Author’s response to reviews

Title: Estrogen and Soy Isoflavonoids Decrease Sensitivity of Medulloblastoma and Central Nervous System Primitive Neuroectodermal Tumor Cells to Chemotherapeutic Cytotoxicity

Authors:

Scott Belcher (smbelch2@ncsu.edu)
Caleb Burton (cburton@u.northwestern.edu)
Clifford Cookman (cookmacj@mail.uc.edu)
Michelle Kirby (kirby_mich@yahoo.com)
Gabriel Miranda (Gabriel.Miranda@amaisd.org)
Fatima Saeed (fatima.saeed@uc.edu)
Kathleen Wray (wrayk@musc.edu)

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The authors' response letter has also been included as a supplementary file

Response to reviewer reports:

Ivonne Rietjens (Reviewer 1): The manuscript by Belcher et al. describes the decrease in sensitivity of medulloblastoma and central nervous system primitive neuroectodermal tumor cells to chemotherapeutic cytotoxicity upon coexposure to estrogen and soy isoflavones.

There are some issues the authors should reconsider and modify and these can be summarised as follows.

1) The data as presented do not clearly characterise the effects of the estrogen and isoflavone alone. Since these compounds may increase cell proliferation the effects observed may rather be explained by proliferation of the cells masking the cytotoxicity, and that is not the same as really a decrease in sensitivity. Just two opposite effects. The concentration at low concentration of the chemotherapeutic agents (for example in figure 1A) was set to 100%, masking such a possible effect. For a real adequate conclusion, the actual proliferation in the absence or presence of the estrogens at zero concentration of the chemotherapeutic agents should also be presented. This holds for all figures. Depending on the outcomes the text at
many places and even the title of the paper should be reconsidered and modified: If the cells proliferate more in the presence of estrogen that may be the underlying explanation; just two opposite events happening at the same time; Estrogen induced proliferation and drug induced cytotoxicity both with a somewhat different EC50 and this is not the same as a modified sensitivity towards the chemotherapeutic agents. Figure 3 and 4 present data that such proliferation indeed occurs. This implies that all figures should be extended including bars for the situation - and + E2 at zero concentration of the test compound. And that the conclusions and all text on decrease in sensitivity should be modified. Preferably the EC50 of the two effects should be determined: EC50 for proliferation and IC50 for cytotoxicity and then the sum may be the curves now provided. All this is needed to answer the question how the authors can exclude the conclusion that the effect is a reflection of a combination of two separate effects instead of a decrease in sensitivity.

Response: In response to the possible role of proliferation.

In Doay cells (shown here in Figure 4) and PFSK-1 cells (Figure 3A of Kirby et al, 2004) estrogen does not have any effects on the growth of these cells.

The impacts and mechanism of estrogen action in the D283 Med cells has been characterized in detail in our previously published studies. There we demonstrated that estrogen upregulates anti-apoptotic mechanisms through an ERβ-dependent mechanism both in vivo and invitro (Cookman and Belcher, 2015; Belcher et al., 2009). That effect was also observed in the PFSK 1 cells (Kirby et al, 2004 ; Cookman and Belcher, 2015). Importantly, detectable increases in the cytoprotective mechanisms, and no increase in D283 Med cell numbers are found during the exposure period (18-24 hours) used in the current study. This lack of an observed proliferative effect is consistent with the slow growth rate of these cells (population doubling time of 52 hours). We have already performed and published the suggested control experiments (the actual growth and proliferation analysis in the absence or presence of the estrogens at zero concentration of the chemotherapeutic agents).

To further clarify, methods of the clonogenic assay, we have edited the methods section to more clearly explain how we treated the cells with ER ligands to ensure effects were not the result of differences in cell numbers following pretreatments with various ligands.

Line 193 now reads as follows: Clonogenic/colony forming assays were adapted from published protocols [41, 42]. Exposure to ER ligands were started 24 hours prior to determining cell numbers and diluting the cells to a concentration of 500 cells per ml and 1000 cell were seeded into 6-well tissue culture plates in 2 mL of 10% CSS supplemented phenol red free MEM/EBSS.

2) Page 6 line 106: add the ER specificity of fulvestrant, is it and antagonist for ERalpha,beta or both?
Response: The following edits were made to clarify at this agonist is non-selective.

Lines 111 now reads Results from additional studies have also demonstrated that the non-selective ER antagonist fulvestrant and the ERβ selective antagonist PHTPP inhibited MB cell growth in cultured human MB cell lines [27].

-and- line 127: Additional experiments were done to determine whether the ER nonselective antagonist fulvestrant or the selective ER antagonist tamoxifen blocked the cytoprotective actions of 17β-estradiol

3) The authors should add data on the actual levels of ERalpha and ERbeta in the cells studied. This is essential for understanding the results observed. For example do the cells express ERalpha? This would provide an explanation for estrogen induced cell proliferation, especially if ERalpha levels dominate over those of ERbeta. This information is essential to fully understand the effects reported.

