Author’s response to reviews

Title: Involvement Of Seladin-1 In Goniothalamin Induced Apoptosis On Urinary Bladder Cancer Cells

Authors:

Afifah Radiah Fauzi A.R. F (yaya_pinkygurl89@yahoo.com)
Kai Yen Heng K.Y H (hkyheng@gmail.com)
Fadilah Nor Rajab N.F R (nfadilah@medic.ukm.my)
Kok Meng Chan K.M C (chan@fsk.ukm.my)
Salmaan Hussain Inayat-Hussain S.H I-H (salmaan@medic.ukm.my)

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Author’s response to reviews: see over
COVER LETTER FOR SUBMISSION OF REVISED MANUSCRIPTS

To: Editor Board of BMC Complementary and Alternative Medicine

Subject: SUBMISSION OF REVISED MANUSCRIPT

I am enclosing herewith a revised manuscript entitled, Involvement Of Seladin-1 In Goniothalamin Induced Apoptosis On Urinary Bladder Cancer Cells submitted to BMC Complementary and Alternative Medicine for possible evaluation. With the submission of this revised manuscript I would like to describe changes that I had make according to the comments from 3 reviewers as below:

Reviewer 1: Sreenivasan Sasidharan

1. Please indicate the concentration of DMSO (Ajax Finechem) which was used to prepare the stock solution of goniothalamin.
   Answer: Absolute DMSO was used to dissolve goniothalamin, which stock concentration prepared was 50 mM and for all experiments treatment of goniothalamin, percentage of DMSO was confirmed less than 1 %.

2. Please provide RT4 urinary bladder cancer cell line passage number.
   Answer: RT4 cells used for all experiments were within 10 passages.

3. The author should mention the references for the assay they use to study the anticancer activity. Please provide the MTT Assay reference.
   Answer: a) Method of MTT assay was carried out as described by Mosmann (1983). 
   b) Method of Annexin V-FITC/PI labeling assay was carried out as described by Chan et al. (2010).
   c) Method of Western blot was carried out as described by Inayat-Hussain et al. (2003).

Reviewer 2: Özlem Sultan Aslantürk

1. There is no information about Etoposide, which was used as positive control in this study. Furthermore, in abstract and methods, it was not expound that etoposide was used as positive control.
   Answer: Etoposide is anti-cancer drug, proved to exert cytotoxicity and apoptosis on variety of cancer cell lines. The purpose of etoposide used in this study was to act as positive control for experiment validation of MTT & apoptosis assay. This is to confirm the experiment that was carried out is valid. Thus the introduction of etoposide was added as the background of the study.
2. There is no information about H2O2 and Etoposide and their concentrations used in Western Blot Assay.
Answer: New Western blot result was updated and treatment of hydrogen peroxide and etoposide was excluded due to the resolution of the blot.

3. Authors must write source (reference) about assays in Methods, which were used in the study.
Answer: Refer to answer of reviewer 1 (3).

4. MTT assay result and Apoptotic effect result of IC50 concentration of etoposide are contradictory. Why is the apoptotic effect of IC50 concentration of etoposide 25.1% but cytotoxic effect 50%.
Answer: The differences of cell viabilities values obtained by MTT assay (IC50) and apoptosis assay is due to the different end point of both assays. MTT assay only measured the activation of the mitochondria enzyme in live cells, while annexin v-FITC labeling able to detect the flipping of PS on the apoptosis cells, which was known as an early event in apoptosis. In addition, the flipping of PS may subject to cell type and treatments. Thus, this explained the different of result in both experiment of etoposide treatment.

5. Authors have not compared results of Goniothalamin and Etoposide treatments each other.
Answer: In this study we are not comparing etoposide with goniothalamin, as both of these are 2 different cytotoxic agent. Moreover, etoposide is not a clinical drug used for bladder cancer and it was used only for experiment validation. Thus, we think that in this study it is not appropriate to compare both agent.

6. Result about apoptotic effect of etoposide (25.1%) is present but necrotic effect value is not present.
Answer: Necrotic event induced by etoposide was 5.6 % as assessed by annexin-V-FITC/PI labeling assay.

7. In Western Blot assay, Authors have presented the treatment time as 24 hrs, but in Results section, treatment times of the substances (Goniothalamine, H2O2 and Etoposide) are different from each other. Furthermore, treatment times of IC50 dose of Goniothalamine is 24 hrs but treatment time for 50 and 100 µM doses are 4 hrs. Why?
Answer: New Western blots result (Fig 4) was added. The reason of replacing it is to give a much relevant result to discuss or support the finding of this research. It is also to give more concrete evidence on the involvement of Seladin-1 in apoptosis induced by goniothalamin. In the new result, Seladin-1 expression was accessed in a series of time point (2,4,8,16 & 24 hrs) upon treatment by goniothalamin (IC50). By using the time point model, expression of Seladin-1 was demonstrated clearly compared to previous data and the treatment dose of goniothalamin was standardize; in addition treatment of etoposide and hydrogen peroxide was taken out to prevent any confusion, as it was not relevant to the objective of study.
Reviewer 3: Benjamas Wongsatayanon

1. The introduction about etoposide should be added.
Answer: Refer to reviewer 2 (1).

1. In Fig. 3a, VC has high viable cell (81.9% in Q3) and low apoptosis cells but in the Western blot experiment, why in Vehicle control; VC which has high expression of seladin-1 both at 60 KD and 40 KD which suggests high in apoptosis induction. Please explain the evaluation of adding H2O2 into the experiment. In addition, the validity of the loaded protein should be checked with the house keeping gene such as actin control so please shows the control result.
Answer: New Western blots result (Fig 4) was added. The answer is similar to the Reviewer 2 No 7.

At the vehicle control (VC) or untreated cells, the cleavage of Seladin-1 from 60 kDa to 40 kDa was observed; this result was consistent over 3 different experiments as carried out by us. We suggest the different of our result from previous studies was due to the different characteristic of cells used for different experiment, as in this case RT4 was primary bladder cancer cell line which bearing wild type p53, this is different from previous studies which done on neuron cells and fibroblast cells, which is not cancerous and the effect of wild type p53 may cause the cells to react differently in cell culture system.
Western blot result for loading control or house keeping gene beta actin was added to proved that experiment was carried out correctly.

2. The result of the article demonstrated more on the effect of the goniothalamin on the apoptosis induction on RT4 cancer cell so the title should be revised.
Answer: The aim of this study was to investigate the role of Seladin-1 in bladder cancer cells. From our literature review, we believe that Seladin-1 may involve in apoptosis pathway, which may function in formation of bladder cancer. In addition, goniothalamin was shown as potent apoptotic inducer on variety of cancer cell line, however the cytotoxicity of goniothalamin on bladder cancer was never demonstrated before. Thus in this study goniothalamin was used to access the cytotoxicity on bladder cancer follow by determination of the involvement of Seladin-1. So we would like to maintain the original title, as we feel that both are equally important in this study.

Thank you for your kind review and comments.