Author's response to reviews

Title: Benzyl butyl phthalate induces migration, invasion, and angiogenesis of Huh7 hepatocellular carcinoma cells through nongenomic AhR/G-protein signaling

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Manuscript entitle: Benzyl butyl phthalate induces migration, invasion, and angiogenesis of Huh7 hepatocellular carcinoma cells through nongenomic AhR/G-protein signaling

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Dear Editor:

Greetings of Peace!

I am pleased to return my revised manuscript, and I have carefully checked and retyped the paper according to the checklist you sent me previously. It would very much appreciate if the referees can read the article soon, and I hope to hear from you again in this regard. Thank you for your time and attention to this matter.

Sincerely yours,

Eing-Mei Tsai

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Reviewer: Ashwin Kotnis

1) Figure Legend. Fig.3: Line 12. It is unclear how “at least ten cells” were measured?

As suggested, “The experiments were performed using the same method as in a previous study [19] with some modifications” are included in the Methods part. (Page 8). As shown in the figure, we choose the cells (n=11) to measure the fluo-4 intensity, which indicated the calcium level. ROI 2 12 and ROI 2 13 indicated the background intensity.
2) For uniformity, use italicized form of AhR in Ahr mRNA

As suggested, we have used italicized form of AhR in AhR mRNA.

3) Spelling errors:

a) Abstract: Methods, last line- angiogenesis

b) Discussion: Second paragraph-subsequentaly-->subsequently,

ligan-induced-->ligand-induced

c) Figure Legend. Fig.3: Line 1- casued-->caused, cleaveage-->cleavage

d) Figure Legend. Fig.5: Line 12- magnificient-->magnification

We are very appreciated for the comments and have carefully corrected the spelling errors.

Discretionary Revisions:
1) Please confirm the use of term environmental hormone for TCDD.

We are very appreciated for the comments and have corrected the sentences as “We believe in that the nongenomic action explains cells elicit fast inflammatory responses to the action of the environmental pollutant TCDD and the endocrine disrupting agent, phthalate” in the discussion (Page 23).

2) Please provide full name of TCDD in background.

Thank you for the reminding, we have provided full name of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD) in the background.

3) Several phrases and sentences in introduction and discussion are repeated.

Please check.

We agree with the reviewer’s comment, thus we deleted the sentence, “Previous studies have indicated that BBP causes liver enlargement or damage [3, 24], but the role of BBP on hepatocellular carcinoma progression is poorly understood. The present study found that BBP promotes liver cancer migration, invasion and angiogenesis in vitro and promotes metastasis and angiogenesis in vivo. These findings suggest that BBP can be considered a tumor-promoting agent.” “Given this evidence of AhR involvement in tumor preogression, we further examined cell funtions related to tumor progression, including migration, invasion and angiogenesis in discussion. Also, we rewritten “Previous study shows that by interacting with
AhR-interacting protein, Gα_{13} negatively regulates the stability of ligand-induced transcriptional activation of AhR via the ubiquitin-proteasome pathway” as “Previous study shows that by interacting with AhR-interacting protein, Gα_{13} destabilized AhR via the ubiquitin-proteasome pathway [18]” (Page 23) and rearranged the sentence as “Previous reports revealed that phthalates promote cancer cell migration, invasion and epithelial-mesenchymal transformation, which may explain the cancer progression observed in both ER-dependent and AhR-dependent pathways [10, 42, 43, 44]. Several reports have shown that AhR regulates cell migration, invasion and plasticity, all of which contribute to tumor progression [10, 36, 45, 46, 47]” (Page 25) in the discussion parts.

Reviewer: Rituraj Konwar

Reviewer's report:

In this manuscript, the authors documented significant amount of work demonstrating that benzyl butyl phthalate promotes hepatocellular carcinoma cells and their metastasis under in-vitro and in-vivo experimental conditions. I have the following comments on this manuscript.

Major Compulsory Revisions:
Abstract:

# Background: “a novel non-genomic AhR mechanism”, was this pathway novel or information that BBP acts through this pathway in new.

We agree with the reviewer’s comment. The the nongenomic AhR mechanism have been reported by our previous study (Hsieh et al., 2012). The present study is the first to show nongenomic AhR mechanism which involved with G-protein pathway. However, the word of “novel” may be too arbitrary that we have deleted the world “novel” in the abstract for the reviewer’s concern.


Background:

# Can be improved and made more specific to current investigation of BPP and AhR. Citations on prior report of nature of BBP and AhR binding or interaction can be incorporated, if any.

