Author's response to reviews

Title: Cucurbitacin B inhibits human breast cancer cell proliferation through disruption of microtubule polymerization and nucleophosmin/B23 translocation

Authors:

Suwit Duangmano (sduangmano@hotmail.com)
Phorntip Sae-lim (s_phorntip@yahoo.com)
Apichart Suksamrarn (s_apichart@ru.ac.th)
Frederick E Domann (frederick-domann@uiowa.edu)
Pimpicha Patmasiriwat (mtppm@mahidol.ac.th)

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Author's response to reviews: see over
Dear BMC Complementary & Alternative Medicine Editors,

We would like to submit our revision manuscript entitled “Cucurbitacin B inhibits human breast cancer cells proliferation through disruption of microtubule polymerization and nucleophosmin/B23 translocation” for being published in BMC Complementary & Alternative Medicine.

Our thanks to the reviewers and the subject editor for thoughtful critiques of our manuscript; we have adopted all of the suggestions in response to reviewer’s comment as shown in the manuscript, including word collection and highlight the changes we have made.

We think that the manuscript has been greatly improved by these revisions and we hope that you will now find it suitable for publication in BMC Complementary & Alternative Medicine.

Best regards,

Pimpicha Patmasiriwat, Ph.D.
Associate Professor
Medical Science and Technology Building (Lab 704),
Mahidol University at Salaya Main Campus, Tumbon.Salaya, Nakhorn Pathom, 73170, Thailand.
Tel: 662-441-4370 ext 2703
Fax: 662-441-4380
E-mail: mpbmpm@mahidol.ac.th
Response to reviewer

We have adopted all of the suggestions in response to reviewer’s comment as shown in the manuscript, including word collection and highlight the changes we have made

Reviewer: Slimane AIT-SI-ALI

Response1:
-Cucurbitacins, an oxygenated tetracyclic triterpenoid compound extracted from cucurbitaceae plants
-Cucurbitacins, oxygenated tetracyclic triterpenoid compounds extracted from cucurbitaceae plants
(in Abstract-background)

Response2:
-Our findings suggest that cucurbitacin B may inhibit the proliferation of human breast cancer cells through disruption of the microtubule network and downregulation of c-Myc and nucleophosmin/B23 as well as the perturbation in nucleophosmin/B23 trafficking from the nucleolus to nucleoplasm, resulting in G2/M arrest
-Our findings suggest that cucurbitacin B may inhibit the proliferation of human breast cancer cells through disruption of the microtubule network and downregulation of c-Myc and nucleophosmin/B23 as well as alters trafficking of nucleophosmin/B23 from the nucleolus to nucleoplasm, resulting in G2/M arrest
(in Abstract-conclusion)

Response2:
-Breast cancer is the most common cancer and the leading cause of cancer deaths among women in all races
-Breast cancer is the most common cancer and the leading causes of cancer deaths among women in all races
(in Background-paragraph1)

Response3:
-However, undesired side effects may occur during breast cancer treatment
-However, undesired side effects may be occurred during breast cancer treatment
(in Background-paragraph1-line7)

Response4:
-Microtubule, a component of the cytoskeleton, is a dynamic heterodimer of α-tubulin and β-tubulin subunit
-Microtubule, a component of the cytoskeleton, is dynamic heterodimer of α-tubulin and β-tubulin subunit
(in Background-paragraph3)

Response5:
- We now replace the sentence “Cells in exponential growth were used” with the following sentence “Cells used in the study were in the exponentiel phase”
(in Method-paragraph1)
Response 6:
-Briefly, the fruits of C. cucumerina, after ripening and became dry naturally, were collected from Nakhon Sawan province, Thailand
(in Method-paragraph1)

Response 7:
-The polymerization of tubulin was measured at each consecutive minute during a period of 30 minutes at 37°C
(in Method-paragraph3)

Response 8:
-Protein extracts were separated according to their isoelcetric point (pI) followed by their molecular weight
(in Method-paragraph6)

Response 9:
-Goat anti-rabbit IgG and goat anti-mouse IgG (BD Transduction Laboratories, San Diego, CA) were used as secondary antibodies against all primary antibodies
(in Method-paragraph8)

Response 10:
-The cover slip was mounted with 50% glycerol in PBS
(in Method-paragraph9)

Response 11:
-Cells (1x10^5 cells/well) were seeded on cover slips in a 6-well plate overnight as mentioned above
(in Method-paragraph10)
Reviewer: Gail Fraizer

Minor Typos:
In discussion 2nd paragraph Pg 13 refers to staining showing disruption of microtubules as Figure 6a, whereas Figure 8a is correct citation. Conversely 1st paragraph Pg 14 refers to translocation of NPM as Figure 8, whereas Figure 6 is correct.

