Author’s response to reviews

Title: Up-Regulated MicroRNA-181a Induces Carcinogenesis in Hepatitis B Virus-Related Hepatocellular Carcinoma by Targeting E2F5

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Version: 3
Date: 16 December 2013

Author’s response to reviews: see over
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Version: 3
Date: 6 December 2013

Author’s response to reviews: see over
Author's response to reviews

Title: Selenosemicarbazone complex with copper efficiently downregulates Heat Shock Protein 90 and its client proteins in cancer cells

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Version: 3 Date: 13 October 2013

Author's response to reviews: see over
Response to the Editor’s Comments

1. Please copyedit your manuscript: Further consideration of your manuscript is conditional on improvement of the English used - please bear in mind that as we are a free-access publisher, we cannot bear the costs of copyediting English ourselves. Please ensure particular attention is paid to the abstract. You should have a native English speaking colleague help you with this, if possible, or you may need to use a professional language editing service.

   We would like to thank the editor’s suggestion addressing to the English improvement. We asked a native English speaking colleague for help. He went through our manuscript and corrected many improper expressions to make it better.

2. Please also ensure that your revised manuscript conforms to the journal style (http://www.biomedcentral.com/info/ifora/medicine_journals). It is important that your files are correctly formatted.

   We would like to thank the editor for pointing this out. We revised manuscript format according to guideline of the journal requirements.
Response to the Reviewers’ Comments

Referee #1 (Remarks to the Author): 2113976461105020.pdf

Major Compulsory Revisions

Based on the above comments, two reasonable questions arise:

1) What are the differences between thio- and seleno-semicarbazone compounds in terms of biologic activity, making selenosemicarbazone derivatives important to be studied? The authors should add a comment on this issue (Introduction, page 3; and/or Discussion, page 11).

   We would like to thank the Reviewer’s suggestion. In the Background part (Page 3, the last paragraph), we introduced the role of selenium and selenosemicarbazones in cancer prevention and cell death.

2) The main cell killing effect of 2-24a/Cu seems to be due to ROS production by copper (Results, page 7; Discussion, page 11). But then, are Selenosemicarbazone compounds only used as vectors for copper? Or does 2-24a exhibit its cytotoxic effects due to additional inhibition of ribonucleotide reductase? No tests on possible ribonucleotide reductase inhibition were carried out in order to assess its input in cytotoxicity. The authors should test ribonucleotide reductase levels. In fact, they should already have some relative data from RNA sequencing, and a western blot would supplement the answer.

   We would like to thank the reviewer for pointing these out. Based on our current raw data, novel selenosemicarbazone compounds without copper can efficiently induced cell death in breast cancer cells at nmol level. These results suggest that selenosemicarbazone compounds could become a novel class of anti-cancer compounds. Since these data is quite preliminary, we wish we could dig deeper and maybe find stronger evidence in the future.

   Then, we examined whether the expression of ribonucleotide reductase was inhibited in 2-24a/Cu-treated cells. Although RNA seq results showed that the transcription of RRM2 was decreased by 0.39 (the value of RPKM decreased from 126.4 to 96.1) in 2-24a/Cu-treated cells compared with that in control cells, and the
transcription of RRM2B was decreased by 0.72 (the value of RPKM decreased from 35.7 to 21.6) in 2-24a/Cu-treated cells compared with that in control cells, the expression of RRM2 was increased in 2-24a/Cu-treated cells (Figure below, (A) U2os cells, (B) HeLa cells). These results indicated that 2-24a/Cu did not inhibit the expression of RRM2.

Since we don’t have the instrument for the detection of ribonucleotide reductase activity, we cannot provide more experimental evidence. We are sorry for that and wish there is chances to do it in the future.

