Author's response to reviews

Title: Constitutively active c-Met kinase in PC-3 cells is autocrine-independent and can be blocked by the Met kinase inhibitor BMS-777607

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Author's response to reviews: see over
Dear Dr. Bristow,

We thank the reviewers for their helpful critiques of our manuscript entitled "Constitutively active c-Met kinase in PC-3 cells is autocrine-independent and can be blocked by the Met kinase inhibitor BMS-777607". We have submitted our revised manuscript via online to be considered for publication in BMC Cancer. The assigned manuscript number is 1439906866652620. The responses to the reviewers’ questions are indicated below and are included in the revised manuscript.

Specific comments and our responses:

- **Editorial Requirements:** We would like to ask for methods section in abstract.
  
  **Response:** We have added the methods in the abstract.

- **Reviewer 1:**

  **Minor Essential revisions:**
  (1) In the Background section, most reports indicate 85% of prostate cancer bone metastases are positive for c-Met expression, not 100%.
  
  **Response:** We have scrutinized the references and agree that although the % of c-Met positive in bone metastases is very high it is not always 100%. We have revised the text accordingly.

  **Discretionary Revisions:**
  (2) A positive control using cells that secrete HGF would be important for the analyses in Figure 1.
  
  **Response:** Although adding a cell line that has an active HGF/c-Met autocrine loop would be a plus, we believe that the use of pure recombinant HGF as the functional active exogenous and endogenous HGF ligand is likely to be a better positive control in this study.

  (3) While the culture medium (CM) was not functional in activating c-Met or inducing c-Met functions, e.g. migration, a true test would be to determine if the CM can activate purified c-Met.
  
  **Response:** We agree that the use of purified c-Met to test CM function would be one approach. Instead of a cell free system we have chosen to evaluate the CM effect by treating DU145 cells, a cell line whose
c-Met receptor is commonly not phosphorylated under regular culture conditions (Fig.2B) with the CM from PC-3 cells. The results showed that in this setting CM failed to stimulate c-Met autophosphorylation in DU145 cells, indicating the absence of functional HGF in the CM of PC-3 cells; finding consistent with our ascribed

(4) The experiments that show an anti-HGF neutralizing antibody did not block constitutive c-Met signaling are among the most interesting in this manuscript. However, they would be greatly strengthened using a cell line that does produce and respond to HGF, and demonstrating in this instance, c-Met phosphorylation decreases in the presence of the neutralizing antibody.

**Response:** We agree. We have used exogenous HGF to mimic a paracrine activation mode and have shown that the anti-HGF antibody inhibited exogenous HGF but not PC-3 CM induced c-Met phosphorylation, confirming the inability of PC-3 cells to generate a functional HGF (Fig.4A).

(5) The expected downstream c-Met targets are affected; this adds little to the manuscript unless the authors determine which are important for the effects they see.

**Response:** We have shown that the Met inhibitor BMS-777607 suppresses multiple c-Met downstream pathways (Fig.6) as support for the argument that this compound interferes with constitutive c-Met activation. We agree that it is important to further identify which pathways are critical for the observed cell functions. Indeed we are pursuing this line of experiments in PC-3 and other highly metastatic cancer cell lines and these data will be included in a follow-up manuscript.

(6) The authors note in the discussion (as this reviewer did above) that the prostate is a rich source of HGF. Thus, an in vivo experiment in an appropriate mouse model would be important and should be feasible, although the reviewer recognizes mouse HGF does not strongly activate human c-Met.

**Response:** Although we have shown the impact of BMS-777607 on c-Met activation in the absence of HGF in vitro, it is difficult to mimic this scenario in vivo. While we recognize the importance of investigating this phenomenon in situ, its pursuit raises significant experimental difficulties. Normal immunodeficient mice that generate host mouse HGF are likely not a suitable model as also noted by the reviewer. Perhaps HGF knockout mice would be a better choice. However, such an approach would require an entire new set of investigations which is beyond the scope of the present studies.

