Author’s response to reviews

Title: Activation of the steroid and xenobiotic receptor, SXR, induces apoptosis in breast cancer cells.

Authors:

Suman Verma (sverma@uci.edu)
Michelle M. Tabb (michelle.tabb@gmail.com)
Bruce Blumberg (blumberg@uci.edu)

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Author’s response to reviews: see over
Editors, BMC Cancer

Dear Editors –

We are pleased to re-submit to BMC Cancer the revised version of manuscript # 6577047992157375 entitled “Activation of the steroid and xenobiotic receptor, SXR, induces apoptosis in breast cancer cells.” Suman Verma, Michelle M. Tabb and Bruce Blumberg.

In response to the queries and suggestions of the referees, we have undertaken several new experiments and so it has taken us some time to comply with the requests. This has resulted in addition of two new figures in the MS (Figure 4C and 5D) and 3 new figures in the supplementary data (Supplementary Figure 3B and 4A and C). To maintain the flow of the manuscript, we moved the original Figure 4C and Figure 5B in the manuscript to supplementary data as supplementary Figure 3A and supplementary Figure 4B.

We believe that we have faithfully answered both the Major and Minor Revisions required by all three reviewers, and these are detailed in the following pages. In one or two cases, we felt that the request was well outside of the reasonable scope of the current paper, and we have indicated our reasons in the detailed reply. We have also provided additional data for the reviewers in the following detailed point by point response to reviewers to more closely scrutinize the data provided in the original manuscript. We hope that the manuscript will now be suitable for publication in the Journal.

Sincerely yours,
Bruce Blumberg, Ph.D.
We would like to thank reviewer for taking the time to review our MS and for many useful comments on our work. We think reviewer’s insightful comments have helped in improving the manuscript a lot. Here is the point by point discussion of the comments.

Reviewer’s report:
This study by Verma and colleagues employs two widely used breast cancer cell lines to demonstrate that SXR activation has anti-proliferative and pro-apoptotic effects, mediated by NO-dependent induction of p53 and its target genes. This is a well-written report and at first glance the data appear compelling. Yet on closer scrutiny, several important questions and methodological concerns remain:

Major Compulsory Revisions
• The level of proof of the proposed pathway would be much improved if cell systems were employed that naturally lack components of the proposed pathway. For instance, are there SXR-negative cell lines? What are the responses to SXR activators in p53-deficient breast cancer cells, such as EVSA-T and MDA-MB-231?

We agree that it would be a good idea to use SXR-null cell lines to further support our results. However, our extensive search for SXR null cell lines using (MDA-MB-435, MDA-MB-231, SKBR3, MDA-MB-175-7, MCF-7, ZR-75-1, T47-D, MDA-MB-468, 578-T and HBL100), we found only MDA-MB-468 completely lacks SXR mRNA expression. All other cell lines express SXR to some degree. Our results are consistent with a previously published report showing that MDA-MB-468 and BT-20 lack SXR mRNA expression (Dotzlaw, Leygue et al. 1999). However, since both of these cell lines have non-functional p53, we will not expect them to respond to SXR activators. Considering these points, we would argue that using siRNA-mediated knockdown of SXR is a valid approach to confirm the involvement of SXR in the proposed mechanism.

Our initial results with p53 deficient cell lines suggest that there is no significant effect of SXR activators in p53 deficient breast cancer cell lines such as MDA-MB-231 and MDA-MB-435, which is consistent with our model. But, as both of the cell lines tested are also ER negative it is difficult be certain about the role of p53 or ER in our mechanism by using this model. This point requires further exploration using more cell lines but we think it is beyond the scope of this paper.
• The reason for using different compounds in different experiments is entirely unclear to me, and undoubtedly confusing to the reader. What is the point of using Tam in these ER-positive cells? Similarly, why use RU486 and do these cells express PR/AR/GR?

In the initial experiments to test the effects of SXR activators on proliferation or cell cycle of cultured breast cancer cells we used many different classes of compounds such as the antibiotic rifampicin, the anti-fungal clotrimazole, fatty acid ethanolamide anandamide, anti-estrogen tamoxifen, calcium channel blocker nifedipine and anti-glucocorticoid RU486. The point was to use as diverse a set of compounds as possible that are all SXR activators to ascertain whether they have similar effects on proliferation and could act through a common mechanism (figure 2A and 2B). We recognize that some of these compounds can have effects on the breast cancer cells through other mechanisms. Tamoxifen is a both estrogen receptor antagonist and SXR activator, similarly RU486 can also affect PR/GR and SXR. To avoid confusion, we have used the antibiotic rifampicin, the anti-fungal clotrimazole and the fatty acid ethanolamide anandamide consistently in all further experiments. We have added additional discussion regarding the rationale for this experimental design in the revised manuscript to reduce confusion (Line 5-9, P14).

