Author's response to reviews

Title: ENMD-1198, a novel tubulin-binding agent reduces HIF-1 and STAT3 activity in human hepatocellular carcinoma (HCC) cells, and inhibits growth and vascularization in vivo

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Author's response to reviews: see over
Dear Prof. Norton,

we are submitting our revised manuscript entitled “ENMD-1198, a novel tubulin-binding agent reduces HIF-1α and STAT3 activity in human hepatocellular carcinoma (HCC) cells, and inhibits growth and vascularization in vivo”, to BMC Cancer to be considered for publication as a research article. We thank the reviewers for their careful review and critiques, which now led to an improved version of our manuscript.

We have responded to each reviewer’s comment and edited the manuscript accordingly. In addition, further in vitro experiments have been performed (MTT, HIF-1α expression in vitro and in tumors), however, we decided not to create another figure as suggested by reviewer#1 in order to keep this manuscript in a reasonable format and length. Importantly, regarding the point raised by reviewer#2 (i.e. lack of comparison to 2ME2 and further ENMD1198 experiments and description of compound), we now have included the relevant reference, as the study on ENMD-1198 has recently been accepted for publication in the AACR journal Molecular Cancer Therapeutics. We have cited this new study without adding further data to our manuscript in order to avoid double publication of data on ENMD-1198.

All authors concur with the submission of this manuscript and we assure that the material submitted for publication has not been previously reported and is not under consideration for publication elsewhere.

We appreciate your time in handling and reviewing our manuscript, and look forward to receiving your final decision.

Kind regards,

Oliver Stoeltzing, M.D.
Associate Professor of Surgery

Regensburg, April 28th 2008
Reviewer #1:

Comment 1) Title. Authors did not show the relationship between most data (Fig.1, Fig. 2, Fig. 3, and Fig. 6) and HIF-1 inhibition. If these data are independent of HIF-1 inhibition, the title should be changed because the present title strongly focuses on HIF-1 inhibition. If authors want to keep the present title, they should do more experiments using HIF-1 expression and knock-down methods. Micro-tubulin inhibitors such as 2ME2 are known to block HIF-1α function, we therefore had stated this in the title. However, the reviewers point is very correct and we changed the title accordingly, as also STAT3 is inhibited (essential for HIF-1 function), which represents a new important finding.

Comment 2) Data quality. Fig. 4A Western blot and Fig. 6A&B. Immunohistochemistry results should be replaced with higher quality ones.

The IHC figures have now been re-formatted and replaced by ones with higher resolution (Figure 4A and Fig 6).

Comment 3) In Fig. 6, authors should measure HIF-1alpha levels by immunohistochemistry in order to examine whether reduced vascularization in ENMD-1198-treated tumors is related with HIF-1 inhibition activity of ENMD-1198

We understand this reviewer’s point, however, tumor sizes are significantly different among treatment groups, a factor which per se substantially influences HIF-1 activation in tissues. Furthermore, staining for HIF-1α does not necessarily provide information on HIF-1α activation (Stoeltzing et al., JNCI 2004) Nevertheless, we now have performed HIF-1α Western blotting of tumor samples and included these results in Results (page 11) Figure 6 and Discussion section (Page 13). In addition, we performed Western blotting for STAT3 and found diminished STAT3 activation in ENMD-1198 treated tumors (statement included on page 13).

Comment 4) Fig. 4B. Normoxic values should be present in this figure, and the experimental number should be also mentioned in its legend.

Figure reflects results from normoxia experiments, as these cell lines do not secrete high levels of VEGF in vitro. Respective changes have been made (Figure 4, legend) and this aspect is addressed in the results (page 12). Western blots have now been repeated to improve quality (HUH7).

Comment 5) Authors mentioned there were no differences in body weights of mice between control and drug treatment group, but they did not show the data. Since the body weight change is important in drug evaluation, they should present the changes in body weights as a figure or in text.

In order to reduce overall numbers of figures, we did not include these non-significant data. We would appreciate if the editor could accept this explanation.

Reviewer #2:

1. The range of the antiproliferative activity of ENMD-1198 is very narrow, practically from 1 to 4 μM and did not exceed 60%?? These effects of ENMD-1198 are with lower potency than have been reported before (AACR meeting 2006).

We have referenced the appropriate journal (MCT in press), which provides further information about this compound. However, HCC is a tumor entity that biologically is quite different from other cancers, thus it is not surprising that MTT assays reflect different IC50 from those reported at AACR. This aspect has now been integrated in the Results section (page
2. The data on HIF-1alpha protein are not convincing. The quality of Western blots is low and there is no loading control. TOPO-I can be used as a loading control in nuclear extracts. Furthermore, it is possible to show normoxic levels of HIF-1alpha protein, at least in HepG2 cells. There is no data on the effects on HIF transcriptional activity, neither by reporter gene assay nor on HIF-downstream genes. The presented VEGF mRNA data were measured only under normoxic conditions and the inhibitory effects of ENMD-1198 were minimal although statistically significant. We repeated the Western blot using whole cell lysates proteins and probing for β-actin as a loading control, thereby confirming the results. The figure has been edited accordingly.

3. The study lacks in vivo evidence on the mechanism of ENMD-1198. The authors don’t show whether STAT3 and/or HIF signaling pathway are indeed disrupted in the xenografts. It is expected to show in vivo that microtubules are destabilized, inhibition of HIF-1alpha expression and inhibition of STAT3 activation!

We now included new data on HIF-1α reduction in ENMD-1198 treated tumors, as determined in additional Western blot analyses of tumor tissues (Methods, Results, and Figure 6 are now modified). In STAT3 was also a decrease detectable (data not shown).