3) In the Abstract (page 2) and the Introduction (page 3), the authors state that 2-24a induced cell-cycle arrest, but this has not been assessed in the present work. In order to assess this, they have to perform cell cycle analysis, using PI/FACS, very much like in the experiments they have carried out for viability testing, but present the percentages of treated and untreated cells in G1, S and G2 phases of the cell cycle. In addition, in the Results (page 8), the Discussion (page 11), and the caption of Figure 2, the authors mention that proliferation of U2os and HeLa cells was inhibited by 2-24a. Again, this has not being tested in the present work. If they wish to comment on cell proliferation they have to carry out the experiment described above.

We would like to thank the Reviewer for pointing it out. We analyzed the cell cycle in 2-24a or 2-24a/Cu-treated cells (Page 9, the last paragraph, Fig. 2). 2-24a induced G1 cycle arrest in U2os cells and HeLa cells, whereas copper complex 2-24a/Cu induced the increase of G2 cycle. These results indicated that 2-24a/Cu had a different effect on cell cycle of cancer cells compared with 2-24a.

4) The PI exclusion/FACS test (pages 5 and 7) is significant for providing information on cell cycle arrest, but not very accurate for the detection of cell death. MTTs would be more suitable here.

Regarding to the detection of cell death, we adopt the reviewer’s suggestion to
analyze the viability of cells by MTT (Page 9, the last second paragraph, Fig. 1). The results showed that 2-24a efficiently inhibited cellular viability in various cancer cells.

5) The authors have to provide a reason why the particular biologic system (U2os, HeLa cells) has been chosen (Materials and methods, page 5).

We would like to thank the Reviewer for pointing it out. As shown in Fig.1C, other cancer cell lines such as H1299, A549 were also sensitive to 2-24a/Cu. U2os and HeLa cells had similar IC\textsubscript{50}, so we choose them for further research.

6) Are Chinese Kun Ming mice nude? Please, provide some data on the mouse strain (Materials and Methods, page 7).

We would like to thank the Reviewer for pointing it out. Chinese Kun Ming mice are not nude mice. The Kunming (KM) mouse is an outbred stock derived from Swiss albino mice with a high heterogeneity of genes and is widely employed in studies on neuroscience, immunology, genetics and pharmacology in Chinese laboratories (Chen GH, Wang YJ, Zhang LQ, Zhou JN: Age- and sex-related disturbance in a battery of sensorimotor and cognitive tasks in Kunming mice. Physiology & behavior 2004, 83(3):531-541.).

7) Please, add a comment to discuss the absence of cleavage in Hsp90, and the presence of cleavage in Hsp70 (Results, page 9).

We would like to thank the Reviewer’s suggestion. We added the comment as the suggestion (Page 11, the third paragraph). Besides, we repeated our experiment for several times. There were two bands below Hsp70 in 2-24a/Cu-treated cells, however, similar bands were showed in the control cells (Fig. 4). We could not be sure that these bands were from the cleavage of Hsp70, so we decided to delete the comment on the presence of cleavage in Hsp70 (Page 11, the third paragraph).

Minor Essential Revisions

1) Adding one reference to the work of Andelkovic, Radulovic and colleagues would be a fair action (Results, page 7 or Discussion, page 11).
We would like to thank the Reviewer’s suggestion. We added the reference in the manuscript (Page 3, the third paragraph).

2) Hsp90 and client protein degradation due to ROS generation is no longer a surprise. Please, better use “interestingly” (or an equivalent) in the place of “surprisingly” (Results, page 9).

We would like to thank the Reviewer’s suggestion. We changed the expression “interestingly” instead of “surprisingly” (Page 11, the third paragraph).

3) In Figures 4A and 4B, the gels for Hsp70 should have run longer. At least, please, put arrows to distinguish Hsp70 from cleaved Hsp70 bands.

We would like to thank the Reviewer’s suggestion. We repeated our experiment again. Although there were obvious two bands below Hsp70 in 2-24a/Cu-treated cells, similar weaker bands were showed in the control cells. We were not sure that these bands were from the cleavage of Hsp70, so we deleted the comment on the presence of cleavage in Hsp70 (Page 11, the third paragraph, Fig. 4).

