Author's response to reviews

Title: Targeting tissue factor on tumour cells and angiogenic vascular endothelial cells by factor VII-targeted verteporfin photodynamic therapy for breast cancer in vitro and in vivo in mice

Authors:

Zhiwei Hu (zhiwei.hu@yale.edu)
Benqiang Rao (benqiangrao@sina.com)
Shimin Chen (shimin.chen@yale.edu)
Jinzhong Duanmu (duanmujz@163.com)

Version: 5 Date: 6 February 2010

Author's response to reviews: see over
January 11, 2010

Sabina Alam, PhD
Senior Scientific Editor
BMC series journals

Dear Dr. Alam, Senior Scientific Editor:

We thank you and the reviewers for the careful and thoughtful review of our manuscript, titled “Factor VII-targeted verteporfin photodynamic therapy for breast cancer in vitro and in vivo in mice”. The comments helped us to improve the manuscript. Enclosed are a revised version of the paper and responses to the reviewers’ comments, both of which have been edited by American Journal Experts, a professional copyediting company (editing certificate enclosed). We thank you again for the opportunity to submit our manuscript to BMC Cancer and look forward to its publication.

Reviewer: Muriel Barberi-Heyob

Reviewer’s report:

General comments:
The purpose of this study is to develop a new ligand-targeted PDT to improve selectivity, using coagulation factor VII and tissue factor as target. This strategy would be a pertinent choice to increase vascular effect of PDT (vascular targeted PDT). But, the question posed by the authors is not really well defined. Indeed, several studies have described that tissue factor is an angiogenic maker and its expression is related to angiogenic endothelial cells of vessels associated with solid tumours.

Concern: Adding additional target cells such as breast cancer cells, leads to confused message.

Response: This is an insightful comment from the reviewer. Since TF is selectively expressed in tumour cells (for instance, in the breast cancer cells in this paper) as well as in tumour vascular endothelial cells (VEC), the goal of fVII-TPDT is to target and eradicate both the tumour vasculature and the tumour cells for cancer therapy. To clarify this key point, the title...
of the manuscript has been revised as “Targeting tissue factor on tumour cells and angiogenic vascular endothelial cells by factor VII-targeted verteporfin photodynamic therapy for breast cancer in vitro and in vivo in mice”. This principle of concept has been tested for the development of fVII-targeted immunotherapy by fusing fVII as targeting domain to an IgG1 Fc as an effector domain (fVII/IgG1 Fc or called as Icon), which has shown dramatic effect on the eradication of pathological neovasculature (tumour vasculature, choroidal neovasculature and endometrial neovasculature) and tumour cells for the treatment of cancer (Hu and Garen, 2000, 2001; Hu et al., 1999; Tang et al., 2007), macular degeneration (Bora et al., 2003; Tezel et al., 2007), and endometriosis (Krikun et al., 2009) in preclinical studies. In addition, fVII-targeted VP PDT has also shown an improved effect for the treatment of choroidal neovasculature in a rat model as compared to non-targeted PDT (Lu et al., 2009). Moreover, fVII-targeted Sn(IV) chlorin e6 PDT has been developed and is being tested for the treatment of various types of cancer including breast and lung cancers (Hu et al., to be submitted). Taken together, fVII-targeted immunotherapy and PDT are designed to target both the tumour neovasculature and tumour cells for novel therapies of cancer.

**Concern:** The methods to estimate PDT effect are not appropriate:

**Response:** Dr. Barberi-Heyob probably refers to the cell viability assay by crystal violet staining and

![Comparison of clonogenic assays](image)
the OD595nm readings to determine the PDT effect. Thanks for pointing out the difference between the conventional clonogenic assay and the alternative assay by crystal violet staining. We were fully aware of the difference and compared these two assays for determining the effect of fVII-targeted SnCe6 PDT on the breast cancer MDA-MB-231 cells. The results in Fig. 1 in this cover letter show that the cell survival percentages determined by the conventional assay were slightly lower than that by the cell viability assay with crystal staining and OD595nm reading, but the difference had no statistical significance ($p>0.05$). Since the alternative of the conventional colony formation assay is much labour- and time-consuming but had no statistical difference, we decided to use the alternative cell viability assay to determine the effect of fVII-tPDT and ntPDT and the EC50 concentrations.

