Author's response to reviews

Title: Human Sulfatase 2 inhibits in vivo tumor growth of MDA-MB-231 in human breast cancer xenografts

Authors:

Sarah Peterson (peters4@mmc.org)
Andrea Iskenderian (aiskenderian@shire.com)
Lynette Cook (lcook@shire.com)
Alla Romashko (aromashko@shire.com)
Kristen Tobin (KTobin@hmc.psu.edu)
Michael Jones (jonesm@mmc.org)
Angela Norton (anorton@shire.com)
Alicia Gomez-Yafal (agomezyafal@shire.com)
Michael W Heartlein (mheartlein@shire.com)
Micheal F Concino (mconcino@shire.com)
Lucy Liaw (liawl@mmc.org)
Paolo GV Martini (pmartini@shire.com)

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

We are pleased to submit a revision of manuscript 143290538341742 “Human Sulfatase 2 inhibits in vivo tumor growth of MDA-MB-231 human breast cancer xenografts” by Peterson et al. for consideration in BMC Cancer. We appreciate the helpful comments of the reviewers, and have revised our manuscript in response to the comments. We have included a point-by-point overview of our responses (in bold) below. We agree that the manuscript has been significantly improved due to these changes. Thank you very much for consideration of this manuscript.

Sincerely,

Paolo G. V. Martini, Ph.D.

RESPONSES TO COMMENTS

Review #1 (Dr. Yasuhiro Miki)
The great majority of breast carcinomas arising in postmenopausal women are estrogen dependent or positive for estrogen receptor in carcinoma cells. In this study, the authors employed estrogen-independent breast cancer cell line. MDA-MB-231 is an estrogen receptor negative highly invasive human breast cancer cell line and has been used as a relevant model system to evaluate drugs with chemopreventive potential against highly invasive breast cancer phenotypes. The authors should describe the reason to focus on ER negative breast carcinoma. It was also reported that MCF-7 (ER positive) and MDA-MB-231 differ in their responsiveness to FGF-2. Therefore, It is necessary to discuss the relation between estrogen and your findings.

Response: We thank the reviewer for bringing up this point. We agree with Dr. Miki that it is important to better clarify the experimental model. We have revised the background on p. 5 and the results text on p. 10 to clarify the rationale. As the reviewer mentioned, the MDA-MB-231 cell line is a commonly used model for invasive cancer, and we did a first screen to determine levels of sulfatase expression in several breast cancer cell lines. The MDA-MB-231 cells were notable because they did not express detectable levels of sulf1 or sulf2 (Fig. 1), rendering it a desirable candidate for studying the therapeutic effect of forced expression of human sulfatases. Secondly, while we recognize the importance of estrogen regulation in breast cancer we felt that using an estrogen-independent line was an advantage to focus specifically on effects of the sulfatase enzymes. Further studies will certainly direct the focus on estrogen regulation and...
sulfatases inhibition of breast tumor growth and will be part of new publication. New references [14] and [15] were added to clarify this on p. 10. We therefore chose the MDA-MB-231 cell line for gain-of-function studies by creating transfected pools that stably express human sulfatases.

It was reported that ER negative MDA-MB-468 breast carcinoma transfected with hSulf-1 leads to reduced proliferation in vitro and reduced tumor burden in athymic nude mice in vivo. In this study, the authors established both hSulf-1 and hSulf-2 -expression MDA-MB-231. The authors should clarify the difference between the previous report and your findings. The invasive capacity of MDA-MB-231 has been established. Do the authors have any data on metastasis and hSulfs expression in MDA-MB-231 athymic mice in vivo?

In histological analysis, I could not understand this section. The authors described “increased connective tissue and collagen” in xenograft tumor tissues. Can this be evaluated accurately? Staining of Masson’s trichrome should be clearer. The authors also described higher cellular density and mitosis in control tumor. The mitosis can be counted (mitosis index). The authors can give more accurate/complete information from the results described above.

Response: This is an important point raised by the reviewer. Given that overexpression of hSulf1 has been established as an inhibitor of tumorigenesis, we used overexpression of hSulf1 as a positive control for overexpression of hSulf2 as well as testing hSulf1 in conjunction with hSulf2 (both of which add new information to our current understanding). We included the description of the MDA-MB-468 study in the results on p. 10 to clarify the differences in these studies. We do not have additional data on metastasis and hSulfs expression, but will certainly consider adding this into the experimental design of any future studies in this area. In response to the histological analysis critique, we utilized the expertise of a pathologist, who validated the description of the pathology of our tumors (p.13). In addition we added the Ki-67 staining for objective quantification of mitotic index. We have also added arrows to Figure 5 to clarify regions of interest, and to further describe the pathological findings.