Response:
We now changed Figure 6a with Figure 8 in discussion 2nd paragraph Pg 13 and Figure 8 with Figure 6a in 1st paragraph Pg 14.
Reviewer: Pratibha Nerurkar

1. Quality assurance criteria are critical for natural products, for reproducible results. Provide information about the method of authentication of the raw material and “voucher specimen” preparations for dried fruit fibers that verifies and confirms the identity and nature of the plant material used, including the stage of ripeness of the fruits and the drying conditions.

Response: The manuscript has been modified according to kind query from the reviewer.

2. Define what is “dried fruit fibers”

Response: “fruit fibers” are the fiber in the fruits of plants especially those in the Trichosanthes species. The fibers become more apparent and hardened when the fruits become ripening. After ripening, the fruits are slowly getting dry and only the outer part (peels), the fibers and the seeds are the fruit contents. Fruit fibers occupy most of the volume of the dry fruits.

3. What is the stability of Cucurbitacin B?

Response: This agent is stable. The stock solution had been used for testing IC50 of the cell lines studied for sequentially every 6 months (we evaluated for 3 years). The stability results were no changed from the beginning throughout the evaluation period. We convince that this agent is stable for 3 years or more. We also used the same lot of cucurbitacin B solution for all experiments. In addition, the cucurbitacin B used were purified from exactly the same lot of the fruits of T. cucumerina.

4. What is NPM? Is it nucleophosmin? It is not mentioned anywhere, including abbreviations. Keep terminology consistent use one or the other.

Response: Yes, NPM is alternative name of nucleophosmin. As the reviewer’s suggestion, we now change all the word “NPM” to nucleophosmin/B23 so that readers will not be confused with the different terminology.

5. Clarify the rationale for using 2.5 and 5 μM for studies other than MTT assay.

Response: Since the IC50 of cucurbitacin B for the two cell lines studied, MCF-7 and MDA-MB-231 are 4.12 μM and 3.68 μM, respectively (mentioned in the first paragraph of ‘result’), the reason of using concentrations 2.5 and 5 μM because these concentrations are near the IC50 concentration of both cell lines which are suitable to evaluate the cellular biological changes within the cells. Cucurbitacin B concentration higher than 5 μM could cause much of cell death due to toxicity and concentration lower than 2.5 μM might be too mild to evaluate any change within cells.
6. For Figure 5B, provide densitometry scans providing a ration for gene of interest: house keeping gene, GAPDH. Tubulin protein appears to have significantly increased in MDA-MB-231 cells at 5 μM. Provide a rationale and explanation for this observation.

**Response:** Authors do not actually understand this question. In fact, in Fig 5 A, we compared gene expressions of cucurbitacin B treated cells versus the untreated cells. The obvious reductions upon the treatment were seen in expression of nucleophosmin and c-Myc. Tubulin and STAT3 expressions in MDA-MB-231 were not significant at 5 μM. In Fig 5B, the observation of proteins levels by western blots revealed the parallel results. The supplement data of western blot-densitometry scan is provided as the reviewer's requested; to show that there is not significantly increased in tubulin protein.

7. Besides nucleophosmin/B23, table 1 shows differential regulation of chaperonin, prolyl 4-hydroxylase and HsP70 by CuB. However, they are not followed further either in the data or discussion. Either delete the Table 1 and the corresponding experiment or provide discussion for the observed effects.

**Response:** The effect of cucurbitacin B on the changing levels of some cellular proteins is quite useful information for the readers of this area. The authors want to keep this table in this paper. We agree with the reviewer that some more explanation for table 1 must be added. We therefore add this
paragraph in the results (page 10, line 14). “As shown in Table 1, the result revealed that among the three proteins whose expression was up-regulated, two of them, Hsp70 and β-subunit of prolyl 4-hydroxylase, belong to the heat shock protein class which could be up-regulated during stress conditions [22, 23]. This might include the stress which induced by cucurbitacin B”.

8. CuB induces apoptosis in MCF-7 and MDA-MB-231 cells. Were any apoptotic proteins differentially regulated in the 2D gel analysis?

**Response:** Since there are many small spots in 2D gel which hardly to be retrieved for further analysis. Also, with some other limitations, we chose only the 4 most obviously altered spots from the 2D-PAGE. These peptide spots showed significant changes upon the CuB-treatment, comparing to the untreated cell. These 4 spots were well separated from other spots, making the spot collecting from gel the most reliable than others. By these limitation natures of this kind of experiment, we could not give data of apoptotic proteins from 2D gel analysis.
Reviewer: Gail Fraizer

Discretionary Revisions:
One weakness is the immunofluorescent images in Figure 6, showing the shift in location of Nucleophosmin/B23 from the nucleolus to nucleoplasm. For untreated cells very little NPM staining is observed and it is localized, unlike the staining in treated cells that is strong and diffusely spread through nucleoplasm. The conclusion that treatment is associated with altered trafficking of NPM seems sound, but a better image might show nucleolar staining better. Also since treatment seems to greatly increase expression detected by immunofluorescence, the author may want to discuss in light of the modest decrease in total protein levels shown by western analysis.

