Author's response to reviews

Title: Equol enhances tamoxifen's anti-tumor activity by induction of caspase-mediated apoptosis in MCF-7 breast cancer cells

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Author's response to reviews: see over
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Dear Dr Chap:

Thank you for considering publishing our manuscript entitled “Equol enhances tamoxifen’s anti-tumor activity by induction of caspase-mediated apoptosis in MCF-7 breast cancer cells” (MS # 1736673833876503) pending revision. Please find below a point by point response to the reviewers’ concerns including the page numbers where changes were made in the revised manuscript.

Please note that the manuscript has been formatted to conform to the journal style. Also, in the revised manuscript we have added a new author who was omitted by mistake in the original.
Reviewer 1: Daniel R. R. Doerge

This reviewer expressed only one concern: the high concentration of equol required to induce apoptosis. We are in full agreement with the reviewer that equol concentration is too high, and we are fully aware that the 100 µM concentration is not achievable through diet or supplements in animals or humans \textit{(in vivo)}. We are also aware of the limitations of applying our results \textit{in vivo} due to the low plasma concentrations and low bioavailability of the aglycone forms of isoflavonoids and their derivatives. We must admit that agents whose \textit{in vitro} effects can be seen at very low, physiologically relevant, concentrations perhaps are more interesting. However, one cannot dismiss the value of agents expressing their \textit{in vitro} effects at concentrations higher than those reached physiologically in the serum/plasma for the following reasons:

1. Methods are being continuously discovered to increase the serum/plasma concentrations. One such method, which involved complexation of genistein with high-amylose corn starch, doubled the concentration of genistein in the plasma of rats compared to the controls (Cohen R et al., J Agric Food Chem., 2011, 59: 7932-7938).

2. There are numerous methods for delivering agents of interest to the target tissues (i.e. tumor) at concentrations that are over a thousand fold higher than the plasma concentrations. Some of these methods had been used extensively and one is referenced in our paper (Uckun FM et al., Science, 1995, 267: 886-891).

3. The induction of measurable apoptosis (or other biological effects) in \textit{in vitro} systems generally requires much higher concentrations than what is required \textit{in vivo}. This is because in the \textit{in vivo} systems agents may be present for
longer periods of time (months to years) while in the in vitro systems only hours to days. A very small effect of the low concentration in the in vivo system can result in a substantial effect over a long period of time while it may not be measurable in the in vitro system. Such an example is the effect of 17β-estradiol (E2) which is known to effectively bind to its receptor and induce its biological effects at 1-10 nM in vivo but still requires concentrations 1-10 µM (1000 times more) to bind to the same receptor in in vitro systems (Gurer-Orhan H et al., Int. J. Environ. Anal. Chem., 2005, 85: 149-161).

4. The use of high concentrations of natural agents (such as flavonoids) and their metabolic derivatives is a common practice for determining biological responses in in vitro systems. References cited in our manuscript #2 and #8 refer to equol concentrations up to 100 µM. Also, references #20-24 refer to genistein, daidzein and other flavonoids exerting their in vitro apoptotic effects at these high concentrations. There are hundreds of other reports in the literature referring to the in vitro effects of high concentrations of flavonoids.

Finally, taking into consideration the reviewer’s concerns and to tone down our claims we modified the “Discussion” section (page 13) as follows: “Even though high concentrations of equol (100 µM) were required to activate MCF-7 apoptosis, which are not physiologically achievable in human plasma due to metabolic conversion of the active aglycone equol to the inactive conjugated form (Allred et al, 2005), our results may find applications in targeted immunotherapies, which may enable maximal delivery of equol into the cancer cells. This strategy was previously
used successfully for genistein, which was immunoconjugated with a monoclonal antibody and targeted to a B cell-specific receptor for treatment of an animal model of B-cell precursor leukemia (Uckun et al, 1995)". 
Reviewer 2: Rémy Bosviel

Only “Minor Essential Revisions” were suggested by this reviewer. These were made as follows:

1. The phrase “in vitro” was converted in italics. In all cases this word was converted to italics except the title of the references.

2. Our objective was to show statistical significance and we choose the SEM for at least 95% confidence interval reflecting a significance level of <0.05. This is a widely acceptable method for scientific data presentation. The number of repetitions of each experimental group which, as the reviewer points out is very important, was mistakenly omitted from the legends of figures 1 and 3, is now added (pages 25-26).

3. In the “Results” section, in the sub-title “Equol and 4-OHT induce MCF-7 cell death via apoptosis”, the P value for [Equol+4-OHT] vs. [Equol] is now also cited (pg 9).

4. According to the reviewer’s suggestion, in the “Results” section, in the sub-title “The combination equol and 4-OHT promotes cytochrome-c release and reduction of bcl-2 expression”, we now point out that cytochrome-c release is not detected by equol or 4-OHT alone (page 11). We also added a relevant sentence in the Discussion section (pg 14).

5. The inhibition of equol and 4-OHT induced apoptosis by the pan-caspase inhibitor Z-VAD-FMK “isn’t complete” as the reviewer correctly points out. This suggests that equol and 4-OHT induce apoptosis not only via the caspase-dependent pathway but also via the caspase-independent pathway. Although we had mentioned in the original article that both pathways were involved, we now state more clearly this point in both the “Results” section (page 10 under
sub-title “Z-VAD-FMK…” and the “Discussion” section (pages 13 – 14, several places), according to the reviewer’s suggestion.

6. The unnecessary space in the “bcl-2:bax” phrase in the “Discussion” section was removed.

7. The labels of the horizontal axis on Figure 1A were fixed so that they are now visible after the PDF conversion.

8. In Figure 1A, the P values were shown for all results that were significantly different from the control, as the reviewer suggested (with * for P < 0.05, ** for P < 0.005 and *** for P < 0.0005).

9. In Figures 1B and 1C, we labeled with asterisks points where viability was significantly different from the control, as the reviewer suggested (with * for P < 0.05, ** for P < 0.005 and *** for P < 0.0005).

10. The number of repetitions of each experiment as well as the number of repetitions of each experimental group within the same experiment was added to the legends of Figures 1 and Figures 3-6, as the reviewer suggested. The number of repetitions for western blot results is also indicated in the Figure legends.

11. In the legend of Figure 2, zero was removed and 4-OHT was properly written.
Reviewer 3: Omer Kucuk

1. This reviewer suggested that no “compulsory revisions” are required.

2. A minor revision was to change the word “warrant” to “warranted” which was done (page 15).

3. As a “Discretionary revisions” he suggested that it will be interesting to compare combinations of equol, genistein and diadzein together with tamoxifen. These experiments are within our future plans.

Once again we would like to thank the reviewers for their constructive comments and suggestions and we look forward to publishing this revised and improved manuscript in BMC Cancer.

Sincerely,

Andreas I. Constantinou