Author’s response to reviews

Title: PAMs Inhibits Monoamine Oxidase A Activity and Reduces Glioma Tumor Growth, a Potential Adjuvant Treatment for Glioma

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Author’s response to reviews:

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Dear Editor

Thank you very much for the comments from three reviewers. We have considered each point raised by the reviewers carefully, performed additional experiments, and incorporated them in the revised manuscript. The responses to each question are described below (the reviewers’ comments are in italics).

All the changes are incorporated to the revised manuscript as shown by track changes in red.

Response to Reviewer #1:

Reviewer reports:
Dr. Zaridatul Aini, Ph.D. in Medicine (Reviewer 1): My major comments on this manuscript are related to the design and methodologies and findings of the study:

1) PAMs are a mixture of Chinese herbal medicine. However, the detail composition of this herbal medication was not described in this study.

Response:

The detailed composition of this herbal medication is described.
“PAMs is a mixture of Chinese herbal medicine consists of plants, including Carthamus tinctorius, Cymbopogon distans, Lithospermum erythrorhizon, and Solanum indicum, and Blumea balsamifera. We had identified two active compounds from PAMs including Hydroxysafflor yellow A (HSYA) in Carthamus tinctorius and Allantoin in Cymbopogon distans. HSYA exhibited anti-cancer and anti-inflammation function [1] while Allantoin has wound healing function [2]. Recently, we found Shikonia from Lithospermum erythrorhizon inhibited MAO A catalytic actively (unpublished data). Using network pharmacology from three database (TCMSP, Batman and YaTCM), we identified 158 compounds from the herb plants present in PAMs which may be the active components. This information will help us purify and identify additional active ingredients in PAMs by HPLC, GC, and Mass Spectroscopy.” See text, page 4-5, line 93-102.

2) Several appropriate controls were not included in the study. For instance, the study involving cell lines, normal glial cells for human and mouse should be included to show that the anti-cancer effects of PAMs were selective. For in vivo study - the animal model for clorgyline should also be included in Figure 4, 5 and 6 as comparison.

Response:

“We showed previously that normal human or mouse glial cells, did not have MAO A catalytic activity [3], they were not affected by PAMs in MTS assay.” See text, page 9, line 197-199.

For in vivo study, clorgyline was included in Figure 4. For Figure 5 and 6, the effect of clorgyline itself (Figure 5) and combined TMZ and clorgyline (Figure 6) were published previously [3].

3) Figure 1 - It was not clear why the MAO Inhibition assay was conducted in human prostate LNCaP cell while it is more logical to perform this in the U251S and U251R cells, parallel with GL-26 cells.

Response:

The reviewer’s point is well taken. LNCaP cells express high level of MAO A activity, we use this cell line as a standard for comparison of unknown cell lines. Since both U251S and U251R cells had lower MAO A activity, it is difficult to do inhibition curve with the sensitivity of our assay. Thus, GL-26 cells were used as a representative for glioma cells.

4) Figure 2 - The three different cell lines were treated with PAMs for 48hr. How this time-point was determined was not described. Has any pilot study been conducted by the group/others to determine this specific time-point?

Response:

When we do in vitro cytotoxicity study, we always started with different times of treatment and different concentrations of the drug for each cell line to find the optimum condition. “In this
study, we treated the cell with PAMs at 24hr and 48hr, with different concentrations. We found 48hr was the optimum condition; thus, we showed the data at 48 hr.” See text, page 9, line 199-200. The data at 24hr is shown below for your information.

Figure 1 Effect of PAMs on cell growth in human glioma TMZ-sensitive (U251S), TMZ-resistant (U251R) and mouse GL-26 cells as determined by MTS assay.

5) Figure 4 b - the error bar for this graph is huge, please describe the stat analysis that had been used for this data and what are the p values for them?

Response:

As suggested, we re-analysis the data by using t-test (two samples and paired) and present the p-value in Figure 4b, see below. Also, we used the One-way ANOVA to confirm the stat analysis.

