Author's response to reviews

Title: The association of N-palmitoylethanolamine with the FAAH inhibitor URB597 impairs melanoma growth through a supra-additive action

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Author's response to reviews: see over
Dear Editor,

Please find here the modifications that were made in answer to the reviewer comments.

**Referee 1: Virginia S Seybold**

1. page 13, results/1: The experimental design did not measure a "rate" of hydrolysis which implies a variable of time. A more precise statement is that the experiment measured hydrolysis of the selected compounds.
   The term “rate” was removed.

2. page 16, results/4: In the absence of a statistical value, it is unclear whether 10 uM capsazepine increased the effect of PEA. Given that the effect may be isolated, in that it was not mirrored by capsazepine+URB597, its significance can be argued. Nonetheless, a more accurate statement, consistent with the goal of the experiment, would be: none of the antagonists reduced the effect of PEA or URB597 alone or in combination.
   Also in this section, clarify that 10 uM cannabidiol enhanced the cytotoxic effect of PEA and URB597 when each of these drugs was used alone.
   The text has been modified and clarified in accordance to this point and the formulation proposed by the reviewer has been added in the manuscript.

3. Discussion, paragraph 2: the authors state: "Surprisingly, at 10µM, we found PEA to decrease cell viability at least equally, if not more, than AEA and 2-AG."
   Statistical analysis only reports difference from vehicle. There is no statistical confirmation that effect of PEA is different from AEA or 2AG. This statement needs to be revised.
   The second part of the sentence, evoking the comparison between PEA effect and AEA/2-AG, has been removed.
Referee 4: Guillermo Velasco

Regarding the response to the other reviewers’s comments I would like to underline that the explanation about the changes on the levels of acylethanolamines and the expression of FAAH and NAAA provided to reviewer 2 is, in my opinion, not satisfactory as: (i) the authors do not measure protein levels of these enzymes and (ii) a 4-fold difference in mRNA levels could produce striking differences in protein levels and enzymatic activity. However the authors include the opposite interpretation in the manuscript.

What we wanted to show here is that there is not huge difference between these two enzyme mRNA expressions. We agree with the hypothesis that this does not strictly reflect protein levels. However, even in the case of equally expressed proteins, there might exist differences in the enzymatic activities. Therefore, the pharmacological experiments are in our view the most relevant information to assess the relative implication of FAAH and NAAA in PEA hydrolysis in our melanoma model. Since PEA hydrolysis is almost fully inhibited by the URB597 and since CCP only produces a modest inhibition, we can conclude that FAAH is mainly responsible for PEA degradation in our system. This point is addressed in the discussion.

Previous report (version 1)

1. The correlation between pharmacological inhibition of FAAH and MAGL and levels of PEA and the other acylethanolamines needs to be strengthened. Levels of PEA, AEA and 2-AG should be determined in the absence and the presence of – at least – some of the other inhibitors of FAAH and MAGL, so a correlation between levels of each these lipids and the observed decrease in cell viability can be established. Likewise, these results should be confirmed (levels of acylethanoamines and viability should be determined) in melanoma cells treated with PEA and other acylethanolamines upon selective silencing of FAAH and MAGL using for example shRNA or siRNA.

In the revises version of the article, we had already included additional experiments measuring the levels of 2-AG and PEA after URB597, MAFP and CAY10499 incubation in order to give a rational explanation to the poor efficacy of the two MAGL inhibitors. Levels of AEA were too low to be properly determined. Attempts were made to silence the enzymes but despite long settings, we were never able to suppress their expression in our cell model.

2. The mechanism of PEA-induced cell death is unclear. Selective silencing of CB1, CB2, GPR55, TRPV1, PPARα and PPARγ should be undertaken in order to completely rule out the participation of any of these receptors on the reduction of cell viability observed in vitro.

We agree that the silencing of all these receptors may strengthen the results obtained with a given antagonist. However and as previously answered in the first revision of our manuscript: “Many experiments were performed in order to check the implication of these five receptors in PEA/URB597 cytotoxicity but, because we wanted to focus on our message, we only showed selected data. In addition, in many papers, comparable approaches were used to study the involvement of receptors in the cytotoxic and antiproliferative properties of cannabinoids and endocannabinoids. The authors generally concluded to an absence of implication of these receptors when no reversion of effects could be evidenced. Furthermore, similar concentrations of antagonists were used that those already published (Patsos, Int J Oncol 2010; Matas, J Biol Chem 2007; Hinz, Mol Pharmacol 2004; Jonsson, Arch Toxicol 2003).”
In addition and once again as previously suggested in our first revision, a subtle combination of several receptors activation could also be at the origin of the effect has been considered. However, so far, we could not validate this hypothesis.

Finally, we would like to underline that the elucidation of the potential targets involved in the cytotoxicity of PEA and URB597 do not constitute the main message of the manuscript, though it is a matter of interesting perspective.

3. The combined administration of PEA and URB597 enhances apoptosis in vitro (Fig 3A) but not in vivo. In order to confirm these results, an additional method to measure apoptosis (for example DNA fragmentation and active-caspase 3 immunostaining in vitro and active caspase 3 in vivo) should be used. Likewise, changes in the number, size and distribution of vessels should be analyzed in these tumors (for examples using CD31 immuno-staining) to confirm whether angiogenesis is affected by the treatment with PEA and URB597.

Apoptosis has been evidenced to involve caspase-3 independent pathways, this is the reason why we measured the translocation of phosphatidylserine, which occurs in the early stages of apoptosis. We first attempted to measure caspase-3 activity using appropriate controls but we could not observe any response.

An additional figure (Figure 7E) displaying the results obtained with CD31 immunostaining has been added. These results confirm that angiogenesis is not significantly affected in vivo either.

4. The authors perform all the study using a single type of mouse melanoma cell line (B16). A few crucial experiments should be also performed with at least one additional human melanoma cell line.

Given the deadline for the submission as well as Belgian and university regulations to observe for using human material, we are not able to perform additional experiments using human melanoma cell lines.