**Concern**: The main criticism concerns the lack of control;

**Response**: It was unclear to us what control Dr. Barberi-Heyob was referring to as lacking. In the *in vitro* experiments, e.g., Fig. 5, we did include the controls of verteporfin alone, laser light alone and no treatment to rule out the potential direct effect of verteporfin and laser light, and we found that those controls did not have any effect on killing cancer cells (Fig. 5c) nor on inducing apoptosis and necrosis (Fig. 5a & 5b). Those controls have also been tested in other experiments, and we found that laser alone, VP alone, and fVII-VP alone had no effect on cell survival (not shown).

**Concerns**: conjugation of verteporfin to mfVII protein changes the chemical properties of the conjugated photosensitiser increasing its solubility. In this condition, cellular uptake kinetics will be completely modified. No experiment (for instance binding by competition or using a scrambled peptide) was performed to check the non specific uptake brought by the mfVII. Before to compare photodynamic effects between tPDT and ntPDT, the authors have to determine dark cytotoxicity of their conjugates; it is surprising to express PDT efficiency in µM and not in light dose (Fig. 4a). Fig. 4b: please give the photosensitiser concentration.

**Response**: Thanks for the thoughtful concerns. Based on the experiments described in Fig. 1d and Fig. 4c in the manuscript, we concluded that conjugation with VP did not [at least not significantly] change the binding activity of fVII in the fVII-VP conjugate. As determined by flow cytometry (Fig. 1d), the VP-conjugated mfVII had similar binding activity to breast cancer MDA-MB-231 cells as compared to unconjugated fVII at the same concentration (20 micrograms/ml), suggesting that the binding activity of fVII did not change due to conjugation with VP. In addition, we showed in Fig. 4c that unconjugated fVII protein could inhibit the effect of fVII-targeted VP PDT, presumably by competitively binding to TF on the target MDA-MB-231 cancer cells.
Concerns: The authors compare the effects of tPDT and ntPDT on VEGF-stimulated and unstimulated HUVEC (Fig. 3c & d), but no information is given concerning the growing effect of VEGF and the probable impact on verteporfin cellular uptake.

Response: 1.1 nM of VEGF diluted in human endothelial serum free medium was incubated with HUVECs only for four hours to induce TF expression, and then the serum-free medium containing VEGF was removed, followed by an incubation with fVII-VP or free VP for PDT. Considering that the doubling time of HUVECs is about 17-24 hours (Marin et al., 2001) or even 30 hours (R & D Systems) in complete growth medium (normally M199 supplemented with 20% FBS and 1:100 diluted ECGS), 4-hr incubation with VEGF in serum-free medium would not have significant effects on the cell growth. Moreover, the experiments described in Fig. 3c & d had separate controls for VEGF-stimulated and unstimulated HUVECs, i.e., no treatment and maximal controls were also stimulated with VEGF for fVII-tPDT and ntPDT with VEGF-stimulated HUVECs and those controls for unstimulated HUVECs were not stimulated with VEGF. By using its own controls, the growth effect of VEGF would be normalised if a 4-hr incubation of VEGF did have any effect on the growth of HUVECs.

