Author's response to reviews

Title: Anti-oncogenic and Pro-differentiation Effects of Clorgyline, a Monoamine oxidase A Inhibitor, on High Grade Prostate Cancer Cells

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Author's response to reviews: see over
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RE: MS: 4810759952137743

Dear Dr. Edmunds,

Thank you for the thoughtful comments on our manuscript entitled "Anti-oncogenic and Pro-differentiation Effects of Inhibition of Monoamine oxidase A on High Grade Prostate Cancer Cells" (MS: 4810759952137743). We have revised it based on the critiques of the reviewers in a point-by-point manner. A detailed explanation of the revisions with an indication of where the changes have been made in the text is attached.

Thank you for your consideration of publishing our manuscript. Please let me know if there is anything else we can do to improve it.

Sincerely,

Donna M. Peehl, Ph.D.
Response to comments by Dr. Emma Guns:

Major compulsory revisions:

1. By treating with clorgyline, you cannot definitively say that you are confirming that gene expression changes are due to inhibition of MAO-A, there may be off-target effects of clorgyline which account for these effects, independent of MAO-A. The use of siRNA to MAO-A to specifically knock-down MAO-A would be more convincing that the direct links of this effect of clorgyline are due to MAO-A inhibition. Also, this type of conclusive statement is misplaced in the results section e.g. sentence on p.8, starting on line 11. Also, sentence starting on p.9, line 5. The sentence starting on p. 9 line. 21 more accurately reflects the conclusions which can be made, however, again I feel that these conclusive statements are misplaced in the results section. I would recommend that this definitive assignment of the effects of clorgyline being MAO-A inhibition be revised and simply state that clorgyline treatment causes these effects – this recurs and is repetitive throughout the results section. Once again in the discussion section, the effects observed have been assigned directly to MAO-A inhibition and this is not definitive.

We attempted to selectively remove MAOA expression using siRNA in our primary prostate epithelial cells. To determine the efficacy of MAO-A siRNA in knocking down the mRNA expression of MAO-A, we transfected the cells with MAO-A siRNA (h): sc-35847 from Santa Cruz Technology Inc., and assessed the MAO-A transcript level after 24 and 48 hrs. MAO-A mRNA level was decreased 300-fold at 24 hrs after transfection compared to the control cells, and 30-fold at 48 hrs (Figure 1 insert). However, no significant changes in the protein levels of MAO-A were detected by Western Blotting after 24, 48, and 72 hrs (Figure 1 insert). These results suggest that the turnover rate of MAO-A protein in primary prostate epithelial cells is low and RNAi-mediated knockdown is not a suitable method to remove MAO-A in these cells. Discussions with experts on MAO-A confirm that they have had similar experiences with other types of cells, and indeed we are unaware of any published studies using siRNA to knock down MAO-A expression. One could try to repeatedly transfect the cells with MAO-A siRNA to determine the time required for protein reduction. However, the cells would be in a very unhealthy state because of the toxicity of the transfection reagents. Because of the limited life span of primary epithelial cells, generation of stable lines expressing shRNA by viral transfection is also difficult.

We revised statements attributing the effects of clorgyline being due to MAO-A inhibition and simply stated that clorgyline treatment caused these effects at the following places throughout the text.

- We changed the title from “Anti-oncogenic and Pro-differentiation Effects of Inhibition of Monoamine oxidase A on High Grade Prostate Cancer Cells” to “Anti-oncogenic and Pro-
differentiation Effects of Clorgyline, a Monoamine oxidase A Inhibitor, on High Grade Prostate Cancer Cells”.

- In the Abstract, we replaced “MAOA-inhibition” with “clorgyline” on page 2 line 15 and 22. Similar changes were made at the following places:
  - In Background, on page 5 line 18.
  - In Results, on page 11 line 3, page 12 lines 11 and 23, page 13 line 21, page 14 line 2, page 15 line 10, page 16 lines 3 and 17, and page 17 line 4.
  - In Discussion, on page 19 lines 8 and 18, page 20 line 9, and page 21 line 25, and page 22 line 14.
  - In Conclusion, on page 23 line 2.