Figure 4b Qualitative representation of bioluminescence imaging conducted at day 8. Each dot, square, and triangle represent one mouse in each group. P-value is the comparison to the vehicle. See Figure 4b in revised manuscript.

6) The study claimed that PAMs reduced tumor growth and MAO A activity, similar to the MAO A inhibitor clorgyline. However, PAMs only showed about 20% inhibition of MAO A catalytic activity while clorgyline inhibition was achieved at a much higher level, ~90% inhibition. This huge difference between the two agents suggests that PAMs might have a different mechanism of action, suggesting that the conclusion of the study might not be entirely true, and requires further validation. Co-administration of PAM and TMZ. Did not provide additional anti-cancer effects and MAO A inhibition when compared to TMZ or PMA alone, suggesting that the conclusion made “PAM might be a promising adjuvant to reduce the toxicity of TMZ.” Might also need further validation.

Response:
Clorgyline is more effective than PAMs on MAO A inhibition under these conditions. However, if we increase the PAMs concentrations, MAO A activity could be inhibited to over 90% (see Figure 1 in text). We agree with the reviewer that there are multiple mechanisms for PAMs’ inhibition of cancer growth. The other mechanisms involved in glioma cancer growth are currently under investigation.

In addition to MAO A as one pathway leading to the reduction of glioma cell growth. Our previous work showed PAMs regulates other pathways and reduced cancer growth in other cancers. PAMs reduced liver cancer growth by regulating apoptosis in HepG2 cells [4]. Also, we showed the anti-cancer effect of PAMs in leukemia cells was mediated by anti-proliferation [5].

Figure 1c shows co-administration of PAMs and TMZ provided additional anti-cancer effects than alone with statistic significant in all three cells (U251S, U251R, and GL-26). Figure 6d shows PAMs and TMZ in combination provided more MAO A inhibition than alone but did not reach statistic significant differences.
As suggested, we rewrite the conclusion “PAMs alone or co-administration with low doses of TMZ may be a potential promising adjuvant to reduce the toxicity of TMZ and to abrogate drug resistance for the effective treatment of glioma.” See text, page 3, line 66-68.

General comments:
N numbers for each experiment and error bars should be included in the Figures and Figure legends.

Response:
As suggested, we now included this information in every figure and figure legend. “Experiments were performed in triplicates and repeated three times with similar results (n=3)” in vitro “each group n=5 for in vivo. All the changes are shown by track changes in red in revised figures and figure legends.

Other comments:
1) Some of the fonts of the manuscript were inconsistent

Response:
We made sure all fonts of the manuscript are consistent now.

2) A typographical error on the statistical analysis section on the method - standard error (SE) not SED.

Response:
The typographical error was corrected. “All data were presented as the mean ± standard error (SE) values and analyzed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). “See text, page 8, line 181-182.

3) It was not mentioned what type of post-hoc test was used for the one-way ANOVA stat analysis

Response:
We re-analyzed and consistently performed the statistics using t-test by GraphPad Prism in all figures.

4) The study had used two types of stat analysis - one-way ANOVA and student t-test: it should be described in detail when this was used, i.e., comparison with multiple groups/ only to control?

Response:
As suggested, we re-analyzed the data by t-test for all the statistical analyses, and revised the method section in Statistical Analysis, and showed a” T-test was performed for comparison with multiple groups. In brief, we analyzed the data using Prism (two samples and paired) by t-test
analysis. A p-value of \( p \lt 0.05 \) was considered statistically significant.”, See text, page 8, line 182-184.

5) Statistical analysis needs to be reviewed since there are certain data that displayed huge error bars, but they managed to achieve stat significance.

Response:

We re-analyzed all of the data and consistently used t-test for statistical analysis. We analyzed the data by using the Prism t-test (two samples and paired). A p-value of \( p \lt 0.05 \) was considered statistically significant.