4) Although the language is acceptable, minor linguistic, grammatical and syntax errors exist throughout the manuscript and have to be corrected.

We would like to thank the Reviewer for pointing it out. We asked a native English speaking colleague for help. He went through our manuscript and polished expression to make the manuscript better.

Discretionary Revisions

1) Increase in observed Pim1 transcription levels could be explained by a putative counterbalancing mechanism induced by Pim1 protein degradation (Results, page 10).

We would like to thank the Reviewer’s suggestion. We added it as the suggestion (page 12, the first paragraph).

2) Transcriptome analysis for 2-24a-treated cells in comparison to 2-24a/Cu-treated ones would be very interesting (Tables 1 and 2).
We would like to thank the Reviewer’s suggestion. We wish we could finish it in our next manuscript.
Referee #2 (Remarks to the Author): 1788815610496935_comment.pdf

1) Introduction section

The authors do not explain the oxidative stress and regulation of hsp protein’s family relationship. It would be very interesting to try to rationalize the use of such complex in a study on the modulation of these proteins.

We would like to thank the Reviewer’s suggestion. We added some explicit content in the introduction part to be informative. (Page 3, the second paragraph):

For example, a citation on cleavage of Hsp90 by ROS generated by vitamin C and K3 in the introduction. So, we suggest a a possible explanation which bridge the oxidative stress and Hsp90 protein.

2) Method synthesis section

Which is copper/semicarbazone complex stoichiometry?

We would like to thank the Reviewer for pointing it out. 2-24a/Cu was freshly prepared by mixing parallel mol of 2-24a and CuCl₂, then diluted to the appropriate concentrations before treatment (Page 5, the second paragraph).

3) Results and Discussion

The RNA-seq analysis data show, as indicated for authors, a significant up-regulation of the metallothionein family genes as well as genes encoding hsp70 and hsp 40 proteins and to a lesser extent hsp90. Why do the authors have chosen to focus on hsp90? Some comments on this would be appreciated.

We would like to thank the Reviewer for pointing it out. As Hsp90 family proteins play an important role in the survival of cancer cells, we investigated whether Hsp90 proteins increased in 2-24a/Cu-treated cells (Page 11, the third paragraph).

Cellular data in Figure 4 must be quantified to mark the observed differences between the compound complexes and free and especially the different behavior in the expression of the hsp90 and hsp70? As the authors explained the weak up-regulation of the HSP90AA1 gene and the down-regulation of Hsp90 protein? In addition, data of the assay time could be included.
We would like to thank the Reviewer for pointing it out. Western blot experiments were redone as suggested (Fig. 4). Hsp90 protein was significantly decreased in 2-24a/Cu-treated U2os and HeLa cells in a dose-dependent manner, while 2-24a or Cu alone did not have the same effect (Fig. 4A and B). There was not obvious cleavage band of Hsp90 in 2-24a/Cu-treated cells (Page 11, the third paragraph). In addition, data of the assay time were included in Figure 4.

4) Finally other remarks to be considered
Sections of the manuscript contain major grammatical errors that makes it difficult for the reader to focus on the content of the paragraphs. The manuscript needs be edited to be grammatically correct before publication

We would like to thank the editor for pointing this out. We asked a native English speaking colleague for help. He went through our manuscript and polished many expression of our manuscript.

5) All references need to be verified and credit given accordingly, especially in the section introduction

We would like to thank the editor for pointing this out. We double checked all the references accordingly and make sure they are correct.
Referee #3 (Remarks to the Author): 1768390631050949_comment.pdf

I) Major Compulsory Revisions

1) Inside Abstract and Introduction section it is stated that novel selenosemicarbazone compound 2-24a induces cell cycle arrest. This is not stated, mentioned nor illustrated in main text body. There is no experiment inside results section depicting or illustrating cell cycle arrest. Cell viability assay is performed on two cell lines, but one can’t claim that demonstrated cytotoxicity is followed by cell cycle arrest. The experiment should be performed.

