Dolores Boyano (lola.boyano@ehu.es)
Gorka Pérez-Yarza (gorka.perezyarza@ehu.es)
Yusuf A Hannun (hannun@musc.edu)
Aintzane Asumendi (aintzane.asumendi@ehu.es)

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Author's response to reviews: see over
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Dr. Alistair Reid,
Associate Editor,
BMC Cancer

Dear Dr. Alistair Reid,

Thank you for your positive letter concerning our manuscript “Evaluation of bioactive sphingolipids in 4-HPR-resistant leukemia cells” MS: 6073482485758454. Please find enclosed a revised version of the manuscript.

The reviewers made a number of helpful comments, and we respond to their specific concerns below. The reviewers’ specific comments are indicated in bold and most significant changes throughout the new version of the manuscript have been highlighted in red. Thank you for this opportunity to submit a revised manuscript.

Responses to reviewer: Riccardo Ghidoni

We thank the reviewer for the positive assessment of our manuscript and all the suggestions and ideas for future studies. We would also like to mention that language corrections have been performed in order to improve the English level of the manuscript.

Regarding the reviewer’s recommendations, we completely agree that events leading to resistance acquisition are an interesting subject for future studies. As for the connection between reduced metabolic activity and altered sphingolipid profiles in resistant cells, new data added as Figure 6A clearly show that long-term removal of the drug does not affect cell resistance (by means of cell survival). Nevertheless, in resistant cells, a clear correlation certainly exists between the endogenous sphingolipid pattern and metabolic activity. In this sense, a situation with no apparent cell death but clear alterations in sphingolipids, metabolic activity, and even proliferation (please see new data in “Additional file 1”) provides us with an adequate scenario for studies focused on sphingolipids and metabolism.

Minor comments:

1. In the abstract: please list all the used chemotherapeutic agents and avoid the suspension marks.

The abstract has been changed accordingly.

2. In the methods section, please add one reference of the “XTT” mitochondria activity evaluation method.
The reviewer has highlighted an interesting methodological point. Tetrazolium salt reduction-based assays, especially first generation MTT, have been largely linked to the activity of mitochondrial succinate dehydrogenase [1]. In this regard, MTT [2] and XTT [3] have been used to determine the activity of enzymes implicated in mitochondrial respiration. Nevertheless, recent data point to several non-mitochondrial oxidoreductase activities mediating tetrazolium salt reduction. As broadly reviewed by Berridge et al. [4], cell permeable tetrazolium salts (e.g., MTT) would better represent intracellular NAD(P)H oxidoreductase activity than selective mitochondrial dehydrogenase activity. On the other hand, reduction of second generation tetrazolium salts (e.g., WST and XTT) occurs at the plasma membrane level. In this case, reduction of the salt seems to be dependent on the malate/aspartate shuttle linking mitochondrial TCA (tricarboxylic acid) cycle-mediated NADH with the extra-mitochondrial space. Therefore, XTT-based data could be interpreted as mitochondrial metabolic data. Nevertheless, these data are indirect and, as a matter of precaution, we would prefer to change “mitochondrial metabolic activity” to “metabolic activity” (in section “Methods”).

3. In figure 2 legend. Please correct “cuadruplicates”.

The figure legend has been corrected.

4. In all the figures. Please remove the purple writing.

The figures have been changed accordingly.

5. In figure 5 and 7. Please use comparable size for the graphs columns (B panels).

The figure size has been corrected.

6. Discussion section ,second page, last lane. Please invert “. [ref]” to the appropriate order.

This mistake has been corrected.
Responses to Reviewer: Bo Yang

We thank the reviewer for finding our manuscript “interesting”. We hope that the performed experiments, together with our responses, answer all of the specific comments made by the reviewer.

1. According to the sentence in P5L10, “the R cell line revealed a significantly lower proliferation rate compared to parental CCRF-CEM cells”, So whether this property will affect the result of resistance test?

We thank the reviewer for this question because it’s true that results obtained with the XTT assay may differ depending not only on the metabolic state of the cell, but also the cell number. In this regard, we are pleased to add new data in order to better characterize the described resistance process.

Concerning the effect of proliferation on resistance test, we would like to mention that in the XTT assay-based resistance tests, each piece of data was calculated based on values obtained in untreated cells from the same cell line. Therefore, treated cells were always compared to untreated cells with the same proliferation rate. Alternatively, we performed two new assays: a) annexin V-propidium iodide staining and b) CFSE-mediated proliferation analysis.

