Author's response to reviews

Title: Gamma tocopherol upregulates peroxisome proliferator activated receptor (PPAR) gamma expression in SW 480 human colon cancer cell lines

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PDF covering letter
We would like to thank the reviewers for taking the time to carefully review our article and make productive comments.

Reviewer 1. Dr. Robert Cooney

1. Explain why the values on the Y axis are so different in the two experiments shown in figure 1a and 1b (relative copy number).

According to Gupta, [Gupta RA, Dubois RN: Controversy: PPARgamma as a target for treatment of colorectal cancer. Am J Physiol Gastrointest Liver Physiol 2002, 283; G266-G269.], the highest levels of the receptor expression in colonic tissue occur in postmitotic cells. The variation in relative copy number is representative of differences in the PPAR expression in cell populations seeded and treated on different days. Figure 1A and Figure 1B were resultant from two different cell populations. The population in Figure 1B had a larger number of cells that had undergone mitosis during the treatment than the population of cells in Figure 1A. We have in turn analyzed the data a little differently, by normalizing the relative copy number to the vehicle treated cells for each experiment and expressing the number as a increased fold over the vehicle treated sample. These increased fold numbers for the triplicate experiments were averaged. We have included 3 figures per tocopherol concentration to further demonstrate the data analysis.

2. The data appear to be sound and well controlled.

3. Data are clear but:

   a. Table 1 should have a label over the second column to define what the mean is representing “tocopherol concentration”. This was changed.

   b. The fact that gamma tocopherol is accumulated at 20 X over alpha should be discussed to greater detail in the conclusions. We have added further discussion about this as well as clarified our interpretation of results in the Ingles study and how it relates to our data. See p. 12 & 13.

   c. Western blot quantification using a densitometer. We included a bar graph for each Western blot image that depicts the densitometry units normalized to actin and the vehicle treated cells.

4. In the conclusions on p. 9 authors state that two clinical studies show an emerging role for γ-tocopherol in the reduction of cancer.

   a. The first study cited from Washington County MD is an epidemiological study, not a clinical one. We agree and the wording here has been changed.

   b. The second study by Ingles et al runs counter to their argument as they report decreased plasma gamma tocopherol is protective. The authors must clarify these issues and make their arguments consistent prior to publication. We have addressed this on p.13 and explained
how our data aids in further interpretation of their data. In addition, we have added our thoughts about Ingles data based on our data and review of the most recent literature.

4. The authors draw attention to the similarity between PPAR regulators and γ-tocopherol. Providing structures of the various compounds in question would strengthen the paper and the authors statements.

Agreed, additional Figure added. See Figure 1.
1. Provide further evidence regarding the consequence of the up-regulation of PPAR γ expression. We have included more background information in the discussion on the known consequences of the upregulation of PPAR γ and how it applies to the results of the paper. See pp. 10 and 11.

2. Relatively higher concentrations of γ-tocopherol and α-tocopherol should also be tested for instance 20-50 µM because tocopherol levels could be relatively high in the colon as result of supplementation (Stone, WL et al. (2002) Cancer Detection Prevention, 26 78-84). When we have used higher concentrations, we have noticed that it compromises the viability of the cells. We have further tested this observation at several higher concentrations in 3 different cell lines. It is possible that other cellular changes occur at higher concentrations. Studies on cell cycle changes, necrosis and apoptosis are underway to further understand the effects on these cell lines at higher concentrations of tocopherols.

3. Results could be stronger if more than one cell line is used. We agree and have completed one experiment with HCT-116 cells and have obtained similar QPCR results. In addition, we are in the process of making comparisons of results in various cell lines based on the carcinogenic mutations inherent to the cell line.

Some minor issues:

1. In the abstract, the statement in the background that “γ-tocopherol may be a more potent form of vitamin E is not clear. Some additional words have been added to reflect that we are referring to chemopreventive potential of the vitamin E isoforms alpha and gamma.

2. The statement in the conclusion that “ligand binding assays are being pursued to determine if vitamin E is a ligand for PPAR γ” should not be in the conclusion but in the discussion of the text. Revised sentence structure to reflect future work that needs to be done with vitamin E isoforms.

3. In the methods, how was actin, measured, by stripping the original membrane and reincubation with the actin antibody? Co-incubation with PPAR γ and β-actin antibodies. This has been corrected in the manuscript.

4. In conclusions, page 9 line 18 “gamma tocopherol may be superior” compared to what? Edited the manuscript to make the point clear we are comparing the chemopreventive activity of gamma tocopherol with that of alpha tocopherol.

5. In conclusions, page 9 line 22 what is MD? MD is the official United States Postal Service abbreviation for the state of Maryland.

6. Figures, in Figure 2, please identify the one band of PPAR γ as PPAR γ 1 or PPAR γ 2. It is also better to label the mw of the bands.
Agreed, in contrast to adipocytes which express both PPAR γ 1 and PPAR γ 2, colon tumors specifically express PPAR γ 1 [ DuBois RN, Gupta R, Brockman J, Reddy BS, Krakow SL, Lazar MA: The nuclear eicosanoid receptor, PPARgamma, is aberrantly expressed in colonic cancers. Carcinogenesis 1998, 19: 49-53.]. We have included figure labeling that identifies the PPAR γ as PPAR γ1 and included mw labels for both PPAR γ and β-actin.