Discretionary comment:

The results described are contradictory based on the conclusions made - If you induce AR and PSA production you are likely sensitizing cells to the effects of androgens, thus driving cell proliferation. However, all other effects of clorgyline treatment support a pro-differentiation and anti-oncogenesis as is stated in the conclusions.

Androgen signaling not only drives proliferation but also promotes prostatic differentiation, therefore, inducing androgen signaling is not contradictory to a pro-differentiation effect of clorgyline treatment. In fact, AR and PSA expression is a hallmark of secretory epithelial differentiation. Consistent with the induction of secretory differentiation as shown in Figure 5B and 7B, the proliferation potential of E-CA cells was significantly decreased by clorgyline treatment. Therefore, although increased AR might in theory sensitize cells to the pro-proliferation effect of androgen, it seemed to be overcome by the pro-differentiation effects of clorgyline. However, it should be noted that these experiments were performed in a medium which is not conducive to growth, in that it lacked the mitogen EGF and also contained growth-inhibitory factors (vitamin D, retinoic acid and TGF-beta). Indeed, even the control cells lost proliferation potential over time in this medium, although not to the degree to which clorgyline-treated cells did.

Whether AR/androgen promote proliferation or differentiation of primary adenocarcinomas of the prostate is a subject of debate, but our experimental system may provide a new model to explore this further in future studies.

Minor essential revisions:

1. **Table 1 title typo ‘regulated’**

   Corrected

2. **Also, table 1 requires more detailed description regarding the citation source for information validating the oncogenic pathways affecting the genes identified by SAM.**

   A footnote was added to the table to describe the citation source of the oncogenic pathway genes.

3. **Table 3 title typo ‘clorgyline’**

   Corrected

Minor comments:
1. **Please include some rationale of choice of treatment concentrations and time points selected for clorgyline.**

The following has been added to the result section page 11 starting at line 6 to explain the rationale of choice of treatment concentrations and time points selected for clorgyline.

“The concentration of 1 µM was chosen because previous studies have shown that it is an effective dose to elicit a variety of effects in cultured animal cells ¹⁻³. Our earlier study using normal primary prostatic basal epithelial cells also showed that 1 µM clorgyline induced secretory differentiation ⁴. Total RNA was isolated at 6, 24, and 96 hr. In normal cells, secretory differentiation occurred by 96 hr after clorgyline treatment. Therefore, the three time points chosen for profiling are sufficient to capture the gene expression changes elicited by clorgyline at early and late stages.”

2. **Prostate ‘secretory cells’ would be more appropriately described as ‘epithelial cells’ throughout the manuscript.**

We have replaced “secretory cells” with “secretory epithelial cells” throughout the manuscript for clarification. We feel “epithelial cells” is not equivalent to “secretory cells” since the term includes both secretory epithelial cells and basal epithelial cells of the prostate epithelium.
Response to comments by Dr. Audrey Player:

Major

1. I feel the study demonstrates "Examination of a MAO-A inhibitor on transcriptional control of high grade epithelial PC-A cells and possible anti-oncogenic implications".

   The tile has been modified to state that the effects were caused by clorgyline.

2. The data appears to be generated using 1 patient sample? Should these data be generated using other (i.e., at least 1 other for validation)?

   We determined the effects of clorgyline on another primary culture derived from a Gleason grade 4 prostatic adenocarcinoma, E-CA-90. As shown in Figure 7A, all top 10 genes on the SAM list generated from E-CA-88 cells were significantly upregulated after 24 hr of clorgyline treatment of E-CA-90 cells. At 96 hr, 7 of the 10 top SAM genes were significantly upregulated except RB1CC1, ZNF292, and CEP70. In addition, both APC and FAS, the 24th and 50th SAM genes, respectively, were significantly upregulated at 24 and 96 hr. Finally, secretory cell markers including AR, PSA, and PSMA were induced at both time points. Consistent with the notion that secretory differentiation was induced, the proliferation potential of treated cells was dramatically decreased compared to control (Figure 7B). These results suggest that the effects of clorgyline on primary E-CA cells from high grade cancers were reproducible.

