Author's response to reviews

Title: Comparative actions of progesterone, medroxyprogesterone acetate, drospirenone and nestorone on breast cancer cell migration and invasion

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Author's response to reviews: see over
To Melissa Norton, MD
Editor-in-Chief
BMC Cancer

Dear Dr. Norton,

I am pleased to resubmit to BMC Cancer the revised manuscript 2939652831784705 R1: “COMPARATIVE ACTIONS OF PROGESTERONE, MEDROXYPROGESTERONE ACETATE, DROSIPRENONE AND NESTORONE ON BREAST CANCER CELL MIGRATION AND INVASION”.

According to all the queries raised by the reviewers, we have finished several new experiments and added new relevant contents in this revised version of the MS. Detailed responses can be found in the enclosed point-by-point reply to the editor and reviewers.

The MS complies with all the BMC Cancer editorial rules, and we thus hope that it will be now fit for publication on the BMC Cancer.

Please, be assured that the manuscript has not been published nor is being considered for publication elsewhere in whole or in part, in any language.

Yours Sincerely,

Tomasco Simoncini, MD, PhD,
(on behalf of the authors)

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“COMPARATIVE ACTIONS OF PROGESTERONE, MEDROXYPROGESTERONE ACETATE, DROSPIRENONE AND NESTORONE ON BREAST CANCER CELL MIGRATION AND INVASION”.

RESPONSES TO REVIEWER 1:

We would like to thank the reviewer for taking the time to review the MS and for the useful suggestions on our work. Detailed responses:

Major Compulsory Revisions:
1) The authors indicate in Figure 1 that the addition of any of the four progestin induced formation of cell membrane structures involved in cell adhesion and movement, including ruffles, focal adhesion complexes and pseudopodia. This result would be greatly reinforce with the addition of the progestin inhibitor ORG 31710 in combination with the four compounds to evaluate whether the formation of this cell membrane structures is now impaired.

2) The authors indicate in Fig 2 that E2 induced actin rearrangement in T47-D breast cancer cells. However they claimed that each progestin, when added to E2, did not significantly change the effect of E2 itself, although the cells often displayed a somewhat more evident rearrangement of actin fibers and cell membrane structures formation as compared to treatment with the progestin alone. This affirmation is too vague and the effect of the two hormones together should be addressed more precisely using each progestin in combination with an ER inhibitor such as ICI 182,780.

3) Page 8 paragraph 4: the authors proposed that the PR antagonist ORG31710 significantly reduced cell migration associated with the four tested progestins (Fig. 6A-E), but also significantly decreased the effect of the combination of E2 with any of the progestins (Fig. 6A-E). However if they were not able to find any significant additive effects of E2 on cell migration during co-treatment with any of the progestins the correct form of this affirmation should as follows. “Interestingly, the PR antagonist ORG31710 significantly reduced cell migration associated with the four tested progestins both in the absence and in the presence of E2 (Fig. 6A-E)”.

As the reviewer suggested, we also added experiments where the association of estradiol with the progestins is used in combination with the ER inhibitor ICI182,780. These data can now be found in Fig.2A.

1) Sorry that the labeling of Fig.1 was unclear. Actually last box in every row (“15’+ ORG”) already showed the effect of ORG31710 in combination with the four progestins. Now we changed the labeling in Fig.1 to make this clearer.

2) As requested, we have quantitated the actin cytoskeleton modifications, measuring the mean cell membrane thickness and the mean fluorescence intensity of the cell membrane by using the Leica QWin image analysis and image processing software (Leica Microsystems, Wetzlar, Germany) and the analyzed data are presented in Table. 1. These data show that actin remodeling to the cell membrane, as shown by increased membrane thickness and intensity, is further augmented when cells were treated with E2 plus each progestin, compared to progestin alone, although this does not reach statistical significance.

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3) Thanks for the reviewer’s suggestion. Now we corrected this sentence in the context as the reviewer suggested.
4) The authors state that the induction of breast cancer cell horizontal migration and invasion by progestins was related to the differential ability of these steroids to activate the actin-binding protein moesin, leading to effects on actin cytoskeleton remodeling and on the formation of cell membrane structures that mediate cell movement. However, this affirmation is not based on these group pf results. A sounder demonstration should be given. Authors should evaluate the effect of blocking moesin expression using moesin specific asense-oligodeoxinucleotides or iRNA on cell fate or the migration and invasion capacity induced by progestins.