• The authors have chosen very disparate time-points to analyze the functional consequences of treatment with SXR activators. This makes it difficult, if not impossible, to closely scrutinize the data. For example, proliferation was measured after 7 days (Fig 2A), cell cycle progression after 24 h (Fig 2B), apoptosis after 48 and 72 h (Fig 2C), and gene expression at different time-point, depending on the cell line. It would be a whole lot more informative if kinetics of the responses are analyzed in each cell line in a proper designed time-course experiment.

Proliferation assays were continued until 7 days because we wanted to see an integral effect on cell cycle and cell survival by SXR activators and this was experimentally determined to be the optimal time to measure this endpoint as the cells in the solvent control wells are just reaching confluency at 7 days(Line 16-18,P12). Obviously, this is too late to observe earlier molecular events. To observe early effects of activating SXR within one or two cell cycles, we did a time course study, taking time points at 12HR, 24HR and 48 HR. We observed G1/S arrest by SXR activators starting at 24 HR except clotrimazole in which cells got arrested at G1/S phase as early as 12 HR post-treatment. The cells treated with SXR activators accumulated more in G1/S phase at 48 HR but the cells in solvent control also increased in G1/S phase at 48 hr time point (44.6% at 24 HR vs. 66.9 % at 48 HR) reducing the difference between control and treatment compounds. That is why 24 HR was determined to be the optimum time for this experiment and was repeated again twice and is shown as Figure 2B in the original manuscript. The data from previous time-course is provided below
for the reviewer and the time-points have been discussed in more detail in the revised manuscript (Line 16-22, P13 and Line 1-3, P14)

<table>
<thead>
<tr>
<th></th>
<th>12HR</th>
<th>24HR</th>
<th>48HR</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>%G1</td>
<td>%S</td>
<td>%G2</td>
</tr>
<tr>
<td>DMSO</td>
<td>40.5</td>
<td>37.1</td>
<td>20.7</td>
</tr>
<tr>
<td>RIF</td>
<td>41.1</td>
<td>37.4</td>
<td>18.5</td>
</tr>
<tr>
<td>ANA</td>
<td>34.6</td>
<td>42</td>
<td>19.6</td>
</tr>
<tr>
<td>CLO</td>
<td>52</td>
<td>30.1</td>
<td>13.9</td>
</tr>
</tbody>
</table>

As we did not see effects on the cell cycle by all SXR activators until 24 hours, we conducted pilot, time course experiments for the apoptosis assay from 24-72 hours. This experiment revealed that there was no strong effect on apoptosis until 48 hours. Results from the previous pilot experiment have been provided below for reviewers.

Based on these results from the cell cycle and apoptosis experiments, we conducted gene expression assays using a time course with data points at 24, 48 and 72 hours in MCF-7 and ZR-75-1 cells, but did not show all of these points in the original manuscript. These data have been provided below for the reviewers and the time points are discussed in more detail in the revised manuscript (P14-P15).
MCF-7

**p53**

**p21**

**BAX**

**PUMA**

ZR75-1

**p53**

**p21**

**BAX**

**PUMA**
• In figure 1, the authors show that SXR levels in ZR-75 and MCF-7 cells are comparable, yet Figure 2A suggest that MCF-7 cells are considerable more sensitive to SXR activators. What is the explanation?

We agree with the reviewer that although MCF-7 and ZR-75-1 cells express almost equal amount of SXR, the MCF-7 cells are considerably more sensitive than ZR-75-1 cells. There can be many reasons for differences in the sensitivity of these cells towards SXR activators. One plausible and likely explanation is that ZR-75-1 cells express much lower levels of p53 in comparison to MCF-7 cells ([http://www.mdanderson.org/departments/cancerbiology/dlIndex.cfm?pn=31062032-B0EB-11D4-80FB00508B603A14](http://www.mdanderson.org/departments/cancerbiology/dlIndex.cfm?pn=31062032-B0EB-11D4-80FB00508B603A14)). The topoisomerase I inhibitor, camptothecin can only induce a ~3 fold increase in p53 protein levels in ZR75-1 cells compared with an ~18 fold increase in MCF-7 cells (please compare figure 3C and Figure 3D). This is consistent with the possibility that differences in the sensitivity of these cells to SXR activators is more closely related to the inducibility of p53, than to the levels of SXR. We have added further discussion of this point on P26 in the revised manuscript.

