Author's response to reviews

Title: Flavones inhibit breast cancer proliferation through the Akt/FOXO3a signaling pathway

Authors:

Chia-Hung Lin (james07212001@yahoo.com.tw)
Ching-Yao Chang (cychang@asia.edu.tw)
Kuan-Rong Lee (krlee@mx.nthu.edu.tw)
Hui-Ju Lin (d2396@mail.cmug.org.tw)
Ter-Hsin Chen (thc@dragon.nchu.edu.tw)
Lei Wan (leiwan@mail.cmu.edu.tw)

Version: 3 Date: 29 May 2015

Author's response to reviews: see over
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Version: 2 Date: 29 May 2015
Author’s response to reviews: see over
Reviewer’s report

**Title:** Flavones inhibit breast cancer proliferation through the Akt/FOXO3a signaling pathway

**Version:** 1  **Date:** 13 January 2015  
**Reviewer:** Asoke Banerji

**Reviewer’s report:**

1. The discussions in the mss could have been better in terms of language.

The manuscript has been edited for English usage by a professional editor at Editage. A Certificate of English Editing is provided on the last page of this letter.
2. Extended discussion on activities of flavonoids/phenolics could have been avoided as these are well-known facts.

We have added the discussion sentence in paragraph 2, line 4 (Accumulating evidence indicates flavone compounds have been shown to have anti-cancer and anti-proliferative activities in *in vitro* and *in vivo*.), paragraph 3, line 5-9 (Many cancers acquired drug resistance by PI3K/Akt pathway activation, which has been observed during the administration of paclitaxel in breast cancer. Our results showed that flavone, apigenin and luteolin treatment substantially suppressed Akt phosphorylation at Ser473 in MCF-7 cells.), paragraph 4, line 4-15 (FOXO3a are important targets of PI3K/Akt signaling pathway. The Akt mediated phosphorylation of FOXO3a is known to transport FOXO3a out of nucleus and retain FOXO3a in the cytoplasm. FOXO3a has also been shown to regulate cell cycle arrest and apoptosis through the activation of transcriptional targets such as p27 and p21. The nuclear localization of FOXO3a and its subsequent transcriptional activity were shown to be a prognosis marker for breast cancers. Our results demonstrated that treatment of breast cancer cells with flavone, apigenin, and luteolin for 12 h led to an inhibiting Akt activation and increase in the expression levels of FOXO3a, which subsequently increase the expression levels of p27 and p21 to inhibit the proliferation of breast cancer cells.), paragraph 5, line 1-13 (Metastatic is complex processes and accounts for the death of most cancer patient. In wound healing assay, we found that treatment of the flavone, apigenin and luteolin suppressed MCF-7 cells migration. Inhibiting Akt signaling reduced the migration and invasion of gastric cancer cells, which may be due to up-regulation FOXO3a. In renal cancer cells, FOXO3a has been identified as a key factor in metastasis. Overexpression of FOXO3a in renal cancer cells could inhibit tumor metastasis as a key factor in renal cancer cells metastasis. Flavone, apigenin and luteolin inhibit breast cancer cells migration was through inhibiting Akt activation and increasing FOXO3a expression.), and paragraph 6, line 1-3 (Epidemiologic and clinical studies suggest that higher intake of plant flavonoids can prevent cancer through their interaction with various genes and enzymes.).

