ANSWERING REVIEWERS

Feb 24, 2014

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 7830-original article.doc).

Title: Clobenpropit enhances anti-tumor effect of gemcitabine in pancreatic cancer

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ESPS Manuscript NO: 7830

The manuscript has been improved according to the suggestions of reviewers:

1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer

Reviewer #1

The present study is reported that Clobenpropit enhanced anti-tumor effect of gemcitabine in pancreatic cancer cells through inhibition of EMT process. Authors' experimental methods including in vitro & in vivo, are not only appropriate but also clear for getting results. Accordingly, these results about anti-tumor effect via inhibition of EMT would be helpful for new paradigm of pancreatic cancer therapy. Therefore, I recommend that this report would be accepted as an original research.

Reviewer #2

In their manuscript, Kon Ryu and collaborators have investigated the effect of the H3R antagonist / H4R agonist Clobenpropit on the behavior of pancreatic cancer cell lines, alone and in combination

with the classical chemotherapeutic treatment, Gemcitabine. They performed a series of in vitro assays including cells growth and migration, and also in vivo tumor growth assays using the model of xenografts in nude mice. They concluded that Clobenpropit increases to antitumor effect of Gemcitabine. This study presents interesting results with a potential is the field of therapeutic treatments. However a huge number of issues, including correcting erroneous conclusions, must be addressed to ascertain the general conclusion and make this work suitable for publication in WJG.

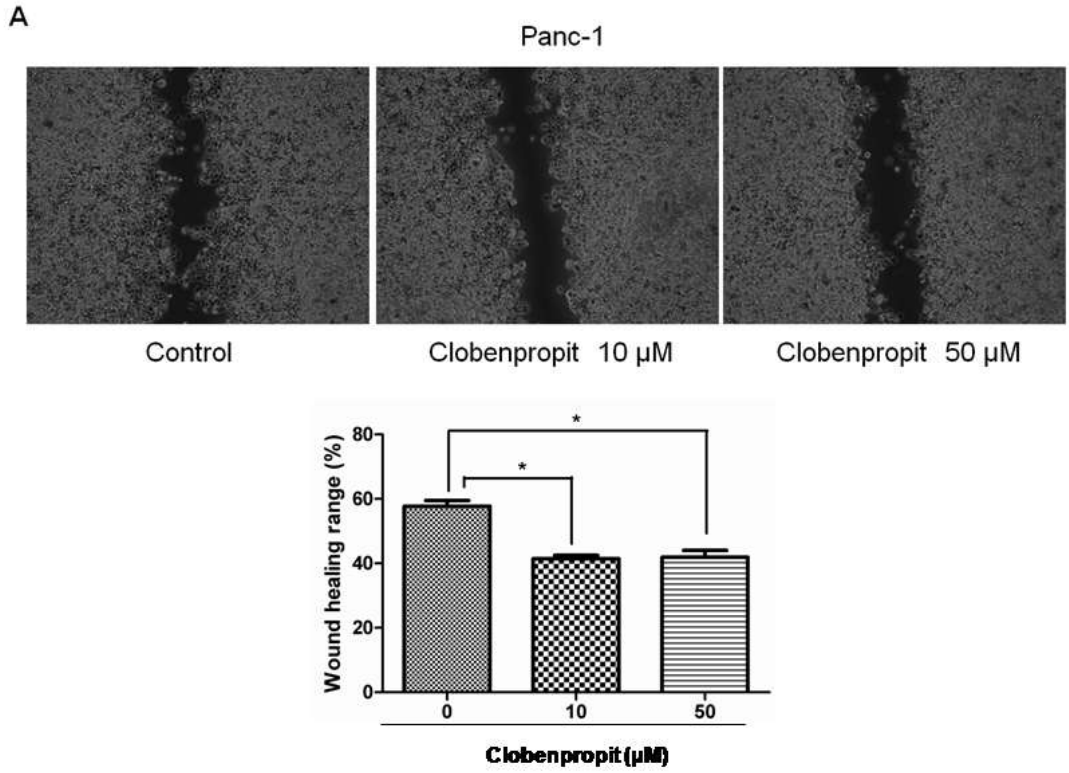
1) The quality of the figures is generally poor, especially in the case of western blots and histological pictures. In particular, the specificity of the H4R antibodies seems to be questionable from the results shown in Fig1. In the A panel, the gel is cut to focus only on the expected bands of 70kDa and 43kDa. However in the panel B, although the gel is also cut, it appears that the antibody also detects at least one strong band with a higher molecular weight than 70kDa (see the first lane). Hence, the entire gels have to be shown to clearly demonstrate that the antibody selectively detects only the H4R bands. In addition, there is no indication at which stage of the cell culture protein levels were analyzed. Data on the levels of H4R must be provided in cell cultures during the proliferation phase and at confluence. This is especially important because the next assays use wound healing, which means that cells are initially at confluence and then can re-enter cell cycle at the wound edge.

