Author's response to reviews

Title: Validation of a VEGFR2 antagonist peptoid in vivo

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Version: 3 Date: 16 June 2010

Author's response to reviews: see over
June 16, 2010

Dr. Melissa Norton
Editor-in-Chief
BMC Cancer

Dear Dr. Norton:

My colleagues and I are pleased to submit a significantly revised version of our manuscript entitled “GU81, a VEGFR2 antagonist peptoid, enhances the anti-tumor activity of doxorubicin in the murine MMTV-PyMT transgenic model of breast cancer”. We request that the manuscript be considered for publication in BMC Cancer as a Research Article.

We are grateful to the reviewer’s who carefully evaluated the manuscript and offered many comments and suggestions that have helped to improve the overall quality of the study. We have specifically addressed reviewer comments as detailed on the following pages and have highlighted the changes made in the manuscript.

Thank you for considering our re-submission

Sincerely,

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Response to Review:

Reviewer 1:

Minor Essential Revisions

1. Although the authors state that the development strategy of GU81 will be published in another paper, the rationale for obtaining a derivative of GU40C is unclear. Why was GU81 designed?

RESPONSE: GU81 was designed as a higher affinity derivative of GU40C4. We have now highlighted the fact that GU81 has a 3-fold increased binding affinity to VEGFR2 compared to GU40C4 in the text (pg. 9)

2. Genetic background can significantly affect latency of tumor formation and aggressiveness of tumor progression. The PyVmT model is available in different backgrounds. Please specify which genetic background is used.

RESPONSE: We agree and have now added this detail into the Material and Methods section. The MMTV-PyMT model used in these studies were on a Fvb genetic background (pg. 7).

3. The mechanism of action for peptoids, especially for GU40C or GU81 are unclear for those who are unfamiliar with the subject. More background in the intro is needed for GU40C and GU81. For example, does GU81 specifically bind to VEGFR2 or does it affect VEGFR1 also? What previous studies were done on GU40C to describe the mechanism of action?

RESPONSE: Great question, thank you. We know that GU40C4 and GU81 bind to VEGFR1 and VEGFR2. GU40C4 has been tested against numerous other cell surface receptors by ELISA and by immunocytochemistry against various cell lines to demonstrate its specificity (1). Three chemistry based studies have been published on GU40C4 (1-3). The Bioorg Med Chem study (3) documents that GU40C4 binds to the extracellular domain of VEGFR2 but does not inhibit VEGF interaction with the receptor. Thus, the peptoid has a unique mechanism of inhibition, which we propose but have not proven is inhibition of ligand-induced dimerization. Structural studies on are going to explore this possibility.

We have found that GU81 competes with GU40C4 for binding to both receptors, indicating that these peptoids recognize the same epitope. This data has been added to Fig. 1B and is discussed in the text on pg. 9.

4. It is interesting that GU81 does not significantly affect angiogenesis in vivo but does affect VEGF phosphorylation in vitro. Does GU81 inhibit VEGF induced sprouting or proliferation in vitro?

RESPONSE: After careful analysis of the staining for microvessel density, we have found that although total vessel number is not reduced following treatment with GU81, the total vascular area and vessel size is reduced, suggesting that GU81 may be “normalizing” the tumor vasculature. This data has
been added to the manuscript (Fig 6). Although the effects of GU81 on VEGF-induced endothelial cell proliferation were not directly analyzed for this manuscript, GU81 and GU40C4 recognize the same epitope on VEGFR2. The IC$_{50}$ value for VEGFR2 phosphorylation is improved 2-fold for GU81 (430 nm) compared to GU40C4 (1 µM) (1). Furthermore, GU40C4 does significantly reduce VEGF-induced endothelial cell proliferation in vitro (1) and we would expect that GU81 will do the same. We have demonstrated that GU81 as a single agent decreases tumor growth and microvessel density and vascular area in Balb/C mice bearing orthotopic 4T1 tumors (4). This information has been added to the manuscript (pg. 9).

5. Given that the in vitro dosages in Figure 1 differ from the in vivo dosages given and the in vitro results differ from in vivo results, does the in vivo dosage correlate with the in vitro dosage?

RESPONSE: The in vitro dose used in Figure 1 ranged from 0.001-25 µM with the IC$_{50}$ value calculated at approximately 430 nM. The in vivo dose of 260 ug/day is approximately equal to the delivery of 92 µmol/day. Given the approximate mouse blood volume of 1.4 ml the concentration of GU81 in the blood could reach concentrations of 65 µM, which would exceed the in vitro IC$_{50}$. However, we have not completed pharmacokinetic evaluation of GU81 and therefore do not know clearance or half-life parameters, which are critical for accurate description of an effective in vivo dose.

6. For clarity, a separate description in the methods section of how the statistics were performed is advised.

RESPONSE: We agree and this information has been included (pg. 8).

7. How was microvessel density measured?

RESPONSE: Microvessel density was measured using Immunohistochemistry with either Meca32 or endomucin followed by analysis with Nikon Elements Software. The total number of fluorescent vessels as well as the percent fluorescent positive area was analyzed. This information is present in the legend for Fig 6.

