Author's response to reviews

Title: Selective Inhibition of Fatty Acid Synthase by (-)-Epigallocatechin-3-gallate Against Lung Cancer Xenografts

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Author's response to reviews: see over
Selective Inhibition of Fatty Acid Synthase by (−)-Epigallocatechin-3-gallate Against Lung Cancer Xenografts. Joana Relat, Adriana Blanchafort, Glòria Oliveras, Sílvia Cufí, Diego Haro, Pedro FMarrero and Teresa Puig.

We appreciate the reviewers’ criticisms which we believe will contribute to improve the quality of our re-submitted manuscript.

We have concisely addressed point-by-point the reviewers’ comments (provided below) and we have highlighted in bold all the modifications in the new revised manuscript version.

Reviewer’s 1 comments:

1) Major Compulsory Revisions

In in vivo tumor therapeutic experiment, authors said that two out of five animals of control group were discarded because of not enough tumor growth. Omitting the animals after starting the experiment is a serious violation. This means that the xenograft model used was not rigidly established. Authors should perform the experiment over again. The number of animals in each group should be same and be more than five. Statistical analysis should be done using appropriate method, preferably, Wilcoxon test on final day.

We agree with the Reviewer on the fact that to draw any conclusion from the in vivo experiments, all animals should have been analyzed. Then, we have performed statistical analysis including all control animals and we use the Wilcoxon test (as referee suggests) as the best appropriate statistical method to analyze the data because is a non-parametric statistical test used when comparing two related samples, matched samples, or repeated measurements (tumour volume) on a single sample to assess whether their population mean ranks differ. It can be used as an alternative to the paired Student’s test when it cannot be assumed the population is normally distributed.

On final day (day 33) the median of the tumour volume from control animals group (median of Tumour Volume_{day33} = 519 mm$^3$) was significantly different (p<0.05) from starting day (median Tumour Volume_{day0} = 33 mm$^3$) and this trend was the same from day 12 to 33 in control animals group (see table below). Interestingly, on C75- and EGCG-treated animals there weren’t differences between the median of the tumour volume on day 33 (290 and 224 mm$^3$, respectively) respect to the starting day (40 and 36 mm$^3$, respectively) those pointing out that the treatment with the anti-FASN compounds C75 and EGCG markedly blocked the growth of A549 xenografts.
According to these results we have re-submitted a new Figure 4A and we have revised and re-written the material and methods section (see the new "Statistical Analysis" section, page 7), the in vivo results section (see the new In Vivo results section, page 8 and 9) and the discussion section (page 10 and 11).

2) Minor Essential Revisions

**Fig.4A: Expressing Y-axis by tumor volume is highly recommended, preferably, logarithmically.**

We have re-submitted a new Fig4A expressing Tumour Volume in Y-axis.

3) Minor Essential Revisions

*In Methods: It is unclear when the drug was started to be injected into mice. Immediately after the tumor cell inoculation? or when tumor volume reached around 200mm³?*

We have now clarified in the “Material and Methods” section (page 7) that the compounds were injected when the tumour volume were palpable (reached around 35-40 mm³).

4) Minor Essential Revisions

*In Methods, Growth Inhibition Assay: The calculation method for IC50 is unclear. The equation does not indicate the calculation of IC50 value. In addition, authors said the IC50 of C75 and ECGC were 100microM and 300microM, respectively. However, judging from the additional file figure, it seems that they are IC70.*

We have revised the Methods and Results sections and we have detected two mistakes regarding the equation to calculate the IC50 values (page 5) and the IC50 values of C75 and ECGC (page 7). We have now included the correct equation to calculate the IC50 value in the new re-submitted manuscript (see page 5). The correct IC50 values for C75 and ECGC are 72 ± 2.8 µM and 265 ± 7.1 µM, respectively (see this data in page 7).

Reviewers’ 2 comments:

*In this manuscript, the authors have investigated the anti-cancer effects of fatty acid synthase inhibitors C75 and (-)-epigallocatechin-3-gallate (EGCG) in a lung cancer model using A549 lung adenocarcinoma cells and xenografts. The authors found that C75 and ECGC blocks fatty acid synthase activity, induces apoptosis, and affects EGFR-signaling in cells in culture. In lung cancer xenografts, both C75 and ECGC blocked tumor growth, but C75 had other adverse affects on the host animal. The authors conclude that inhibition of fatty acid synthase can be*
achieved through effects of ECGC on EGFR signaling and that ECGC may be a good candidate for future pre-clinical drug development.

This is a well written manuscript that describes an interesting study. The results clearly demonstrate a positive effect of fatty acid synthase inhibitors C75 and ECGC on lung adenocarcinoma cell line growth in vitro and in vivo, and dissects some of the pathways that may be involved. The authors have utilized excellent methods and the results are clearly presented in the text and figures. This study will be of interest to investigators in the field, and advances our knowledge of how lung adenocarcinoma might be therapeutically addressed. The major issue with this study is that it involves one lung adenocarcinoma cell line (A549) and derived xenografts. Hence, it is impossible to know if this is a generalizable effect, or if this effect is specific to this particular cell line.