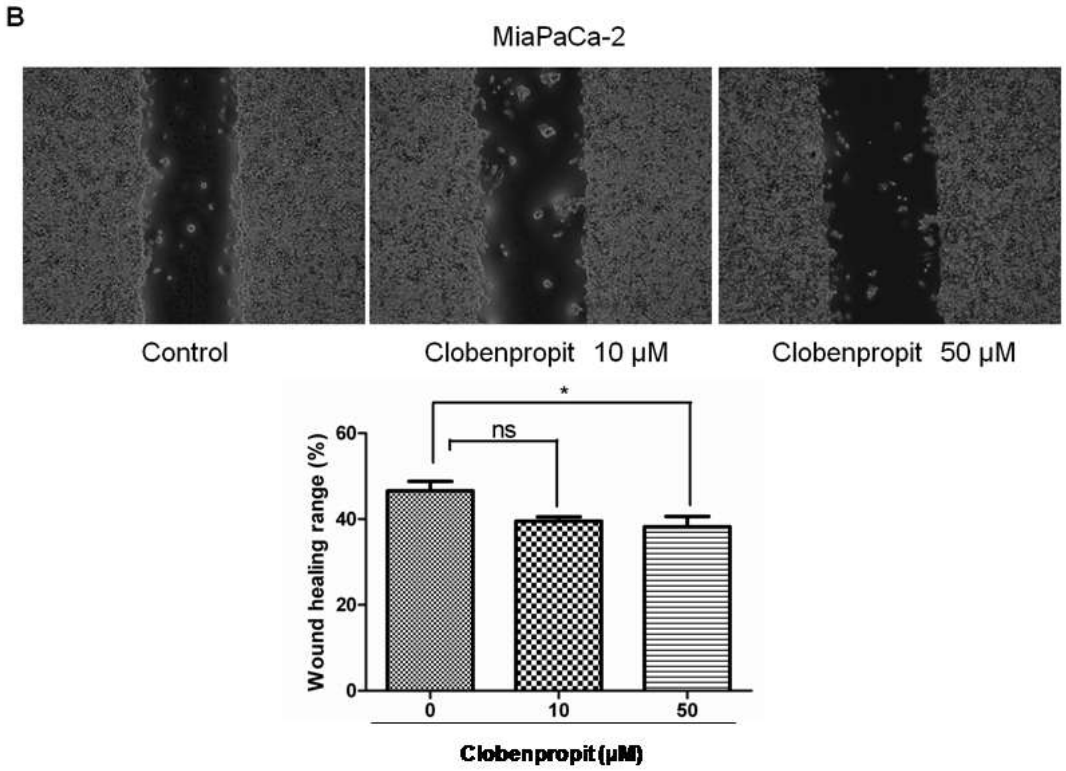
According to the reviewer's comment, we performed the Western blot again, however, the result was not changed and the quality of picture was not better than previous one. The data of Western blot and Wound healing assay were obtained at 70%-80% of confluence level, and we mentioned about the phase of cell cultures as follows:

