Author's response to reviews

Title: The prospective application of a hypoxic radiosensitizer, doranidazole to rat intracranial glioblastoma with blood brain barrier disruption

Authors:

Hironobu Yasui (yassan@vetmed.hokudai.ac.jp)
Taketoshi Asanuma (asanuma@cc.miyazaki-u.ac.jp)
Junichi Kino (radjimu@vetmed.hokudai.ac.jp)
Tohru Yamamori (yamamorit@vetmed.hokudai.ac.jp)
Shunsuke Meike (meike@vetmed.hokudai.ac.jp)
Masaki Nagane (nagane@vetmed.hokudai.ac.jp)
Nobuo Kubota (n-kubota@pola-pharma.co.jp)
Mikinori Kuwabara (kuwabara@vetmed.hokudai.ac.jp)
Osamu Inanami (inanami@vetmed.hokudai.ac.jp)

Version: 4 Date: 15 February 2013

Author's response to reviews: see over
Professor Brad Wouters  
Associate Editor  
BMC Cancer  

8 February 2013  

Dear Prof Wouters  

We appreciate the opportunity you have given us to revise our manuscript (MS: 6883067337522996) entitled “The prospective application of a hypoxic radiosensitizer, doranidazole to rat intracranial glioblastoma with blood brain barrier disruption”.

We are very grateful to the reviewer for their useful suggestions, which have helped us to improve our paper. We have revised the text in accordance with reviewer’s comments, and significant changes that have been made to the text have been colored red in the revised manuscript. Further, please find below our point-by-point responses to the reviewer’s comments.

We hope that the revised version of the manuscript will be suitable for publication in BMC Cancer, and we look forward to hearing from you. Thank you very much for your time and consideration.

Yours sincerely,

Professor Osamu Inanami, D.V.M., Ph.D.

Laboratory of Radiation Biology, Department of Environmental Veterinary Medical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Kita-18 Nishi-9, Kita-ku, Sapporo, Hokkaido 060-0818, Japan  
Tel: +81-11-706-5236 / Fax: +81-11-706-7373  
E-mail: inanamio@vetmed.hokudai.ac.jp
Author's response to Reviewer #2

General Comment
The authors have addressed the scientific concerns, but several editing issues remain. The authors are encouraged to have the manuscript edited by a second English language proficient writer. In particular the authors should address the following:

Response
In accordance with the reviewer's suggestion, we have had the manuscript edited by Edanz, the language editing service recommended by BioMed Central. In addition, we have responded to the comments point-by-point as follows.

Comment 1
Page 5, Line 6 - 9
The sentence structure needs editing.

Response
As requested, we have rewritten the sentence (P5, L6-8) as follows: “Several factors are considered to be responsible for the radioresistance of glioblastomas such as hypoxia [4], the up-regulation of the EGFR pathway [5] and the existence of glioma stem cells [6].”

Comment 2
Line 11
Use of phrase ‘especially WHO grade IV glioblastoma’ suggests there are other WHO grade glioblastomas which is inaccurate? glioblastoma is WHO grade IV

Response
As suggested, we have removed the term "WHO grade IV" (P5, L11).

Comment 3
Page 7, Line 1
BBB restricts hydrophilic or high molecular drugs what do they mean by ‘highmolecular drugs’?
Response
As suggested, we have changed the phrase to "high-molecular-weight compounds" (P7, L4).

Comments 4 and 5
Line 4
Instead of use of terms such as `intrabrain` regions, a more specific scientific description is recommended.

Line 6
The argument is that doranidazole crosses the BBB and it would follow that it will penetrate the areas where BBB is disrupted, but why would it specifically go to hypoxic regions of the tumor?

Response
In this section of text, it was our intention to indicate that doranidazole cannot penetrate the normal brain, but can penetrate the BBB-disrupted tumor region. The relevant sentences in the original manuscript could potentially confuse readers. Therefore, we have rewritten the text (P7, L5-9) as follows:
"Therefore, doranidazole, which has a hydrophilic residue, could not cross the BBB and cause any toxicity to the intact brain. However, in many advanced malignant gliomas, disruption of the BBB has been reported [23-25]. These facts led us to consider the possibility that doranidazole might only reach the tumor regions and not the surrounding healthy brain."

Comment 6
METHODS
Page 8-9
Hypoxic conditions are provided? what percentage hypoxia is this equivalent to?

Response
In line with the reviewer’s suggestion, we have provided the value of the percentage hypoxia equivalent to 10 mmHg (P9, L1).