Discretionary decisions

1. While is it mentioned that no differences in vivo angiogenesis were observed between GU81 treatment and controls, showing the results anyway may help the reader to further understand the effects of GU81 in vivo.

RESPONSE: We agree and this information has now been added to Fig 6.

2. Given that increased VEGF expression is increased in tumors with GU81 treatment, could GU81 enhance VEGF expression in cultured tumor cells?

RESPONSE: We have found that treatment of met-1 cells, which were derived from a primary PyMT tumor, with GU81 increases VEGF expression at both the mRNA and protein levels (Fig 7C&D)
Reviewer 2:

Major Compulsory Revisions:

(3) The insights gained from this study are not clear; a deeper analysis of the mechanistic basis for the in vivo effects of GU81 and the additive effects of doxorubicin is required.

RESPONSE: Further analysis of vascular parameters in MMTV-PyMT tumors in the study reveals that although vessel number was unchanged following treatment with GU81 either alone or in combination with doxorubicin, there was a significant effect on the total vascular area (measured as the % fluorescent area/200x field) and on vessel size. These results have been added to the manuscript Fig 6 and Discussion pg. 15. The results are consistent with anti-VEGF strategies increasing the delivery of chemotherapy by increasing vascular function. Normalization is typically thought to proceed by ‘pruning’ of inefficient blood vessels that are not associated with pericytes; however, a reduction in vessel size/area may also facilitate an increase in vascular efficiency without a change in microvessel density (5). Additionally, as pointed out by the reviewer, we found that anti-VEGF strategies are not that potent at reducing vascular parameters in MMTV-PyMT tumors (4). GU81, however, is quite effective at reducing vascular parameters in orthotopic 4T1 tumors (4). The reason underlying this lack of efficacy of GU81 and other anti-VEGF agents in these tumors is unclear. The MMTV-PyMT tumor in particular is challenging as it develops de novo in a well vascularized fat pad and may not require an exuberant angiogenic response for progression. We are very interested in understanding the mechanism of the additive effect of GU81 and chemotherapy and will investigate improved drug delivery as a potential explanation in the near future.

(4) The title of the study needs to be changed. The current title, “Validation of a VEGFR2 antagonist peptoid in vivo”, is misleading in that the use of the peptoid for treatment of tumors in vivo has already been published (PLoS One 2009). The only new in vivo validation aspect of GU81 in the present study is that for the MMTV-PyMT breast cancer model it does not work when administered alone. Results from in vitro experiments show that it represses VEGFR2 phosphorylation in endothelial cells but no data are presented to indicate that such repression is the primary action of Gu81 in vivo. In fact, the increased levels of VEGF and lack of effects on tumor vessel density would be consistent with the hypothesis that GU81 has no suppressive effects on VEGFR2 phosphorylation in vivo.

RESPONSE: We agree and have changed the title to better describe the data presented in this study. Although we were unable to determine directly whether VEGFR2 phosphorylation is reduced in vivo following treatment with GU81, we did determine that total vascular area and vessel size but not vessel number is reduced following treatment with GU81 (as detailed above). These results demonstrate that GU81 is impacting the vascular system of MMTV-PyMT tumors. However, we agree with the reviewer that the efficacy of GU81 is dependent upon co-treatment with chemotherapy, in this case doxorubicin.
While the data in this study are intriguing, they leave open the question of what the mechanism of action of GU81 on tumor vasculature or tumor cells may be in vivo. The authors assume that it acts by blocking activation of VEGF receptors R1 and R2, but based on the absence of any effect on vessel density and the increased levels of VEGF in the tumor as a result of treatment with the peptoid, one wonders whether the compound has other effects on cells that may be unrelated to suppression of VEGFR signaling. Additional mechanistic data to explain the ability of GU81 to stimulate VEGF levels in the tumor are required.

RESPONSE: The change in total vascular area and vessel size following treatment with GU81 alone and in combination with doxorubicin suggests that GU81 is able to target the VEGFRs in vivo. The suggestion to evaluate the effect of GU81 on VEGF production was excellent and we explored this in vitro using Met-1 cells, a cell derived from a primary PyMT tumor. The data presented in Figure 7 demonstrate that Met-1 cells upregulate VEGF expression following treatment with GU81. Met-1 cells and primary PyMT tumor cells express VEGFR2 at low but detectable levels (data presented below). However, it is difficult to argue that either VEGF or GU81 have a direct effect on tumor cell proliferation or survival as we see no change in either proliferation or apoptosis markers following treatment with GU81 as a single agent. The increase in VEGF expression after GU81 treatment in vivo and in vitro suggests that there may be an intact negative VEGF:VEGFR2 feedback loop in these cells. Alternatively, the increase in VEGF levels in vivo could be due to an increase in macrophage levels post GU81 treatment.

Immunohistochemistry

MMTV-PyMT Tg (8 wk)

RT-PCR