Review #2 (Dr. Wen Jiang)
Main concerns:
1. Given the inconsistency of the results: i.e. in vivo tumour growth rate in models injected with sulfatase-2 transfected cells and injected with recombinant sulfatase-2, one would anticipate that more than one cell lines are to be tested in order to consolidate a valid argument and that refined strategies were attempted (also refer to points 3 and 4).

Response: We have now included data from the MDA-MB-435 cell line in the revised versions of Figure 1 and Figure 2. In addition, the MDA-MB-231 cell line is a heterogenous cell line that we have maintained in the transfected cell populations, rather than undergoing clonal selection. To address the reviewer’s point, we have expanded the text to include more discussion of studies done in other cell lines, including MDA-MB-435 and MCF-7, to provide support for our
conclusions. These revisions are in the results section on p. 10 and in the discussion on p. 15.

2. Figure-1C and D: recombinant hSulf2 was said to have a relatively low stability, <2 days. In fact, more than 50% of the activity seemed to be lost with 5 hours. It is not clear how this reflected in the in vitro and in vivo models.

Response: The activity of hSulf2 is relatively stable over the course of 2 days (Fig. 1D) if diluted in mouse serum, which has a protective effect on rhSulf2, and extends its biological activity over time compared to rhSulf2 stored in cell medium at 37 °C. These results suggest that rhSulf2 could potentially be used for in vivo studies due to its long lasting activity in serum. While it is beyond the scope of this paper to identify potential serum binding proteins that may provide a protective effect on enzymatic activity, we are hypothesizing that potential binding partners would also be present in the tumor microenvironment. The fact that a short-term intratumoral injection could not compare to the endogenously, constitutively expressed sulfatase proteins suggest that either protein degradation or local availability was indeed playing a role in this effect.

3. Figure-2 showed an inhibitory effect of sulfatases on the growth of the cells by an MTT method. It will be useful to demonstrate the nature of the inhibition: change of cell cycle, apoptosis or necrosis. Given the limited data from the study, this will be a desirable and welcoming addition.

Response: We thank the reviewer for this suggestion. We have revised Figure 2 to include the viability data corresponding to the MTT assays in (panels C and D), demonstrating that the inhibition is due to slowed proliferation as opposed to apoptosis or necrosis (no significant decrease in cell viability between the two groups).

4. Figure-3. Although ERK phosphorylation was shown to be inhibited by hSulf2, it will be necessary to show if the phosphorylation of receptors for FGF and EGF, two cytokines used in the study, were also affected, to establish a context. In addition, phospho-ERK in the figure are seen as double bands. This needs further clarification.

Response: Because heparin sulfate proteoglycans act as co-receptors for a wide variety of signaling receptors, we analyzed phospho-ERK as a downstream mediator. This more general approach was favored due to the ability to capture a signaling readout that is downstream of many transmembrane kinase receptors. In the text, we cite references to the well-established paradigm of sulfatase activity in regulation of FGF and HGF (ref #10), and our study shows sulfatase inhibition of both FGF2 and HB-EGF-mediated ERK1/2 phosphorylation (Fig. 3). It is well understood that phosphoERK antibodies recognize two species of MAPK, p44 and p42 when they are phosphorylated on T and Y residues. This has been clarified in the legend for Fig. 3.

5. In vivo delivery of rSulf2 resulted in no change in tumour growth. Although the
Response: The points of the reviewer are well taken, and reflect some of our studies to address this. We did in fact complete three in vivo experiments with lower doses of sulfatase 2 prior to the experiment shown, with the maximal dose at 5mg/kg. It was not necessary to include these earlier experiments given that the experiment with the highest administered concentration was still ineffective. Duration of treatment was limited by the amount of purified hSulf2, which is challenging to purify. We did a pilot study in 7 mice to assess feasibility of administration of hSulf2 by intravenous tail vein injection. Due to the comparatively high osmolarity (secondary to high salt content) of the hSulf2 buffer vehicle, injection of the 5mg/kg dose of hSulf2 into the distal tail vein (in order to allow subsequent proximal daily injections) caused local osmotic bursting of the vessel and decrease in subsequent access.