Response to Reviewer #2:

AHM Khurshid Alam, Ph.D. (Reviewer 2): The findings of the manuscript are interesting but need to solve the following queries before being submit the revised manuscript.

Response

We thank the reviewer for considering this manuscript interesting. Each query is answered below and incorporated into the revised manuscript.

1) IC50 of PAMs is 80.0 μg/ml in GL-26 cells and 112.1 μg/ml in LNCaP cells, but 50 μg/ml of PAMs showed significant activity. Author needs to clarify it.

Response:

Figure 1a, shows IC50 of PAMs for MAO A inhibition is 80.0 μg/ml in GL-26 and 112.1 μg/ml in LNCaP cells. In Figure 1b shows IC50 of PAMs for cell viability is 102.4 μg/ml for GL-26 cells. Figure 1c, PAMs at 50ug/ml inhibited~25% of cell viability as expected.

2) Author could check the GC mass for identify the compounds present in the sample

Response:

Yes, we agree with the reviewer that we could check the GC mass for identify the compounds present in the sample. We included new information on the composition of PAMs in the revised manuscript, as below.

“PAMs is a mixture of Chinese herbal medicine consists of plants, including Carthamus tinctorius, Cymbopogon distans, Lithospermum erythrorhizon, and Solanum indicum, and Blumea balsamifera. we had identified two active compounds from PAMs including Hydroxysafflor yellow A (HSYA) in Carthamus tinctorius and Allantoin in Cymbopogon distans. HSYA exhibited anti-cancer and anti-inflammation function [1] while Allantoin has
wound healing function [2]. Recently, we found Shikonia from Lithospermum erythrorhizon inhibited MAO A catalytic actively (unpublished data). Using network pharmacology from three database (TCMSP, Batman and YaTCM), we identified 158 compounds from the herb plants present in PAMs which may be the active components. This information will help us purify and identify additional active ingredients in PAMs by HPLC, GC, and Mass Spectroscopy. “See text, page 4-5, line 93-102.

3) Author provided evidence that NF-κB is a key transcription factor for the proliferation and survival of glioma cells. Therefore, it is necessary to check the effect of the sample on transcription and translation level of NF-κB.

Response:
We agree with reviewer, it is necessary to check the effect of the sample on transcription and translation level of NF-κB. In addition, we are studying other pathways based on our previous work [3], MAO A inhibitors regulate the secretion of these growth factors, such as TNF-α expression, proliferation ki67, macrophages F4/80…etc. Also, we have found these genes: NF-kB, HIF1α, IKBKB, and p65, were co-expressed with MAO A and NF-kB from the TCGA dataset. Currently, we are studying all these pathways as the potential mechanisms for PAMs’ inhibition of cancer growth (Figure 2).

Figure 2 Correlations between MAO A and NF-kB-associated markers (NF-kB, IKBKB, HIF-1α, p65) using Pearson's correlation. MAO A were significantly correlated with the NF-kB pathway in Glioma cancer samples. (A total of 248 samples, data was analysis by TCGA dataset, Cell 2013).

Response to Reviewer #3:
Oluyomi Adeyemi (Reviewer 3): Study is of topical interest.
1) It’s good that PAMs showed promising anti-cancer prospects. The phytochemical and/or microbial characterizations of PAMs are necessary to enrich findings. As it is, findings are preliminary as there is no information on likely constituents of PAMs.

Response:
We thank the reviewer for agreeing with us that PAMs showed promising anti-cancer prospects. As suggested, we added phytochemical/or microbial characterizations of PAMs to enrich findings. We also add the constituents of PAMs in the revised manuscript, as shown below.