• Figure 2B. How many times was the flow cytometry experiment repeated? Does the percentage of cells in different phases of the cycle represent an average of several measurements? Why is there no apparent induction in <2N cells upon treatment with SXR activators?

In pilot experiments, the time course was done in duplicate and the data shown are the average of duplicates. The time course showed the most pronounced effect at 24 hours; therefore, the 24 hour time point (Figure 2B) was repeated twice in duplicate and the numbers represent the average of four independent determinations. This has been clarified in the figure legend of the revised manuscript now.

To accurately measure the percentage of cells in a particular phase of cell cycle the flow was gated on live cells. This can explain why we do not see an increase in <2N cells. This has also been clarified in the figure legend of the revised manuscript now.

• The authors logically examined the expression of calmodulin in response to one SXR activator and seemingly only in MCF-7 cells. The rationale of this odd experimental design is unclear. More importantly, is the very modest and transient induction of calmodulin mRNA translated into sustained increase at protein level or of little consequence?

We have tested the response of SXR activator rifampicin on CaM mRNA in a time course experiment in ZR-75-1 cells and the data is now provided as supplementary Figure 3B.
We have also tested the effect of two SXR activators rifampicin and anandamide on CaM protein level in both MCF-7 and ZR-75-1 cells and the data is provided as Figure 4C and has been discussed on P17 in the revised manuscript.

The previous Figure 4C showing mRNA level of CaM in response to rifampicin in a time course experiment in MCF-7 cells has now been moved to supplementary data as Figure 3A.

• Figure 5 is important as it should provide functional evidence for the proposed pathway. Thus, the authors should show how overexpression of a constitutively active SXR mutant induces p53 expression, its downstream target genes and apoptosis. Conversely, it only seem logical to examine the induction of p53-dependent genes and the proliferative and apoptotic responses to SXR activators in cells where the expression of the endogenous receptor in silenced by siRNA. These data are essential and currently missing. The data presented in Figure 6 are merely confirmatory and can be omitted.

The effects of constitutively active SXR on the proliferation assay have been shown in Figure 5A. We have also further confirmed the effects of constitutively active SXR mutant on iNOS, p53 and its down-stream target genes and the data has been provided as supplementary Figure 4A and has been discussed on P20-P21 in the revised manuscript.

We have also tested the induction of p53 and its down-stream target gene p21 in response to SXR activators rifampicin and anandamide in untransfected MCF-7 cells or in MCF-7 cells transfected with scrambled or SXR specific siRNA. These data are now provided as supplementary Figure 4C in the revised manuscript.

We have also tested the apoptotic response of SXR activator rifampicin in untransfected MCF-7 cells or in MCF-7 cells transfected with scrambled or SXR specific siRNA. The data are now provided as Figure 5D and discussed on P21 in the revised manuscript.

Discretionary Revisions
• On page 11, the authors refer to data regarding the induction of CYP3A4 mRNA in ZR-75 and MCF-7 treated with 3 compounds. If this is such a good marker gene for SXR activity, why was it necessary to treat the cells for 48 h? Is CYP3A4 induced in response to Tam/RU486?

Cyp3a4 is a bona-fide target gene of SXR. We previously found that it takes 48 hours for cyp3a4 to be induced significantly by SXR activator in osteosarcoma cells (Tabb, Sun et al. 2003). Bone and breast are not primary organs for drug metabolism; moreover, we have previously published that SXR can selectively regulate expression of its target genes in different tissues depending on the ratios of co-regulators in that particular tissue. Therefore, it may not be completely unexpected that it takes longer to induce cyp3A4 in bone or breast
compared to liver or intestine (Zhou, Tabb et al. 2004). We also note that previous reports demonstrate that tam and RU486 can induce cyp3A4 through activation of SXR. These points have been further discussed in the revised manuscript (P11).

• In figure 2A, the authors use rutin as a control compound. What is the rationale for using rutin? Why was this control omitted in the subsequent experiments?