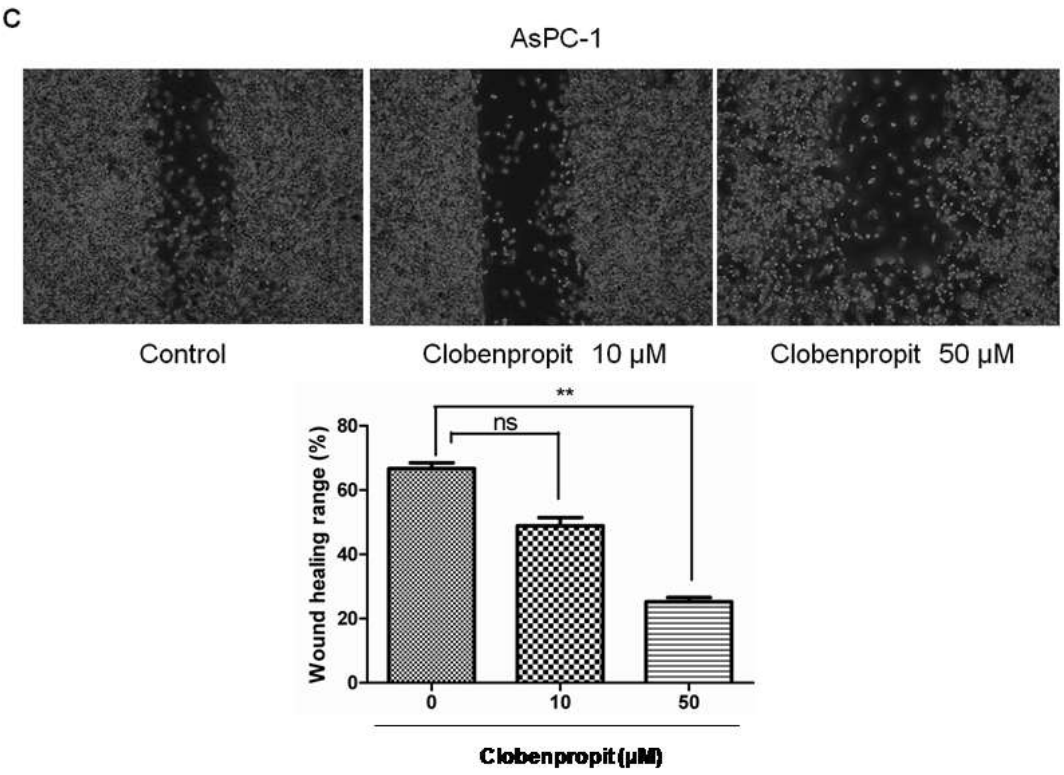
"After washing with PBS, three kinds of pancreatic cancer cells (Panc-1, MiaPaCa-2 and AsPC-1) at 70%-80% of confluence level were processed…"

2) The effects of Clobenpropit in the wound healing assays alone (Fig 2) or in combination with Gemcitabine (Supp Fig 2) are not obvious. Moreover, they are not quantified and there is no indication of the number of repeated assays performed and no statistical data on the significance of the results. This should be provided absolutely. A reduction of the wound healing capacity can be the result of a decreased cell migration, of a decreased in the burst of cell proliferation induced by the wound, or of an increased in cell death. Which one of these effects is targeted by Clobenpropit?

According to your comments, we described the statistical analysis of wound healing assay as follows:







As we mentioned in supplementary data, clobenpropit alone has little effect on cell proliferation or cell death. Therefore, we think that the decreased cell migration by Clobenpropit resulted in the reduction of wound healing.

3) As above for cell pictures, the data of real-time-PCR of EMT markers in wound healing assays must be repeated and analyzed statistically. In addition, it is not clear if the RNA used for these RTqPCR were extracted from the whole cultures or if they came from cells dissected out from the wound edge. To strengthen these observations, the authors should make immunocytochemistry to really illustrate, for instance, if E-cadherin is actually overexpressed by Clobenpropit in those cells at the wound edge. The results illustrated in this section of the manuscript show an increase of Ecad and a decrease of Vimentin and MMP9 after Clobenpropit. Nevertheless, the authors conclude:" clobenpropit down-regulated epithelial marker, while up-regulated mesenchymal markers". This is obviously an erroneous conclusion. Besides the results presented in this study, the authors should provide data on actual drivers of EMT (and not only on downstream targets), like Zeb1, Snail, Slug,

Twist. They should also analyze b-catenin by immunocytochemistry to address if Clobenpropit changes the nuclear/membranous ratio of this important factor in cells at the wound edge.

We performed real-time PCR in pancreatic cancer cells to find any suspicious EMT markers associated clobenpropit, therefore, we did not analyzed statistically. Instead, we statistically proved the elevation of E-cadherin by immunochemistry in vivo. Among a variety of EMT related markers, we focused on the E-cadherin and Zeb1 in this study. As the reviewer mentioned, further study about other EMT markers will be helpful clarifying the mechanism of clobenpropit in pancreatic cancer.

4) The authors present data on the effect of Clobenpropit on apoptosis, which are supported by a statistical analysis. They state that the combination of Clobenpropit with Gemcitabine significantly increases apoptosis in comparison with controls (untreated). However, the real comparison must be made versus Clobenpropit alone and Gemcitabine alone. Is this difference significant? From the data presented, it is hard to believe

The difference of apoptosis between control and combination therapy was significant in vitro. The apoptosis tended to increase in combination therapy compared to gemcitabine or clobenpropit alone, but the difference did not reach statistical significance. However, the animal study showed significant enhancement of apoptosis in combination therapy compared to clobenpropit or gemcitabine alone. Therefore, we concluded that combination therapy of clobenpropit and gemcitabine increased the apoptosis of pancreatic cancer.

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology*.

Sincerely yours,

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