   We would like to thank the Reviewer for pointing it out. 2-24a induced G1 cycle arrest in U2os cells and HeLa cells, whereas copper complex 2-24a/Cu induced the increase of G2 cycle (Fig. 2). These results indicated that 2-24a/Cu had different effect on cell cycle of cancer cells compared with 2-24a. (Page 9, the last paragraph, Fig. 2).

2) Inside Materials and methods section the following is stated: “HeLa cells were pretreated with 4 mM NAC for 2 hours and then treated with different concentrations of 2-24a/Cu for analysis of cell viability”. According to the Results section, HeLa cells were treated with anti-oxidant NAC only in experiment dealing with ROS-inducing properties of the tested compounds. This should be clarified.

   We are sorry to make this mistake and delete it (Page 6, the second paragraph).

3) Inside Materials and methods section it is stated that Western blot analyses methods were described previously. Reference should be included.

   We are sorry to make this mistake and add the reference (Page 8, the second paragraph).

4) It is stated that OriginPro tool was used for statistical analyses, as an online statistical tool. However, it has to be outlined which particular statistical test was used?

   We would like to thank the Reviewer for pointing it out. The statistical significance of differences was assessed using the Student’s t test in OriginPro 7.5
5) Table 1 states “IPA analyses of U2os cells treated”, although inside Materials and methods section it is stated that cDNA was “libraried” and further sequenced from HeLa cells. This is confusing and needs to be clarified in more details.

   We are sorry to make this mistake and correct “U2os cells” instead of “HeLa cells”. (Page 7, the first paragraph)

6) Which is the rationale for using HeLa and U2os cells? Should the U2os/Cisplatin resistant cell line be used for comparison purposes, due to the cisplatin-based widespread anticancer and onco-thrapeutic activity?

   We would like to thank the Reviewer’s suggestion. Indeed, U2os/Cisplatin resistant cell line would be more suitable than HeLa cells, but we did not have U2os/Cisplatin resistant cell line. HeLa cell line is also resistant to cisplatin, so we choose it for experiments. We hope that we can use U2os/Cisplatin resistant cell line to test the effect of 2-24a/Cu in the future.

7) The discussion is short, and should deal with the results in more details. The authors claim that novel Copper complex exerts Hsp 90 and thus Pim1 and Akt degradation, as per Western blot results. This should be discussed in more details, i.e. Akt function in suppression of apoptosis, promotion of cell survival, as well as angiogenesis signaling. The claimed anti-cancer activity of the complex based on the Hsp90/Pim1/Akt degradation is loose, and should be further strengthened with experimental evidence, due to the fact that those proteins are upstream regulators in a variety of cellular processes.

   We would like to thank the Reviewer’s suggestion. The Discussion was rewrite in this version of the manuscript (Page 13-14).

8) Additional experiments on cell type exerted in treated cells (TUNEL assay, differential staining-microscopic assessment, FACS analysis of cell death) are suggested. Additional
experiments on exact molecular pathways exerted are suggested, based on canonical molecular pathways analyses obtained.

We would like to thank the Reviewer’s suggestion. Our preliminary results indicated that 2-24a/Cu did not induce apoptosis in U2os cells. More experiments need to done to confirm the type of cell death. We are planning to carry out in the follow up project.

9) Other literature data on selenosemicarbazone metal complexes activity should be taken in account. The following is stated inside Discussion section: “Little anti-cancer research is done on selenosemicarbazones, in which the Se atom is substituted for the S atom”. This is not correct and following references should be taken into account:


The authors in references listed, dealt with the anti-proliferative activity and detailed molecular mechanisms underlying anti-tumor and anti-angiogenic potential of the complexes.