Concerns: Effect of tPDT for cancer in vivo in mice bearing mouse breast cancer. The authors show that ntPDT with VP did not have any effect. This result appears surprising. Moreover, the results in Fig. 6b in the manuscript showed that tPDT with either 2 or 4 µM VP had significant therapeutic effect as compared to control. But increasing VP concentration did not enhance the efficacy. Determining optimal modalities of PDT could be efficient if in practice, parameters were known, measurable and constant. However, space-time variations in the parameters can occur during the photodynamic treatment. Light dosimetry does not take into account numerous phenomena, regarding one or several of the three main factors involved (i.e. photosensitiser, light and oxygen). These difficulties make the determination of the PDT modalities a nonlinear and multivariate optimisation problem, and a successful dosimetry strategy has to take into consideration these particular aspects of the problem. In practice, the four factors usually considered are: the administered photosensitiser dose, the time interval between photosensitiser administration and light irradiation (drug-light interval, DLI), light fluence and light fluence rate. The determination of the optimal PDT dosimetry can be expressed as the optimal choice of the three remaining factors (photosensitiser dose, light fluence and fluence rate), i.e. selection of values leading to an optimal PDT response.

Response: Thank you, Dr. Barberi-Heyob, for your advice, and we fully agree with your comments. The reason for the lack of an effect of ntPDT in mice bearing mouse breast cancer was probably because, in order to compare to the effect of fVII-targeted verteporfin PDT, verteporfin was used in ntPDT. Since verteporfin was extracted from liposomal Visudyne and the carrier proteins such as
lipoprotein had been removed from Visudyne, the removal of those carriers might have reduced the amount of intravenously injected verteporfin to be transported to the subcutaneous tumours and therefore ntPDT had no effect on inhibiting tumour growth in mice. We did notice that increasing the VP concentration in fVII-targeted VP PDT did not enhance the effect. The reason for this was probably that fVII at 2 µM VP concentration had already saturated the binding of TF molecules on the vascular endothelial cells. Therefore, further increasing VP to 4 µM did not further increase the effect of fVII-tPDT. One of future experiments will be to investigate if increasing the light fluence with the saturated VP concentration (2 µM) could further enhance the in vivo efficacy.

**Minor comments:**
Axes of ordinates are missing in figures 3, 4. Legends do not give sufficient information (concentrations, number of experiments, etc.) to understand the figures.

**Response:** This has been corrected/revised in the figure legends.

The discussion needs to be completely reconsidered by arguing the obtained results by the authors with the targeting strategies already published in PDT.

**Response:** The discussion section has been extensively revised as suggested.

**Reviewer:** Mahendra Deonarain

**Reviewer’s report:**
Hu et al describe an innovative procedure to use a natural tissue-factor ligand, Factor VII to target verteporfin (VP) photosensitiser for the application of cancer PDT. The concept is wholly reasonable and the impact of this work important as this work could lead to the development of improved agents for human cancer therapy. PDT is becoming increasingly attractive due to its many advantages, but many photosensitisers, such as VP have limitations. These are addressed here. However, I find this paper unacceptable for publication in BMC cancer for the reasons below. This mainly due to the execution of the research (minor issues) and writing of this paper (major issues). The pages are not numbered making my referencing difficult.

**Response:** Thank you, Dr. Deonarain, for agreeing with us that our manuscript describes an innovative procedure to use fVII to target VP to its cognate receptor TF for cancer PDT. We have addressed your concerns and comments individually under each of them. In addition, this revision was edited and proof-read by American Journal Experts prior to the submission of this revision, so hopefully the writing of the paper in English is no longer a concern. We apologise for not numbering
the manuscript in the first version, and now this revision is numbered.

**Major Revisions**

1. The introduction is too brief and lacks important detail. For example, what is the affinity of FVII for TF—is it approaching or better than that of antibodies? By how much does the K341A mutation reduce the TF coagulation activity? Is it enough to not be a therapeutic risk? VP is a soluble sensitiser and thus clears rapidly from the circulation. Targeting will certainly alter the pharmacokinetics (PK) *in vivo* and nothing is mentioned of PK effects, which is a key feature. This point is also relevant to the *in vivo* results below.

