Author's response to reviews

Title: The radiosensitising effect of gemcitabine and its main metabolite dFdU under low oxygen conditions is in vitro not dependent on functional HIF-1 protein

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Version: 3 Date: 26 June 2014

Author's response to reviews: see over
Dear Madam, Sir,

Please find enclosed the amended version of our manuscript entitled “The radiosensitising effect of gemcitabine and its main metabolite dFdU under low oxygen conditions is not dependent on functional HIF-1 protein”. We appreciate very much the positive and constructive comments of the referees.

We are pleased to resubmit the manuscript within the requested deadline and we tried to improve our manuscript according to the reviewers’ recommendations, in detail:

**Comments of the first reviewer**

**Major revisions**

1. As correctly pointed out by the reviewer, HIF functions have become increasingly complex and it is likely that non-canonical regulation by HIF-1 is not inhibited, since the dnHIF construct is identical to endogenous HIF except for loss of the oxygen-dependent degradation domains and DNA-binding domains. We added this in the description of the cell lines (page 5, line 108-113).

   We would like to illustrate that the same methods and validation procedures however have already been published in previous research on the contribution of HIF-1 to therapy resistance (Brown et al., Mol Pharmacol, 2006; Roberts et al., Br J Cancer, 2009). We hope this convinces the reviewer further that the system used effectively inhibits HIF-1 function.

   In order to improve the validation of the cell lines, we performed another CA9 western blot and we improved the quality of the blot (figure 1A).

   Also, figure 1B now elegantly shows that the dnHIF construct was localised to the nucleus (colocalisation with nuclear DAPI staining) and was expressed independently of oxygen availability. Transfection with the control vector (EV) resulted in eGFP expression that was confined to the cytoplasm. Moreover, the immunofluorescence images clearly illustrate induction of CA9 in the EV cells under hypoxic conditions, while CA9 staining was absent in hypoxic dnHIF cells.

   As shown in figure 1C, an adenoviral reporter gene assay showed that HIF-1 activity was significantly inhibited, with a huge difference between the EV control and the dnHIF cell line.

   Indeed, as illustrated in figure 1D, the difference in hypoxia-induced VEGF secretion is less pronounced, yet significantly different between dnHIF and EV cells. This however might be explained by the fact that HIF-1 downstream targets (such as VEGF, CA9,...) are regulated by both HIF-1 dependent and independent pathways.

   Finally, as suggested by the reviewer, we included an additional table (table 1) presenting the 11 genes showing a more than two-fold up- or downregulation in mRNA levels between normoxic and hypoxic conditions.
2. We appreciate the reviewers’ suggestion to perform additional experiments with siRNA depletion of HIF-1 and agree that this would be of additional value in view of HIF-1 inhibition. However, by improving the validation data as described above, we hope to have met the reviewers’ expectations of the manuscript. For our future research, we will surely elaborate on this remark.

3. In figure 3, we initially included all data from one cell line (six conditions) in one graph, as this seemed the best way to directly compare normoxic and hypoxic conditions within the same cell line. As the reviewer suggested, this however makes the data a little difficult to understand, as it is hard to distinguish all six conditions from each other. Therefore, we now split the survival data for each cell line in separate normoxic (on the left) and hypoxic (on the right) panels. As such, the three cell lines are shown side by side, with the three normoxic/hypoxic graphs in one column. We hope this makes the survival data more comprehensible.

4. We now explain in more detail in the paragraph on “Statistical analysis” (M&M section, page 8, line 198-201) that two-way ANOVA was used to study the influence of oxygen tension, HIF functionality (i.e. the cell line under investigation) and treatment with gemcitabine, dFdU and/or irradiation on the outcome parameter (i.e. cell survival or cell cycle distribution). Post hoc comparisons revealed which groups differed significantly from one another. As such, we indeed compared wt/EV to dnHIF cells stage by stage. As reported in the results (page 11, line 268-270), these post hoc analyses did not reveal any significant difference in the percentage of G0/G1, S or G2/M phase cells between MDA-MB-231 EV and MDA-MB-231 dnHIF at any condition tested, suggesting that the cell cycle perturbations were not dependent on functionality of HIF-1α.

Minor revisions

1. As suggested, we simplified the presentation of the data. Figure 3 is now more comprehensible and table 2 and 3 were simplified (cfr. comment 4 of the second reviewer). We hope that the explanation on the statistical analysis (major revisions, point 4) clarifies to the reviewer which comparisons were made and which endpoints were used.

Comments of the second reviewer

Minor revisions

1. The rationale for the 18 h exposure to hypoxia in the validation experiments was that this time window yields a robust induction of the expression of HIF-1α, HIF-1-induced downstream targets and HIF-induced luciferase activity. This is now mentioned in the manuscript (M&M, lines 123-124). A similar time point has been used in previous experiments validating transfection with the dnHIF construct (e.g. Brown et al., Mol Pharmacol, 2006; Burrows et al., J Clin Endocrinol Metab, 2011).

2. As suggested, the description of the western blot now includes more details on the antibodies and experimental conditions used (M&M section, page 6, line 125-138).

3. As proposed by the reviewer, we included an additional table (table 1) presenting the 11 genes showing a more than two-fold up- or downregulation in mRNA levels between normoxic and hypoxic conditions.

4. In order to simplify the presentation of the data in table 2 and 3, we now report only the most relevant drug concentrations, i.e. those that are also shown in the radiation dose response curves (figure 3). We hope this makes the data more comprehensible.

5. We thank the reviewer for her highly relevant remark to add a paragraph on biomarkers of hypoxia (different from HIF-1α) and the clinical implications of our results. As such, we expanded our discussion on the prognostic role of HIF-1α and other hypoxia markers (lines 278-282, lines 293-302 and lines 334-336).
6. As suggested, we now further discuss the value of in vivo research in order to enable an enhanced prediction of prognosis, optimisation of (gemcitabine and/or radiation) treatment and information on whether and how to target tumour hypoxia (page 14, lines 355-358).

Taken together, our manuscript in its revised form has clearly benefited from the reviewers’ profound evaluation of the paper. We hope to have convinced you and the reviewers to reconsider our manuscript for publication in BMC Cancer.

Yours sincerely,

dr. An Wouters