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 a list of the modifications that were made to our manuscript in answer to the reviewer comments.

Referee 1: Virginia S Seybold
“I am satisfied with the authors' changes in the manuscript in response to my initial comments.”
The comments concerning precision of language are suggested to improve the clarity of the writing have been taken into account in the present version of the manuscript.

Referee 2: Vincenzo Di Marzo
“The authors have reviewed the article according to my specifications”

Referee 3: Natsuo Ueda
There were no additional comments from this reviewer, we thus believe he was satisfied with our revised version of the manuscript

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. It is well known that protein levels of these enzymes do not always correlate with their activity. Also, regardless of their protein level, the involvement of one or both enzymes in N-acylethanolamine metabolism depends on the tissue (cell), the physiological conditions, and other parameters, beside raw protein levels. 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. Here we show that (i) B16 melanoma cells express FAAH and NAAA mRNA; (ii) melanoma B16 cells hydrolyze AEA and PEA; (iii) URB597 blocks $[^3]$H-PEA hydrolysis in homogenates and whole cells; (iv) URB597 increases PEA levels in intact cells, demonstrating that FAAH, and not NAAA, is responsible for PEA hydrolysis in B16 melanoma cells. Indeed, it was demonstrated by one of the reviewers of this manuscript, Prof. N. Ueda, that URB597 does not inhibit NAAA (Sun, Biochim Biophys Acta 2005).
Since PEA hydrolysis is almost fully inhibited by URB597 and since CCP only produces a modest inhibition, we can conclude that FAAH is mainly responsible for PEA degradation in our system. In addition, because we performed these experiments also in intact cells, this
information reflects an actual situation as opposed to what can be observed when using cells homogenate. These points are addressed in the discussion: “… although the poor inhibition of PEA hydrolysis by the NAAA inhibitor is puzzling at a first glance, the almost full inhibition of PEA hydrolysis by URB597 suggests that FAAH is likely to account for most of PEA degradation in our cellular model”.

In addition, some modifications have been made in the result section in order to state that we do not directly associate mRNA levels with the enzyme expression.

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 acylethanolamines 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.

We had 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 (Additional Figure 5). This was discussed in the revised version of our manuscript. Please note that the levels of AEA were too low to be properly determined.

Attempts were made previously to silence the enzymes but despite long settings, we were never able to obtain a satisfying level of suppression 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 would further rule out the involvement of a given target and strengthen the results obtained with antagonist. We have included this interesting suggestion in the manuscript. Numerous experiments were realized to elucidate the targets mediating the cytotoxic effect of PEA and URB597, not all of them have been described in the manuscript. Pharmacological blockade is a reliable, widely used, and recognized way to study the role of receptors in the endocannabinoid field and, actually, many authors, including Dr Velasco, used this approach in several of their papers. See for instance Oesch, Cancer Therapeut 2009; Salazar, J Clin Invest 2009 or Blazquez, Cancer Res 2008 where authors used selective antagonists without using siRNA. Furthermore, we used concentrations of antagonists similar to those already published by others (Patsos, Int J Oncol 2010; Matas, J Biol Chem 2007; Hinz, Mol Pharmacol 2004; Jonsson, Arch Toxicol 2003) and known to block the activation of the target receptor.

We think the use of gene silencing is an exquisite tool to confirm the implication of a given receptor in a drug’s effect, however our data do not point to such a receptor in the effects of PEA and or PEA+URB597. As previously suggested in our first revision, it is possible that a
The subtle combination of several receptors activation could be at the origin of the observed effects. 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 immunostaining) to confirm whether angiogenesis is affected by the treatment with PEA and URB597.

Similarly to what the reviewer suggests, measurement of caspase-3 activity was the first element we studied once we observed PEA and PEA+URB597 effects. However, we obtained data suggesting that the apoptosis undergone by the cells in our conditions is through caspase-3-independent pathways. Since translocation of phosphatidyserine is an early event in apoptosis, we thought its measurement would bring interesting information on the presence or not of cells undergoing apoptosis. This point is now specified in the manuscript.

Concerning the in vivo angiogenesis, we performed an additional experiment along this reviewer interesting suggestion. We analyzed the formation of new vessels using CD31 immunostaining. Thus 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.

Additional experiments were performed, as requested by this reviewer, on a human melanoma cell line (MZ2-MEL.43) in a view to strengthen the potential interest of using PEA and URB597 in the management of melanoma. These results indicate a significant decrease of cell viability after PEA and URB597 treatments and a potentiation of PEA cytotoxicity by URB597 leading to a reduction of cell viability of more than 50%. A figure (Figure 8) has been added to show these results.

Our approach is thus similar to that followed by Blazquez and colleagues, which published a major article studying the influence of cannabinoid treatments on melanoma growth (Blazquez, FASEB J 2006). Indeed, in the context of a comprehensive study conducted on the murine B16 melanoma cell line, the authors provided interesting in vitro data supporting the relevance of their results on a human melanoma cell line by measuring the number of remaining viable cells after treatment.

In conclusion, we believe that the supplementary information brought in this revised version of the manuscript, in terms of endocannabinoid quantification, tumor vascularization and human cell viability, strengthen our results and further support our initial hypothesis.