**Response:** Thanks, Dr. Deonarain, for your thoughtful comments.

Regarding the affinity and coagulation activity, in the revised manuscript we now include the affinity of fVII (pM level) to TF as compared to those of antibodies to TF (nM level). To directly address your question, Dickinson et al. showed in their 1996 PNAS paper that the TF binding activity of K341A mutated fVII was indistinguishable from wild-type fVII, whereas its coagulation function was reduced about 10 fold (Dickinson et al., 1996). We also included a table (Table 2 from the Hu & Garen PNAS 2001 paper) below, in which we compared the prothrombin time of human and mouse Icon proteins to determine how significantly the K341A mutation reduced their coagulation activities. As shown in the originally numbered Table 2, human and mouse Icon proteins at 10 micrograms/ml (~50 nM based on its molecular weight of 210 kDa) had prothrombin times of 14.7 and 25.8 seconds, respectively, as assayed in fVII-deficient plasma, whereas circulating fVII (0.77 ± 0.19 microgram/ml (Albrecht et al., 1996), ~15 nM based on a molecular weight of 50 kDa) in normal plasma had a PT of 11.8 seconds. The results showed that both human and mouse Icon proteins, even those at three-fold greater molar concentrations, did not reach the same prothrombin time as that of wild type fVII in normal plasma, indicating that the K341A mutation reduced the coagulation activity of fVII by at least three fold or more.

![Table 2: Effect on the PT of the mfVII/hFc and hfVII/hFc icons](image)

<table>
<thead>
<tr>
<th>Icon concentration, µg/ml</th>
<th>Pooled normal plasma</th>
<th>FVII-deficient plasma</th>
<th>Pooled normal plasma</th>
<th>FVII-deficient plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>11.8 ± 0.1</td>
<td>44.0 ± 0.2</td>
<td>11.8 ± 0.1</td>
<td>44.0 ± 0.2</td>
</tr>
<tr>
<td>1</td>
<td>11.7 ± 0.1</td>
<td>29.8 ± 12.5</td>
<td>11.7 ± 0.0</td>
<td>32.1 ± 0.0</td>
</tr>
<tr>
<td>5</td>
<td>11.5 ± 0.2</td>
<td>14.3 ± 0.3</td>
<td>14.2 ± 1.4</td>
<td>27.2 ± 0.1</td>
</tr>
<tr>
<td>10</td>
<td>12.0 ± 0.0</td>
<td>14.7 ± 0.1</td>
<td>14.6 ± 0.5</td>
<td>25.8 ± 0.0</td>
</tr>
</tbody>
</table>

From Hu & Garen. PNAS. 2001

Regarding the PK of fVII-targeted photosensitiser, we fully agree with Dr. Deonarain’s comments. PK study is one of the next experiments we are planning to do, and we will report it in a separate paper.
2. These experiments describe the in vitro cell killing of human VECs, and tumour cells, mouse tumour cells but the control is a CHO (hamster) cell line. Either a human or murine negative cell line should be used. The authors should at least justify the choice of such a negative cell line.

**Response:** Thanks for your suggestion. The choice of CHO as a TF-negative cell line is now justified in the Results section. It was indeed difficult to find a TF-negative immortalised normal mammalian cell line. That was the reason for using a Chinese Hamster Ovary (CHO) line, as noted. Nevertheless, unstimulated HUVECs also served as TF-negative normal human cells.

3. In the methods, the cell viability test described is NOT a clonogenic assay. A true clonogenic assay to measure cell killing involves taking the cells after treatment and seeding a culture to see how many cells grow (forms clones of colonies). This assay should be appropriately renamed.

**Response:** Thanks for this comment. The assay has now been renamed as non-clonogenic cell viability assay by staining with crystal violet. As shown in Fig. 1 in this cover letter, however, we found that there was no statistical significance in determining the cell survival percentages between this cell viability assay (staining with crystal violet for loss of monolayer adherence) and the conventional clonogenic assay (counting the numbers of colonies).