3. Some of the experiments such as PARR cleavage have not been carried out properly. Positive controls were not carried out in many experiments.
The results for PARP, p53, and cytochrome c in Figure 2F has been replaced. We treated MCF-7 cells with flavone, apigenin, and luteolin for 24 h and the expression levels of PARP, p53 and cytochrome c were determined by western blot. The expression levels of cleaved PARP, p53 and cytochrome c increased when treated with flavone, apigenin, and luteolin for 24 h. Yes, We know the positive control is a part of good experimental design. A positive control indicates that drug treatment with a known response, so that this positive response can be compared to the unknown response of the treatment. Previous studies indicate flavonoids can inhibit the growth of tumor cells by inducing apoptosis (Park, S.H., et al., Luteolin induces cell cycle arrest and apoptosis through extrinsic and intrinsic signaling pathways in MCF-7 breast cancer cells. J Environ Pathol Toxicol Oncol, 2014. 33(3): p. 219-31, Lu, J., et al., Luteolin exerts a marked antitumor effect in cMet-overexpressing patient-derived tumor xenograft models of gastric cancer. J Transl Med, 2015. 13(1): p. 42, Mrazek, A.A., et al., Apigenin inhibits pancreatic stellate cell activity in pancreatitis. J Surg Res, 2015, Shi, M.D., et al., Apigenin, a dietary flavonoid, inhibits proliferation of human bladder cancer T-24 cells via blocking cell cycle progression and inducing apoptosis. Cancer Cell Int, 2015. 15: p. 33.). These study showed that apigenin and luteolin inhibited cell viability by apoptosis. Therefore, we did not include a positive control in our experiments.

4. Sources and purities of the test compounds are not mentioned. The structures of flavone and luteolin are incorrect.

The source and purity of flavone, apigenin and luteolin has been added the Methods sentence in line 12. Flavone (HPLC>98%), apigenin (HPLC>95%) and luteolin (TLC>98%) were purchased from the Sigma. The structure of flavone (46380-CN), apigenin (10798-CN) and luteolin (L9283-CN) has been corrected in Table 1 and were according to the manufacturer (sigma).

5. There are confusions in loading controls between PCNA and beta-actin. (e.g. fig 5B & 5D).

These are typos. We have corrected the errors.
6. Some of the assays such as "colony formation" or "wound healing" assays were carried out with very few numbers of cells. Some experiments were carried out for as long as 21 days. I wonder if such long periods are necessary?

The wound-healing assay was performed using Culture-Insert (Ibidi, Martinsried, Germany). According to the manufacturer’s protocol, which suggest seeding the cell at a concentration between $3 \times 10^5$ to $7 \times 10^5$ cells/ml to reach a confluent layer within 24 h. In our experiments, we plating the cell at a concentration of $3.5 \times 10^5$ cells/ml. The colony formation assay is a method used to confirm cellular anchorage-independent growth in vitro. The goal of this protocol is to illustrate a stringent method for detecting the suppressive effects of the drug on cancer cells. We have tested several different conditions on this experiment. As shown in the figure below, cells are seeded at a concentration of 8000, 4000, and 2000 cells for each 25 T flask. The flask with 4000 and 8000 cells/flask were incubated for 14 days. For the flask with 2000 cells/flask was incubated for 21 days. Our results showed that 2000 cells/flask, 21 days incubation has the most visible colonies and stronger staining. Therefore, we chose this condition to reveal the effect of the flavonoids.

<table>
<thead>
<tr>
<th>8000 cells/flask</th>
<th>4000 cells/flask</th>
<th>2000 cells/flask</th>
</tr>
</thead>
</table>

**Level of interest:** An article of limited interest

**Quality of written English:** Not suitable for publication unless extensively edited

**Statistical review:** Yes, but I do not feel adequately qualified to assess the statistics.

**Declaration of competing interests:** I declare that I have no competing interests
Reviewer's report

Title: Flavones inhibit breast cancer proliferation through the Akt/FOXO3a signaling pathway

Version: 1 Date: 2 February 2015

Reviewer: Yongping Bao

Reviewer’s report:
In this manuscript, the authors studied the inhibitory effects of three flavones on the proliferation on breast cancer cell lines and dissected some of the key signalling pathways such Akt/FOXO3a involved in the mechanisms of the action. The work is interesting and the manuscript is well written.

Comments:
1. Figure 1. The concentration of the flavones used from 12.5-100 µM, the IC50 values could be calculated.

The IC50 values of each compound on different breast cancer cells have been calculated and the results were listed in Table 1.

