Author's response to reviews

Title: Characterization of the Apoptotic Response of Human Leukemia Cells to Organosulfur Compounds

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Author's response to reviews: see over
Dear Dr. Neilan,

**MS: 1416345458328806**

We wish to thank you for providing editorial guidance and managing the review of our manuscript, “Characterization of the Apoptotic Response of Human Leukemia Cells to Organosulfur Compounds”. We are very pleased that you and the reviewers felt our research was potentially suitable for publication in *BMC Cancer*, and appreciate the thorough and helpful commentary of the four reviewers. To that end, please find below point-by-point responses to each request or comment.

We hope this letter addresses the concerns of the reviewers and that you will find this resubmission to be acceptable for publication.

Sincerely,

Dr. Linda Z. Penn

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**Reviewers’ Comments:**

**Reviewer 1: Lennane Michel Espinoza-Fonseca**

We would like to thank Dr. Espinoza-Fonseca for the thoughtful review of this manuscript, and helpful suggestions.

“1. *It has been suggested that similar compounds have been synthesized and tested. However, I would like to see studies addressing the toxicity of this compounds (or lack thereof). This will improve the manuscript significantly.*”

We definitely agree that toxicology studies will be essential to the further develop OSCs as potential anti-cancer agents. The current study has focused on chemical synthesis and activity, so a thorough study of OSC pharmacodynamics, pharmacokinetics, and toxicology is beyond the scope of the current work. Nevertheless, this important aspect needs to be raised in the manuscript, so We have added the following text into the Discussion (page 23):

Moving forward, we believe these compounds are suitable for preliminary toxicology testing. These studies will be an essential component to further advance these OSCs as potential anti-cancer therapeutics. Additionally, an improved understanding of the pharmacokinetics of these compounds may allow determination of the potential therapeutic index required for tumor specificity *in vivo*.

“2. *Although the discussion is appropriate for the results presented in the manuscript, I feel it could be enriched (i.e., by making a thorough comparison with other compounds and indicate what are the advantage of these new compounds compared to previous ones).*”
We thank the reviewer for this valuable suggestion. We have added the following text to the Discussion (page 23):

Our previous structure-activity studies identified several promising OSCs (F, H, K, L, N, O, P and P). These molecules were all classified as tumour-selective group II compounds. In this manuscript we have characterized the anti-proliferative effects of 18 newly synthesized OSCs, and identified an additional five group II compounds (F1, H2, H5, H6, N2). Combined, we believe F, H, H5, L, N, O and P show the most promise. These six OSCs were able to induce apoptosis and reduce colony formation of leukemic cells. Importantly, they had little effect on colony formation of normal myeloid progenitor cells, suggesting that they have a therapeutic window of activity.

**Reviewer 2: Xie Li-Ping**

“In addition to yielding promising drug candidates, this paper also provides a model for academic drug discovery and design. By integrating separate synthetic chemistry and molecular biology laboratories remarkable progress was achieved towards a novel therapeutic class”

We would like to thank Dr. Li-Ping for their kind review of this manuscript.

**Reviewer 3: Yoshimasa Nakamura**

We thank Dr. Nakamura for a thorough and insightful review. These comments have been very helpful in ensuring that the quality of our manuscript.

“1. It would be better to include structural details for the relatives of OSCs in the manuscript to help the readers’ understanding.”

We thank the reviewer for this valuable suggestion. We have updated Table 2 to include the details of all newly synthesized and previously characterized OSCs evaluated in this manuscript.

“2. P14, L24 - P15, L1: The authors mentioned that Group III compounds, which have tumor-specific activity like compound M, from their previous study, are compounds F1, F4, F5, F6, F7, F8, H3, H4, H7, H8, N1, and N2. All compounds are not tumor-specific but somewhat tumor-selective, because some showed toxic effect on the normal cells. Also, compound H8 showed normal cell-specificity based on the MTT assay data. The authors are invited to improve this point.”

We apologize for this lack of clarity. Group III compounds do not exhibit tumor-selectivity, and have similar anti-proliferative effects on both AML-3 and WI38 cells. The text has been updated accordingly.
“3. The result section is too lengthy and includes some descriptions repeated in the method sections.”

We have edited this section and removed several redundant portions.

“4. P22: Scheme 3 is somewhat attractive but too speculative to include in the text. Is there any evidence to support that peroxy anion is involved, such as intracellular peroxide level is upregulated by the test compounds. Where or how this anion comes from. Activities of methyl disulfide derivatives such as F, H, and N, are low, and especially H, having the alpha-ester/methyl disulfides, shows little effect. Therefore, other structural factors such as lipophilicity should be taken into account.”

We agree with Dr. Nakamura, the mechanism proposed in Scheme 3 is speculative. Through our evaluation of 18 novel OSCs, one of our goals was to better understand the structure-activity relationship of these compounds. We believe that Scheme 3 nicely accounts for the observations that cannot easily be rationalized by previously published protocols. We feel that this scheme provides valuable insight and could serve as a platform from which to design future studies. In appreciation for the above comments, we have edited the text to further emphasize that Scheme 3 provides a hypothesized model of activity that warrants further study.

“5. P23, L3: Scheme 2 should read Scheme 3.”

The text has been changed accordingly.

Reviewer 4: John Pinto

We thank Dr. Pinto for his thorough review of our manuscript and are pleased that the significance of our work and our model of academic drug discovery are appreciated.

“1. Pg 6, MTT assays “were””

The text has been changed.