Response: For all of the cell lines used in this study the results of ER expression analysis has previously been published as Figure 2A of Belcher et al., 2009. Only extremely low levels of ERα were observed in each cell line that express comparatively very high levels of ERβ. The complete absence of ERα in nearly all MB tumor cells has similarly been demonstrated in vivo for D283 xenografts, human tumors and in knock-out mouse models. Further, the analysis in this manuscript (Figures 3A and 3B) as well as numerous experiments presented in the study by Cookman and Belcher (2015) that used both the ERα agonist PPT and the ERα selective antagonist MPP, demonstrate that ERα activity has no role in any of the observed estrogen induced responses. In contrast, ERβ specific agonists have consistently mimicked fully the activities of estradiol. Along with the experiments presented here the results of numerous studies have consistently demonstrate no ERα mediated activities.

Response: We have tried to clarify the results of previously published analysis characterizing the expression patterns and activities of ERs in MB and the cell lines used here by editing and specific reference to those studies.:

Beginning at Line: 103 that now reads:

Results of Western blot and immunohistochemistry analysis using ERα and ERβ specific antibodies, along with pharmacological studies using ER selective agonists and antagonists have demonstrated that human MB tumors and cell line express predominantly ERβ and that the activity of estrogens is dependent on activity of ERβ and independent of ERα [26-28].

-and-
Line 336: We and others have found that Daoy cells express ERβ, with little or no active ERα, but the pattern of ERβ protein isoform expression is distinctive from other MB and CNS-PNET cells in which E2 has cytoprotective activities [26, 54].

Leena Hilakivi-Clarke (Reviewer 2): This manuscript describes results of a study that investigated the effects of estrogen and soy isoflavones on the effectiveness of chemotherapy against medulloblastoma and neuroectodermal tumor cells in culture. Manuscript is well written and the experimental design is appropriate. Conclusions drawn from the data also are adequate and supported by the results. Minor clarifications are needed to the text.

Minor comments:

Similar to Comments of reviewer 1, each of the three comments below address additional clarification of ER expression in the cells used in this analysis:

• Page 3, lines 46-47. Please define what are PFSK1 and Daoy cells. Further, it is not apparent how the data suggest that ERbeta mediated effects may vary in different cancer subtypes. For example, do PFSK1 and Daoy cells exhibit differences in ERbeta?

• Page 6, lines 119-122. Please provide more background of Daoy and PFSK1 cells, especially regarding their ERbeta content.

• Page 16, line 327. Clarify if Daoy cells express only ERbeta, or also ERalpha.

Response: We have tried to clarify the results of previously published analysis characterizing the expression patterns and activities of ERs in MB and the cell lines used here:

Beginning at Line: 103 that now reads:

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-and-

Line 336: We and others have found that Daoy cells express ERβ, with little or no active ERα, but the pattern of ERβ protein isoform expression is distinctive from other MB and CNS-PNET cells in which E2 has cytoprotective activities [26, 54].
Page 11, lines 234 - 237. Please rewrite this sentence, because it is unclear what the authors wish to state. For example, it is not clear what the authors mean by "… determine the effects of estrogens effects."

Response: Rewritten and now reads…… characterize in more detail the effects of estrogen on the cytotoxicity of cisplatin.

Page 17. It is proposed that the differences among cell lines to various treatments could be due to DMSO decreasing efficacy of cisplatin. It is unclear why DMSO would affect only one cancer cell type but not the other.

For clarification: We are not proposing that the cell specific responses are due to differences in responses observed in the cell lines. Rather we are speculating on the cause of differences in response seen in our studies (no increase decrease in sensitivity in the presence of ICI182,780 (fulvestrant) and those published in a previous studies [34]. As detailed in reference #35, DMSO the presence of DMSO may have caused an apparent increase in resistance to cisplatin by directly inactivating of DMSO. We speculate that one possible experimental difference between our studies and those studies could be the use of DMSO as a vehicle. While we accounted for the impact of DMSO in our analysis, sufficient experimental detail is not given in reference [34] to determine if DMSO was used as a vehicle and if so, whether it was controlled for in those studies.

Page 17. This reviewer is not an expert in MB. Since different tumor cell types responded differently to estradiol and isoflavones, it is critical to know that the tumors these cell lines represent are clinically distinguishable. Please clarify.

In the methods we have added much more details regarding the cell lines indicating the clinical nature of the cell lines: addressed at Lines 146:

The D283Med cell line (HTB-185) was established from a peritoneal implant and ascetic fluid of a 6 year old male with metastatic medulloblastoma and grows in multicell aggregates in suspension with some adherent cells or on poly-L-lysine coated culture dishes with an epithelial morphology [36]. The Daoy cell line (HTB-186) was isolated from a desmoplastic cerebellar medulloblastoma of a 4 year old male and grows adherent with a polygonal morphology [37]. The PFSK-1 cell line, (CRL-2060) was established from a PNET from the cerebral hemisphere of a 22 month old boy, and grows adherent with a fibroblast-like morphology [38].