We would greatly like to thank the Reviewer’s suggestion and the help for references. The Discussion was re-written in this version of our manuscript and the list the references mentioned above were corrected. (Page 13, the first paragraph)

10) It is stated that among synthesized selenosemicarbazone compounds (Fig.1), 2-24a has shown the “best activity in cancer cells”. This should be supported with experimental design and results obtained for all synthesized compounds. Which cytotoxicity/cell viability assay was used? Which cell lines were treated? Without these data, the statement is insufficient.

We would like to thank the Reviewer for pointing it out. We think it probably not suitable to say that “2-24a has shown the best activity in cancer cells” based on current limited results in the manuscript, so we delete other compounds in Fig.1A and improved the description (Page 9, the last second paragraph).

11) IC50 values are stated for 2-24a and Cu/2-24a. How were the IC50 values calculated? Which program was used?

We would like to thank the Reviewer for pointing it out. We deleted the description of IC50 in the manuscript. In addition, based on the supplement results of MTT in Fig.1, we can evaluate the cytotoxic effect of Cu/2-24a on the different cancer cells (Page 9, the last second paragraph).

II) Minor Essential Revisions
1) Inside Material and methods section, the following is stated for RNAseq: The 260/280 must above 1.8. Sentences like this are superficial and non-informative, and should be explained in more details or paraphrased throughout the manuscript.

We would like to thank the Reviewer for pointing it out. We revised the “Material and methods section” (Page 4-8). For example, we replaced the sentence mentioned above (“The 260/280 must above 1.8”) with “The quality of RNA is judged by the ratio of OD_{260}/OD_{280}, which must between 1.8 and 2.0.”.

2) The treatment that preceded RNA sequencing must be explained in more details. Which were the concentrations of Cu complex applied, which was the incubation time, why was particular concentration chosen etc.?

We would like to thank the Reviewer for pointing it out. We added the detail as follows: “Briefly, the cells were treated with 5 μM 2-24a/Cu or 0.1% DMSO for 8 h. Then total RNA was extracted from 2-24a/Cu-treated cells and the control cells by using the RNeasy mini Kit (Qiagen, 74104) according to manufacturer’s protocol, and then treated with DNase I (Qiagen, 79254) for 15 min at room temperature to remove residual genomic DNA.” (Page 7, the first paragraph). The reason for the particular concentration is that we wish to choose a time point with specific concentration and incubation time that without obvious cell death, although the cell treated with 2-24a/Cu died finally.

3) English spelling must be double-checked. Misuse of certain words and phrases should be avoided.

We would like to thank the editor for pointing this out. We asked a native English speaking colleague for help. He went through our manuscript and polished many expressions.
1) In general, the results shown are simple and clear. The major weakness of this study, however, is that the authors did not show the effect of copper alone without the selenosemicarbazone in most of the experiments. Therefore, it is not possible to discriminate if the observed effect of the treatment were induced by the selenosemicarbazone-copper complex, or just by selenosemicarbazone and copper additively/synergistically.

We would like to thank the Reviewer for pointing this out. Additional experiments were supplied, and results showed that it was 2-24a/Cu, but not 2-24a or copper alone, induced oxidative stress and down-regulated Hsp90 protein in cancer cells (Fig. 4).

2) The most surprising finding in this article may be that Hsp90 protein was decreased, whereas the Hsp90 gene expression was increased by the treatment. However, the authors did not show any data explaining this apparent discrepancy. It is important and essential to clarify the molecular mechanism of the reduction of Hsp90 protein level by the treatment.

We would like to thank the Reviewer for pointing these out. We are sorry that we did not elucidate the molecular mechanism of the reduction of Hsp90 protein level by 2-24a/Cu. As oxidative stress has been shown to induce Hsp90 cleavage in cancer cells, perhaps oxidative stress induced by copper complex induces degradation of Hsp90 in cancer cells, and the transcriptional increase of Hsp90 is to compensate the degradation of Hsp90 protein. Currently, we discussed the possibility in the Discussion part of the manuscript (Page 14, the first paragraph). This hypothesis will need more experimental evidence to prove for sure. We are planning for this in the future project.