4) We fully agree with the reviewer’s comments. Indeed, we have performed such experiments in T47-D breast cancer cells and we now present these data on P- and MPA- induced cell migration after transfection with specific moesin antisense and sense oligonucleotides in Fig. 6. After blocking moesin expression by using moesin antisense, P- and MPA-stimulated cell migration distance were markedly reduced to the control level, suggesting the pivotal role of moesin protein in these processes. This fits with some recent data from our lab where we have found this same effects in human endothelial cells.

5) The discussion is particularly confusing, with many ideas that are thorny to follow, presenting some difficulties in reaching a conclusion based on the results. Specifically paragraph 5 and 6 should be rewritten paying particular attention in the concentration discussion which is the most unclear issue. In paragraph 8 the idea of the ORG mediated- inhibition of the additive effect of E2 on progestin regarding the migration and invasive capacities of T47D cells in not clearly explained. The grammar should be check in all the discussion.

5) According to reviewer’s suggestion, we revised some parts of the discussion, in particular paragraph 5, 6 and 8.

- Minor Essential Revisions

Abstract:
1) The word “Little” should be replaced for “limited or lacking”.

1) We replaced it as “limited”.

2) In the results paragraph the affirmation “differences were found in terms of potency, with MPA being the most active and DRSP being the least” the word “active” should be replace for “effective” or something similar.

2) We replaced it as “effective”.

3) In the conclusion paragraph the word “tendency” should be replace for “ability” or something similar.

3) We changed the word “tendency” to “ability”.

4) The sentence “we characterized the signaling steps recruited by these progestins” lacks of experimental bases and should be removed. There is no characterization of any signaling steps in the results, only some assumptions based on inhibitor assays.

4) We deleted this sentence in the abstract.

Introduction
5) Page 3 Line 2 delete the phrase “in women with an uterus” and explain more ie. an inappropriate endometrial proliferation caused by estradiol administration.

5) We changed it as “Progestins are required in HRT in women to prevent an inappropriate estrogen-stimulated endometrial proliferation”.
6) Page 3 Line delete “On the other hand, progestins are not equal”.

6) Now the sentence was deleted.

7) Revise the sentence As for the risk of breast cancer, the French cohort study as well as the E3N-EPIC cohort study show that synthetic progestins, but not natural progesterone, increase the relative risk of breast cancer in postmenopausal women receiving continuous-combined HRT. It is confused.

7) We revised this sentence, please see it in Introduction section for details.

8) Link the paragraph 4 with 5 using a period.

8) We did this as suggested.

9) Paragraph 6 line 3 the word “little” should be replaced for “limited or lacking”.

9) We changed it to “limited”.

10) Paragraph 6 line 4 the word “Plus” should be replaced for “moreover”.

10) We changed it as suggested.

11) Last paragraph line 2 add the phrase “in combination” with E2.

11) We added the phrase as suggested.

Material and methods

12) Page 5 paragraph 2 line 4 delete the word “or”.

12) We deleted it in the context.

13) In the immunobloting protocol a detailed composition of the lysis buffer or an indicative reference is missed.

13) Now we described the detailed composition of the lysis buffer in this section.

Results

14) The sentence should be revised for an English expert. Underline parts are wrong. “As P, MPA and DRSP have binding affinities for progesterone receptor (PR) in the same range, while NES is about a 100-fold more effective compared to P in inducing endometrial transformation or binding to PR [11], we used a 100-fold lower concentration of NES”.

14) Now we changed and simplified this sentence as follows: “Based on the evidence that P, MPA and DRSP have comparable binding affinities for progesterone receptor (PR), while NES is about a 100-fold more effective than P in binding to PR [11], we used a 100-fold lower concentration of NES than other three progestins”.

Figure Legends

15) Figure 3: First line “moesin” should not appear between parentheses. The indication of the use of the inhibitor Y-27632 and the concentration used is missing in the legend.

15) We corrected them in Figure Legends.
Discussion
16) Paragraph 1; the word “Little” should be replaced for “limited or lacking”.