As shown in supplementary figure 2A rutin is a phytoestrogen which does not activate SXR. In our initial screening of SXR activators and related compounds, we found that rutin was inactive on SXR, hence, it is an appropriate control. The subsequent experiments were done at different times by a different investigator (Ms. Verma compared with Dr. Tabb). This point has been clarified in the revised manuscript.

• On page 13, the authors state that apoptosis begins in MCF-7 cells at 48 h of treatment with SXR activators. Yet as far as I can deduce from Figure 2C, apoptosis in response to these compounds was only measured at 48 and 72h. A more detailed time-course is required. What happens to SXR levels upon prolonged treatment with these compounds?

The more detailed experiments were done in the early pilot study with 24, 48 and 72 hours time points and there were no significant effects on apoptosis at 24 hours. The data has been provided above for the reviewer.

Our preliminary results in hepatocytes and previously published data have shown that prolonged treatment with SXR activators decreases the level of SXR protein via proteosomal degradation (Masuyama, Inoshita et al. 2002). Detailed studies of SXR turnover in these cells are an interesting avenue for future experimentation.

• The authors do not examine the mechanism of the induction of p53 at protein level, although offer several speculative explanations. A minimum requirement would be to probe the blots presented in Figure 3C and D for Mdm2 expression.

Previously published reports have shown that nitric oxide causes nuclear accumulation of p53 by impairing its nuclear export (Wang, Michael et al. 2002; Schneiderhan, Budde et al. 2003). It remains controversial whether the nitric oxide induced increase in p53 levels is entirely because of impaired nuclear export or because of decreased protein degradation. However, it is well established that this nuclear accumulated p53 is transcriptionally active and can increase expression of Mdm2. We propose that a similar mechanism works here and expect to see an increase in Mdm2 expression in the blots from Figure 3C and 3D where p53 is up in the presence of SXR activators but we wonder how much would that help in providing mechanistic insights of the process? We have presented a substantial amount of data in support of our model (activation of SXR leads to increased iNOS mRNA, NO and induction of p53 and its target
genes; loss of SXR blocks this increase in iNOS, and p53, and inhibition of iNOS blocks p53 induction). A more detailed study of the mechanism of p53 induction in response to SXR activation would be interesting and informative for future experimentation but, we believe, beyond the scope of this paper.

**Level of interest:** An article of importance in its field

**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

Masuyama, H., H. Inoshita, et al. (2002). "Ligands have various potential effects on the degradation of pregnane X receptor by proteasome." Endocrinology 143(1): 55-61.
Title: Activation of the steroid and xenobiotic receptor, SXR, induces apoptosis in breast cancer cells.

Response to Reviewer: Ratna K Vadlamudi

We thank reviewers for taking time to review our manuscript and for useful and thought provoking suggestions. Please review the detailed point by point discussion of the comments below.

Reviewer's report:
This is an interesting manuscript that tests the hypothesis that orphan receptor SXR may play a role in the induction of apoptosis in the breast cancer cells. The rationale for this study is sound. Using proliferation and cell cycle assays, authors provided strong evidence that SXR signaling play a key role in the growth inhibitor action of various compounds that function as activators of SXR in estrogen receptor (ER) positive breast cancer cells. Using mechanistic studies, they have provided evidence that SXR mediated induction of iNOS levels, local production of NO and up regulation of p53 as the mechanism by which SXR signaling contributes to growth inhibition. Even through the manuscript is little bit descriptive, it contains some clinically useful information and these findings have clinical implications in potential combinatorial therapy for breast cancer cells. Addressing the following concerns will further strengthen the manuscript.

1. Even through the manuscript uses estrogen receptor (ER) positive cells, the role of ER in SXR pathway is not addressed. Is SXR mediated inhibition is only limited to ER positive cells. Is ER needed for SXR mediated induction of iNOS. Do the observed effects hold good in ER negative, p53 WT positive cells.

Previously published reports have suggested that there is an inverse relationship between ER and SXR level in breast and endometrial tissues(Dotzlaw, Leygue et al. 1999; Masuyama, Hiramatsu et al. 2003). However, no such relationship was observed in breast cancer cell lines in previously published reports or in our preliminary experiments. It is possible that this may be because insufficient samples have been examined. This point requires future exploration and has been discussed in the revised manuscript (P25-P26).

