Author's response to reviews

Title: Citrus auraptene suppresses cyclin D1 and significantly delays N-methyl nitrosourea (MNU) induced mammary carcinogenesis in female Sprague-Dawley rats

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Author's response to reviews: see over
June 8, 2009

Dear Dr. Sabina Alam,
Scientific Editor
BioMed Central

Re: MS: 1790574927265880

Thank you for considering our manuscript, “Citrus auraptene suppresses cyclin D1 and significantly delays N-methyl nitrosourea (MNU) induced mammary carcinogenesis in female Sprague-Dawley rats.” We appreciate the referees’ comments and have revised the manuscript according to their recommendations.

Comments to Referee-1 Dr. Takuji Tanaka

1. The referee suggested to delete “(MNU)” from the title. The title has been changed accordingly.

2. The referee suggested to provide rationale for the dose-selection of auraptene (200 and 500 ppm). The manuscript has been revised to include the rationale for auraptene’s dose selection and references to previous studies.

3. The referee suggested replacing “immunohistochemical” with “histopathological” in line -7 from bottom of the page-8. The change has been made to the manuscript.

4. The referee suggested to briefly describe the criteria for diagnosis of experimental mammary tumors in ‘Histopathology’ under Materials and Methods section with citing a reference. We have included a brief description of histopathological classification of mammary tumors under ‘Histopathology’ referring to the paper by Russo et al. that describes the histopathology of mammary tumors in rats and compares with that in humans. The histopathology of the rat tumors were analyzed by our pathologist Dr.Marcelo Aldaz who is an expert in rodent mammary tumor histopathology. According to Dr.Aldaz, the “Lactating Adenoma” is a benign tumor with milk like substance filling the lumen. To answer the question of the referee whether we found any fibroadenomas in the rats, we didn’t find any fibroadenomas in the MNU administered rats.

5. The referee recommended replacing “ODC” with “ornithine decarboxylase (ODC)” in line-10 of page-15. The change has been made in the revised manuscript.
6. The referee recommended replacing “ornithine decarboxylase (ODC)” with “ODC” in line-8 of page-17. The change has been made in the revised manuscript.

7. The referee recommended to delete “(TMA)” in line 6 from the bottom, page -17. The word (TMA) has been deleted.

8. The referee recommended reducing the number of figures by combining some figures. We have combined the figures as follows: Figure 4 is figure-1 in the revised manuscript. Figures 1, 2 and 3 have been combined as figure 2a, 2b and 2c. Figures 5, 6 and 8 have been combined as figure 3a, 3b and 3c. The figures 7, 9 and 10 are figures 4, 6 and 5 in the revised manuscript.

Comments to Referee-2: Dr. Gallo Daniela

Major compulsory revisions:

1. The referee suggested many changes to properly present the data and discuss and to improve the quality of the paper. The referee has correctly pointed out the lack of explanation of mechanisms of chemoprevention and removing irrelevant parts under ‘Discussions’, lack of focus on the actual results in the Discussion section, the need for clarifying the data analyzed in page-9 about rat body weight, improving writing style, removing colloquialism and typos. The manuscript has been revised by including the different possible mechanism of action of auraptene in our study. We have included references from previous studies with auraptene that show its effect on Phase-II metabolizing enzymes. The irrelevant paragraph for the ‘Discussions’ has been removed. Improvement has been made in the language by removing typos and colloquialism and by clarification has been given about the data analysis of rat body weight.

2. The referee questioned why we did not analyze the tumors in the auraptene 500 ppm group for auraptene concentration and why we chose different sample sizes for HPLC analysis. The referee’s observation is correct. There were not enough samples to analyze the concentration of auraptene in the auraptene 500 ppm group as majority of samples were used for western blot analysis. Since the samples were scarce we had different sample sizes for HPLC analysis.

3. The referee stated that since auraptene concentration was measured in only 2 samples, mean ± SE cannot be used and therefore it should be removed from the abstract. The referee is right. The value has been revised to indicate mean ± Range and this value has been removed from the abstract.

4. The referee stated that it was not clear why the tumor data was analyzed at both week 16 and 18. The manuscript has been revised to include the fact that the tumor incidence was measured by palpation all through the study and statistical analysis performed every week. We have found significant changes in incidence in Auraptene 500 ppm group only at week16.

5. The referee suggested to report the relevant tumor numbers to support our statement that auraptene 500 ppm group had significantly lesser number of tumors when compared to MNU only group. The relevant tumor numbers have been included in the revised manuscript.
Minor essential revisions:

1. The referee suggested substituting “delay” with “latency” in Abstract, Page-2 “Dietary effects of auraptene on tumor incidence….” The word “delay” has been replaced with “latency”.

2. The referee suggested substituting “formation” with “development” in Abstract, Page-3 and the substitution has been made.

3. The referee advised to include the details of the cell proliferation assay in relation to MCF-7 cells in “Materials and Methods”, Page-6. The details of the cell proliferation assay in MCF-7 cells have been included in the manuscript.

4. The referee suggested to arrange the figures in the order they are discussed in the manuscript. The figures have been arranged in the order in which they are described.

5. The referee asked for clarification of the membrane used in “Materials and Methods” Page-9. We used PVDF membrane with a pore size 0.45 µm and the corresponding clarification has been made in the manuscript.

6. The referee states that in the “Materials and Methods” section, Page -9, the Least Significance Difference (LSD) Test has been used while there are other post-hoc multiple comparison tests which are generally used. The referee is correct that the LSD test emphasizes sensitivity as opposed to some of the more conservative tests. The manuscript has been revised using the Tukey test results.

