Author's response to reviews

Title: 5-allyl-7-gen-difluoromethoxycyrys in enhances TRAIL-induced apoptosis in human lung carcinoma A549 cells

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Author's response to reviews: see over
Responses to the comments raised by Reviewer 1:

Reviewer: 1

I have reviewed the manuscript entitled “5-allyl-7-gen-difluoromethoxychrysin enhances TRAIL-induced apoptosis in human lung carcinoma A549 cells” by Xie et al. The authors have examined the interactive effects of 5-allyl-7-gen-difluoromethoxychrysin (AFMC) and TRAIL on DR5 expression, cell proliferation and apoptosis in A549 and WI-38 cells. AFMC sensitize human NSCLC A549 cells to TRAIL-mediated apoptosis. Combined treatment of A549 cells with AFMC and TRAIL significantly activated caspase-3, -8 and -9. The caspase-3 inhibitor zDEVD-fmk and the caspase-8 inhibitor zIETD-fmk blocked the apoptosis of A549 cells induced by co-treatment with AFMC and TRAIL. In addition, the treatment of A549 cells with AFMC significantly induced the expression of death receptor 5 (DR5). AFMC-mediated sensitization of A549 cells to TRAIL was efficiently reduced by administration of a blocking antibody or small interfering RNAs against DR5. In contrast, AFMC-mediated induction of DR5 expression was not observed in human embryo lung WI-38 cells, and AFMC did not sensitize WI-38 cells to TRAIL-induced apoptosis.

Comments to the Author

1. By looking at the data of DR5 western blot, it appears that there is only one band. Generally, there are 2-3 isoforms of DR5 that can be recognized by the antibody. It is not clear why the authors have seen only one isoform.
2. The data on DR4, DcR1 and DcR2 should also be provided. Are there any effects of drugs on these receptors.

3. It has been shown that the DR proteins can be sequestered in the cytoplasm and thus can not be seen on the surface and also may nor enhance the effects of TRAIL. The expression of DR4, DR5, DcR1 and DcR2 should be measured by the flowcytometry technique. The experiments should also be performed to determine whether it is sequestered in the cytoplasm.

4. The recent literatures on TRAIL sensitivity should be cited. The following articles should be included in the paper.


Dear reviewer:

We appreciate very much your careful review, constructive comments and kind corrections to our manuscript (MS ID: 1427164769499711.) We have revised the manuscript according to your comments and incorporated all corrections which were marked with red font in the revised version. The detailed point-by-point answers to your concerns are below.

1. By looking at the data of DR5 western blot, it appears that there is only one
band. Generally, there are 2-3 isoforms of DR5 that can be recognized by the antibody. It is not clear why the authors have seen only one isoform.

Re: Thanks for your call in question. In our experiment, we used the anit-human DR5 and DR4 monoclonal antibodies, but not polyclonal antibody, which we have explained in the reagents part of my manuscript. So, there was only one band in the data of DR5 western blot.

2. The data on DR4, DcR1 and DcR2 should also be provided. Are there any effects of drugs on these receptors.

Re: Thank you for the valuable suggestion. In our experiment, Western Blot has been used for testing the influence of AFMC on DR4, but no remarkable change. So the part has been omitted in our essay.

3. It has been shown that the DR proteins can be sequestered in the cytoplasm and thus can not be seen on the surface and also may nor enhance the effects of TRAIL. The expression of DR4, DR5, DcR1 and DcR2 should be measured by the flowcytometry technique. The experiments should also be performed to determine whether it is sequestered in the cytoplasm.

Re: Indeed, you opinion is very good, we should measured the expression of DR4 and
DR5 though the flowletometry technique. This was a omission in our preliminary experimental design. We have performed more experiments to perfect our manuscript. The results have been added in the revised manuscript (Figure 3C).


Re: The documents which you have recommended have great revelatory sense for our research and will further strengthen the convince of my essay. Thank you for your direction.

Responses to the comments raised by Reviewer 2:

Comments to the Author

In this manuscript authors reported that the derivative of Chrysin augmented TRAIL-induced apoptosis through upregulation of DR5. The data showing that AFMC enhanced TRAIL-induced apoptosis are interesting and convincing. However, studies of supporting DR5’s role in this combination treatment are too preliminary
and need to be improved.

1. In Figure 2C, the actual FACS data should be shown.

2. It is not known how authors pick DR5 as the primary target of AFMC action (Fig. 3), other potential targets influenced or not influenced by AFMC treatment should be shown and discussed.

3. To support that silencing DR5 would block AFMC plus TRAIL-induced apoptosis, the actual FACS data should show (Fig.4). Western blot analyses of showing cleavage of PARP and caspase-8 should be performed. To support the specificity of DR5 siRNA, the levels of DR4 should be measured.

4. Although authors claimed that AFMC did not increase the levels of DR5 and did not enhance TRAIL-induced apoptosis in WI-8 cells, WI-8 cells express certain content of DR5. It seems that DR5 upregulation-independent pathway is involved in the action of AFMC to enhance TRAIL-induced apoptosis in A549 cells. The levels of DR4 and DR5 as well as the signaling of death receptor-mediated apoptosis should be compared in both cell lines.

5. More cancer cell lines should be used to test the induction of DR5 by AFMC and enhancement of AFMC on TRAIL-induced apoptosis.

