Author's response to reviews

Title: The natural triterpene maslinic acid induces apoptosis in HT29 colon cancer cells by a JNK-p53-dependent mechanism.

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Author's response to reviews: see over
Reviewers’ reports

Title: The natural triterpene maslinic acid induces apoptosis in HT29 colon cancer cells by a JNK-p53-dependent mechanism.

Version: 1 Date: 22 January 2010

The authors thank the editors and their reviewers for the time and pains they have taken in reviewing our paper. We have read their comments carefully and reply individually to each reviewer on separate pages below.
Replies to the criticisms of Reviewer #1

This study provides original information of the anti-cancer ability of natural triterpene maslinic acid in colon cancer HT29 cells. However, the data presented in this study are looks quite interesting, there are, however, a number of critical points. Considering the required scientific quality, this question has to be answered before publication should be considered. Thus, I do not recommend to accept these data for publication in *BMC Cancer*.

The reviewer states that our paper contains original information about the antitumoral capacity of maslinic acid in colon-cancer cells but at the same time cannot recommend publication on the basis of two points. I’m afraid, however, that I can’t agree with the reviewer on these issues. Our work, which is completely original, follows step by step the long-term molecular anti-tumoral mechanisms induced by maslinic acid in HT29 cells.

1. The authors already published almost same story in Cancer letters (Reyes-Zurita FJ, Rufino-Palomares EE, Lupiáñez JA, Cascante M. Maslinic acid, a natural triterpene from *Olea europaea* L., induces apoptosis in HT29 human colon-cancer cells via the mitochondrial apoptotic pathway. Cancer Lett. 2009 Jan 8;273(1):44-54.). This manuscript is only additional study for that.

On the contrary, our intention in this paper has been to examine in depth the long-term mechanisms that lead to the induction of apoptosis in HT29 cells as induced by maslinic acid. The aim of our study has been to put forward a complete molecular mechanism to explain fully the induction of apoptosis in HT29 cells rather than describe superficial observations concerning an activation of the mitochondrial apoptotic pathway, which is where we had got to in the paper mentioned by the reviewer. In the previous paper we published the initial effects of maslinic acid upon HT29 cells, but in fact its levels of activity are considerably higher, for which reason we decided to lengthen incubation times, and did indeed obtain much stronger and more significant evidence. And it was this new data that prompted us to propose a possible complete mechanism for mitochondrial activation.

2. The authors mentioned JNK-p53 dependent mechanism of maslinic acid. However, the authors should study the direct relationship between JNK and p53 using siRNA or p53 knockout cells.

The aim of our paper is to propose a molecular mechanism that might explain the anti-tumoral effects observed in HT29 cells in response to the activity of maslinic acid and also the time frame involved in this response. Our proposed molecular mechanism explains the sequential effects taking place during this time and also the effects of this duration upon apoptosis and differentiation.

We do not consider a description of the relationship between JNK and the activation of p53 to be essential to our paper as it has already been widely discussed in the literature.

Another question is whether the activation of JNK by p53 is necessary for maslinic acid to trigger apoptosis. As we say in our previous paper (Reyes FJ et al. (2006). *FEBS Lett.* 580, 6302-10), maslinic acid is capable of inducing apoptosis in p53-deficient cells, which may take place via a completely different, though complementary, mechanism; we are currently working on this subject and hope to have a paper ready for publication shortly. In fact the reviewer seems to be urging us to demonstrate the molecular mechanism of maslinic acid in the absence of p53, but this, together with his/her suggestions in the following point, constitute a different paper from the present one. Within this very context, we are currently coming to some conclusions from a study of the anti-tumoral effect of maslinic acid upon Caco2 cells, which
do not express p53. In this latest research we have discovered that its molecular mechanism is very different in cells that do not express p53 as it stimulates the extrinsic apoptotic mechanism. But all this belongs to a separate paper.

