Author's response to reviews

Title: Promoting E2F1-mediated apoptosis in oestrogen receptor-alpha-negative breast cancer cells

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Version: 3
Date: 19 June 2014

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Dear Editor and Reviewers:

Thank you for your letter and for the reviewers’ comments concerning to our manuscript entitles “Promoting E2F1-mediated apoptosis in oestrogen receptor-α-negative breast cancer cells” (ID: 2083042115115977). All the comments and suggestions addressed by the Reviewers have been taken into consideration in the preparation of this new version, and detailed answers to the referees are attached to this letter. We consider all the Reviewers’ comments valuable and very helpful for revising and improving our manuscript; therefore, we sincerely thank to them for these comments. The changes in the new version of the manuscript are marked in red for clarity. We hope that the changes made in the new version will enable you to expedite publication.

Responses to the reviewers´ comments:

Reviewer#1:

Comments to the Author
1. At the second graphic in Figure 6, the name of last column “4OHT/TMCG/DIPY” is missing.

Response: The label “TMCG/DIPY/4OHT” has now been included at the second graphic in Figure 6 (new Figure 6A).

Reviewer#2:

Major Compulsory Revisions

1. Page 3, 2nd paragraph, the authors describe the effects of tamoxifen but do not mention the fact that tamoxifen as a competitive inhibitor of estrogen action blocks the cell cycle at the G1. There are references to this action of tamoxifen by both Osborne's group and Sutherland's group in the early 1980s.

Response: The effects of tamoxifen on G1 cell cycle arrest have been reported in the new version of the manuscript and key references from Osborne’s and Sutherland’s groups have been included. The following paragraph has been inserted in the introductory section: “The growth inhibitory effects of anti-oestrogens in ERα-positive breast cancer cells are profound, and this allowed early demonstration of a G1 phase site of action for anti-oestrogens [1,2]. Studies using synchronized cells demonstrated that cells were most sensitive to oestrogens and anti-oestrogens in the early G1 phase, immediately following mitosis [3], compatible with a model whereby oestrogens and anti-oestrogens acting via the ER regulate the rate of progression through the early G1 phase of the cell cycle.”

2. Page 10: I am intrigued why inhibiting MAP-ERK prevents RNA production during 4OHT treatment. Can this be explained a little better, as phosphorylation of the Estrogen Receptor is the target.

Response: Inhibition of ERα-mRNA production by U0126 is consistent with ChIP data (Figure 1D), where this drug impeded transactivation of ERα to its own promoter. It is well known that phosphorylation of ERα at specific residues can stimulate ERα activity in a ligand-independent manner [4]. By this mechanism of action ERα is phosphorylated by active kinases, thereby activating ERα to dimerise, bind DNA, and regulate genes [5]. Taken together, these results indicated that 4OHT may promote this ERα ligand-independent pathway in ERα-negative breast cancer cells, activating the MAPK-mediated phosphorylation of ERα, which may contribute to its own expression (autoregulation). This interesting and previously missing aspect has now been discussed in the new version of the manuscript.
3. On page 11 (top). The authors suggest a novel way that the ER self regulates to produce further ER. This as a fact has been known for 15 years and first published by Pink and Jordan in Cancer Research in 1996.

Response: We agree with the Referee that self-regulation of ER to produce more ER is a well-known mechanism. The publication of Pink and Jordan [6] has now been included to clarify this fact.

4. Page 11, line four from the bottom, the recurrent use of the word 'resistance' seems inappropriate for 4OHT in MDA-MB-231, from what I can gather from the authors' data, this is 4OHT stimulated growth, and a form of resistance first described by Gottardis and Jordan in Cancer Research 1988 when they first discovered that tamoxifen stimulated growth occurs; so this is a principle.

Response: We agree and we made reference to this article [7] as the first discovery of this resistance to tamoxifen in breast cancer cells.

5. Page 12, three lines from the top, the authors focus much attention on the MAPK cascade so that some of the ER is actually membrane bound?