Dear reviewer:

We appreciate very much your careful review, constructive comments and kind corrections to our manuscript (MS ID: 1427164769499711.) We
have revised the manuscript according to your comments and incorporated all corrections which were marked with red font in the revised version. The detailed point-by-point answers to your concerns are below.

1. **In Figure 2C, the actual FACS data should be shown.**

   Re: Thank you for the valuable suggestion. The data of flow cytometry could be shown by statistical chart or typical image, we choose the style of statistical chart.

2. **It is not known how authors pick DR5 as the primary target of AFMC action (Fig. 3), other potential targets influenced or not influenced by AFMC treatment should be shown and discussed.**

   Re: In Figure 2, we confirmed that the apoptosis induced by AFMC and TRAIL co-treatment in A549 cells was dependent on Caspase-8 activation, while, Caspase-8 plays a central role in apoptosis mediated by the death receptors. So we pick DRs as the potential target of AFMC action. The effect of AFMC on DR4 and DR5 expression levels in A549 cells have been determined using flow cytometry(Figure 3C).

3. **To support that silencing DR5 would block AFMC plus TRAIL-induced...**
apoptosis, the actual FACS data should show (Fig.4). Western blot analyses of showing cleavage of PARP and caspase-8 should be performed. To support the specificity of DR5 siRNA, the levels of DR4 should be measured.

Re: Similarly, we choose statistical charts to present the datas of flow cytometry in Figure 4. The cleavage of apoptosis-related proteins (Caspase-3, Caspase-8, PARP) is the favourable index for apoptosis test. In Figure 1, apoptosis was detected by flow cytometry (FCM) after propidium iodide (PI) staining. DNA ladder bands were determined by agarose gel electrophoresis. In Figure 2, Caspase activity assays were evaluated using enzyme-linked immunosorbent assay (ELISA).

4. Although authors claimed that AFMC did not increase the levels of DR5 and did not enhance TRAIL-induced apoptosis in WI-8 cells, WI-8 cells express certain content of DR5. It seems that DR5 upregulation-independent pathway is involved in the action of AFMC to enhance TRAIL-induced apoptosis in A549 cells. The levels of DR4 and DR5 as well as the signaling of death receptor-mediated apoptosis should be compared in both cell lines.

Re: We have adopted DR5-specific blocking chimera antibody and DR5 siRNA to block AFMC-induced DR5 expression and AFMC/TRAIL induced apoptosis in A549 cells. The results demonstrate that the enhancement of AFMC to TRAIL-induced apoptosis in A549 cells depends on DR5-upregulating partly.
But this phenomenon doesn’t observed in immortalized human embryo lung WI-38 cells. All these results indicate that the upregulating of DR5 level may rely on a indirect mechanism which the exact details need further research.

5. More cancer cell lines should be used to test the induction of DR5 by AFMC and enhancement of AFMC on TRAIL-induced apoptosis.

Re: Thank you for your advice. Our previous research only could illustrate AFMC could increase DR5 expression of A549 cells. Whether it could be applicable to other cell lines still need further empirical study. According to your pertinent suggestion, We have performed more experiments to perfect our manuscript. The results have been added in the revised manuscript (Figure 5A, B).

Responses to the comments raised by Reviewer 3:

Comments to the Author …

The manuscript by Xie et al. reports that AFMC sensitizes NSCLC A549 cells to TRAIL-induced apoptosis by up-regulation of DR5 expression. In general, the paper is well-presented and will be of interest to readers of the Journal, but there are a few issues need to be addressed.

1. Fig 3A, how long were the cells treated with different doses AFMC?

2. Fig 4A, How does the DR5/Fc fusion protein decrease the expression of DR5 in A549 cells?

3. Figs 4B, C and D, Control values with the DR5/Fc fusion protein should also
be shown.

4. More cell lines, preferentially, primary cell lines, should be included in the study, to show that if up-regulation of DR5 by AFMC in general applies to NSCLC cells.

Dear reviewer:

We appreciate very much your careful review, constructive comments and kind corrections to our manuscript (MS ID: 1427164769499711.) We have revised the manuscript according to your comments and incorporated all corrections which were marked with red font in the revised version. The detailed point-by-point answers to your concerns are below.

1. Fig 3A, how long were the cells treated with different doses AFMC?

Re: Thank you for the valuable suggestion. We have remarked in the legend of Figure 3 that the reaction time for different dosage AFMC is 24 hours. We have carefully revised the manuscript according to your comments.

2. Fig 4A, How does the DR5/Fc fusion protein decrease the expression of DR5 in A549 cells?

Re: Many documents verified DR5/FC chimera protent(a DR5 antagonist) can
downregulate the expression of DR5.

For example:


3. Figs 4B, C and D, Control values with the DR5/Fc fusion protein should also be shown.

Re: Thank you for the valuable suggestion. According to your pertinent suggestion, we’ll make a few alterations on Figure 4.

4. More cell lines, preferentially, primary cell lines, should be included in the study, to show that if up-regulation of DR5 by AFMC in general applies to NSCLC cells.
Re: Thank you for your advice. Our previous research only could illustrate AFMC could increase DR5 expression of A549 cells. Whether it could be applicable to other cell lines still need further empirical study. According to your valuable suggestion, We have performed more experiments to perfect our manuscript.

The results have been added in the revised manuscript(Figure5A,B).