3. In the same line with number 2, the authors also should suggest the direct evidence between JNK and Bid.

The activation of Bid has been described in response to two proteins: caspase-8 and JNK. Given that Bid is clearly activated by maslinic acid and that there is no effect upon caspase-8 until after 72 h, we set out to determine to what extent maslinic acid affected JNK levels. We observed from our experiments that it was clearly active and have described our results in the paper. Thus we propose that Bid activation occurs in response to the activation of JNK because these two proteins clearly become active in response to maslinic acid, whilst caspase-8 does not. As I have already mentioned, the aim of this paper is to propose a complete mechanism for the induction of apoptosis that takes place in HT29 cells rather than demonstrate a direct relationship between the activation of Bid and JNK, which is widely discussed in the literature.
Replies to the criticisms of Reviewer # 2

The manuscript by Fernando et al. describes an in vitro study on the induction of apoptosis in HT29 colon cancer cells, by the natural triterpene maslinic acid. A mechanism for apoptosis induction is proposed. Overall, the manuscript deals with an issue of interest, since a better knowledge of mechanisms that regulate apoptosis in tumor cells, could lead to the development of new therapeutic strategies against cancer. The authors find that the natural triterpene maslinic acid is genotoxic (Comet assay) and causes apoptosis (Hoechst). They also show an earlier and significant increase in JNK expression. P53 is over expressed. The signal transduction pathway was studied through Western blotting. The results apply to one human cell line, originating from colon cancer (HT29 cells). However, there are methodological aspects that should be considered and/or further clarified during revision of this manuscript.

1-A major point: Why the authors did not study the expression of Bax, Bcl-2, cyt c, and caspases at 12h and 24h?? It is indispensable to “propose a perfect synchronized sequential mechanism”.

We agree with the reviewer that it is not essential to propose a perfectly synchronised sequential mechanism and so we have omitted this phrase from the Abstract of our text. Our main intention in this paper is to investigate more deeply the effects induced by maslinic acid and thus add to our previously published results (Cancer Lett, 2009 Jan 8; 273(1): 44-54.), in which we described the expression of some of the mentioned proteins at the times suggested by the reviewer.

Authors should better discuss some points:

1- At high dose of maslinic acid (IC80) and after 24h, no clastogenic effect was observed. Does it mean that in these conditions cells died? DNA lesions are repaired?

We agree with the reviewer’s comment here in that we can’t really ascertain that the decrease in the clastogenic effect can be put down to repairs to the DNA, and that it might well be a response to the cytotoxic effect of the maslinic acid itself. In accordance with the reviewer’s remark, we have included the following sentence in the Results section: “probably due to the citotoxic effect induced by maslinic acid under these conditions”.

2- Based on references 3 and 4 the authors suggest that Bid activation could be mediated by JNK. Does it mean that Bid could be activated through phosphorylation? Or that JNK could act as a protease?

As far as we know nothing has been published to date about the activation of Bid by phosphorylation. In fact we report a decrease in the expression of complete Bid, a zymogen of the active protein. Thus, as the references suggest, if Bid is activated directly via JNK this latter would have to act as a protease, generating the active fragment j-Bid, although due to its low molecular weight and rapid degradation (ref.) it has never been detected.
Nevertheless, the aim of our work here is not to enter into the controversy of whether JNK could act directly as a protease or whether it might phosphorylase an undescribed protein, which could then act as an intermediary between the activation of JNK and Bid, because the JNK-dependent activation of Bid is amply described in the literature. In the light of our results we merely put forward the possibility of this activation as part of the induction mechanism since caspase-8 is totally inactive until 72 hours after the beginning of treatment.

3. A mechanism is proposed in Fig 5. Authors should insist that is a PROBABLE mechanism.

In accordance with the reviewer’s suggestion we have included the idea that this is a “plausible” molecular mechanism in all references to it, although the verb “propose” also carries this idea within it.
Replies to the criticisms of Reviewer #3

This paper describes the effects of maslinic acid on the genotoxicity, cell cycle, phosphorylation of JNK, and expression of p53, Bid, Bax, Bcl-2, Cyt-c, and four kinds of caspases. These data seem to be new and complement the author’s previous findings (Cancer Lett, 2009 Jan 8;273(1):44-54.). There also seem to be no apparent grammatical or statistical problems.

We thank the reviewer for his positive report and for his recognition that this paper contains new data which complement that found in our previous publication.