According to the reviewer’s comment, we have added citations on prior report of BBP and AhR interaction. “Previous studies investigated that phthalates affect the
activation of the aryl hydrocarbon receptor (AhR)” [7, 8]. Moreover, phthalates suppressed type I interferon expression in human plasmacytoid dentritic cells via AhR [9]. (Page 5)

# “Accumulating evidences showed the effect on liver induced by phthalates”.

Incomplete sentence, which or what effect? Can be deleted if not necessary.

Thank for the precious comments. We take the reviewer’s suggestion to delete the sentence.

# “Hepatocellular carcinoma” is more preferred over old terminology of “malignant hepatoma” which many uses simply as “hepatoma” and gives impression of benign tumor.

As suggested, we have made correction as “Hepatocellular carcinoma” in the manuscript according to the reviewer’s comments.

# “……treated…Huh7 and PLC cells with BBP and the in vivo cancer growth were evaluated”. Only in vitro studies in PLC are reported and only in supplementary results.

As suggested, we moved the supplementary results to the discussion parts and rearranged the sentences as

“We tested the cell function treated by BBP including migration, invasion and angiogenesis. We found that BBP induced migration in Huh7 and PLC cell lines. In
addition, BBP induced invasion and angiogenesis in Huh7 cells (Supplementary figure 2)). This may be due to the higher constitutional AhR level of Huh7 than PLC cells (Supplementary figure 2D). HepG2 cells is not appropriate in animal studies for the non-tumorigenic property in immunosuppressed mice [31]. Therefore, we further investigate the mechanism induced by BBP in Huh7 cells. We used Huh7 cells in the in vivo study, and clarified the effect of BBP induced metastasis and angiogenesis in vivo. (Page 22, 23)

# Author mentions that earlier reports suggests AhR/HDAC6/c-Myc and other signaling pathway for promoting invasiveness of cancer by phthalates, how or why author selected non-genomic signaling or AhR/G protein pathway for this study can be incorporated.

The point raised by the reviewer are important, thus we add the statement to the Background part as:

“Study of nongenomic signaling is of much importance in the toxicology fields. Because scientists have difficulty to find dioxin response element (DRE)-based target genes and many reports suggested the toxic effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is more compatible with the nongenomic signaling of AhR, rather than the genomic action [13]. Moreover, our previous study reported phthalate mediated AhR/HDAC6/c-Myc pathway, which demonstrated the
nongenomic effect of AhR [10].” (Page 6)

Our data suggested that AhR activated at cell membrane upon the stimulation of BBP by the TIRF, confocal and Double immunogold transmission electron microscopy studies. Previous study revealed that AhR negatively regulate G protein (Nakata et al., 2009), suggesting that AhR may involve in G protein signaling. Therefore, we focus on non-genomic signaling as our study pathway.


Methods:

# Authors discuss earlier reports on HepG2 cells in the background section. But used Huh7 and PLC cells. Are there any criteria adopted for choosing liver cancer cell lines, if so please mention.

The point raised by the reviewer are important, hence we add the statement in the discussion part as follows.

“We treated the hepatocellular carcinoma cell lines, Huh7, HepG2 and PLC cells with BBP (1 µM). All cell lines showed the activation of AhR after treatment (Supplementary figure 1). We then tested the functions of cells treated with BBP
including migration, invasion and angiogenesis. We found that BBP induced migration in Huh7 and PLC cell lines. In addition, BBP induced invasion and angiogenesis in Huh7 cells. (Supplementary figure 2). This may be due to the higher constitutional level of AhR in Huh7 than in PLC cells (Supplementary figure 2D). HepG2 cells were not appropriate for further animal studies for non-tumorigenic property in immunosuppressed mice [31]. Therefore, we further investigate the mechanism induced by BBP in Huh7 cells. We used Huh7 cells in the in vivo study, and clarified the effect of BBP induced metastasis and angiogenesis in vivo.” (Page 22, 23)

# 500 mg/kg of BBP intra peritoneal injection to promote metastasis, why this dose and route was selected should be mentioned.

As suggested, we add the statement as follows. “Previous studies reported that administration of BBP by i.p. at dose of 800 mg/kg for 24 weeks resulted in no significant toxic effects [11, 22], which was a higher dose than used in this study. Our resulted showed the significant effect in metastasis under this dose.” (Page 15)

# Animal studies, intrahepatic xenograft experiments are not described adequately.

Groups and numbers in each group, definition of control used, whether it is no Huh7 cells or HuH7-IFP cells or HuH7-IFP cells with BPP, description or citation of surgical procedure, how distant metastasis was determined etc. If, any threshold or
cutoff used for considering metastasis positive organ. Were there any differences in histo-pathological characteristics of BPP promoted tumor versus BPP non-treated tumors can be included.