7. The reviewer has pointed out that the data in figures in figures 9 and 10 did not match in the results section and also asked to rectify the relevant legends. The manuscript has been revised to include these changes.

8. The referee suggested to properly report the values on the X-axis in figure-9. The values on X-axis in figure-9 have been properly modified.

Discretionary Revision:

1. The referee suggested removing figure 4. We have combined some of the figures to reduce the total number of figures. The figure-4 is now figure-1 in the revised manuscript.

2. The referee asked for clarification for using 4-20% gel for western blot experiment. To conserve the samples used in the experiments, we used the 4-20% gel to analyze the changes in proteins with cyclin D1 (36 KDa), S6 Kinase and p-S6Kinase (70KDa) with respect to actin (45KDa). However we couldn’t get the S6 Kinase and p-S6 Kinase antibodies to work with our rat tumor samples. We are currently using cell culture models to identify more targets and mechanisms of action of auraptene in human breast cancer cells.

3. The referee suggested to remove the paper by Yin et al. (# 26 in the manuscript before review) and add details of procedures used for protein extraction. The manuscript has been revised to include these changes.
Major compulsory revisions:

1. The referee pointed out that the subheading under Results section regarding auraptene and tumor incidence did not agree with the data shown. The reviewer is correct in pointing out the lack of explanation. We have provided explanation for this comment by including the fact that the tumor incidence before sacrificing the animals (at week 18) was measured by palpation of the animals throughout the study for 18 weeks. We did the statistical analysis on the palpation data every week and only at week 16 we saw that auraptene 500 ppm had a significant effect on tumor incidence. However, at the end of the study (18 weeks), there was no significant effect on tumor incidence. Therefore we auraptene was only effective against tumor incidence at a certain time period in the study. The subheading has been therefore revised accordingly.

2. The referee asked for clarification about the effect of auraptene on the histopathology of tumors. The aim of histopathological analysis was to verify the malignancy of the tumors which we palpated during the study. We would like to confirm that there was no change in the histopathology of tumors after auraptene administration. Broadly the tumors were classified into malignant and non-malignant. The malignant tumors were carcinomas while the benign tumors were adenomas. We have mentioned in the manuscript that the malignant carcinomas were of different types such as comedo, papillary etc. which have human counterparts. We have provided the reference Russo et al. which describes the mammary tumors in rats and compares them to that in humans. In order to avoid confusion the subheading has been changed to “histopathology of mammary tumors.” The referee asked whether there were any differences in histopathology status of the tumors when harvested at 16 weeks vs. 18 weeks. The tumors were harvested only at 18 weeks which is at the end of the study. Therefore, the histopathology of tumors at 16 weeks was not analyzed.

3. The referee asked for clarification about the cyclin D1 bands in the rat mammary tissues. We agree with the reviewer that there were two distinct bands for cyclin D1 in rat mammary tissues where as there was only one in mammary epithelial cells. We have used the same aliquot of MCF-7 cells treated with IGF-1 as positive control for cyclin D1 in multiple blots. So the cyclin D1 bands in rat mammary tumors corresponding to the molecular weight of cyclin D1 band in MCF-7 cells (the lower band) were quantified. We have normalized the cyclin D1 bands in rat mammary tissues to that in the MCF-7 cells treated with IGF-1 to account for the variability in running different blots.

4. The referee has asked whether we could relate the tumor protective effects of auraptene after MNU-administration to developmental changes in mammary gland structure and/or differentiation status prior to MNU-exposure. We agree with the reviewer that the differentiation status of rat mammary glands prior to carcinogen insult can be considered as predictive of mammary tumor protection of dietary factors. It is well established that early pregnancy is a protective factor against breast cancer. However, in this study we were looking at the chemoprotective effects of auraptene after dietary administration on 3 parameters-tumor incidence, multiplicity and latency. This is the first time that auraptene
has been tested in a mammary cancer model. Since this compound has shown encouraging results further studies need to be done to identify the various aspects of auraptene’s protective effects including the developmental status of the mammary glands.

5. The referee stated that our conclusion of reduction of cell proliferation by cyclin D1 suppression as a direct effect may not as direct and the referee suggested us to consider flow cytometry to demonstrate cell cycle arrest at Go/G1 stage of cell cycle. The reviewer is correct. Our future experiments will be on the mechanism of action of auraptene and we would like to present the results of those studies as a new paper focusing only on the mechanism of action of auraptene which will include the flow cytometry data.

Minor essential revisions:

1. The referee suggested including the auraptene levels in normal mammary tissues. The abstract has been revised as per the suggestion.

2. The referee suggested to correct the statement regarding cyclin D1 expression under Results section, second paragraph, last sentence. In the Figure 2-C, cyclin D1 is almost completely lost with auraptene treatment in the representative figure show in the manuscript, where as in the other blots there was no complete loss of cyclin D1. The overall effect of auraptene in MCF-7 cells was in suppressing cyclin D1 by 40% of the control.

3. The referee suggested to correct the figure number 10 to figure number 9. The correction has been made in the revised manuscript.

4. The referee suggested to provide the data to support the statement under Discussion that “There was no change in the concentration of auraptene………those that did not develop tumors.” Since the number of samples is not enough to provide a statistically significant result, we have removed the above sentence from the revised manuscript.

We thank the referees for the extensive reviews and suggestions. We thank you again for considering our manuscript. Please let me know if any other revisions need to be made.

Sincerely,

[Signature]

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