Previously to perform the study with the A549 lung cancer model, we tested the FASN protein expression levels (by Western Blotting) of three different cellular models of human lung carcinoma: HC827 (a Non-Small Cell Lung Cancer cell line with an EGFR mutation), A549 (an Adenocarcinoma cell line) and H460 (a Large Cell Lung Carcinoma cell line). The FASN expression levels of HCC827 and H460 were few to moderate (+/-) but A549 cells expressed high FASN protein levels (++)

According to the immunoblotting analysis, when we test the anti-cancer effects of the FASN activity inhibitors EGCG and C75 in the three lung cancer cell lines, the IC50 value of C75 and EGCG in A549 cells were 72 ± 2.8 µM and 265 ± 7.1 µM, respectively and the IC50 values of EGCG and C75 in HCC827 and H460 were up to 350 and to 150 in both cellular models, respectively. Thus, we perform the experimental study with A549 human adenocarcinoma lung cancer cells. Another important characteristic related to A549 is due to its high constitutive expression of EGFR those permitting to study the relation between FASN inhibition and the EGFR-signaling pathway. Finally, adenocarcinoma lung cancer (A549 cells) accounts for 40% of non-small-cell lung cancers (NSCLC), the most common type of lung cancer. Adenocarcinoma is one of the most complicated types of lung cancer due to its metastasis to various pulmonary regions, thereby severely affecting lung function. Every tumour has its clinical behavior because of its type and location and in this case, the adenocarcinoma of the lung is considered as truly critical and needs extensive clinical measures to help the patient recover from this deadly carcinoma.

We are currently testing the cellular and molecular effects of the novel FASN inhibitor G28UCM (Puig et al. Breast Cancer Research 13:R131, 2011), structurally related to EGCG, in a xenograft model of adenocarcinoma A549 cells.

Specific Comments:

1. Abstract, page 2 – The abstract summarizes the studies and results with both C75 and ECGC, but the conclusions are restricted to ECGC and the title of the manuscript does not mention C75

   As suggested by the referee, we have changed the title of the manuscript into: “Different Fatty Acid Metabolism Effects of (-)-Epigallocatechin-3-gallate and C75 in Adenocarcinoma Lung Cancer”.

2. Figure 1 is presented in a diffuse fashion. These results could be displayed on a single bar graph (rather than an A and B).
As suggested by the referee, we have performed a new Figure 1 on a single bar graph.

3. **Figure 2 is not mentioned in order in the text of the results.**

   We have exchanged the order of Figure 2 and 3 and we have re-written the results text regarding this change (see page 8).

4. **Results – The authors should insert P values for all references to statistically significant results (such as the significant induction of CPT by C75 on page 7).**

   Revised and corrected (see page 8 and 9).

5. **The in vivo xenograft studies suggest that ECGC or C75 treatment may impair xenograft growth (resulting in a smaller tumor at the end of the experimental period). Nevertheless, the tumors do increase in size from 150-250 mm³ at time zero to nearly 900% that size by day 33. Will these compounds also result in tumor shrinkage under different conditions?**

   We find very interesting the referee comment because we thought to continue the *in vivo* study testing the preventive effect of EGCG and the novel structurally related-FASN inhibitors (G28UCM, see Puig *et al.* Clin Cancer Research, 15, 2009 and Puig *et al.* Breast Cancer Research, 13:R131, 2011) in breast and lung cancer xenograft models inoculating the compound in parallel with the tumour cells. Another interesting point to test could be the effect of the exogenous dietary fatty acids (the mice diet composition) in the tumour shrinkage. *In vitro* we have observed that exogenous palmitate suppressed FASN inhibitor-induced breast cancer cell cytotoxicity (11 and 13).

   We are currently analyzing three different ways to administer the compounds into mice (orally, intraperitoneally or intravenously) to study their pharmacokinetics.

6. **No histological images of the xenografts are included. Was the growth fraction different between control versus C75 or ECGC treated animals? Was there observable/measurable apoptosis in the tumor masses?**

   We were interested in analyze the FASN expression levels and apoptosis in the tumour xenografts. For that purpose, we realized Western Blot analysis of FASN and cleavage of PARP (to assess apoptosis) as we describe in the Material and Methods section in page 6. Results in Fig 4A (right) shows that C75 and EGCG-treated tumors showed apoptosis by induction of PARP cleavage (89 KDa band) without any change in the total levels of FASN protein. In lung cancer, C75 and EGCG inhibit the activity of the enzyme FASN without affecting the levels of FASN protein, as we previously shown in breast cancer (8, 10, 11).