We are very appreciated for the comments, thus we have re-written this part according to the Reviewer’s suggestion and described in the Method part as

“The hepatocellular carcinoma model of direct intrahepatic injection was performed according to a previous study with some modifications [21]. After a small incision was made to explore the liver, Huh7-IFP cells (1×10^6) suspended in PBS were slowly injected into the upper left lobe of liver of the nude mice by using a 28-gauge needle. A transparent bleb of cells was formed through the liver capsule after injection. To prevent from bleeding, a small piece of sterile gauze was placed, and light pressure was applied on the injection site. After implantation, the mice were placed on a heating pad or below a heating lamp until fully active. The mice were randomly divided into two groups (vehicle treatment and BBP treatment), each group containing 18 mice”. The images of vehicle treatment group were shown in Figure 5C. The numbers of the organs (lung, kidneys, spleen) that expressed fluorescence were considered as the metastasis positive organs (Figure 5D). (Page 15, 16)

“Immunohistochemistry showed that liver PI3K and NF-κB levels were significantly
higher in the treatment group than in the vehicle control group (Figure 5E).” (Page 21)

Discussion:

# PLC results were not discussed, and also, why probably BPP is not effective.

We agree with the reviewer’s concern, hence the following statement are included.

“We found that BBP induced migration in Huh7 and PLC cell lines. In addition, BBP induced invasion and angiogenesis only in Huh7 cells (Supplementary figure 2). This may be due to the higher constitutional level of AhR in Huh7 than in PLC cells (Supplementary figure 2D).” (Page 22)
Figure legend:

Supplementary figure 2 (D) Huh7 and PLC cells were harvested for whole cell lysates for AhR protein levels measured by immunoblotting, β-actin was used as an internal control.

Conclusions:

# In methodology, authors have described use of ERK antibodies, they have used ERK inhibitor in some experiments, and there are no results on ERK phosphorylation with BPP treated in either Huh7/PLC/HUVEC cells. But, concludes “BBP promotes angiogenesis via the AhR/ERK/VEGF pathway”, also in “Abstract” section. ERK phosphorylation experiments can be included.

The points raised by the reviewer are important. As suggested, we performed the experiments carefully. The data shows that the phosphorylation levels of ERK were evaluated (Figure 6C). To further confirm if activation of ERK is AhR dependent, we trasfected Huh7 cells with two different AhR shRNA, and the results showed that the phosphorylation levels of ERK induced by BBP were inhibited (Figure 6D). (Page 21)

Figure legends:

Figure 6 (C) Huh7 cells were treated with BBP for the indicated times. p-ERK and
ERK levels were measured by immunoblotting. (D) Huh7 cells were transfected with two different Huh7 shRNA and treated BBP (1 μM) for 15 minutes. P-ERK, ERK levels were evaluated by immunoblotting.

Figures:

# Figure 1 legend: “Data shown in the upper image are” can be replaced with “Agarose gel image is”?? Also can be expanded as “Each value in the graph <obtained through densitometry> is the mean…”. Also, reorganize “BBP (1 μM) or DMSO for 15minutes as control group”

As suggested, the sentences have been reorganized in the figure legend 1.

Figure legend:

Figure 1 (A) Huh7 cells were treated with BBP (1 μM), and mRNA expression of AhR was analyzed by RT-PCR at the indicated time points. The agarose gel image is the expression of AhR mRNA. Each values in the graph obtained through densitometry is the mean of three independent experiments. Each value in the graph is the mean ± SD from three independent experiments. The asterisks indicate a significant difference expression relative to the level at 0 minute, as analyzed by Student’s t-test (p < 0.05). (B) Huh7 cells were treated with BBP (1 μM) or DMSO as control group for 15minutes, and AhR mRNA was stained by fluorescence
carboxyfluorescein, FAM) in situ hybridization. Imaging was performed by confocal microscopy.

# Though supported by GFP intensity graph, Figure 2 A and B image and scale resolutions are poor. Correct “magnificent x600”

As suggested, the scale bar and image have been provided for better resolutions. We have deleted the statement of magnificent x600 and provided the scale bar in Figure 2B.