3) In addition, it remains unclear if the cytotoxic effect of the compound was specific for cancer cells as compare to normal cells. Altogether, I believe that this manuscript requires a significant revision with additional experiments before publication in BMC Cancer.

We would like to thank the Reviewer for pointing these out. Based on our current raw data, novel selenosemicarbazone compounds without copper can
efficiently induced cell death in some types of cancer cells. For example, 2-24a can efficiently inhibited the viability of breast cancer cells at nmol level, while 2-24 at this concentration has no effect on normal cells (unpublished data). These results suggest that selenosemicarbazone compounds could be a novel class of anti-cancer compounds. We hope to publish these results on selective killing cancer cells of 2-24 in the future manuscript.

4) The points to be clarified or modified are described below.

Major Compulsory Points:

1. Figure 2A, Figure 2C, Figure 3, Figure 4, Figure 5: In these figures, there are no data showing the effect of Cu alone. Therefore, it is impossible to estimate whether the observed effect of the treatment were induced by the selenosemicarbazone-copper complex, or just by selenosemicarbazone and copper additively/synergistically.

   We would like to thank the reviewer for pointing this out. We did experiments as suggested (Fig. 1, 2, 3, 4).

2. Page 9, line 4 from the bottom: “Surprisingly, Hsp90 was significantly decreased”; Page 11, line 9: “perhaps oxidative stress induced by copper complex induces degradation of Hsp90” -> The authors should show experimental data indicating that Hsp90 degradation is induced by the drug treatment even when Hsp90 gene was upregulated.

   We would like to thank the Reviewer for pointing these out. We are sorry that we did not elucidate the molecular mechanism of the reduction of Hsp90 protein level by 2-24a/Cu. We discussed it in the Discussion: As oxidative stress has been shown to induce Hsp90 cleavage in cancer cells, a possible explanation is that oxidative stress induced by copper complex induces degradation of Hsp90 in cancer cells, and the transcriptional increase of Hsp90 is to compensate the degradation of Hsp90 protein. Further experiments are needed to testify this hypothesis. We hope we can provide more data regarding to this issue it in the future project.

3. Page 9, line 4 from the bottom: “was not significantly increased” -> The figure seems to show the increase in Hsp70 protein levels.
We would like to thank the reviewer for pointing this out. We repeated our experiment again. There were two bands below Hsp70 in 2-24a/Cu-treated cells, however, similar weaker bands were showed in the control cells too. We could not be sure that these bands were from the cleavage of Hsp70, so, we removed the comment on the presence of cleavage in Hsp70 (Page 11, the third paragraph).

5) Minor Points:

There are many punctuation errors in the text (for example, see page 4, 2nd paragraph, line 5 “thiosemicarbazide A”; line 8 “then removed,”; line 12 “reduced pressure.”; “silica gel of”). The authors should check the text more carefully.

Page 3, line 3: Reference 1 may not be an appropriate review article for Hsp90.

Page 3, line 7: Reference 90 should be 9.

Page 4, 2nd paragraph, line 3: “2.84(20 mmol)” -> 2.84 g (20 mmol)?

Page 5, line 5: “H2DCF” -> Indicate the full spelling of H2DCF.

Page 6, line 1-3 from the bottom: “To detect different expression levels ... Since differentially expressed genes ...” : These sentences are incomplete or broken.

Page 7, 2nd paragraph, line 1: “as described previously.” -> Show the reference.

Page 7, 3rd paragraph (Xenograft study) : This section should be in double-spacing lines.

Page 7, line 3 from the bottom: “cancer types [16]” -> Is this a right reference?

Page 14, Reference 11: This reference seems not appeared in the text.

We would greatly like to thank the Reviewer for pointing out these mistakes, and we have corrected as suggested.