Response: This is a really interesting question and a discussion on it has been included in the new version of the manuscript. It is well known that membrane ER-α plays a role in the temporal coordination of phosphorylation/dephosphorylation events for the ERKs in breast cancer cells [8]. Although, at the moment, the mechanism by which 4OHT activates the MAPK cascade is unknown, it is tempting to speculate that in ERα-negative breast cancer cells may exist some levels of membrane-bound receptor. Related with this, we (in this study) and other groups [9] have detected consistent expression of ER-α-mRNA in MDA-MB-231 cells; however, whether this expression may be related with membrane-bound ER-α is actually unknown. More recently, Zhang et al [10] indicated that 4OHT promoted the proliferation of ERα-negative breast cancer cells via the stimulation of the MAPK/ERK pathway, which is mediated by ER-α-36. Therefore, understand how 4OHT activates the MAPK cascade in ERα-negative breast cancer cells will require further studies.

6. Page 12, five lines down, the discussion about the mechanism of action does not include the fact that fulvestrant binds to the ER and causes rapid ubiquitinylation and destruction of the aberrant receptor complex.

Response: We agree with the Referee that modulation of the stability of ER by the ubiquitin/proteasomal system after its binding to fulvestrant has been reported [11]. We have mentioned this mechanism of action in the new version of the manuscript.

7. Page 12, four lines from the bottom, the ChIP assays are interesting but compared to what? The authors stress that the ER:4OHT complex binds rapidly to the E2F1 promoter; but, how does this work? The 4OHT:ER complex usually weakly binds to any promoter and the major antiestrogenic action of this complex is that it does not bind a coactivator. Can the authors reconcile this with their data?

Response: Binding of ERα to the E2F1 promoter has been proposed to mediate TAM resistance in ERα-positive breast cancer cell [12]. As described in the older version of the article, although the E2F1 promoter lacks the classic oestrogen-response element 5’-GGTCAnnnTGACC-3’, ERα is known to interact with several other transcription factors, including Sp1. In fact, it has been clearly demonstrated that tamoxifen increases ERα/Sp1 interactions, modulating E2F1 expression in MCF7 tamoxifen-resistant cells. As far as we know, we did not specifically proposed that the ERα:4OHT complex bond to the E2F1 promoter. In fact, our results are more consistent with 4OHT inducing the ligand-independent pathway (see point 2) in which phosphorylated ERα may control the expression of several genes, including E2F1. This aspect has also been discussed.
8. The authors visually quantify the amounts of apoptotic cells, but they stress the role of their combination in enhancing pro-apoptotic genes. In the conclusions, they mention some of these. Because their effects are so profound, can RT-PCR now quantify before and after treatment for pro-apoptotic genes that they select?

Response: Consistently with our hypothesis of TMCG/DIPY/4OHT combination inducing E2F1-mediated apoptosis, we observed a significant increase of p73 and Apaf1 mRNAs after treatment (new Figure 6B).

9. The authors do everything in the presence of 4OHT for their combination, but what happens without 4OHT? I see this is in figure 4, but more should be made of this synergistic effect.

Response: The effect of TMCG/DIPY combination (without 4OHT) was previously described in an early publication [13]. We observed that the TMCG/DIPY combination acted as an epigenetic treatment that induced apoptosis in several breast cancer cell systems. We did not include this data in this new manuscript to avoid repetition and to prevent an excessive length of it.

9. It is clear that 4OHT does something to stimulate the growth of these cells, but the authors should consider adding the article by Sipila et al, EJC, vol. 29A, pp2138-2144, 1993, as this fits very nicely into what they are now finding in culture. This group showed that long term TAM treatment of MDA-MB-231 cells increased their growth and became tetraploid and also grew aggressively into tumors in animals. This would be a useful addition.

Response: We completely agree with the Referee’s opinion that inclusion of Sipila’s reference [14] could represent a useful addition. We have discussed this reference in the new version of the article and point out that our results agree with previous observation in which long term tamoxifen treatment of MDA-MB-231 cells increased their growth and their aggressiveness in animal tumours.

10. All of this refers to 4OHT, but what happens with Estradiol and what happens with estradiol after conversion by 4OHT? Does this still all work?

