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,

Thank you for the attention given to our manuscript.

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

Referee 1: Virginia S Seybold

Major compulsory revisions:
1. The experimental design does not meet the criterion to conclude that PEA and URB597 act synergistically. An isobolographic analysis is necessary to make this conclusion. The data, however, do support a supra-additive effect.

We based our explanation on the following definition: “A synergistic effect is one in which the combined effect of two chemicals is greater than the sum of the effect of each agent given alone” (Goodman & Gilman, 1995). In addition, we clearly observed in vivo that the injection of PEA or URB597 alone did not produce any effect, while the co-injection significantly reduced melanoma growth.

However, we agree with the fact that PEA and URB597 does not respond in the strict sense of the pharmacological definition of a synergy that requires an isoblogram as described in other papers (e.g. Berenbaum, Pharmacol Rev 1989; Tallarida, J Pharmacol Exp Ther 2001; Grabovsky and Tallarida, J Pharmacol Exp Ther 2004). Actually, the amplitude of response resulting from URB597 incubation was too weak to properly express an ED50, and thus generate an isobologram analysis. For this reason, we modified the term “synergistic” in the manuscript and, since our article originally contained the word “synergistical”, also propose a revised title. The new title is: “The association of N-palmitoylethanolamine with the FAAH inhibitor URB597 impairs melanoma growth through a supra-additive action”

2. The authors miss-use the word "potent". The author's conclusion that "PEA was equipotent if not more potent than AEA and 2AG in inhibiting tumor growth" is not supported by the data; no concentration-response data were shown. PEA may have had a larger effect at a single concentration (Fig. 1), but a statistically significant difference was not reported. Similarly, the authors conclude that URB597 was most potent in inhibiting PEA hydrolysis when no concentration-response data were shown (p. 18) nor were differences in effects tested.

The word potent was replaced in the manuscript.

3. The authors cannot claim that PEA reduced B16 cell viability at a concentration <10 µM if they do not show the data.

We agree with this reviewer, thus according to her suggestion, a figure (Fig. 1D) has been added to show the data.

4. Methods section 3.1: The authors determined hydrolysis of AEA, PEA, and 2-AG as a measure of the enzymes that degrade the compounds, not as an assay of the compounds themselves. Thus, the introductory sentence needs to be revised.

The sentence was revised and replaced by “In order to detect a hydrolytic activity for N-acylethanolamines (AEA and PEA) or 2-monoacylglycerols (2-oleoylglycerol, 2-OG) in B16 cells, radiolabeled substrates - either [3H]-anandamide, [3H]-2-oleoylglycerol or [3H]-N-palmitoylethanolamine (25µl, 50000 dpm, 1nM) - were incubated in glass tubes for 10min at
37°C with increasing amounts of cell homogenates (160µl, 10mM Tris-HCl, 1mM EDTA, pH 7.4) and 10µl of DMSO.”

5. The authors normalized all of the measures of AEA, 2AG and PEA to control values so no raw data are reported. In order to assess the reliability of their measures, actual values for the control group need to be reported. In addition, data for levels of the compounds in tumors would need to be normalized to some other factor in order to control for variability in samples (e.g., sample wet weight, protein, or total lipid weight). This is not described in the methods.

This is absolutely true, and we apologize for this omission. Control values for endocannabinoid dosages have been added in the figure legends and the way the data were normalized is now described in the method section. The data were normalized by cell number in the *in vitro* experiments and by tumor sample weight in the *in vivo* testing.

6. The data for the receptor antagonists are not compelling. The experiments used high concentrations of PEA and URB597. What are the data that the antagonists effectively block the receptors at concentrations relative to those of the agonists? The data would be more convincing if the authors confirmed that receptor selective agonists had no effects at high concentrations. In addition, the antagonist data are difficult to interpret because there are no data for the antagonist alone. At face value, though, the antagonists did not reverse the effect of PEA +/- URB597, but cannabidiol may have enhanced the effect (suppl fig 3). The authors do not address this in the figure legend or the text.

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. Indeed, as suggested by the referee, higher concentrations of antagonists were tested (up to 10µM), as well as fewer concentrations of PEA and/or URB597. Different time points were also tested. Nevertheless, we have never been able to detect any restoration of the cell viability. For this reason we have chosen to illustrate these observations with the concentrations of antagonists that did not, or only little, influence cell viability when used alone (an additional file (4) has been added to show the 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).

Cannabidiol was shown to be a weak agonist of the TRPV1 receptor (EC50 = 3.5µM – Bisogno, Br J Pharmacol 2001) and this could explain the enhancement of cytotoxicity observed with this compound at 10µM. This point is now addressed in the manuscript.

**Discretionary revision:**

Introduction: P. 4: reference to a cannabimimetic effect of PEA is awkward because "mimetic" effects refer to effectors or receptor mediated events. As acknowledged by the authors, PEA does not bind to CB receptors.

