Author's response to reviews

Title: Mitochondria-targeted vitamin E analogs inhibit breast cancer cell energy metabolism and promotes cell death

Authors:

- Gang Cheng (gcheng@mcw.edu)
- Jacek Zielonka (jzielonk@mcw.edu)
- Donna M McAllister (dmcallis@mcw.edu)
- A Craig Mackinnon Jr (amackinnon@mcw.edu)
- Joy Joseph (jjoseph@mcw.edu)
- Michael B Dwinell (mdwinell@mcw.edu)
- Balaraman Kalyanaraman (balarama@mcw.edu)

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Author's response to reviews: see over
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Authors:
Gang Cheng (gcheng@mcw.edu)
Jacek Zielonka (jzielonk@mcw.edu)
Donna M. McAllister (dmcallis@mcw.edu)
A. Craig Mackinnon Jr. (amackinnon@mcw.edu)
Joy Joseph (jjoseph@mcw.edu)
Michael B. Dwinell (mdwinell@mcw.edu)
Balaraman Kalyanaraman (balarama@mcw.edu)

Version: 1 Date: 23 May 2013
Author's response to reviews: see over
Reviewer's report

Title: Mitochondria-targeted chromanol inhibits breast cancer cell energy metabolism and promotes cell death

Version: 3 Date: 15 May 2013
Reviewer: Yaping Tu

Reviewer's report:

In a manuscript entitled “Mitochondria-targeted chromanol inhibits breast cancer cell energy metabolism and promotes cell death,” authors Kalyanaraman et al. evaluated selective anti-tumor effects of mitochondria-targeted chromanol (Mito-ChM) on breast cancer cells in vitro and in vivo and also explored the possible underlying mechanisms.

Findings were: (1) Mito-ChM had anti-proliferative effects and cytotoxicity in eight breast cancer cells but little effects on non-cancerous MCF-10A cells, (2) Mito-ChM selectively accumulated in tumor tissue and inhibited tumor growth in a xenograft model of human breast cancer, (3) Mito-ChM caused prolonged inhibition of ATP-linked oxygen consumption rate and selectively depleted intracellular ATP in breast cancer cells, but not in non-cancerous cells, (4) inhibition of glycolysis augmented the inhibitory effects of Mito-ChM.

Overall, this study includes a significant amount of work and fits nicely within the scope of the journal. The data are generally clean and the results are of significant value to the field of cancer research. However, I also raised some major concerns about the paper, which needs to be addressed in the revision.

Major Compulsory Revisions

1. Fig. 1 and Supplemental Figs. 2 and 3 showed that EC50 for MCF-7 cells was 20 uM and no toxicity and cell death were seen after 4 h treatment with 4 uM Mito-ChM. However, data shown in Fig. 2 indicated that 4h treatment with 3 uM Mito-ChM was sufficient to induce 75% inhibition of colony formation of MCF-7 cells. Why?

   • The difference in these two assays is the following: the Sytox Green assay measures directly cell killing effects (Figure 1) whereas the colony formation assay measures the anti-proliferative effects of the compound tested (Figure 2). As noted by the reviewer, we did not observe significant cell death after treatment of MCF-7 cells with 4 µM Mito-ChM. However, as shown in Figure 2, the colony formation data indicate that a 4 h treatment with 3 µM Mito-ChM was sufficient to inhibit cell growth and colony formation even without direct killing effects. This is consistent with the observed irreversible inhibition of the mitochondrial respiration in MCF-7 cells after a 4 h treatment with the compound (Figure 3). From these results we infer that a 4 h treatment with 3 µM Mito-ChM was sufficient to inhibit cancer cell growth, while not directly causing cell death at this time point. This has been now clarified in the Results section, as follows:

   “Notably, the colony formation data indicate that a 4 h treatment with 3 µM Mito-ChM was sufficient to induce significant anti-proliferative effects in both MCF-7 and MDA-MB-231 cells without noticeable cell death under those conditions (Figure 1A). Taken together, we conclude that a 4 h treatment with 3 µM Mito-ChM was sufficient to inhibit cancer cell growth, without directly causing cell death at this time point.”
Fig.4 and Fig.5 showed that Mito-ChM could accumulate in both cancer cells and non-cancerous cells, and also inhibited ATP-linked oxygen consumption rate in these cells. As expected, there was rapid depletion of intracellular ATP in MCF-7 and MDA-MB-231 cells. However, little depletion of ATP was seen in MCF-10A.

a) It would be helpful if the authors can comment on the possible mechanism(s) for such difference since ATP depletion is critical for the inhibitory effects of Mito-ChM (Is this true? Any other evidence to support it?).