Table 1. Chemical structures and IC50 values in flavone, apigenin and luteolin on Hs578T, MDA-MB-231, and MCF-7 breast cancer cells.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Structure</th>
<th>Cells</th>
<th>IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavone</td>
<td><img src="image" alt="Flavone Structure" /></td>
<td>Hs578T</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MDA-MB-231</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MCF-7</td>
<td>88</td>
</tr>
<tr>
<td>Apigenin</td>
<td><img src="image" alt="Apigenin Structure" /></td>
<td>Hs578T</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MDA-MB-231</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MCF-7</td>
<td>30</td>
</tr>
</tbody>
</table>
2. What’s the rational to select these three compounds? Are there any structure and activity relationships?

We screened many of flavones (including flavone, apigenin, luteolin, fistein, genistein chrysin, rutin, quercetin and myricetin) on the viability of breast cancer cells. The result revealed that flavone, apigenin and luteolin exhibited highest growth inhibiting activities against breast cancer cells. The IC$_{50}$ of each compound against each breast cell are listed below.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Hs578T; IC$_{50}$ (µM)</th>
<th>MDA-MB-231; IC$_{50}$ (µM)</th>
<th>MCF-7; IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavone</td>
<td>55</td>
<td>44</td>
<td>88</td>
</tr>
<tr>
<td>Apigenin</td>
<td>45</td>
<td>28</td>
<td>30</td>
</tr>
<tr>
<td>Luteolin</td>
<td>28</td>
<td>27</td>
<td>43</td>
</tr>
<tr>
<td>Chrysin</td>
<td>36</td>
<td>49</td>
<td>70</td>
</tr>
<tr>
<td>Rutin</td>
<td>54</td>
<td>67</td>
<td>90</td>
</tr>
<tr>
<td>Fisterin</td>
<td>54</td>
<td>73</td>
<td>80</td>
</tr>
<tr>
<td>Genistein</td>
<td>23</td>
<td>52</td>
<td>62</td>
</tr>
<tr>
<td>Myricetin</td>
<td>48</td>
<td>70</td>
<td>51</td>
</tr>
<tr>
<td>Quercetin</td>
<td>40</td>
<td>83</td>
<td>70</td>
</tr>
</tbody>
</table>

We didn’t find any specific structure activity relationship in our experiments.

3. What’s the metabolic fate of these compounds in the cell lines? And is it different from in vivo studies?
We didn’t perform the metabolite analysis on flavone, apigenin and luteolin in our experiments. Thus, we don’t know the differences in metabolites of those flavonoids in vitro and in vivo. In 1999, a study shown the quercetin metabolism in Hep G2 cell line (Boulton, D.W., U.K. Walle, and T. Walle, Fate of the flavonoid quercetin in human cell lines: chemical instability and metabolism. J Pharm Pharmacol, 1999. 51(3): p. 353-9.). These cells showed a 9.6-fold accumulation of quercetin and the formation of an O-methylated metabolite, isorhamnetin. In 2011, another study indicated that cytotoxic of flavone-8-acetic acid (FAA) is weakly in vitro but is active in vivo. Their conclusion described several cytochrome P450s (Cyps) were shown to be involved in FAA metabolism, particularly Cyps 3a11 and 2b9 which were responsible for the formation of the principal metabolites (5,6-epoxy-FAA, 3’,4’-epoxy-FAA). Therefore, the difference of metabolism between in vivo and in vitro may because presence of these enzymes induced anticancer activity of FAA in vivo. (Pham MH, Rhinn H, Auzeil N, Regazzetti A, Harami DE, Scherman D, Chabot GG: Identification and induction of cytochrome P450s involved in the metabolism of flavone-8-acetic acid in mice). According to these published results, it is possible that flavone, apigenin and luteolin may metabolize differently in vitro and in vivo. An xenograft animal model will be performed in the near future and the blood samples will be collected to determine the compounds and their metabloites.

**Level of interest:** An article whose findings are important to those with closely related research interests

**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' below