Response: In Figure 6, authors tried to focus the difference of nucleophosmin/B23 localization between cucurbitacin B untreated (mainly localize in nucleolus) and treated (mainly in nucleoplasm) MCF-7 and MDA-MB-231 cells. We appreciate reviewer’s suggestion of improving the image for nucleophosmin staining in the nucleolus. Therefore, we make the picture change by replacing the original images with the better images for nucleophosmin-nucleolus staining of the untreated (control) cells. They are now very much better.

The localization of nucleophosmin in nucleoplasm after the treatment is not necessarily due to increased its expression. We used Alexa568 (as fluorochrome ) conjugated to secondary antibody in order to efficiently detected the nucleophosmin signals. This type of fluorochrome gave satisfied strong fluorescent intensity. This nucleophosmin localization study can not be compared with the western analysis for the protein level, because the incubation period for these two experiments were much different; only 20 mins cu.B treatment for the localization study and 2 hrs treatment for the western analysis. Therefore, the reliable protein levels after the treatment are from the western analysis.

Major Revisions:
Another weakness is the lack of in vitro evidence supporting disruption of microtubular polymerization in Figure 8. The author’s conclusion that this indicates a lack of direct interaction seems sound. However the immunofluorescent images showing alpha tubulin staining are difficult to interpret, perhaps a higher magnification would better reveal changes in staining patterns in MDA-MC-231 cells. The authors may want to discuss the relative increase in alpha tubulin staining in the treated MCF-7 cells.

Response: In Fig 8 A, we demonstrated the normal polymerized-network distribution of microtubules throughout the cell in untreated MCF-7 and MDA-MC-231 cells, while in the treated cells we showed the ‘undistributed or ‘aggregated ’ image of tubulin. We do believe that this change of tubulin organization from microtubule network distribution to ‘aggregated’ signals of alpha tubulin could be attributed to reduced nucleophosmin from cucurbitacin B treatment , as mentioned in the discussion part (end of paragraph 4 of discussion) “..... Cells lacking nucleophosmin/B23 exhibited a disrupted microtubule network with a lower level of polymerized tubulin. Therefore, nucleophosmin/B23 plays a protective role in microtubule polymerization. Cucurbitacin B could down-regulate the expression of nucleophosmin/B23,
leading to disrupt the polymerization of mitotic spindle and arrest the cell cycle at G2/M phase and induce apoptosis.”

We accept that the in vitro study did not show any evidence supporting disruption of microtubular polymerization (in Figure 8). Anyway, microtubule polymerization process could be regulated indirectly by nucleophosmin. Factors directly control microtubule organization under drug treatment could be a topic of the future cucurbitacin B study.

**Minor Essential Revisions:**
1. Figure legend for western described results as the average of three experiments, but quantitation is not shown.

**Response:** The sentence “Results shown in A are the average of three independent experiments. * P < 0.05 (treated vs untreated control) ” are described for Fig 5 A (real-time RT-PCR) and not for Fig 5 B (western).

2. Last sentence in results describing Figure 5 B is unclear.

**Response:** We now revised the sentence in results (in result- paragraph 2) describing Figure 5B as below
“The protein expressions of nucleophosmin/B23 and c-Myc were also decreased in dose-dependent manner but STAT3 and tubulin were not significantly different from the control in both cell lines, as demonstrated in Figure 5B”

3. Typos in discussion:
3.1 “MDA-MB-231 was choosed for study” should be “chosen”

**Response:** We now changed choosed to chosen

3.2 “Seldom-filamentous normal microtubule structure could be seen” is unclear

**Response:** We now replace the sentence “seldom-filamentous normal microtubule structure could be seen” with the following sentence “normal filamentous organization of microtubules were scarcely observed (in discussion- paragraph 3)

3.3 “The addition of paclitaxel was shown to increase the polymeric intensity of the reaction which vincristine was shown to decrease this polymerization.” is unclear

**Response:** Adding paclitaxel into the reaction clearly demonstrated the increase in tubulin polymerization, whereas adding vincristine provided the opposite result as the drug decrease tubulin polymeric intensity. (in discussion-paragraph 3)
Reviewer: Slimane AIT-SI-ALI

Major Compulsory Revisions:
1. Authors cited the HBL-100 cell line used as a control “normal” cell line in a previous work (not used in this study). HBL-100 cell line is controversial since it acquires easily a tumorigenic phenotype. Thus, this cell line is not indicated as a control one. Authors must use HMEC or at least MCF10A cell lines as negative control cells.