# Figure 2 D: Whether upper left image is of BPP untreated cells? Localization of AhR and G-alpha in BPP untreated would have been interesting. Are the two lower images having different significance here?

We agree with the reviewer’s comment. As shown in the figure, the upper left image is BPP untreated cells. According to the upper left image, G-alpha localized at cell membrane; However, AhR did not. After treatment, AhR represented at the membrane and interacted with G-alpha. The two lower images showed the same phenomena here. We have reorganized the figure.

# Figure 3 legend: 3A is missing. “BBP casued the cleaveage of PIP2 ....”. Apart from the spelling mistakes, if description needs to be exact to the figure, it should be “reduction of level of PIP2” rather than “cleavage”.

We agree with the reviewer’s concern, thus we have corrected the sentence as
follows.

Figure legend:

Figure 3 (A) BBP caused reductions in the level of PIP2 and activation of IP3R via G\(\alpha_{q/11}\). After Huh7 cells treated with BBP (1 \(\mu\text{M}\)), protein levels were analyzed by immunoblotting at the indicated time points.

# Figure 3 B legend: “Real-time imaging of calcium … by Cell-R microscopy” Please include of what and in which condition? Also, description of vertical axis in Figure B, C & D can be provided in legend.

We have re-written this part according to the Reviewer’s suggestion as follows.

Figure legend:

Figure 3 (B) Real-time imaging of calcium was performed by Cell-R microscopy. The experiment was performed with BSS medium. (C) Elevated intracellular calcium was induced by BBP treatment. The experiment was performed with a calcium free medium. The arrows indicate the time points of BBP (1 \(\mu\text{M}\)) addition (1 minute after the experiment started). The fluorescence intensity indicates the relative calcium levels (Y-axial). (D) Huh7 cells in calcium-free medium were pretreated with various concentrations of 2-APB for 30 minutes before stimulation with BBP (1 \(\mu\text{M}\)). The internal calcium release was inhibited by 2-APB in a dose-dependent manner. The relative intensity of fluo-4 indicates the calcium levels: peak/baseline ratio of
fluorescence intensity. Calcium-free medium was used for each experimental interval.

Each value in the graph is the mean ± SD for repeated six times using at least ten cells”.

# Text of figure 3 B& C not clear due to low resolution. I feel, even B & C can be clubbed.

As suggested, we have provided figure of better resolution of Figure 3 B& C according to the reviewer’s concern. Figure B, C were the two different conditions of experiments. We used the normal medium in Figure 3B but calcium free medium in Figure 3C.

# Figure 4C legend: If it is time points of post-BPP treatment, please incorporate.

As suggested, the time points are included.

Figure legend:

Figure 4(C) Nuclear and cytoplasmic fractions of NF-κB were detected by immunoblotting. Lamin A/C and α-tubulin were used as internal markers for nuclear and cytoplasmic proteins, respectively. Huh7 cells were pretreated with wortmannin (100 nM) and then treated with BBP (1 μM) for 2 hours.

# Figure 4 legend: “#-actin was used as an internal control. Move from description of “4E” legend to be part of “4D” legend of Akt-phopho detection image.

We take the reviewer’s suggestion and re-written as follows.
Figure legend:

Figure 4 (D) The Akt phosphorylation levels were measured and β-actin was used as an internal control. (E) The nuclear and cytoplasmic fractions of NF-κB were analyzed by immunoblotting. Huh7 cells were transfected with two different shRNA and treated with BBP (1 µM) for 2 hours.

# Figure 5B: Upper and lower images should be marked with time points.

We have marked with time points according to the reviewer’s suggestion.

# Figure 5 B: correct vertical axis title “invaded cells/field”

We thank the reviewer for the correction. We have corrected the title as “invaded cells/fields”.

# Figure 5 D & E legend. Mention control if it is BPP untreated or vehicle treated tumor group.

We agree with the reviewer’s concern, and the “vehicle treated tumor group” has been added in the legend part.

# Supplementary figure: It should be described in result or discussion, instead of background.

We thank the reviewer’s comment, thus we have moved the Supplementary figure to the discussion as follows. “To further investigate, we treated the hepatocellular carcinoma cell lines, Huh7, HepG2 and PLC cells with BBP (1 µM). All cell lines
showed the activation of AhR after treatment (Supplementary figure 1). We then tested the functions of cells treated with BBP including migration, invasion and angiogenesis. We found that BBP induced migration in Huh7 and PLC cell lines. In addition, BBP induced invasion and angiogenesis in Huh7 cells (Supplementary figure 2). This may be due to the higher constitutional level of AhR in Huh7 than in PLC cells (Supplementary figure 2D).” (Page 22)

Minor essential revisions:

Lacks page numbers, make difficult to specify comments on the manuscript. The manuscript also uses lots of abbreviations, full form of which should be incorporated at the time of first use. eg. DEHP etc. Also, giving full forms of abbreviations in abstract can be helpful. In addition, few spelling and grammatical mistakes needs to be corrected.