We agree with the reviewer's suggestion that ER negative, p53 WT cells will be a good model to further explore the role of ER in SXR activator induced apoptosis. However, our extensive literature search revealed that only CAL51 cells are ER negative and have functional p53. CAL51 cells are also PR negative and Her2 negative; therefore, we could not be certain about the effects of ER on SXR-induced apoptosis using this model. It is likely that the best way to examine the interactions between SXR and ER would be to stably knock down ERα and ERβ in MCF-7 and ZR75-1 cells and then test the effects of SXR activators in that cell
model. This would be an interesting avenue for future studies but beyond the scope of this paper.

2. Figure 3 C, 3D: the quality of the figure is not good. The numeric values of p53/GAPDH ratios do not correlate very well with the intensity of the bands shown in the figures.

The figures have been re-scanned at higher resolution and put in the revised manuscript. The numeric values are the average of two independent runs and were derived from direct imaging of the blot itself. The procedures have been more fully described in the figure legend of the revised manuscript.

3. Figure 4F: The data using 1400W is little bit confusing and contradictory and the explanation given on page 18 does not clearly explain the effects seen in lane 3 and lane 6. If 1400W is an activator of SXR transcription, then why it is inhibiting RIF mediated induction of P53 in lane 6 is not clear.

The referee is correct that the results from 1400W are a bit confusing. One possible explanation is that rifampicin is a higher affinity ligand for SXR than is 1400W. Thus, in the absence of rifampicin 1400W can bind to and activate SXR. However, in the presence of rifampicin the 1400W is competed out and functions only as an iNOS inhibitor. The point has been discussed in more detail in the revised manuscript (P19-P20).

4. Figure 5C; It would be better if authors show the expression of SXR as effected by control SCR or SXR specific siRNA in one gel rather than as two separate pieces attached together. in addition use of single siRNA for down regulating SXR and p53 is a concern

The blot has been repeated and replaced with the new blot in the revised manuscript.

The siRNA used for knocking down SXR has been used successfully in previously published reports (Bhalla, Ozalp et al. 2004; Ichikawa, Horie-Inoue et al. 2006). We also tested 3 other siRNAs for SXR. Each of these achieved various degrees of SXR knockdown but all were less efficient than the one used in the study. Similarly, the p53 siRNA used in our study was chosen for its effectiveness from 4 siRNAs. The data provided in the manuscript support our pathway by showing both gain- and loss-of-function experiments. Moreover, we knocked down SXR using siRNA at the lowest concentration required to achieve efficient knockdown (36nM). Therefore, we argue that the results shown are real and not non-specific effects of siRNA. Using additional siRNAs to show the same results would be repetitive and not provide any new information or confirmation of the results shown.
**Level of interest:** An article of outstanding merit and interest in its field

**Quality of written English:** Acceptable

**Statistical review:** Yes, and I have assessed the statistics in my report.


Title: Activation of the steroid and xenobiotic receptor, SXR, induces apoptosis in breast cancer cells.

Response to Reviewer: Rakesh K. K. Tyagi

We thank reviewer for taking time to review our manuscript and for his enthusiastic feedback on our manuscript. Please review below point by point discussion of the comments.

-Minor essential revisions
1. Refer to Figure 4F: While pretreatment with iNOS inhibitors blocked the rifampicin-activated p53 up regulation, the effect of iNOS inhibitor 1400W on basal p53 levels is confusing. Apparently, when used alone, both 1400W and rifampicin are potent SXR activator as well as inducer of p53 level. But when used in combination (1400W + rifampicin) lead to down-regulation of rifampicin-activated p53 level. These results are not as would be expected, and therefore, need to be discussed in the text. Is it possible that as a ligand, rifampicin competes out 1400W for SXR binding?

The referee is correct that the 1400 W results are confusing and has given an excellent idea for explaining the results. We have increased discussion on this point in the revised manuscript on P20-21.

2. Refer to Figure 5C: Though the Western blot results supports the claims of the authors when considering the SXR/GAPDH ratio, the amount of total protein loaded in lane SCR appears to be more than in lane UT that initially gives a contradictory/confusing impression. Also, lane SiRNA is a portion from other blot. This figure, if replaced by a single composite blot with equal protein loading will be more convincing to your claims.

The blot has been repeated and replaced with a new blot in the revised manuscript as Figure 5B.

- Discretionary revisions
3. While the present manuscript is under review another article dealing with the role of SXR in ovarian cancer has been highlighted in the reference below. A reference to the study may be incorporated in the present manuscript.
We have incorporated the above mentioned reference in the discussion of the revised manuscript on P27.