   The above results were added to Results on page 17 starting from line 21.

3. Study lends itself very nicely to a cell-culture-model for validation of the authors theory that "inhibiting MAO-A can effect differentiation and reverse the tumorigenic behavior of the cancer cells."

   To address whether clorgyline can reverse the tumorigenic behavior of the cancer cells would require the establishment of an in vivo animal model. Unfortunately, our primary cells do not form tumors in mice, therefore impeding our ability to test this hypothesis. We have initiated studies of clorgyline with tumorigenic prostate cancer cell lines to explore the effect of this drug in advanced cancers.

4. The top 10 genes were selected for validation of the microarray. Might the authors consider validation of select (a) genes suspected as important in a particular pathway as done with APC?

   We validated the 50th gene on SAM list, FAS, a member of the TNF receptor superfamily, which plays a central role in the physiological regulation of programmed cell death. It has been shown that FAS is hypermethylated and downregulated in prostate cancer and is a potential therapeutic target. As shown in Figure 3A, FAS was upregulated by clorgyline by 3.0-, 1.4-, and 2.7-fold as determined by qRT-PCR at 6, 24, and 96 hr, respectively, consistent with our microarray results. The above results were added to Results on page 13 starting from line 21.

Minor
1. **Page 6)**: 37,340 clones. Were these clones or the result of hybridization signal intensities on microarray?

Thirty-seven thousand three-hundred and forty is the number of clones whose signal intensities were >150% above background in either Cy5- and Cy3-channels.

2. **Can the authors address the proliferative capacity of the chlorgyline-treated cells used for RNA extraction (as all 'SAM-genes' were upregulated).**

We assessed the proliferation capacity of the chlorgyline-treated E-CA-88 and E-CA-90 cells. As shown in Figure 5B and 7B, in both cases, chlorgyline dramatically decreased the proliferation potential of E-CA cells. This is consistent with the notion that secretory differentiation was induced in these cells. The results were described on page 16 lines 12 and 18, and page 18 line 4.

3. **Statement "In fact, expression of many.... this statement was unclear. How many is "many".**

Specifically, expression of 4029, 5606, and 2299 genes was upregulated at least 2-fold at 6, 34, and 96 hr, respectively. In addition, expression of 3576, 2486, and 597 genes was downregulated at least 2-fold at 6, 34, and 96 hr, respectively. These genes are listed in additional data file 2. To clarify, the following was added to the result section on page 12 line 1.

“In total, expression of 4026, 5606, and 2299 genes increased and 3576, 2486, and 597 genes decreased at least 2-fold in response to chlorgyline at 6, 24, and 96 hr time points, respectively.”

4. **First paragraph of this section was difficult to follow. Table 1= clear, but derivation of Table 2 was not clear. How were the genes (1839, etc...) derived? from Creighton study or this study?**

The oncogenic genes are derived from the Creighton study. The numbers represent named genes compiled in that study. To clarify, a footnote has been added to the table (now Table 1 because the original Table 1 was removed as supplemental material) as the following.

*The named oncogenic pathway genes used for comparison were compiled by Creighton.*

5. **Consider Table 1 as supplemental material?**

It was removed from the manuscript and designated as additional data file 3.

