Author's response to reviews

Title: Inhibition of DNA methyltransferase activity and expression by treatment with the pan-deacetylase inhibitor panobinostat in hepatocellular carcinoma cell lines

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Author's response to reviews: see over
Dear Editors of BMC Cancer,

We appreciate the comments of the reviewers and thank you for the opportunity to revise our manuscript “Inhibition of DNA methyltransferase activity and expression by treatment with the pan-deacetylase inhibitor panobinostat in hepatocellular carcinoma cell lines” accordingly.

Reviewer 1, Chunmei Wang:

Major Compulsary Revisions:

1. The results in Figures 1, 3 and 4 are shown as relative expressions against untreated results which were given a value set at 1.0. We therefore consider the presentation of an additional control of untreated cells at 0h as redundant, as these bars would also be shown at 1.0. Instead, we have included a dotted line in the bar diagrams to better demonstrate the level of untreated controls under each experimental condition. Figure legends have been revised accordingly.

In Figure 2 an untreated control for each point in time is demonstrated. As no difference was established as compared to time point 0h without any relevant treatment, there is no need to show these data.

2. The results in Figure 4 are illustrated as relative expressions against untreated controls which were given a value set at 1.0. We therefore consider the presentation of untreated controls as redundant, as these bars would also be shown at 1.0. Instead, we have included a dotted line in the bar diagrams to better illustrate the level of untreated controls under each experimental condition. Figure legends have been revised accordingly.

3. We have discussed our own results and findings in more depth by comparing them with other findings in the discussion part.
4. We agree with the reviewer that Figure 6 does not demonstrate the findings of this paper; however it was our intention to outline a possible context in which our findings could be interpreted. The figure should be interpreted as a hypothesis for combining our results on inhibition of DNMT activity and transcriptional control mediated by panobinostat in order to show a more general mechanism of action and as a basis for discussion and further experimental approaches. Should this be undesirable, it is possible for Figure 6 to be deleted. In the new revised version we have added APC and RASSF1a into the figure to incorporate the results of this work.

Minor Essential Revisions:

1. We have amended the punctuation from “,” to “.” of the number labels on the Y axis.

2. We have changed the label to "normalized expression" and deleted the term “normalized to GAPDH”.

We have added the symbol “-” to the untreated lines.

3. We have carried out a new scan and densitometry analysis of the blots for DNMT3a and DNMT3b blot in Hep3b.

4. We have labeled “1” on the Y axis of Figure 3.

5. We have changed “?-actin” to “β-actin” in the protein extraction and western blot analysis.

6. We have changed “approx.” to “approximately”.

Discretionary Revision:

1. We have added the cell line names above Figures 1B and 1C.
Reviewer 2, Huanjie Shao:

Major Compulsary Revisions:

1. We have used the “EpiQuick DNA Methyltransferase Activity/Inhibition Assay Kit” (Epigenetek, Brooklyn, NY, USA) for detecting the DNMT activity. This assay detects the activity of all human DNMTs including DNMT1, DNMT3a and DNMT3b and the manuscript therefore contains the results of all investigated DNMTs. On the whole, we observed a strong reduction of the protein expression of DNMT1 and DNMT3a, while the de novo DNMT3b showed only a minor reduction. Overall, a residual expression of DNMTs could be observed under the described experimental conditions, confirming the findings of the DNMT activity assay. Moreover, it has been shown that loss of DNMT1 may be compensated by DNMT3b (Rhee I et al., Nature 2000; 404:1003-7; Rhee I et al., Nature 2002; 416:552-6). This has now been included in the discussion section.

2. We have performed new blots for DNMT3a and DNMT3b in Hep3B and altered the scans and densitometry values.

3. We agree with the reviewer that additional genes may also be targets of DNA methylation in HCC. However, the overall literature on this topic is still unclear and the majority of papers investigate the methylation status from primary patient’s samples, but not from cell lines. Our findings on RASSF1 are in line with a previous paper from Lambert et al. (J Hepatol 2011;54:705-15) which shows that this gene is the most commonly hypermethylated gene in HCC. In contrast to other cancers and to primary specimens, the methylation level of other genes, e.g. p16 (CDKN2a), was low in our cell lines and was therefore not investigated in detail as we do not consider this gene to be an optimal candidate gene for our experiments. Other researchers also reported low
methylation for e.g. retinoic acid receptors (RAR) in HCC (Yang et al., Am J Pathol 2003;163:1101-7). Yu et al. also demonstrated that, for example, CDH13 is unmethylated in HCC patients from Asia with or without cirrhosis, whilst RASSF1A was shown here to also be commonly hypermethylated (Yu et al., BMC Cancer 2002;2:29). We therefore concentrated on APC and RASSF1A, which are described as hypermethylated in a high percentage in HCC (Lit. 13-17; 32-36). Our results clearly show the effect of lower DNMT activity due to panobinostat treatment on two representative hypermethylated genes in HCC cell lines. These aspects are included in the discussion section.

4. We performed our investigations on HCC tumor cells with a high and fast cell turnover. In our opinion, this fact explains the results available at this early point in time given that the new developing cells are affected by the changed DNMT activity and its effect on the methylation status of APC. We have included this in the discussion section of the manuscript.

5. As previously mentioned in the comments to reviewer 1, it was not our intention to reveal our findings in Figure 6, rather to demonstrate a possible context in which our findings could be interpreted. Should this be undesirable, it is possible for Figure 6 to be deleted.

General remarks:

1. The manuscript has been revised by a professional language editing service.