4. In the in vivo therapy, the authors say that one control mouse had metastases. This is an important observation. Was this seen in one mouse per study or one mouse overall (n=4 ?).

**Response:** Yes, it was one control mouse overall that had metastases (n=5 in each group). Metastases in control mice were also observed in our previous tests of Icon immunotherapy for prostate cancer (Hu & Garen. PNAS 2001), and copied below is an excerpt from page 12182 in the Hu & Garen 2001 PNAS paper.

“The control and Icon-treated mice were examined at necropsy for evidence of bleeding, clotting, or tumor metastasis. A bone tumor was present on the spine of one of the seven control mice, presumably derived by metastasis of the original skin tumor.”

5. The discussion is too brief and lacks many important points. For example, FACSs is not quantitative (as done here), so any decrease in FVIII affinity is not measured. Does the conjugation method used here alter the binding affinity? The authors do not comment on the quality of the conjugate—the coupling ratio described is around 13:1. Is the all covalent or is there some non-covalently bound material? The authors cite the work of 2 other groups who have made targetable VP
conjugates but do not evaluate their work with this other published work. How does it compare in terms of efficacy? Another key discussion point is the choice of 90 minutes for the drug-light interval. This was also used for the \textit{in vitro} work, but the PK is completely different \textit{in vivo}. The authors do not show that the conjugate localises \textit{in vivo} or how fast the uptake/clearance is. The rationale behind the \textit{in vivo} work should be explained and justified in the discussion.

\textbf{Response}: We agree with Dr. Dr. Deonarain that FACS is not quantitative and does not directly measure fVII affinity. One of the direct measurements of affinity of fVII to TF is the Biacore assay (Dickinson et al., 1996), which we plan to use for comparisons of unconjugated fVII protein and the fVII protein in the conjugates with photosensitising. These results will be reported in a separate paper. Nevertheless, as shown in Fig. 1d, the peak shift of the mfVII-VP conjugate at the 20 \( \mu \)g/ml protein concentration was similar to that of 20 \( \mu \)g/ml of unconjugated mfVII protein, indicating that the binding activity of fVII in the VP conjugate to the breast cancer MDA-MB-231 cells was not significantly changed by conjugation with VP.

The molar ratio was calculated using the fVII-VP conjugates that had been separated by Sephadex G-50 spin columns. In Fig. 1c, we showed that there was no VP after separation on Sephadex G-50 spin columns if only PBS buffer was added to the conjugation with VP by covalent cross-linker EDC, indicating that free VP could not go through the size exclusion spin columns. Therefore, the VP calculated in the molar ratio should represent the covalently fVII-bound material.

In the revised manuscript, we included a discussion of the work of the other two groups. In addition, we also discussed the choice of 90 minutes for the drug-light interval. This observation was repeatedly seen while using fVII-targeted chlorin e6 PDT (Fig. 2 in this cover letter), which will be
reported in another to-be-submitted manuscript.

6. Figure 1c: I don't understand what PBS-VP control is. Is this VP-there is no Q-band

**Response:** The PBS-VP control in Fig. 1c was the collected material after being separated by Sephadex G-50 spin columns, as was the mfVII-VP conjugate in Fig. 1c. After spin column separation, the collected solution was colourless from the spin column loaded with the PBS-VP conjugation mixture. In contrast, it was green from the spin columns loaded with the fVII-VP conjugation mixture, as observed by naked-eye. After spectral scanning (200-800 nm) on the spectrophotometer, there was no Q-band in the collected PBS-VP control solution but there were both Q-band and protein peaks in the collected fVII-VP conjugate, indicating that free VP could not but fVII protein-conjugated VP could go through the spin columns and therefore indicating that the Q-band in the fVII-VP conjugate (Fig. 1c) was solely from the fVII-conjugated VP. We conclude that Sephadex G-50 spin columns can separate free VP from the fVII-conjugated VP.