While this could potentially be avoided by cannulation of the internal jugular vein for central intravenous access as opposed to peripheral intravenous access, we felt that this required undue discomfort and distress in the experimental animals to justify progression to IJ access without first demonstrating success directly to the tumor site with intratumoral injection. Had we been able to demonstrate efficacy of intratumoral injection, we would certainly have moved forward with exploring advanced models for central intravenous access. Similarly, we felt that progression to a minipump was unlikely to be successful in the absence of direct intratumoral injection efficacy.

We have included this information in the discussion of the manuscript on p. 16-17 to enhance the discussion.

6. Conclusion is not supported by the data. It is concluded that ‘in vivo progression of human breast cancer xenografts can be inhibited with sulfatase treatment’. In fact, the study showed that intratumour injection of sulfatase-2 had no effect on tumour growth.

Response: We regret this inconsistency within our manuscript. In agreement with the reviewer, the term ‘treatment’ has been changed to ‘expression’.

Review #3 (Dr. Paul Foster)
Major Compulsory Revisions
1) How do the authors explain that the S1 and S2 xenografts established
tumours initially (at day 5) but then regressed (figure 4)? Why did these tumours form tumours at all considering the loss of volume over 35 days?

2) Why did the control-vector MDA-MB-231 tumours (figure 4) not grow larger by day 35? MDA-MB-231 xenografts are usually extremely aggressive and fast growing. This is why they are used routinely to produce rapid in vivo drug efficacy models. Can the authors explain why their MDA-MB-231 cells grew so slowly?

Response: There is wide variation published in the literature pertaining to the growth rate of MDA-MB-231 tumor xenografts in athymic nude mice. Published tumor size ranges from $150\text{mm}^3$ at 24 days (Fraker et al with implantation of 10 million cells per tumor) to approximately $5000\text{mm}^3$ at 4 weeks (J Wang et al with implantation of 1 million cells per tumor). Our tumor sizes are within range of what has previously been published for MDA-MB-231 tumor xenografts. These references have been provided in the text (ref #18, #19). We were also intrigued by the fact that all tumors in sulfatase transfectants initially developed, but sulfatase expression correlated to regression of those tumors. While the answer to this question is not clear, we hypothesize that there is threshold of sulfatase enzymatic activity that is required for the anti-tumor activity, and that this requires stabilization in vivo of injected tumor cells and a number of critical mass of metabolically active tumor cells. This is discussed on p. 12.

Minor Essential Revisions:

1) How were the tissue samples collected (e.g. in formalin)?

Response: The methods section has been revised to include details about the collection of tissue samples with fixation in 4% paraformaldehyde.

2) Can the authors clarify why the rhSulf2 was administered intratumorally? This does is not a standard approach to treatment. Would it have been more appropriate to administer rhSulf2 i.v.?

Given that sulfatases are active enzymes in modifying the cell surface and the extracellular matrix, we hypothesized that a more direct injection in the tumor and its microenvironment would have provided a better diffusion and efficacy of hSulf2 especially considering that breast tumors could be pharmacologically targeted through an intratumoral route. As described above, we also did a pilot study in 7 mice to assess feasibility of administration of hSulf2 by intravenous tail vein injection. Due to the comparatively high osmolarity (secondary to high salt content) of the hSulf2 buffer vehicle, injection of the 5mg/kg dose of hSulf2 into the distal tail vein (in order to allow subsequent proximal daily injections) caused local osmotic bursting of the vessel and decrease in subsequent access. While this could potentially be avoided by cannulation the internal jugular vein for central intravenous access as opposed to peripheral intravenous access, we felt that this required undue discomfort and distress in the experimental animals to justify progression to IJ access without first demonstrating success directly to the tumor site with intratumoral injection. Had we been able to
demonstrate efficacy of intratumoral injection, we would certainly have moved forward with exploring advanced models for central intravenous access.

3) Throughout the document the authors use both MB 231 and MDA-MB-231. This should be standardised.

Response: All instances of MB 231 in the text have been changed to MDA-MB-231. The only exception is for labeling in the figures due to space considerations with explanation of the abbreviation in the text.