“2. The volume of acetone used in control cultures should be noted in the MTT assay and microarray assay.”

As stock solutions were prepared by dissolving approximately 20 mg of each compound in ACS grade acetone (5.00 mL), the molar concentrations were not identical for all OSCs. For MTT analysis the volume of acetone used in control cultures corresponded to the highest concentration utilized in the experiment and was maintained below 1% (v/v) to limit solvent toxicity. For the microarray analysis, the concentration of acetone in control cultures was 0.67% (v/v),
corresponding to the largest volume required for treatment with the desired concentrations of OSCs. The methods section now includes this information.

“3. The use of a plural verb should be provide when using the word “data”. As …data were. This misuse of “data was” occurs periodically throughout manuscript.”

The text has been changed accordingly.

“4. Pg 7. The method of preparation or ingredients of “conditioned” media should be indicated. Preparations of conditioned media vary among investigators.”

Conditioned media was prepared by growing the 5637 cells to confluency, replacing with fresh media and harvesting the media after three additional days of incubation. The conditioned media is used at 10% (v/v) with aMEM +10% FBS. The text (page 7) has been updated to include this information.

“5. Pg 8. Plural verb with “data” at two locations.”

The text has been changed accordingly.

“6. Pg 12. The compound “Q” has been introduced and repeatedly used throughout the manuscript. Although it has been presented in a previous publication, it would be important for clarity and scientific comparison with the other organosulfur derivative that the structure be included in this manuscript. Preferably located in Table 2 listing compounds examined in study.”

Thank you for this suggestion. We have updated Table 2 to include the details of all 18 compounds newly synthesized and presented in this report, as well as all OSCs (including F, H, K, L, M, N, O, P and Q) synthesized previously and further evaluated in this manuscript.

“7. Last paragraph: a “than” needs to added to the sentence. “…more potent than the parent structure”.”

The text has been changed accordingly.

“8. Pg 14. Identifying compound Q (cited at 2 locations) with its structure would help clarity of this section which compares the organosulfur compounds.”

We have updated Table 2 to include the details of all 18 compounds newly synthesized and presented in this report in addition to all OSCs (including F, H, K, L, M, N, O, P and Q) synthesized previously and further evaluated in this manuscript.

“9. Pg 15. F1 has been listed here (1st line) as a member of group III compounds yet on previous page 12, it was listed as a group II compound. This needs clarification. Also, compound K was previously listed as a group II member on page 14 and, on page 15, it’s listed as a group III compound. Please clarify.”
We thank Dr. Pinto for this observation. In the original text there were a few inconsistencies defining the three groups of OSCs. We have thoroughly reviewed the manuscript and have addressed this concern for both accuracy and consistency within the text.

“10. Compound Q again cited without access to molecular structure.”

Compounds Q, as well as other cited OSCs previously synthesized, is provided in Table 2.

“11. Last sentence of pg 15 should iterate the criteria assigned to each of the three groups.”

We have edited the text to more clearly define the criteria used to determine the three groups.

“12. Pg 16, Where Table 2 is indicated, do the authors mean Table 1 instead?”

The text has been changed.

“13. Should the term “metrics” or “matrix” be used?”

“Metrics” is the intended term and refers to a set of measurements that quantify results.

“14. Pg 19, Last paragraph should contain a listing for clarity of the 18 compounds with their numbered subscripts”.

We agree that clarity would be provided by listing each of the 18 newly synthesized OSCs within the three groups. The text has been updated to incorporate this change.

“15. H5 was indicated as displaying the most tumor-specific activity yet it was provided at the highest dose level (200 uM) than the other compounds. Should potency be based on mole per mole relationship or dose and time-dependency of exposure?”

We agree with Dr. Pinto’s concern, and dose and time dependency are very important considerations when evaluating drug activity. Accordingly, we have removed this statement from the discussion in this manuscript.

“16. Pg 20, For consistency, “F” rather than “Q” should be cited as representing a compound that alters expression of a few genes. See page 15 where F was originally cited.”

Compound Q was incorrectly used to represent a molecule that alters expression of a few genes. As Dr. Pinto identifies, compound F is the appropriate comparison, and the text has been adjusted accordingly.

“17. Pg 22, Regarding the proposed mechanism shown in scheme 3, would activity of the CC-linked alpha ester disulfide be compromised if catalase were introduced into the culture media?”
As mentioned previously, Scheme 3 provides a hypothesized mechanism of activity. As suggested by Dr. Pinto, the presence of catalase in culture media could have activity against the peroxide linkage. As Scheme 3 provides a possible explanation for our structure-activity results in order to provide a platform for future studies, we believe it is beyond the scope of the discussion to speculate further.

“18. Figure 1: legend should indicate which compounds are represented within each box in Fig 1A. Group III only shows presence of F6.”

For clarity and simplicity we did not indicate all Group III compounds in Figure 1A. We agree that providing this information in the figure legend would be beneficial and have adjusted the text accordingly.

“19. Figure 3 “Clusters” should be in the singular”

The text has been adjusted to reflect this change.

“20. Table 2: It would help clarity of the manuscript to include an additional column that shows which group each compound has been categorized.”

Thank you for this excellent suggestion. We have altered Table 2 to include an additional column that indicates the group each compound has been categorized. We feel this will provide important clarity.

“21. Figure 3: Should depict on charts, the phases of the cell cycle indicated by percentage”

We agree that providing this information on the figure itself would provide ease in interpretation. We have altered Figure 2 to show that the specified values represent the percentage of cells stained in the pre-G1 region.