7. Figure 2b: Why is the TF receptor not evenly and completely expressed on the stimulated cells?

**Response:** Thanks for noticing this phenomenon. We did not understand why the TF receptor was not evenly and completely expressed on the stimulated HUVEC. This remains to be determined.

However, we recently carried out a similar experiment, as described in Fig. 2b, using microvascular endothelial cells. As shown in Fig. 3 in this cover letter, TF was EVENLY AND COMPLETELY expressed on VEGF-stimulated human microvascular endothelial cells (VECs, green staining). Briefly, microvessel VEC HMVEC-LBI cells (Lonza) were isolated from human lung microblood vessels. The staining procedure was similarly described in Fig. 2b in the manuscript. After staining, the cells were observed and photographed under green and red channels of a confocal microscope. The results show that TF was specifically expressed by the VEGF-stimulated HMVECs, representing angiogenic tumour microvessel VECs, but not by the unstimulated VEC, representing quiescent normal VEC. This experiment was repeated once, and the results were the same as shown above. Original magnification: 400×. (Unpublished data)
these VEGF-stimulated microvascular ECs. Consistently with the observation in Fig. 2b in the submitted manuscript, TF was NOT expressed on unstimulated microvascular ECs. Taking the results together, we conclude that TF is selectively expressed on angiogenic VECs (either from large veins or from microvascular vessels) stimulated by VEGF.

8. Figure 3b: How were EC50s determined when the killing did not reach 50% for the ntPDT?

Response: The EC50 was determined as projected EC50 by the equation as shown in Fig. 3b.

Minor revisions

9. The English grammar and formatting needs to be checked. For example, in the methods, the washing description of the confocal imaging paragraph is poor. The use of ‘u’ instead of the proper ‘micro’ symbol is also not right. There are numerous other examples.

Responses: The revised manuscript has been edited by American Journal Experts and should be improved. The description of confocal imaging has been revised, and “u” has been changed to the symbol of micro (µ).

10. In the introduction, the VP is ‘laser-activatable’ not ‘activated’, as it is not yet activated!

Response: Thanks for pointing this out. It has been changed to laser-activatable.

11. In the methods, what is the relevance of vitamin K1 for post-translational modification?

Response: FVII is one of the vitamin-dependent coagulation factors. Vitamin K is required for the gamma carboxylation modification at the N-terminus of the fVII protein. The lack of this post-translational modification will cause the inactivation of fVII binding activity (unpublished observation).

12. Curve fitting by adding a trendline is not appropriate as true EC50s cannot be determined from this, sigmoidal-regression fitting or equivalent should be used.

Response: Thanks for your comments. We have now used Prism (the latest Version 5, Graphpad Software) to re-plot all of the figures in Figs. 3-6. Particularly in Fig. 4a, the points are separated well and look much better.
13. In the discussion, what is meant by ‘third-level treatment’?

Response: This has been revised, as seen in the discussion. It was derived from the definition from online Wikipedia and, as noted at the time of this revision, it also needs clarification on the website of Wikipedia.

14. Figure 1b: The gel is a poor quality with fuzzy protein bands

Response: The protein markers have been replaced with bars on the left side of the mfVII protein lane.

15. Figure 4 should use log scale X-axes to show the difference better and not squash the points together

Response: Thanks for your suggestions. As suggested, we now used log scale X-axes in Fig. 4a and indeed they look much better.

16. Figure 6a: The Y-axis needs a label.

Response: Sorry for missing the Y-axis label. It probably disappeared when converting the ppt files to TIFF files. This has been fixed.

Level of interest: An article of importance in its field

Quality of written English: Needs some language corrections before being published

Statistical review: No, the manuscript does not need to be seen by a statistician.

Declaration of competing interests:
As well as my academic university position, I am a co-founder and director of a university spin out company developing targeted PDT. The company is developing targetable prostate cancer PDT using a different approach, so there is no direct conflict. However, given the similarities and small commercial field, this potential conflict should be noted.

Sincerely yours,

Zhiwei Hu
References:


