Author's response to reviews

Title: The NF-kappa B inhibitor celastrol could enhance the anti-cancer effect of Gambogic acid on oral squamous cell carcinoma

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Version: 2 Date: 5 July 2009

Author's response to reviews: see over
Dear Editors,

Enclosed please find the revised version of our manuscript “The NF-κB inhibitor celastrol could enhance the anti-cancer effect of Gambogic acid on oral squamous carcinoma” by Dr. He, et al. We are so grateful for those considerable suggestions from both reviewers and editors. Accordingly, in the revised version, we included two extra oral squamous cell carcinoma cell lines, TSCC and NT, which were gifted from two different university, to validate the synergistic effect of GA and celastrol. We also add some new data to further support our observation that GA treatment induced the activation of NF-kappa B pathway and minimal cytotoxic dose of celastrol could inhibit the GA induced NF-kappa B activation and significantly increased the anti-cancer effect of GA on oral squamous cell carcinoma cells. We believed that these data would greatly enhance our conclusions that minimal cytotoxic dose of celastrol could effectively suppress the GA-induced NF-kappa B pathway activation and increase the anti-cancer effect of GA on oral cancer combination of GA, and celastrol may be a promising modality for treating oral squamous cell carcinoma.

A point-by-point response was attached and marked the revised contents with underline in the re-submitted manuscript.
We do appreciate editors and reviewers taking over 6 months for judgement of our manuscript and providing so many suggestions. We do hope that the interesting story will be published soon.

Best regards,

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A point-by-point responses

Reviewer 1:

1. **using only one established oral squamous cancer cell line to generalize what will happen in oral cancer is insufficient.**

   In the revised version, we included two new cell lines, TSCC (gifted by School of Stomatology, Wuhan University, China) and NT (gifted by Nagasaki University, Japan). With these two new OSCC cell lines, we got the similar results that minimal dose of celastrol could significantly increase the anti-cancer effect of GA on oral squamous cell carcinoma cells.

2. **Furthermore, there are places in the paper demonstrated inadequate English writing.**

   In the revised version, we asked a friend from American to edit the English language, and correct all of the errors.

3. **Figure 3a and Figure 5a: There was no obvious NF-κB nuclear translocation exhibited in these figures. In fact, there was only intensified immunofluorescence staining in the cytoplasm upon GA treatment.**

   In the revised version, we improved the quality of those figures, and marked the nuclear translocation cells with arrows. There was increased
immunofluorescence staining in the nuclei upon GA treatment compared to GA plus celastrol group and transfected dominant mutant pBabe-SR–IκBα plus GA group.

4. It will be helpful to use TNF-α as a positive control since TNF-α is known to induce NF-κB activity in almost all cell systems. In the revised version, we used 50 ng/ml TNF-α (treatment for 60 minutes) as the positive control to confirm the nuclear translocation of p65. Images was included in Figure 3a (E&F).

5. There are many grammatical mistakes in the writing. It will be a good idea to prove read the paper if not by a native speaker. In the revised version, we asked a native English speaker to edit our paper and correct errors.

6. The authors used an oral cancer cell line relatively unknown to the cancer research field outside of China. It will be beneficial to conduct similar studies in additional oral cancer cell lines in order to relate to other studies in the field. In the revised version, we added two new cell lines, one was from other university in China, and another was from Japan, to further prove our observations.
7. Furthermore, it will be interesting to see if Celastrol can also improve the anti-tumor effect on other chemotherapeutic drugs commonly used in oral cancer in the clinics.

We totally agreed on this point and we will investigate if celastrol can also improve the effects of other chemotherapeutic drugs in the future.
Reviewer2

1. The experiment needs to be repeated in multiple cell lines and some more convincing means used to prove that NFkB inhibition is the mechanism of synergy.

In the revised version, we included two new cell lines, TSCC (gifted by School of Stomatology, Wuhan University, China) and NT (gifted by Nagasaki University, Japan). With these two new cell lines, we got the similar results that minimal cytotoxic dose of celastrol could significantly increase the anti-cancer effect of GA on oral squamous cell carcinoma cells. In the revised version, we added the dominant mutant pBøbe-SR–IkBa, a specific inhibitor of NF-kappa B pathway, as a positive control, to support our observation that GA treatment induced the activation of NF-kappa B pathway and minimal cytotoxic dose of celastrol, as well as pBøbe-SR–IkBa, could inhibit the GA induced NF-kappa B activation and increased the anti-cancer effect of GA on oral squamous cell carcinoma cells.