**Reviewer 2:**

**Major Compulsory Revisions**

(1) As mentioned above, the authors need to characterize the abnormal HGF AND/OR carry out some in vivo experiments to confirm their in vitro findings. For example, The authors bring up some reasonable suggestions for exploring MET signaling in the discussion, which should really be acted on in the paper to improve the science therein.

**Response:** As indicated above, we agree that animal studies are important to confirm the findings in vitro. However it is clear that the direct assessment of the drug’s effect in this setting in vivo will be very difficult. We suggest that HGF knockout mice that lack the host HGF may be the most suitable model for
such studies, which will require significant efforts to accomplish. In addition, we agree that uncovering the mechanism behind the phenomenon of constitutive c-Met activation in this model is of particular interest. As discussed a number of avenues could be pursued. However we believe that the completion of such studies will require extensive detailed investigations which is too large to address in a single manuscript and that the data presented here should stand on their own.

**Minor Essential Revisions**

(1) The effect on cell proliferation with BMS 777607 would be better off depicted as an IC50 curve. It seems that effects at 3 or 10 um actually indicate that the drug is not that potent - wouldn't an siRNA approach be a good confirmatory experiment?

**Response:** We agree that siRNA is a good tool for validation. Nevertheless, we think it is more informative to apply the pharmacological inhibitor to directly test the efficacy of c-Met targeting.

(2) The first paragraph contains some errors - I would suggest that references 4,5 imply that HGF has been identified as a independent prognostic factor FOR advanced disease NOT of advanced disease. I failed to find a reported figure of 100% in references 6,7 - can the authors suggest where that figure is exactly in those references?

**Responsive:** We have reviewed the references and find that although a 100% c-Met positive staining of bone metastases is reported in Ref 7 (second paragraph of Abstract in *Pisters et al, J Urol, 1995*), Ref 6, quotes a positive value of 91% of bone metastases (41/45, page 1115 in *Knudsen et al, Urology, 2002*). We have revised the text accordingly.

(3) Is it unclear what the 2 control lanes in Figure 2B represent, these should be better described in the legend; the rationale for the anti-HGF is not explained in the text or the legend and why this was also not added to the 10% FBS experiment; Finally whether the CM (PC-3) bar should have extended over the HGF column too?

**Response:** We have clarified the figure (Fig.2B) and figure legend based on these comments.

(4) The relevance of an anoikis experiment is unusual in the usual panel of tests of a malignant phenotype, whilst I have no objection to its inclusion, it would be better placed if the authors justified its importance to a greater extent. Figure 1A - NT as an abbreviation for Not determined is peculiar. Was it in fact, non detectable?

**Response:** Anoikis is a mode of anchorage-independent cell death that negatively affects cancer cell dissemination. Anoikis-resistance has been considered as a key feature associated with prostate cancer progression and metastasis. We have strengthened this statement in the text. Also we have revised the Fig.1A and its legend.

(5) The clinical relevance of Fig 6 would be better supported if some measure of apoptosis was also included or at least the scientific justification for the experiment was better explained. Why is characterizing these changes important? Do they differ in other cell lines? Is apoptosis possible what accounts for the decreased proliferation seen?
Response: We observed that BMS-777607 treatment didn’t induce apoptosis in PC-3 cells, suggesting apoptosis is unlikely the reason for the reduced proliferation. The c-Met downstream pathways monitored in the current study have been reported to be strongly associated with c-Met-mediated functional behaviors by us and others. Interestingly, our data suggest that some pathway alterations seem to be cell type dependent. For example c-Src/FAK signaling is impaired by BMS-777607 in PC-3 (Fig.6) but not in KHT sarcoma cells (Dai et al, Clin Exp Metast, 2012).

Again, we thank the reviewers for their thoughtful reviews and suggestions. We have taken their suggestions into account and believe that as a result the revised manuscript has been considerably strengthened. We hope that with these changes the manuscript will now be suitable for publication in BMC Cancer.

Sincerely,

Yao Dai, PhD