“PAMs is a mixture of Chinese herbal medicine consists of plants, including Carthamus tinctorius, Cymbopogon distans, Lithospermum erythrorhizon, and Solanum indicum, and Blumea balsamifera. we had identified two active compounds from PAMs including Hydroxysafflor yellow A (HSYA) in Carthamus tinctorius and Allantoin in Cymbopogon distans. HSYA exhibited anti-cancer and anti-inflammation function [1] while Allantoin has wound healing function [2]. Recently, we found Shikonia from Lithospermum erythrorhizon inhibited MAO A catalytic actively (unpublished data). Using network pharmacology from three
database (TCMSP, Batman and YaTCM), we identified 158 compounds from the herb plants present in PAMs which may be the active components. This information will help us purify and identify additional active ingredients in PAMs by HPLC, GC, and Mass Spectroscopy.” See text, page 4-5, line 93-102.

“This study shows the potential use of PAMs for the treatment of glioblastoma via MAO inhibition. There are multiple functions of PAMs have been reported. We have shown previously the antimicrobial effects of PAMs, on Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia. coli, Canidia Albicans, and Aspergillus niger [6]. PAMs remarkably inhibits the growth of Staphylococcus aureus and enhance the wound-healing by increasing the permeability of bacterial cell membranes, leakage of contents, and eventually the death of Staphylococcus aureus. PAMs reduced liver cancer growth by regulating apoptosis in HepG2 cells [4]. Also, we showed the anti-cancer effect of PAMs in leukemia cells was mediated by anti-proliferation [5]. Recently, we reported that PAMs could also inhibit the tumor growth of cancers by downregulating the expressions of inflammation and vascular growth associated with TNF-α and VEGF [1].” This new information is in discussion, page 13-14, line 309-318 in revised manuscript.

2) Toxicity in normal cell lines necessary to aid estimation of therapeutic window. Toxicity towards the cancer cell lines could have been general.

Response:

Normal human or mouse glial cells do not have MAO A activity [6], they were not affected by PAMs in MTS assay. We showed previously that the cell proliferation of other normal cell lines HACAT (human skin keratinocyte cell line) was not inhibited by PAMs at 1-3 % concentration of PAMs [1] (16ug/ml), see Figure 3. Most complex herbal medicines exert effects through multiple components and target multiple sites; they have synergistic and balanced effects with low toxicity PAMs has been used as an herb medicine over hundred years; no toxicity or side effects was reported.

Figure 3 No inhibition of cell viability was seen in HACAT cells as determined by CCK-8 assay. The effect of various concentration of PAMs was investigated for both 12 and 24hr.

3) Combination treatment effect was determined at a single dose. Could authors justify this? In combination treatments, authors pre-treated with TMZ for 48 h before incubating with PAMs. Why? Treatments could have been simultaneous.

Response:

We determined the IC50 for cell viability of PAMs to be 115.1, 115.0, and 102.4 µg/ml for U251S, U251R, and GL26 cells, respectively. We tested various concentrations for the combination effect. We chose single dose 50ug/ml based on IC50 because we are studying if there is a synergistic effect between PAMs and TMZ.
In combination treatments, we pre-treated with TMZ for 48 h before incubating with PAMs. Because TMZ is the first line of treatment for glioma patients we like mimic the clinical situation. Further, TMZ effect is not stimulated by low dose or inhibited by high dose upon incubation. Simultaneous treatment could be another condition to study.

4) For the MAO A inhibition assay, inclusion of a reference inhibitor would have been appropriate to validate assay. For example, CLORGYLINE, a known inhibitor of MAO could have been included to benchmark.

Response:

We do use clorgyline, a known specific inhibitor of MAO A as benchmark to compare other unknown inhibitors. Clorgyline are used routinely to validate our assay. In this study, we did run MAO A inhibition assay using PAMs side by side with clorgyline and demonstrated clearly that PAMs inhibits MAO A glioma cell with IC50: 80µg/ml in Gl-26 glioma. We did not show the clorgyline inhibition data which were published previously [3].

5) Why did authors include CLORGYLINE for in vivo experiments only?

Response:

In addition to in vivo experiments, we have also included Clorgyline in vitro experiment, as shown in Figure 1d.

Reference


Also attached in the file.