Response: To our knowledge, MCF-10A and HMEC can display epigenetic inactivation of the key cell cycle regulatory genes, notably p16 and others, and therefore the use of these two cell lines as control could be also questionable. Also, we could not get availability of the other non-tumorigenic cell types for using as negative control better than HBL-100. The use of newly purchased, well–controlled HBL-100 has been acceptable as non-tumorigenic negative control cells in many published articles and this cell serve as an adequate control against the “tumorigenic” MCF-7 and MDA-MB-231. However, we will try the MCF-10A for our future work and compare with the use of HBL-100. Unfortunately, we could not make the change as reviewer comment this time.

2. Authors claim that cells lacking NPM have disturbed microtubule network with a lower level of tubulin polymerization. However, authors did not actually demonstrate this even in their in vitro test (Figure 8B). Consequently, authors cannot make a link between NPM and microtubule polymerization. Authors are encouraged to study another actor of microtubule network such as actin polymerization which can be informative.

Response: The result in Figure 8B show that cucurbitacin B did not act directly on microtubule polymerization in vitro but we suggested that cucurbitacin B could inhibit microtubule polymerization in vivo (cell system). Nucleophosmin is not the only factor that involves polymerization of microtubules. There are many regulatory factors that affect the microtubules polymerization such as survivin. Previous study reported that cucurbitacin B downregulate the expression of surviving and hence this agent might indirectly disrupt the polymerization of microtubule via affecting some regulatory proteins that function on microtubule assembly (such as survivin).

3. Figure 4B: what do you mean by % of apoptosis (which quadrants have been selected?)

Response: We selected Quadrant 2 (right upper) and Q4 (right lower) for calculating the % apoptosis because Q4 is the area of early apoptotic cell and Q2 is the area of late apoptotic cell and necrotic cell. % of apoptosis is the percentage of apoptotic cells that calculated by combining the percentage of cells in Q2 and Q4.

4. Figure 3A: explain the increase in G0/G1 sub-population for the cell line MDA-MB231.

Response: Increase of cell in sub-G0/G1 phase shown in DNA content histogram is the indicative of DNA fragmentation and apoptosis. Apoptosis was confirmed by Annexin V-FITC as shown in Figure 4. the result indicated that cucurbitacin B could induced apoptosis in MDA-MB-231 cell line.
5. According to 2D gel, RT-PCR and western blot analysis, authors notice a decrease in NPM levels (both at RNA and protein levels) in Cucurbitacin B-treated cells (Figure 5 A and B), while the immunofluorescent NPM staining illustrate a modification in the distribution profile (from nucleoli to nucleoplasm, Figure 6). Authors should make a coherent link between these two results.

Response: For the nucleophosmin immunofluorescent study, cucurbitacin B could induce translocation of nucleophosmin from nucleolus to nucleoplasm within as fast as 20 minutes. However, the 2D-gel, RT-PCR and western blot analysis, the level of nucleophosmin decreased markedly after cucurbitacin B treatment for 48 hrs. We believe that the observed translocation from nucleolus to nucleoplasm and the decreased gene expression as well as protein level of nucleophosmin could be linked to each other. (1) Since one function of nucleophosmin in nucleolus is to regulate the ribosome biosynthesis, reduction of this protein in nucleolus could reduce amount of ribosomes and consequently reduced synthesis of all proteins (non-specific) (2) We do not think that the translocation of nucleophosmin as early as 20 mins after CuB treatment is specifically involved or related directly with the lower nucleophosmin protein expression levels studied at 48 hrs. Alternatively, the translocation of nucleophosmin might involve apoptotic process because dissociation of nucleophosmin from nucleoli is the initial step of nucleolar segregation. This process renders the nucleolar DNA vulnerable to attack by nuclease. (3) Down-regulation (reduced nucleophosmin protein level) during cucurbitacin B treatment could be a consequence of the decreased of c-Myc level due to CuB , and decreased binding of c-Myc to nucleophosmin promoter could therefore lead to reduced nucleophosmin expression level. [c-Myc is known to regulate the expression of nucleophosmin]

6. Authors should show the 2D gel, this can be informative for the readers.

Response: We will add the 2D gel results in the supplemental data since the 2D gel has not commonly appeared in research articles published presently.

Minor Essential Revisions:
Authors must choose either nucleophosmin/B23 or the abbreviation NPM, in the latter case you should add it to the abbreviation list.

Response: We agree and choose the term “nucleophosmin/B23” throughout the article.