Response: To ask this question we include experiments performed in hormone-deprived conditions. For such experiments cells were maintained for three days in phenol red-free DMEM plus 2.5% dextran-charcoal-stripped foetal calf serum and then they were treated in the presence or absence of 4OHT. Stimulation of cancer cell proliferation in the presence of 4OHT was also observed in hormone-deprived conditions, which indicated that this effect was independent of oestrogens in the culture medium. We are pretty confident with these results because experiments from other group [10] showing 4OHT-induced proliferation of ERα-negative breast cancer cells were also carried out in hormone-deprived conditions. Although we had these data, we agree with the Referee that this important control was missing in the previous version of the manuscript.

11. In figure 1B, shows Western blots for the 4OHT modulated MDA-MB-231 cells, but 1C shows the relative levels of mRNA compared to MCF-7. To me the Western blot in 1B is an enormous amount of ER not consistent with 3.3 copies of mRNA per million. Can the authors reconcile this?

Response: We believe that Referee misunderstood this result. Control MDA-MB-231 expressed 3.3 copies of mRNA per million but this was not accompanied of visible ERα protein expression (Control lines in PCR and WB experiments). Only after 4OHT treatment the ERα protein could be visualized by WB, but in this case the number of copies of ERα mRNA corresponded to close to 20 copies of mRNA per million of β-actin. In any case, the relation between quantities of mRNA and protein is always complicated because several factors related to mRNA and/or protein stability are also important. As observed in Figures 1A and B, ERα protein levels in MDA-MB-231 after several treatments were consistent when analysed by both confocal microscopy and WB, respectively.
Minor Essential Revisions

1. The MDA-MD-231 cells should be genotyped to produce fidelity with these studies.

Response: The MCF-7 and MDA-MB-231 cell lines were purchased from ATCC and are routinely authenticated with genotype profiling according to ATCC guidelines by our University Culture Service.

2. Page 3, first sentence of 2nd paragraph, the word 'allosterically' is incorrect as tamoxifen and other SERMs are competitive inhibitors within the ligand binding domain of the ER. Allosteric defines a separate site other than the active site. This must be changed.

Response: We agree; the term “allosterically” has been removed.

3. Page 13, 2nd paragraph, the MDA-MB-231 cells are classified as triple negative breast cancer. They have a mutated fas that is unusual. Important for the authors hypothesis concerning apoptosis, do these cells also have mutated p53?

Response: MDA-MB-231 cells have a p53 gain-of-function and hyperactive mutation. p53 in MDA-MB-231 presents a homozygous mutation at gene sequence 839G>A and with a protein residue change R280K.

Discretionary Revisions

1. This is a suggestion to the authors for future studies that they should consider this combination in ER positive breast cancer cells that have acquired resistance to 4OHT. These data would be extremely valuable as this is what is occurring in the clinic and requires a solution.

Response: This is a very interesting suggestion in which we are working in. As the referee may perfectly know breast cancer is considered one of the most chemosensitive solid tumours; however, initially responsive tumours relapse and develop resistance to a broad spectrum of drugs, including, of course, tamoxifen. In a previous study [13], we observed that TMCG/DIPY treatment positively influences E2F1-mediated cell death, and we hypothesized that this combination might represent an attractive strategy to target overexpressed E2F1 in tamoxifen resistant cells. The observation that TMCG/DIPY treatment was highly effective on MCF7-tamoxifen resistant cells [13] confirmed this hypothesis and suggested that this combinational therapy could be extended to the treatment of patients with anti-oestrogen resistant breast cancers. In addition, this treatment could also be of interest for cells harbouring a p53 deficient pathway. Although MCF7 cells show a wild-type p53 phenotype, deficiencies in caspase-3 make these cells difficult to kill by apoptosis. We have also observed that treatment of MCF7 with TMCG/DIPY induced efficient E2F1-mediated apoptosis in these cells [13]. In fact, we have recently reviewed several strategies to target the epigenetic machinery of cancer cells using combinations such as TMCG/DIPY which affect the folic and methionine metabolism in cancer cells [15].

References:


At last, we sincerely appreciate the reviewers’ comments, which in our opinion have greatly helped to improve the clarity and quality of this article.

Sincerely yours

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