The term cannabimimetic was removed from the manuscript.
Referee 2: Vincenzo Di Marzo

1) It is indeed puzzling that the authors could not see any effect of the selective NAAA inhibitor on both PEA degradation and PEA-induced inhibition of cell viability, since the authors show that NAAA is, in fact, expressed by these melanoma cells. The problem here is that the authors have not used a quantitative method to measure enzyme expression. They should provide quantitative RT-PCR data and/or western blot data to allow for an assessment of the relative amounts of FAAH vs. NAAA. Additionally, since the authors also have unexpected results with MAFP and CAY10499, they should have measured the effect of these all inhibitors per se also on 2-AG levels. In fact, I disagree with the authors' interpretation of these experiments. MAFP also inhibits PLA2 enzymes and DAGLs, the latter of which catalyse 2-AG biosynthesis rather than degradation. I think there is very strong evidence in the literature (not all of which has been quoted here) that by inhibiting 2-AG hydrolysis one can observe anti-cancer effects, either by indirectly stimulating CB1 receptors (see below) or by inhibiting the production of arachidonic acid and other fatty acids that may act as pro-tumor agents (see recent papers from Cravatt's group). Quantitative measurements of FAAH and NAAA mRNA expression were performed by qPCR and we found that the expression of FAAH was only four times higher than that of NAAA. This difference is minor and we can consider that the two enzymes are expressed in the same order of magnitude, ruling out the possibility that a disproportionate amount of FAAH is at the origin of the inefficiency of CCP. This point has been addressed in the manuscript. The levels of 2-AG, as well as PEA, were also measured after URB597, MAFP and CAY10499 incubation in order to give a rational explanation to the poor efficacy of the two MAGL inhibitors. The data are now reported as “additional figures” and discussed in the corresponding section of the manuscript.

2) Some relevant literature on the effect of inhibitors of endocannabinoid degradation on various aspects of cancer growth and metastasis in vitro and in vivo has not been quoted. In particular, Ligresti et al Gastroenterology, 2003; Nithipatikom et al Cancer Res 2004 and Biochem Biophys Res Commun. 2005; ; Endsley et al Int J Cancer. 2007 and 2008; Bifulco et al FASEB J 2004, should be quoted. The latter authors also reported that intratumor administration of a FAAH inhibitor could increase the levels of anandamide, 2-AG and PEA, opposite to what found here by the authors, and this difference should be briefly discussed. Indeed, FAAH inhibition or knock-down can also cause elevation of 2-AG levels (as shown also by Endsley et al, 2008), and not only in tumors. All these reports have been quoted and discussed in the revised manuscript.

Minor: Refs 30 and 37 are identical
This has been corrected in the manuscript.
Referee 3: Natsuo Ueda

Discretionary Revisions:

1. **URB 597 is a FAAH inhibitor. Administration of this compound should increase endogenous levels of not only PEA but also other N-acyl ethanolamines. Furthermore, the authors showed that antagonists of CB1, TRPV1, PPAR-alpha, PPAR-gamma or GPR55 did not alter the effect of PEA and/or URB597. Therefore, the authors need to discuss possible molecular mechanism(s) mediating the anti-tumor activity of PEA and URB597.**

   As explained in the response to referee 1 (point 6), we were not able to significantly reverse the cytotoxic effect using receptors antagonists and therefore to elucidate the mechanism by which the cytotoxicity is produced, despite the fact that many experimental conditions were tested.

   We agree that the elevation of the level of other endocannabinoids is of interest in order to elucidate the potential molecular mechanism underlying PEA and/or URB597 effect. As pointed out by the referee, URB597 is indeed able to increase other N-acyl ethanolamine levels in our system. On the one hand, OEA concentration was increased after incubation with URB597 (data not shown). On the other hand, we could not detect AEA in non-incubated cells but detected it in URB597-treated cells (data not shown), indicating that its concentration also raised. These data were omitted in this paper in order to simplify the message, to focus on the effect of URB597 upon PEA degradation and to validate the hypothesis that this inhibitor could act through an increase elevation of PEA levels.

   Furthermore, as mentioned in the manuscript, PEA may also influence AEA mediated effects through a downregulation of FAAH or a modulation of TRPV1 receptor activity. Along this line, the hypothesis that a subtle combination of several receptors activation could be at the origin of the effect has been considered. However, so far, we could not validate this hypothesis.

   Besides this, other experiments (not shown here) using methyl-β-cyclodextrin clearly indicate that lipid rafts are not involved in the effect of PEA/URB.

   Last, the implication of other enzymes such as COX or the liberation of reactive oxygen species could also be envisaged.

2. **(page 4) The endocannabinoid system should also contain cannabinoid receptors CB1 and CB2 in addition to endogenous ligands and enzymes/transporters.**

   The sentence describing the endocannabinoid system has been modified in order to emphasize this notion.

3. **(page 4) Biological activity of PEA unrelated to binding to CB1 and CB2 may not be “cannabimimetic”.**

   The term cannabimimetic was removed from the manuscript. (see also Referee 1 discretionary revision)

4. **(page 7) Concerning radioactive substrates used for enzyme assays, there is no information about concentrations. Therefore readers cannot compare activities among different enzymes.**

   Values have been updated in the manuscript.
5. (Fig. 5C) Why does not the administration of PEA increase the endogenous level of PEA?
We can explain this result by the fact that PEA, as well as other endocannabinoids including AEA, is rapidly hydrolyzed in the absence of the FAAH inhibitor URB597. In addition, it is possible that an earlier resection of tumor, just after treatment injection, could have shown more elevated PEA levels.