16) We replaced it as suggested.

17) Paragraph 5 line 3 “induce” instead of “inducing”.

17) We corrected it now.

18) Last paragraph, rewrite the sentence “These differences in biological efficacy are to some extent linked to partially discrepant recruitment of extra-nuclear signaling pathways by PR in the presence of each progestin” using potential.

18) We rewrote the sentence as suggested.

19) Last paragraph; delete the last part of the sentence from the coma.

19) We deleted it now.

- Discretionary Revisions
20) What happened with moesin expression or activation at 24 or 48 hs? I would be interesting to evaluate the effect of progestin addition at long times to differentially characterized short and long terms effects.

20) We previously found in human endothelial cells that E2 and progestins not only induce a rapid activation of moesin, but also up-regulate moesin expression after longterm treatments (T.S Mol Endo; 2006, Fu XD, JCEM, 2008). Hence, an upregulation of moesin expression will be expected in T47-D breast cancer cells exposed to progestins for 24 or 48 h. The issue will be investigated in the next future.
RESPONSES TO REVIEWER 2:

We are grateful to the reviewer for taking the time to look at our paper and for the many useful comments. Here are the detailed responses to the reviewer’s comments:

Minor Essential Revisions

1. E2 mediated enhancement of progestin activation of actin rearrangement is overstated and is not borne out on comparing data shown in Figures 1 and 2. For definitive conclusions, data in Figures 1-2 must be quantitated. While Figures 1-2 are impressive representative pictures of morphology changes, a quantitation of the % cells with spatial modifications of actin fibers and cells that form specialized cell membrane structures (longitudinal actin fibers, pseudopodia, ruffles) would permit a more rigorous comparison among treatments with statistical analysis.

1) Now we quantitated actin cytoskeleton modification, including mean cell membrane thickness and the mean gray level of cell membrane, % cell with spatial modifications of actin fibers by using Leica QWin image analysis and image processing software (Leica Microsystems, Wetzlar, Germany) and the analyzed data are presented in Table. 1.

2. A panel of three additional representative breast cancer cell lines is needed in Figures 1 and 2 to demonstrate the generality of these morphology changes for breast cancer. These data should be quantitated as described in #1 above and data compared among the cell lines. T47-D is not the best representative model of estrogen receptor positive breast cancer and one needs to know whether these ligands have similar or different effects in other breast cancer cells.

2) As suggested, we added new experiments in MCF-7 ER+/PR+ breast cancer cells and progestins, including P and MPA, together with E2, induced the actin remodeling as in T47-D cells. On the contrary, these compounds have no impacts on MDA-MB-468 ER-/PR- breast cancer cells. Furthermore, when MDA-MB-468 cells were transfected with PRA and PRB, progestins regain the potential to induce the actin reorganization. These results now are presented in Fig. 2B and the analyzed data on spatial modification are shown in Table. 1.

3. The additive effects of E2 and Progestins on moesin phosphorylation are not convincing based on the data presented (Figures 4A-C). Densitometry data of moesin and phosphorylated moesin bands normalized to an internal control with statistical analysis are needed for definitive conclusions to be made.

3) The reviewer is right, although moesin is slightly more activate when Progestins + E2 is administered respect to Progestins alone, this is never statistically significant. We quantitated densitometry data by using our quantitative chemiluminescence acquisition machine (ChemiDoc, Bio-Rad), by building up graphs that report the numbers of photons emitted by the single bands. We are now comparing the intensities of the bands to Moesin in the graphs in Fig. 4. In addition, we rephrased the results and discussion to highlight that the difference between estrogen + progestin or progestin alone is not significant.

4. Combination of E2 and Progestins does not appear to significantly alter the cell migration index compared to effects of Progestins alone, although E2 alone significantly increases the invasion index compared to Progestins alone. The Discussion on the combinatorial effects of E2 and Progestins on moesin phosphorylation, cell migration and cell invasion should be modified. Although E2 induces actin rearrangement, cell migration and invasion E2 does not appear to further enhance Progestin induced actin rearrangement moesin phosphorylation, and cell migration and invasion.

4) We rewrote the Discussion on the combinatorial effects of E2 and Progestins on moesin phosphorylation, cell migration and cell invasion. Please see the details in Discussion section.