Author's response to reviews

Title: Neutral sphingomyelinase 2 modulates cytotoxic effects of protopanaxadiol on different human cancer cells

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Responses to reviewers’ comments and editorial ones

Reviewer 1

Comment: The authors are suggested to perform more mechanistic investigations in the future addressing how PPD affects the integrity of lipid rafts, is the activation of caspase-8 by PPD dependent on cell death receptors on cellular surface? Are intracellular membrane structures, such as ER, also affected by PPD? etc.

Response: Thank you for mentioning the potential directions where the next projects of our PPD study should explore in the future. We’d like to investigate the changes of components in lipid rafts using western blotting, fluorescent labeling, etc. of lipid raft components. Participation of cell death receptors also can be tested with western blotting, knockdown or inhibition studies of death receptors. In addition, ER, a continuation of cell membrane structures, will be explored using confocal microscopy.

Reviewer 2

Comment 1: Finding PPD depletes neutral sphingomyelin from the cell membrane is important. But the evidence is only by fluorescent image and the authors did not show the sphingomyelin is raft associated. Centrifugation separation of lipid rafts and measure the sphingomyelin content in the rafts quantitatively will be more convincing.

- Yes, the reviewer made a good point on the analysis of sphingomyelin contents in lipid rafts using ultracentrifugation. However, given the fact that high contents of sphingomyelin in lipid rafts has been well known, the disappearance of sphingomyelins from cell membranes are very likely to be from lipid rafts,
although some of ceramides, degradation products of sphingomyelins, could be from non-lipid raft membranes. Honestly, analyses of sphingomyelins in rafts with ultracentrifugation are quite limited in our lab at the moment, but they should be considered as one of the analytical tools in our studies for lipid rafts in the future. Finally, as shown in Fig 4.B, rather indirect evidences such as disturbances in levels of highly lipid-raft associated IGF-1R, pAkt, etc supports our notion that the sphingomyelins depleted from cell membranes are likely to be from lipid rafts.

Comment 2: Although increase of ceramides was seen at high concentration of PPD (50uM), I am not convinced that is the mechanism of PPD caused cytotoxicity since the toxicity by PPD was evident at 25uM at 24hr when no significant increase of ceramides were seen at 16hr. In addition, knockdown or inhibition of sphingomyelinase by siRNA or GW4869 (an inhibitor) did not really abolish the effect of PPD (only 5-10%).

- As the reviewer implied somewhere in the comments, intracellular accumulation of ceramides from hydrolysis of sphingomyelins are not the only mechanism by which PPD caused cytotoxicity. Moderate levels of ceramides can cause apoptosis of cancer cells, even though they are not greatly high. Concentration of ceramides quite increased in 25 uM at 6 hr, decreasing to the basal level in 25 uM at 16 hr. In our MTT assay, we looked at the death of cancer cells at 24 hr, but actually those cancer cells started dying in a few hours after adding PPD to the cells (unpublished data). Thus, we are just looking at the total cell death by 24 hr after PPD treatment in Figure 5. Secondly, we have been aware that hydrolysis of sphingomyelins is only one of the cytotoxicity mechanisms by PPD. However, it still has its own novelty in that we identified the production of ceramides from sphingomyelins as one of the multiple cytotoxic mechanisms by PPD, although only a portion of total cell deaths can be explained by the neutral sphingomyelinase.

Comment 3: The authors claim that MbCD significantly enhanced PPD’s cytotoxicity (Fig 4). I cannot agree that point. Based on Fig.4A, 0.5mM of M#CD alone has 30% inhibition on K562 cells and 20% on HT29 cells. 25uM PPD alone had 60% inhibition on both cells. But 0.5mM of M#CD and 25uM PPD together still only had 60% cell killing. Therefore, it is clearly that PPD and M#CD at those concentrations had the same mechanism of action and 25uM PPD may be more potent than 0.5mM M#CD (that is why the same degree of inhibition as PPD alone). Similarly, combination of 25uM PPD with 1 mM M#CD did not show any additive effect of the both (actually less additive). My point is supported by Fig.4B, that clearly showed that M#CD at 1mM had almost identical effect on various proteins to that of 25uM PPD, further suggest the two work on the same mechanism at these concentrations.

Response 3: Yes, I agree with your point of view on this matter in that PPD showed only marginal enhancements in inhibition by MbCD on cell growths. But I’m not sure that it means that PPD has almost common cytotoxic mechanisms with MbCD, because there were a little of enhancements in other sets of MTT.
assays as well as some differences in the Western blot analyses on lipid raft-associated proteins.

Comment 4: By the way, the authors should give statistical results of those western blots as one result of western blotting is not sufficient to draw the conclusion.

Response 4: Yes, I have provided the average numbers of 3 sets of Western blots in Fig 3. D and Fig 4.C as statistical results. Even though some of the bands don’t have enough significances, they were still visible in their Western blots. And the Western blot figure is a representative of three sets of experiments.

Editorial comments: We note that one of the authors on your manuscript is associated with BTGin Co Ltd, the suppliers of the protopanaxadiol for your study. We would ask you to clearly state this affiliation in the Competing Interests section of your manuscript.

Response: Thank you for suggesting changes again in “Competing Interest” section to state that the authors hereby declare that BTGin (Daejeon, Korea) had no role or interest in the funding, design, conduct and interpretation of our study and findings, although the private company is the manufacturer who has provided the protopanaxadiol for free for conducting this scientific study.