6. **Paragraph beginning "Genes downregulated by beta catenin...." is unclear. I understand derivation of the '156genes', but others are somewhat unclear (ie, 1839,etc...).**

These oncogenic pathway genes are derived from the Creighton study. To clarify, the following modification has been made.
Page 13 line 1: “The oncogenic pathways regulated by beta-catenin, Src, ERBB2, and Ras overlap and have common target genes. For example, 1110 of the 1839 (60%) named genes downregulated by beta-catenin as complied by Creighton [15] are also downregulated by Src. Similarly, 595 (32%) and 308 (17%) of genes downregulated by beta-catenin are also downregulated by Ras and ERBB2, respectively [15]. In our dataset, these genes downregulated in oncogenic pathways are the most enriched in the SAM list of clorgyline-induced genes and the enriched genes also overlap considerably. Specifically, 21, 19, and 12 out of the 36 genes (58%, 53%, and 33%) that overlap between beta-catenin downregulated genes and the SAM list of clorgyline upregulated genes were also downregulated by Src, Ras, and ERBB2, respectively.”

7. Consider validation of the differentiation associated genes, not all, but a few.

We validated three secretory cell markers including AR, PSA, and PSMA in E-CA-88. As shown in Figure 5A, transcript levels of all three markers were significantly upregulated at 24 and 96 hr except for PSMA at 24 hr. In addition, consistent with the notion that secretory differentiation was induced, the proliferation potential of treated cells was dramatically decreased compared to control (Figure 5B). These results suggest that clorgyline promoted secretory differentiation in E-CA-88 cells. The above results were described on page 16 lines 12 and 16. Similar results were obtained using E-CA-90 cells as described on page 18 line 3.

8. EZH2 expression appears to be transient, but associated Polycomb signatures appear enriched. The authors might consider Q-PCR validation of a few of these? 13 of 87 appear consistent across 6-24hr time points. this considered enrichment?

We attempted to validate four Polycomb signature genes that have been implicated in the differentiation of various cell types, namely MYO6, SATB2, SOCS2, and RGC32 [7-10]. As shown in Figure 5A, expression of three of the four genes was significantly upregulated in treated E-CA-88 cells compared to control, suggesting that clorgyline induced genes suppressed by the Polycomb complex. While 23 and 29 out of 87 genes upregulated by clorgyline at 6 and 24 hr, respectively, was statistically significant enrichment, 13 of 87 upregulated at both 6 and 24 hr was not statistically significant. Nonetheless, these represent a subset of the Polycomb repression signature consistently upregulated by clorgyline. These results were described on page 17 starting from line 13.

9. All PCR primers included (ie, APC?)

Primer sequences for APC and other additional genes were added to the method section on page 8 line 12.

10. (Figure 2):(B) is unclear- difference averaged across 6, 24, 96hr, SAM then, PCR?

Figure 2 (B) compared the fold-changes for the top 10 SAM genes calculated by SAM using microarray measurements to those determined by qPCR. For both SAM and qPCR method, the fold-change is an average across the three time points. However, for qPCR, fold-changes at each time point were calculated first and the three fold-changes were averaged. For SAM, the average of gene expression across the three time points
for treated (T) and control (C) was calculated first and then the fold-change was determined as T/C. To clarify, the following has been added to the figure legend on page 31 line 19.

“For qPCR, fold changes at each time point were calculated first and the three fold changes were averaged. For SAM, the average of gene expression across the three time points for treated (T) and control (C) was calculated first and then the fold change was determined as T/C.”

10. **(Figure 3): (D) and (E) are somewhat confusing. red and green designate up and downregulation, respectively. What does "below the "0" designate, even though red and green? Same question for Figure 4**

In Figure 3 (D) and (E) as well as Figure 4 (B), red represents upregulation by the oncogenes and green downregulation. Bars above the X-axis represent upregulation by clorgyline and bars below the X-axis downregulation. To clarify, the following has been added to the figure legends on page 32 lines 13 and line 24 for Figure 3 and 4 respectively.

“…..bars above the X-axis represent genes upregulated by clorgyline and bars below the X-axis downregulated.”