• We thank the reviewer for pointing out this apparent discrepancy. The different response to mitochondrial inhibition may be interpreted in terms of the differences in the potential to stimulate glycolysis (to compensate for inhibition of ATP production by mitochondrial respiration) in cancerous MCF-7 cells and non-cancerous MCF-10A cells. In fact, we have tested this possibility by monitoring the extracellular acidification rate (ECAR) after treating both MCF-7 and MCF-10A cells with oligomycin (mitochondrial complex V inhibitor). The results show that MCF-7 cells exhibit a lower glycolytic response to oligomycin as compared to MCF-10A cells. These results have been published in our previous paper (ref. [4]) and we have clarified this aspect in the revised manuscript as follows:

“Interestingly, Mito-ChM did not significantly deplete intracellular ATP levels in non-cancerous MCF-10A cells, even though it inhibited mitochondrial respiration upon direct treatment (Figure 3B). This may be interpreted in terms of the differences in the potential to stimulate glycolysis (to compensate for inhibition of ATP production by mitochondrial respiration) in cancerous MCF-7 cells and non-cancerous MCF-10A cells. We have recently shown that MCF-10A cells have significantly higher glycolytic potential, as compared to MCF-7 cells [4]. Other mechanisms of selective retention of ATP upon direct treatment with Mito-ChM in non-cancerous MCF-10A cells cannot be excluded.”

b) Is this a general observation in other non-cancerous cells? The authors should strongly consider additional studies using more non-noncancerous cells.

• We agree with the reviewer that this topic is worthy of further investigation. In fact, we have tested the cytotoxic effects of Mito-ChM in H9c2 cells (cardiomyoblasts). There was no dramatic increase in cell death (~10% of dead cells) after a 24 h treatment with 10 µM Mito-ChM (not published), while in case of cancerous MCF-7 cells we observed over 80% of dead cells under the same conditions (Figure 1). These results are consistent with the reported lack of significant toxicity of a structurally similar compound, Mito-Vit E succinate at low micromolar concentrations in several non-cancerous cell lines including H9c2 cells (Free Radic. Biol. Med. (2011) 50:1546-55). We are in process of testing more cancerous and non-cancerous cell lines to test the generality of this important observation.

Minor Essential Revisions:

1. Abstract section: Lane 11, “Mito-ChM …. inhibited intracellular ATP” is not correct. May change it to “depleted”

• This correction has been made.
2. Abstract section: Lane 17, Mito-ChM did not cause tumor regression. It inhibit tumor growth.

- This correction has been made.

**Level of interest:** An article of importance in its field

**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

Title: Mitochondria-targeted chromanol inhibits breast cancer cell energy metabolism and promotes cell death

Version: 3 Date: 20 May 2013
Reviewer: Warren Ladiges

Reviewer's report:

1. Major Compulsory Revision

One concern is the lack of data on the effects of the two compounds on normal cells. This should be emphasized, and a more rigorous comparison of the MCF-10A breast epithelial cell line with normal primary cells needs to be made. A similar concern is the absence of any discussion on the effect of the two compounds on activated stromal cells. Stromal cells play an active role in promoting cancer cell progression, but may respond differently to mitochondrial targeted compounds than tumor cells or normal epithelial cells. The Discussion Section needs a paragraph to discuss this in relation to the data presented.

• As discussed above (response to reviewer's 1 comment 2b) we are currently testing more cell lines, both cancerous and non-cancerous (including primary cells) in their response to mito-chromanol. Recent results indicate that mito-chromanol did not elicit significant toxicity in H9c2 cells (cardiomyoblasts), as compared to MCF-7 cells. Additionally, the results of in vivo experiment indicate the selective accumulation of the compound in tumor tissue (Figure 6) and the lack of apparent toxicity to normal tissue (Supplementary Figure 4 and Supplementary Table 5). We agree with the reviewer that the effect tumor microenvironment on the tumor response to the compounds tested may be significant and is clearly worthy of further pursuit. This will include both testing the effect of the compounds on stromal cells, as well as testing the effect of stromal cells on the sensitivity of cancer cells. This aspect of the anticancer effects of the mito-chromanol has been added to Discussion section, as suggested by the reviewer:

“The role of stromal cells and the tumor microenvironment in general in modulating tumour sensitivity is crucial to developing successful anticancer therapeutics. Future studies should focus on investigating the effect of mitochondria-targeted small molecules on stromal cells alone and on the combination of cancer cells with stromal cells.”

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 interests