Annexin V-propidium iodide staining is extensively used to evaluate apoptotic cell death and represents an appropriate method for evaluating resistance to cell death. Moreover annexin V-propidium iodide staining has been used to evaluate cell resistance in a proliferation-independent method (i.e. individual cell analysis by flow cytometry vs. cell population analysis by XTT assay). The results are in Figure 6A in the revised manuscript and clearly show that long-term drug removal does not sensitize resistant cells to 4-HPR exposure. Therefore, we must conclude that, in resistant CCRF-CEM cells (i.e. R10 cells), long-term removal of the drug (i.e. R10 long WD) does not reverse cell resistance by means of cell survival, but 4-HPR treatment does affect the metabolic activity of the mentioned cells.

On the other hand, CFSE-mediated evaluation of cellular proliferation (additional Figure 1 in the revised manuscript) reveals that the decrease in cell proliferation is reversible upon long-term drug removal.

Taken together, the data prove that acquired resistance to 4-HPR is not reversible, whereas endogenous sphingolipid profiles, cell proliferation, and metabolic activity are clearly dependent on 4-HPR presence in the medium.

2. According to the result illustrated in Fig.2, the R cells are cross-resistant to H2O2 (both 10 uM and 50 uM), cisplatin (0.5 ug/ml) and Adr (100 nM), these results strongly suggest the resistance may result from the adaptation of oxidative stress induction, why the authors choose the sphingolipid (SL) profiles for further research?
This is certainly an interesting question. As mentioned above, we observed increased resistance against H$_2$O$_2$ and low dose Adriamycin, but obtained resistance levels were much lower comparing to the ones obtained against 4-HPR. On the other hand, data on cisplatin revealed a partial resistance of R0.5 cells (i.e. CCRF-CEM cells under sustained exposure to 0.5 µM 4-HPR). Nevertheless, R10 cells (i.e. CCRF-CEM cells under sustained exposure to 10 µM 4-HPR) did not behave in the same manner. Taken together, the data indicated a lack of strong cross-resistance to all of these compounds and not as proposed by the reviewer. Nonetheless, we find the reviewer’s interpretation interesting and are planning to further study this subject in the future. On the other hand, we must add that the cross-resistance analysis was performed once all of the SL assays were finished. Therefore, the possible implication of sphingolipids in acquired resistance to 4-HPR was the principal aim of the study from the beginning.

3. **If the DES activity was inhibited in R cells and this change seems play a major role in the resistance to 4HPR, the authors should overexpress the DES in R cell line and then perform the resistance assay.**

Lymphoid cells (including leukemia and lymphoma cells) are a difficult target for transfection techniques. Our efforts to transfect CCRF-CEM and derivate R cell lines using theoretically adequate techniques (e.g., Nucleofector) did not work out. However, we would like to mention that data on R10 (long WD) cells (R10 cells released from drug for long term) indicate that inhibition of DES activity is not required to keep cell resistance as their SL profile is comparable to the one obtained in parental sensitive CCRF-CEM cells (Figure 5) while R10 (long WD) cells clearly keep mentioned resistance to 4-HPR (Figure 6). Nonetheless, our data do not rule out a possible role of sphingolipids in the initial resistance development.

4. **Considering the SK inhibitor DHS and SAF also inhibit the PKC, so I do recommend the authors should using SiRNA to exclude the PKC inhibiton.**

Lack of specificity is definitely a problem when working with chemical inhibitors and, unfortunately, previously mentioned technical difficulties limit some aspects of our studies. Therefore, for the present study, we chose a new generation sphingosine kinase inhibitor (i.e. SKI-II) in order to support our previous findings. This inhibitor has been described as a specific sphingosine kinase inhibitor with no PKCalpha, PI3K, or ERK2 inhibitory effect *in vitro* at concentrations up to 60 µM [5]. Moreover, SKI-II shows *in vivo* antitumor activity and oral bioavailability [6]. All of these characteristics indicate that this sphingolipid modulator is an interesting chemotherapeutic compound.

The results we obtained with SKI-II are shown as Figure 8C. As explained in the Results and Discussion sections, combining SKI-II with glucosylceramide synthase inhibitor (PPMP) clearly decreased cell viability of both 4-HPR-sensitive CCRF-CEM and derived R10 cells. As observed with safingol (Figure 8B), addition of 4-HPR does not have a major effect on tumor cell cytotoxicity. The differential antitumor capacity observed among safingol-based and SKI-II-based drug combinations may be due to the non-specific kinase inhibitory effect of safingol. Nevertheless, results obtained with SKI-II support our hypothesis that sphingolipid modulators are suitable chemotherapeutic strategies.

5. **There is lack a bar for CCRF-CEM in Fig1 (left one), please check it.**
6. The discussion section was not well organized, the authors should improve it.

Addition of new data has forced us to revise the entire manuscript. Hopefully, we offer a clearer discussion in the new version. In case the reviewer’s criteria are not satisfied, we would be grateful if we could be given specific points for improvement.

References