Comment 7
Page 10, Line 18
Administered `to` intravenously should be corrected

**Response**
In accordance with the reviewer's comment, we have corrected the sentence (P10, L18).

**Comment 7**
Page 22, Line 1-2
Where the animals perfused with saline ? (what volume) ? and brains removed and sectioned following fixation or OCT or freezing? Please clarify details of brain tissue preservation

**Response**
We could not find the relevant section of text on Page 22, Lines 1-2 referred to by the reviewer, but believe it to be on Page 13, Lines 3-4 of the original manuscript. In the autoradiography experiment, the rats were not perfused with saline. Fresh frozen brains were sectioned. The following text has been added (P13, L4-5): “…. rats were decapitated without prior perfusion with saline. Their brains were immediately removed and frozen.”

**Comment 8**
Page 12, Line 13
Please provide details of the software that was used to calculate the ratio of signal intensity to normal brain of non-tumor region.

**Response**
We used ImageJ software for the quantification of the MRI signal intensity. In the revised manuscript we have added the relevant information to the Methods section (P12, L14-15).

**Comments 9 and 10**
Page 13, Line 11
This first line is unclear and has a grammatical mistake
Line 17
The method of brain collection and fixation is different from what was described
Response
As mentioned above, we cannot find the relevant text referred to by the reviewer on Page 22 and believe they are referring to the text on Page 13, Lines 3-4 of the original manuscript.

In response to the comment; we used different methods of tissue preparation in the autoradiography and immunohistochemistry experiments. In the autoradiography experiment, the brain was not fixed and was sectioned at 20 µm thickness for clear detection of [$^{14}$C]-radiolabeled doranidazole. After the collection of autoradiographic images, the sections were fixed with 4% formaldehyde and stained with H/E. Conversely, in the immunohistochemistry experiment, first we fixed the brain with 4% formaldehyde and sectioned it at a thickness of 5 µm. In the text of the original version, the description of the autoradiography method was potentially confusing, so that we have rewritten it in the revised manuscript as follows: “…. rats were decapitated without prior perfusion with saline. Their brains were immediately removed and frozen.” (P13, L4-5). ”After the acquisition of autoradiographic images, parts of sections were fixed with 4% buffered formaldehyde and stained with hematoxylin/eosin (H/E).” (P13, L9-11).

Comment 11
RESULTS
Page 18, Line 4
Please clarify what is meant by - `to clarify how doranidazole expressed its radiosensitizing effect on C6 glioma.

Response
In accordance with the reviewer’s comment, we have changed the sentence (P18, L5-6) to: "To examine the suppressive effect of doranidazole on the hypoxic region in the C6 glioma".

Comment 12
DISCUSSION
Page 19, Line 11-13
What do the authors mean by this concluding statement? C6 glioma which has a relatively high tumor grade compared to what? Their experiments did not test effects of doranidazole to a lower grade glioma? this is not the study design for the set of experiments they report and this statement is not supported by their own or literature referenced data.

**Response**
We mentioned that C6 glioma has a relatively high tumor grade on the basis of a previous study by Towner et al. Free Radic Biol Med 2010, 48(5):691-703. They classified three gliomas as "RG2 (grade IV tumor) > C6 (grade III–IV tumor) > ENU-induced glioma" (reference 25). However, we did not evaluate the effects of doranidazole on various grades of gliomas, so we have removed this concluding statement from the revised manuscript.

**Comment 13**
Line 14 - 16
The sentence structure needs to be corrected.
Are the authors suggesting that the level of hypoxia within individual GBM tumors has to be determined prior to treating with doranidazole.
The basis of the introduction and background to this study however has been as the authors indicate that GBMs are highly hypoxic tumors. Therefore individual levels of hypoxia in each patient will not be necessary to decide whether this drug will work as a radiosensitizer? and I would argue in fact the general high level of hypoxia known to be present in every GBM is why this study has potential and is interesting results? there is generalized applicability to all GBMs potentially.

**Response**
We agree with the opinion of the reviewer that a high level of hypoxia is present in every GBM, so doranidazole has the potential to radiosensitize every glioma to some extent. As suggested by the reviewer, we have clarified our comments by removing the text relating to the fact that the level of hypoxia within individual GBM tumors has to be determined prior to treating with doranidazole.
Though the statements about identifying levels of hypoxia in tumors is accurate the results of this study do not demonstrate a variability in response to the drug based on levels of hypoxia of the C6 glioma models. Perhaps a second cell line to create GBM xenografts or other GBM models to make a comparison would more definitively support their statements.

Response
Again, we could not find the relevant text on Page 22, Lines 1-4 referred to by the reviewer. We agree with the reviewer’s view that a comparison between C6 glioma and another GBM model would be useful in demonstrating the variability in tumor response to doranidazole based on hypoxic levels. However, it will take time to obtain results from additional experiments involving other glioma models. Therefore, at this stage, we would like to present data exclusively obtained from the C6 glioma. In line with the reviewer’s comment, we added the following sentence to the revised manuscript (P20, L8-10): "To reveal the variability in tumor response to doranidazole based on levels of hypoxia, further investigation using other glioma models will be required."