Author's response to reviews

Title: Comparative Effects of Alpha- and Gamma-Tocopherol on Proliferation and Apoptosis in Human Colon Cancer Cell Lines

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Version: 2 Date: 13 October 2005

Author's response to reviews: see over
Reply to Reviewer’s Report

Title: Comparative Effects of Alpha and Gamma-Tocopherol on Proliferation and Apoptosis in Human Colon Cancer Cell Lines.

Version 1 Date: October 10, 2005

Reviewer: Kimberly Kline

Reply:

First, I would like to thank both reviewers for taking the time to thoroughly review my manuscript and make comments that have significantly improved the quality of the paper.

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Major Compulsory Revisions:
NONE

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Minor Essential Revisions
1. Tables giving statistics on numerous comparisons are confusing. Authors need to decide what is the central (primary) outcome of a particular study and provide statistics on this comparison. Also biological relevance needs to be considered. Just because something is significant from a purely mathematical standpoint, this does not make it important from a biological standpoint. For example, what is the biological relevance of Fig 2 Panel A statistically significant data at 25 and 50 microMolar concentrations of the various pro-death agents? In this same set of experimental data, some type of evaluation of whether or not the pro-death agents produce a concentration-dependent response needs to be made.

   Reply: We agree, the tables were difficult to read. All tables (with the exception of one) have been removed. The statistically significant data (p values <0.05) have been identified on the graphs with an asterisk. The data in Figure 2 has been reevaluated and the results section have been reworded to reflect that the γ-tocopherol treatment of each cell line is concentration dependent, while α-tocopherol was not effective at inhibition of cell proliferation at any concentration tested.

2. More caution needs to be exercised in conclusions drawn. For example, the statement: “The amount [of] growth reduction was dependent upon the molecular signatures of the cell lines suggesting more than one pathway can be modulated by gamma-tocopherol to inhibit cell proliferation.” An equally likely speculation is some of the cells express elevated levels/enhanced functions of survival factors that block apoptosis by gamma-tocopherol. Also it is not justified to draw “conclusions” about the involvement of regulatory
pathways [for example, Cox-2, lipoxygenase and sphingolipid metabolism),
PPAR and wnt], based on descriptive data from non-isogenic cell lines.

Reply: We agree the conclusions contained statements of speculation
that the data did not confirm. We have confined these statements
regarding potential pathways by which γ-tocopherol could act to the
discussion section. The conclusions contain only statements that can be
supported by the data in this manuscript.

3. There are two completely different types of alpha-tocopherol: RRR-alpha-
tocopherol, a naturally occurring form and all-rac-alpha-tocopherol, a
combination of eight steroisomers only 1/8th of which is RRR-alpha-tocopherol.
Thus, all references to alpha-tocopherol in the manuscript need to be
clarified. For example, in the Background section the following statement is
made: “The isoform found in highest concentration in the serum and dietary
supplements is alpha-tocopherol. ”RRR-alpha-tocopherol is the form found in
highest concentration in the serum and synthetic all-rac-alpha-tocopherol is
the form frequently used in dietary supplements and clinical studies.

Reply: We have changed the terminology from the simple α-tocopherol or
γ-tocopherol to RRR-α-tocopherol or RRR-γ-tocopherol throughout the
text. Discussion involving the data of other investigators, included this
terminology as well where the other investigator has indicated in the
manuscript referenced. When the manuscript referenced did not stipulate
which form of α-tocopherol (RRR- or all-rac), we have indicated that in
parenthesis after the isoform or analogue term using the words (RRR- or
all-rac- not specified).

4. What data from animal models of colon cancer support the hypothesis that
gamma-tocopherol can act as either a chemopreventive or chemotherapeutic
agent? If there is none, this needs to be clearly stated.

Reply: We have added a section in the introduction that describes the
evidence that indicates gamma-tocopherol may be chemopreventive. It is
on page4 ¶2. The animal model data that measures mean survival and
tumor size are non-existent. There are some animal studies that measure
surrogate end markers. They are mentioned and referenced in this
paragraph.

5. Figure 1 needs to be omitted since this information has been published
previously.

Reply: Done
6. Clarification of RRR versus all-rac form of alpha-tocopherol from Eastman Chemical is needed in the Methods section

   Reply: All forms of Vitamin E used in our laboratories are the RRR isomer. We have indicated this in the methods section (page 6 ¶1).

7. Clarification of whether or not bovine serum albumin was included in the vehicle is needed in the Methods section.

   Reply: BSA was included in the vehicle treatment. We have indicated this in the methods section (page 6 ¶3).

8. Clarification is needed whether or not CCD-112CoN cells are truly normal or if they are really are an immortalized cell line. If the later it is not accurate to refer to them as normal.

   Reply: These cells are primary, non-immortalized cells. We have clarified this in the methods section (page 6 ¶2).

