Author's response to reviews

Title: A phenolic ester from Aglaia loheri leaves reveals cytotoxicity towards sensitive and multidrug-resistant cancer cells

Authors:

Else G Dapat (elsegdapat40@gmail.com)
Sonia D Jacinto (soniajac2008@gmail.com)
Thomas Efferth (efferth@uni-mainz.de)

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Author's response to reviews: see over
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Letter to the Reviewer: Vivian M. Rumjanek

Dear Madam Rumjanek:

This is to respectfully submit my answers to your comments regarding my paper entitled: “A phenolic ester from Aglaia loheri leaves reveals cytotoxicity towards sensitive and multidrug-resistant cancer cells”.

Please find my answers below each question.

Sincerely,
Else G. Dapat

Reviewer's report:
Version: 1 Date: 16 February 2013

A phenolic ester from Aglaia loheri leaves reveals cytotoxicity towards sensitive and multidrug-resistant cancer cells by Dapat et al. The authors have studied the anti cancer effect of isolates from crude extracts of A. loheri. It was already known that a number of other compounds obtained from different Aglaia species were found to be cytotoxic to tumor cells, supporting the possibility that A.loheri should be investigated. The active fractions obtained from the extracts of A.loheri were selected by bioassay-guided isolation and the active principle was characterized by mass spectroscopy and NMR. The authors tested the isolated active component against a sensitive and a multidrug resistant cell lines, suggesting it as a promising new drug for further development to be used for MDR tumors. The problem of multidrug resistance is, presently, the major drawback of chemotherapy and many efforts are being invested in finding new drugs that are not substrates for MDR transporters. Despite the potential interest of the work the authors must address the criticisms before a decision can be reached. It is fundamental to distinguish an anti-tumor effect from a non-specific general toxicity.

Major compulsory revision-
It is necessary to clarify some points, related to the bioassays, in the Results section:

1- To be able to distinguish between non-specific cytotoxicity and an anti-tumor effect, it is necessary to perform a control using normal cells. It would be important to use normal peripheral blood mononuclear cells activated with a mitogen such as PHA to see if the effect is specific towards tumor cells.
When Maldi531.2[M+H]+ was assayed against non-cancer cell line (AA8) no toxicity was observed. When crude extract of A. loheri which yielded Maldi531.2[M+H]+ was tested against AA8 no toxicity was observed. It is to be noted that A. loheri is edible and used by indigenous people of Bataan, Philippines as staple vegetable.

AA8 is used in many studies as a basis to compare performance of cytotoxic samples between cancer and non-cancer cells. Still, AA8 is a murine cell line (as it is from Chinese hamster ovary); it is recommended that these results of Maldi531.2[M+H]+ against non-cancer cells be tested with non-cancer, human cell lines such as normal peripheral blood mononuclear cells.

Although it is important that the cytotoxic agent be determined to be specific only to cancer cells, it is an established fact that many of the presently used cancer chemotherapeutic agents are not specific against tumor/malignant cells only. They also affect fast dividing normal cells such as those in the blood, in the digestive tract and other organs leading to a number of side effects like vomiting, hair loss, and the like. Hence, a number of studies that seek to mitigate these effects on normal cells such as on drug delivery specifically targeting cancer cells are being actively explored. We can also suggest this as an offshoot of this study as part of the recommendation or future directions/follow up studies.

2- Figure 3 tries to establish that exposure to Maldi 531.2[M+H]+ induces cell death via mitochondria membrane depolarization. It is necessary to explain how, using a cell line resistant to doxorubicin, depolarization can be observed in the presence of doxo (used as a positive control!!). Unless an excessive concentration has been used, doxo should not have an effect. The concentration used as a positive control should be stated.

Answer: Pages 12, 33 & 34

5000ng/mL was only used in the maintenance of the cells’ drug resistance, the concentration used in the treatment (as positive control) was twice this concentration, thus doubled (10µg/mL); and cells have longer exposure to doxorubicin than CCCP (as per instruction from the manufacturer) because of the latter’s immediate effect on the intrinsic membrane potential).