We are very appreciated for the kind comments. As reviewer suggested that we have added the page numbers, full form of abbreviations eg. DEHP, TCDD at the time of first use and abbreviations in abstract, corrected the spelling error and grammatical mistakes carefully.

Discretionary Revisions:
Hepatocellular carcinoma is added to Keywords according to the reviewer’s suggestion.

Conclusions: “… useful for developing approaches to prevent and treat liver cancer”.

Please remove and give prospect of immediate impact.

We are very appreciated for the kind comment. As suggested, we have removed the sentence and rephrased as “The results imply that BBP has a detrimental impact on hepatocellular carcinoma, and that phthalate should be avoided in these patients.”

Reviewer: Ghoshal Kalpana

1. The authors showed BBP increases AhR mRNA expression but they didn’t provide any mechanism of BBP induced AhR mRNA upregulation. Figure 1A shows AhR mRNA level peaks at 15 minutes after BBP treatment and then goes down gradually. However AhR protein doesn’t follow the same trend as mRNA, unlike mRNA protein is stable for 2 hours. There is no discussion about it in the manuscript. Instead of end-point RT-PCR, real-time RT-PCR for AhR at different time points will be more quantitative.

The points raised by the reviewer are important. We have added the statement to the
discussion parts “After BBP treatment, the AhR mRNA levels were unregulated. Some molecular eg. NF-κB, IL-27, IL-6 were investigated that can regulate AhR [32, 33, 34]. However, the mechanism of BBP induced AhR mRNA upregulation remain unclear so far.” (Page 23)

Following the reviewer’s suggestion that we measured the AhR mRNA levels by using real-time RT-PCR. As shown in the figure, AhR mRNA levels peaks at 15 minutes and stable for 2 hours. The trend is compatible with AhR protein levels.

2. In figure 2, the negative control and transfection control (pGFP alone) for pAhR-GFP is missing. They calculated AhR-GFP relative intensity, they didn’t mention relative to “what”? Figure-2C requires quantification, since it is hard to see significant difference in G# q/11 protein level after BBP treatment. Similarly, quantification is necessary for the data in Figure 2F.

Thank reviewer for the precious comments. The statements of “AhR-GFP relative
intensity” were corrected as “AhR-GFP intensity” in Figure 2. We are very sorry for our negligence of negative control and transfection control (pEGFP-C1 alone). As reviewer’s suggestion; we have showed the data of negative control and transfection control in Figure 2A. There is no change of GFP intensity during the experiment of negative control and transfection control (pEGFP-C1 alone) groups. Also, Figure 2C and Figure 2F have been quantified. As Shown in Figure2C, $\alpha_{q/11}$ protein level increased from 1.27 (after 30 min) to 1.39 (after 2 hours) folds after BBP treatment. As shown in Figure 2F, transfection with AhR shRNA reduced the levels of $\alpha_{q/11}$ and $\beta$ induced by BBP.

Methods:

pEGFP-C1-AhR, a kind gift of Dr. Hsin-yu Lee (Department of Life Science, National Taiwan University), was cloned the AhR gene into pEGFP-C1 (Clontech). (Page 7)

Huh7 cells were transfected with pEGFP-C1 as plasmid control or pEGFP-C1-AhR, treated with DMSO as vehicle control or BBP and viewed by TIRF microscopy. AhR-GFP expression peaked 2 minutes after BBP treatment (Figure 2A). (Page 17)
Figure legends:

Figure 2 (A) Huh7 cells were transfected with pEGFP-C1-AhR or pEGFP-C1 as plasmid control. Cells were stimulated by adding DMSO or BBP (1 µM) and then analyzed by real-time TIRF microscopy. Scale bars: 10 µm. The left panel showed pEGFP-C1 plasmid control treated with DMSO (upper) or BBP (lower). The middle panel showed Huh7 cells transfected with pEGFP-C1-AhR, treated with DMSO (upper) or BBP (lower). The increased intensity of GFP indicates AhR expression at the cell membrane induced by BBP. The right panel showed the GFP intensity analyzed by Axio Vision Rel 4.8 software.