9. Vertical axis for Figures 2 and 3 need to be the same for all panels to permit easier comparisons among cell types.

   Reply: We have changed the y-axis for Figure 2 so that the units are the same for all three bar graphs. Figure 3 is a bit problematic because HCT-116 and HT-29 cells grow much more rapidly than the SW480 and the HCT-15 cells. The slower growing cells were less dense than the rapid growing cells by a factor of 5. We did change y-axis on the graphs such that the two rapid growing cells have the same units and the two slower growing cells have the same units.

10. Horizontal axis for Figure 3 and Figure 5 need to be labeled in days for easier analysis.

    Reply: Time was converted to days for both figures.

11. Figure 4 data is a repeat of data in Figure 3 except for normal cell data.

    Reply: We have added 4E to Figure 3 and omitted the other bar graphs, which reduced the total number of Figures.

12. What was the rationale for using different positive controls for induction of cell death for the different cells types? Is there no single pro-apoptotic agent to which all these cell types respond?
Reply: Our initial investigations included determining if PPAR gamma was up regulated by these vitamin E isoforms. The 15PGJ₂ and Troglitazone are PPAR gamma ligands that cause apoptosis and cell death in colon cancer cells. Some of our cell death experiments and PPAR gamma experiments have overlapped. After the counting, we either isolated mRNA or protein to determine PPAR gamma expression. This is why we used the PPAR gamma ligands. The later experiments concerned only with cell death used camptothecin as a positive control (it is less expensive than the PPAR ligands).

13. Vertical axis of Figure 5 needs to be labeled in some easily understandable measure.

Reply: We have changed the vertical axis label to read the same as in Figure 3 (now figure2).

14. Figure 3 legend refers to 5 day data that is not in Figure.

Reply: This has been removed.

15. Discussion / second paragraph. Since colonic epithelium is exposed to the contents of the gastrointestinal tract lumen it would seem that this exposure to dietary vitamin E forms would be as important as exposure to plasma levels. It would be helpful if this exposure was addressed. Also according to Maret Traber and colleagues there is no bioselective processes of vitamin E forms by the GI tract just by the liver alpha-tocopherol transfer protein.

Reply: You are correct. We have revised this paragraph (page 16 ¶1) to incorporate your point, that plasma levels are important, but direct exposure from gastrointestinal lumen can also occur. Thank you for your point.
Reply to Reviewer's Report

Title: Comparative Effects of Alpha and Gamma-Tocopherol on Proliferation and Apoptosis in Human Colon Cancer Cell Lines.

Version 1 Date: October 10, 2005

Reviewer: Qing Jiang

Reply:

First, I would like to thank both reviewers for taking the time to thoroughly review my manuscript and make comments that have significantly improved the quality of the paper.

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Major Compulsory Revisions:
The study is largely observational. Although based on the publications by others the authors mentioned several possible mechanisms that may be involved in gamma-tocopherol induced effect, none of the mechanisms were examined in the current study.

Reply:
We agree that the conclusions had too many speculations and were not based on our data. We confined the discussion to comparisons of our data to that of other publications. We have removed all speculative remarks from the conclusions and made statements that can be derived from our data only. Thank you for pointing this out.

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Minor Essential Revisions

1. Please clarify the amount of serum present when cells were treated with tocopherols.

   Reply:
   The serum present at the time of tocopherol treatment was 10% FBS. We have corrected this omission in the methods section page 6 ¶ 3.

2. Reference 18 indicated gamma-tocopheryl quinine, but not CEHC is a potent inhibitor of apoptosis.

   Reply:
   Reference 18 belonged to a sentence that was deleted while we were editing the manuscript prior to submission. This reference should have been deleted from the text. We have done so now on page 5 ¶ 2.
3. Gysin et al. did not find apoptosis induced by gamma-tocopherol in colon cancer cells, which is different from the results in the current study. Please discuss the reasons for the discrepancy.

   Reply:
   We have made revisions to this on p. 15 ¶1. Our previous statements were based on reading the FASEB express version of the article. After obtaining the (20 page) expanded version of the article and reading it, we have found that Gysin did not report testing for apoptosis in the colon cancer cells. They tested apoptosis in the prostate cancer cells. We have made revisions in this section to reflect the testing by Gysin reveal reasons for differences between the Gysin study and our own. In short, we feel that the higher concentration we used allowed for more tocopherol up take into the cell.

4. The discussion of Jiang et al. is not correct as in their study dihydroceramide, but not ceramide was found to increase.

   Reply:
   Our apologies for misrepresenting your data so carelessly. We have corrected this on page 18 ¶1.

5. Relatively high concentrations of gamma tocopherol have been used in order to induce cell death, which raises a question regarding the physiological significance of the study. Although the authors mentioned the intracellular levels of gamma tocopherol was 15-20 femtomoles/cell, it is not clear if this level can be achieved in vivo by gamma tocopherol supplementation. This issue should be further discussed.

   Reply:
   To date, no studies have been performed to verify if these concentrations can be achieved in vivo. We have added a statement as such to the manuscript page 16 ¶2. We have also added some discussion on the fact that colonocytes can receive exposure to tocopherols that pass through the digestive tract which would allow for more tocopherol uptake than would be available in the plasma, page 16 ¶1.