11. **There are 126 genes listed representing the 156??**

   Table 1 has 72 genes listed. The last gene in the table is the 126th gene in the SAM list. There were an additional 11 genes missing because of a cut-and-paste error. Now Table 1 has 83 genes which are common between the list of 156 genes upregulated by clorgyline identified by SAM and the lists of genes downregulated by oncogenic pathways compiled by Creighton (PLoS ONE. 2008 Mar 19;3(3):e1816.). To clarify, the title of the table was modified as the following.

   “Eighty-three genes upregulated by clorgyline identified by SAM that are downregulated by oncogenic pathways”

   This table was designated as additional data file 3 as suggested by Dr. Player.

12. **(Additional data file 2): Are all of the genes listed here upregulated?**

   There are six gene lists in the file with each list as a separate Excel sheet. The name of each sheet indicates whether the genes are up or down regulated and at what time point. For example, “6 hr up” indicates the genes are upregulated after 6 hr of clorgyline treatment. To clarify, the following has been added to the additional files section page 37 line 9.

   “The name of each Excel sheet indicates whether the genes are up or down regulated and at what time point.”

13. **(Figure 5): "(A)" noted twice in legend**
The legend for Figure 5 (now Figure 6) was modified to the following as shown on page 33 starting at line 12.

“(A) Expression of EZH2 was decreased and its target, ADRB2, was increased at 24 hr after clorgyline as determined by qRT-PCR. The relative expression levels of the top 10 genes in clorgyline-treated cells compared to control. (B) Expression of PcG signature genes was upregulated after clorgyline treatment at 6 and 24 hr determined by microarray. “
Response to Dr. Anne Collins:

Major compulsory revisions:

1. My major criticism is that only one high grade prostate cancer was used for this study. I do have some sympathy as it is a move in the right direction, away from studies on cells lines. However, microarray studies should be based on more than one sample.

   We determined the effects of clorgyline on another primary culture derived from a Gleason grade 4 prostatic adenocarcinoma, E-CA-90. As shown in Figure 7A, all top 10 genes on the SAM list from E-CA-88 were significantly upregulated after 24 hr of clorgyline treatment. At 96 hr, 7 of the 10 top SAM genes were significantly upregulated. In addition, both APC and FAS, the 24th and 50th SAM genes, respectively, were significantly upregulated at 24 and 96 hr. Finally, secretory cell markers including AR, PSA, and PSMA were induced at both time points. Consistent with the notion that secretory differentiation was induced, the proliferation potential of treated cells was dramatically decreased compared to control (Figure 7B). These results suggest that the effects of clorgyline on primary E-CA cells from high grade cancers were reproducible. The above results were described on page 17 line 21.

2. The functional effect of inhibition of MAO-A activity is alluded to, but not tested. The authors demonstrate differentiation of prostate cells, but it would strengthen the paper to study the functional consequence of Clorgyline treatment. For example, what is the effect on clonogenic recovery following treatment? If differentiation is induced, it would be expected that the colony forming activity would be affected.

   We determined the effects of clorgyline treatment on the proliferation capability of both primary cultures. As shown in Figure 5B and 7B, clorgyline dramatically decreased the proliferation potential of both cultures, consistent with notion that secretory differentiation was induced. These results were described on page 16 line 18 and page 18 line 4.

3. Figure 5A shows error bars but is the difference statistically significant? On pg 16 of the discussion (pg 16, last paragraph Ln6) the authors state that the downregulation of EZH2 was significant, but it is not clear that a statistical test was carried out.

   Student’s t-test has been performed for both EZH2 and ADRB2, and asterisks have been added to Figure 5A (now Figure 6A) to indicate this as stated in the figure legends. In addition, the following has been added to the result section on page 17 line 2.

   “Both EZH2 and ADRB2 expression changes were statistically significant by student’s t-test.”

Minor compulsory revisions:

1. Figure 2A, Label Y-axis.

   The Y-axis is labeled with “Relative expression compared to control”.
References