3. Figure 3F requires quantification, upregulation of COX-2 upon BBP treatment compared to control in first two lanes (Control and BBP) is not obvious, any explanation? It is hard to understand significance of the results without quantification. As reviewer’s suggestion, the data have been quantified and shown in Figure 3F. After BBP treatment, COX-2 expression increased from control (1) to 1.23 (BBP) folds.

4. PI3K/Akt/NF-κB is known to enhance cell migration and invasion, phenotype of BBP treated Huh7 cells in presence of AhR shRNA and NF-κB shRNA is very
convincing. However, it would be more informative if the expressions of NF-κB, pAkt and PI3K in Control and BBP treated cells in presence of AhR shRNA were provided. This will show if activation PI3K/Akt/NF-κB is specific via stimulation of AhR by BBP. Similarly, it would be nice to measure ERK/pERK, VEGF in Control and BBP treated cells/tissues in AhR depleted cells.

The points raised by the reviewer are important. Considering the reviewer’s suggestion, we have transfected Huh7 cells with two different AhR shRNA, and the pAkt (Figure 4F), PI3K (Figure 4F), NF-κB (Figure 4G) and ERK/pERK (Figure 6D) levels were provided. As shown in Fig. 6B, the VEGF levels were measured by using ELISA. PI3K, pAkt, NF-κB pERK, VEGF induced by BBP were suppressed after transfection with AhR siRNA or shRNA.

To further confirm whether the activation of PI3K/Akt/ NF-κB is specific via the stimulation of AhR by BBP, we transfected Huh7 cells with two different AhR shRNA. PI3K and p-Akt (Figure 4F), the translocation of NF-κB into nucleus (Figure 4G) that was induced by BBP was found to be inhibited by transfection of the shRNA. (Page 20)

Moreover, the phosphorylation levels of ERK were evaluated (Figure 6C). To further
confirmed if activation of ERK is AhR dependent, we transfected Huh7 cells with two
different AhR shRNA, and the results showed that the phosphorylation levels of ERK
induced by BBP were inhibited (Figure 6D). (Page 21)

Figure legends:

Figure 4 (F) PI3K, p-Akt, Akt levels were measured by immunoblotting. β-actin was
used as an internal control. (G) The nuclear and cytoplasmic fractions of NF-κB were
analyzed by immunoblotting. Histone H3 and α-tubulin were used as internal
markers for nuclear and cytoplasmic proteins, respectively.

**Figure 6** (C) Huh7 cells were treated with BBP for indicated times. p-ERK, ERK
levels were measured by immunoblotting. (D) Huh7 cells were transfected with two
different Huh7 shRNA and treated with BBP (1 μM) for 15 minutes. p-ERK, ERK
levels were evaluated by immunoblotting.

5. Some more explanation about methods used for intrahepatic injection of Huh7-IFP
cells in mice in the Supplement would be helpful.

We agree with the reviewer’s concern, thus we have re-written in the Method “**In
vivo tumor xenograft experiments**” part according to the Reviewer’s suggestion.

The descriptions were shown as follows: “The HCC model of direct intrahepatic
injection was performed according to a previous study with some modifications [21]. After a small incision was made to explore the liver, Huh7-IFP cells \((1\times10^6)\) suspended in PBS were slowly injected into the upper left lobe of liver of the nude mice by using a 28-gauge needle. A transparent bleb of cells was formed through the liver capsule after injection. To prevent from bleeding, a small piece of sterile gauze was placed, and light pressure was applied on the injection site. After implantation, mice were placed on a heating pad or below a heating lamp until fully active. The mice were randomly divided into two groups (Vehicle treatment and BBP treatment), each group containing 18 mice.” (Page 15)

6. All experiments are performed using only one HCC (Huh7) cell line. Use of several other HCC cell lines would elucidate whether BBP mediated Ahr activation is a general phenomenon or restricted to only one cell line.

According to the reviewer’s suggestion, we have re written in the discussion part as follows. “To further investigate, we treated the hepatocellular carcinoma cell lines, Huh7, HepG2 and PLC cells with BBP (1 \(\mu M\)). All cell lines showed the activation of AhR after treatment (Supplementary figure 1).” (Page 22)
Supplementary Figure 1. Effects of BBP on AhR expression of hepatocellular carcinoma cell lines.

Huh7, HepG2, PLC cells were treated with BBP (1 μM) for 24 hours and AhR protein levels were analyzed by immunoblotting. β-actin was used as an internal control.