2. Poor English and editing

In the revised version, we edited the English language, and corrected all the errors.

3. Quantify apoptosis demonstrated in figure 2.

In the revised version, we added the percentage of sub-G1 peak in Fig. 2b, the percentage of early apoptosis (LR region) and late apoptosis (UR region) in Fig. 2c.
4. Only one cell line used. The results should be repeated in a second cell line.

In the revised version, we included two new cell lines, TSCC and NT. We got the similar results with them.

5. Page 9. The interaction effects of celastrol and GA were considered highly significant (P < 0.01) by the factorial ANOVA test. This sentence is vague. What exactly is being compared? Which concentration of celastrol is being assessed for interaction? The classical way to assess interaction of 2 drugs is an isobologram.

We used the factorial ANOVA test to investigate if there were any interaction effects between celastrol and GA with the help of SAS software. Celastrol was administrated with three concentrations: 0 \( \mu \) M, 0.5 \( \mu \) M, 1 \( \mu \) M. GA. GA was administrated with four concentrations: 0 \( \mu \) M, 2 \( \mu \) M, 3 \( \mu \) M and 4 \( \mu \) M. \( P < 0.05 \) was regarded as statistical significance. Just like the classical isobologram method, factorial ANOVA could also be used to test the interaction effects between two or more factors, and it is easier to carry on with SAS software.

6. Page 4 line 5: [chemotherapy pre/post surgery improves local control and survival [in head and neck cancer]. This is not thought to be true, and is not standard of care. Sometimes radiation (with or without) chemotherapy is combined with surgery; but not chemotherapy alone.

We agree with the reviewer’s opinion. The description of “chemotherapy pre/post surgery improves local control and survival in head and neck cancer”
is not reasonable. In the revised version, we have modified the descriptions related to chemotherapy.

7. This paper focuses down on the specific interaction between gambogic acid and celastrol. I would have preferred the authors to look at either: 1) the effect of multiple NFkB inhibitors (which work through different mechanisms) on gambogic acid induced cell death. This is especially true since Celastrol has multiple actions aside from NfkB inhibition (antioxidant, proteasomal activity etc). or 2) the effect of Celastrol on multiple cytotoxic/anti-cancer agents.

In the revised version, we included another specific inhibitor of NF-kappa B pathway, dominant mutant pBabe-SR–IκBα in our experiments. We found that similar to celastrol, pBabe-SR–IκBα effectively inhibit the GA-induced nuclear translocation of p65, which followed with significantly increasing the effects of GA. We totally agreed with the point that celastrol may have multiple actions aside from NF-kappa B inhibition, such as antioxidant, proteasomal activity et al. While based on our data, we believed that the inhibition of minimal cytotoxic dose celastrol on the GA-induced NF-kappa B pathway activation, did play an important function on its synergistic effects with GA, although some other mechanisms may be also involved. We will investigate other possible mechanisms and explore if celastrol can also improve the effects of other chemotherapeutic drugs in the future.

8. Needs some language corrections before being published
In the revised version, we edited the English language and correct errors.

**Reviewer3:**

1. **Analysis of additional OSCC cell lines is required.**

   In the revised version, we included two new cell lines, TSCC (gifted by School of Stomatology, Wuhan University, China) and NT (gifted by Nagasaki University, Japan). With these two new cell lines, we got the similar results that minimal cytotoxic dose of celastrol could significantly increase the anti-cancer effect of GA on oral squamous cell carcinoma cells.

2. **these compounds appear to have multiple cellular targets, making direct linear conclusions suspect.** For example, celastrol does appear to inhibit NF-κappaB, as judged by their gel shift assay and the p65 nuclear localization. And celastrol does increase cell killing by Gambogic acid. But it does not follow that the reason cell killing is enhanced is because of the NF-kappaB inhibition. For this conclusion to be strengthened, additional evidence would be required using more specific reagents (eg IkB that cannot be phosphorylated and degraded, or p65 siRNA).
In the revised version, we included another NF-kappa B inhibitor, dominant mutant pBøbe-SR–IkBα, which is specific targeting on NF-kappa B pathway. We found that both celastrol and pBøbe-SR–IkBα could effectively inhibit the GA-induced nuclear translocation of p65, and followed with significantly growth inhibitory effect on Tca8113 cells. Celastrol might have multiple actions aside from NF-kappa B inhibition, while based on our data, we thought that the inhibition on the GA-induced NF-kappa B pathway activation by celastrol, although not the only one, played an important mechanism on its synergistic effects with GA. We will investigate other possible mechanisms in the future.