3- Figure 4 states that the positive control for the induction of apoptosis was 5000ng/ml doxorubicin. Again an unrelated chemotherapeutic drug should have been used considering that the cells are resistant to doxorubicin (however, from the figure nearly 8% are necrotic and 13% apoptotic what is significant for a 24h
assay, if these are cells normally maintained in 5000ng/ml). On the other hand, significant apoptosis with Maldi 531.2[M+H]+ was only observed using extremely high concentrations (500nM- 1000nM)

Answer:

On the issue of using unrelated chemotherapeutic drug in the experiment, I used 10µM camptothecin alongside with 10µg/mL doxorubicin as one of my positive controls.

Considering the IC50 values of Maldi 531.2[M+H]+ in HCT116 (data not shown), CCRF-CEM and CEM/ADR5000 which are 3.9µg/mL, 0.02µM and 0.03µM respectively, the isolate could be considered a potential cytotoxic agent against cancer cells.

Concerning the right concentration to consider a compound as an anticancer drug: there is not fixed concentration. However, in our own investigations, we use 10 µg/ml as cutoff (Efferth et al., Molecular Cancer Therapeutics, 2008;7:152-161).

4- Maybe statistical significance should be stated in a clearer way.

Answer: Pages 33 and 34

Figure 3  Determination of mitochondrial membrane potential (ΔΨm) in multidrug-resistant CEM/ADR5000 cells after 24 and 48 h exposure to Maldi 531.2[M+H]+. Data are means±SEM of the three replicates of two independent experiments; P<0.05. Untreated cells served as negative control and 10µg/mL doxorubicin (doxo) and carbonyl cyanide 3-chlorophenyl hydrazone (CCCP)-treated cells as positive controls. Significant differences were indicated by letters. After 24 h treatment mitochondrial membrane potential (ΔΨm) of untreated cells (UT-S), indicated by subset “a” is significantly different from the CCCP and Maldi 531.2[M+H]+ treated cells as indicated by subset “b”. Values for CCCP and Maldi 531.2[M+H]+ treated cells don’t show significant differences from each other as belong to the same subset. “b”. Mitochondrial membrane potential (ΔΨm) of untreated cells (UT-S), indicated by subset x” is significantly different from the CCCP and Maldi 531.2[M+H]+ treated cells as indicated by subset “y” and “z” for 48 h.

Figure 4. Measurement of apoptosis induction by Maldi 531.2[M+H]+ in CEM/ADR5000 determined by incubation with annexin V. Data are means±SEM of three replicates of two independent experiments with reference to their controls: Untreated cells served as negative control; 10µM camptothecin (CAMPT) and 10µg/mL doxorubicin (DOXO) - treated cells as positive controls. Significant differences in apoptotic activity between treatments are indicated by various groups of subsets: Early - “abc”; late apoptosis - “mno” and dead cells – “xy”. Cells treated with 0.05µM Maldi 531.2[M+H]+ did not show significant apoptotic activity compared to negative control cells in all stages but significantly different from cells
treated with the positive controls and those cells treated with 0.5 and 1.0µM Maldi 531.2[M+H]^+ (P<0.05). However, early apoptosis in cells treated with 0.05µM Maldi 531.2[M+H]^+ is not significantly different from early apoptosis in cells treated with 1.0µM but significantly different from 0.5µM Maldi 531.2[M+H]^+ treated cells. Bars of same subsets are not significantly different from each other.

Minor essential revisions-

1- Legend of Fig.2. In the legend it should be stated that the measurement was performed at 72h, to allow comparison with the other assays.

**Answer: Page 33**

**Figure 2** The effect of Maldi 531.2[M+H]^+ on CCRF-CEM and CEM/ADR5000 cells determined by XTT assay after incubating the cells with the isolate for 72 hr. in a humidified environment. Values are means±SEM of six replicates each of two independent experiments.

2- Legend of Fig.3. What is the concentration of doxo used as a positive control in the assay, considering that the cell line used is resistant to 5000ng of doxorubicin?

**Answer: Page 33**

I have stated this in number 4 above

The letters used to indicate significance are very confusing. In my copy of the manuscript they seemed to be misplaced in the figure.

**Answer:**

Please see revised Figures in separate files

*Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that I have no competing interests*