**Author's response to reviews**

**Title:** Molecular Mechanisms of Action and Potential Biomarkers of Growth Inhibition of Dasatinib (BMS-354825) On Hepatocellular Carcinoma Cells

**Authors:**

Alex Y Chang (alexchang@imc.jhmi.edu)
Miao Wang (wangmiao@imc.jhmi.edu)

**Version:** 3  **Date:** 10 May 2013

**Author's response to reviews:** see over
Author's response to reviews

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Authors:
alexchang@imc.jhmi.edu
wangmiao@imc.jhmi.edu

Version: 3 Date: 08 May 2013

Author's response to reviews: see over
Reviewer's report

Title: Molecular Mechanisms of Action and Potential Biomarkers of Growth Inhibition of Dasatinib (BMS-354825) On Hepatocellular Carcinoma Cells

Version: 2 Date: 26 March 2013

Reviewer: Jorge Martin-Perez

Reviewer's report:

Comments to revised version

Major Compulsory Revisions

If I asked you to determine the ration p-EGFR/EGFR, pFak/Fak, p-Stat3/Stat3, pAkt/Akt, pMapk42-44/Mapk42-44 is because, as a reviewer, I think that is the correct method to determine activation of these molecules, and also, because your scientific document will improve in quality. However, you have not modified the figures to include the statistical analyses. This would be something that will greatly help the reader to interpret the data. For the ratio pSrc/Src the result could be indeed misleading as the anti-pY418-Src recognizes all members of the Src Family of Kinases (SFKs) and unless you immunoprecipitate each one of them and do in vitro kinase reaction, you may not know which member(s) are activated in your model system. Therefore, it is probably more correct to set the ration pY418-Src/beta-actin, assuming that beta-actin does not change upon your treatment. For the other phospho-proteins the appropriated is to determine the ration between the phosphorylated forms versus the total protein.

I have modified all the figures to include the statistical analyses. We understand that it is the standard method to test the ratio of phospho-proteins to the related total proteins for determining their activation, but at this moment we are not able to retest the total proteins because the project has already closed and no more protein samples left. We will always do that in our future project. The primary reason that we didn’t test the total proteins is that dasatinib is considered as a TKI and does not regulate total protein expression as far as we know, and we also refer to some previous research studies to design our experiments, such as Johnson FM, Saigal B, Talpaz M, Donato NJ: Dasatinib (BMS-354825) tyrosine kinase inhibitor suppresses invasion and induces cell cycle arrest and apoptosis of head and neck squamous cell carcinoma and non-small cell lung cancer cells. Clin Cancer Res 2005, 11(19 Pt 1):6924-6932. Therefore we quantified the ration between the phosphorylated forms versus β-actin and calculated the p-values by Students t-test (Additional file 1).
In Figure 9 A Co II it is surprising that no standard deviation is detected. In this figure as well as in Figure 10, to show statistical differences an eventually significance among treatment within the graph would also be very useful.

There is a very small standard deviation in CoII of Fig 9. It is not displayed in the figure because we shrink the figure from its original size. However, we repeat the experiment one more time and modified the chart. We have added the statistical analyses in both of Fig 9 and 10.

The invading analyses should quantified and results presented in a graphic (number of assays, SD and significance) together with the pictures.

Thanks very much for your suggestion. We have modified Fig 11.

Thank you very much for considering our publication on J Biol Chem 2006, 281(30):20851-64 (reference 30 in your document), nevertheless the title of that publication is not “A non-catalytic function of the Src family tyrosine kinases controls prolactininduced Jak2 signaling”, it is: “Role of c-Src in Human MCF7 Breast Cancer Cell Tumorogenesis”. In that scientific document we described the observation that conditional expression of SrcDN in MCF7 human breast cancer cells reduces adhesion, migration and spreading; concomitantly, SrcDN expression inhibits activation of Src (pY418-Src), Fak (pY925-Fak) and p130CAS. Because expression of SrcDN alters the shape of MCF7 cells, immunofluorescence confocal analyses showed concentrated focal adhesion proteins. However, the adhesion of cells was reduced. Furthermore, in MDA-MB-231 human metastatic breast cells, we have shown that Dasatinib inhibits proliferation, migration and invasion, as well as inhibits activation of Src, Fak (Y925), paxillin, caveolin-1 and p130Cas phosphorylation/activation [Cellular Signaling 2012, 24(6):1276-86]. In contrast, in Huh-7 hepatocellular carcinoma cells (your reference 29), which you showed that are the most resistance cells to Dasatinib, increased adhesion and migration occurs at the same time upon treatment with prostaglandin E2 by increasing activation of Fak, paxillin and Erk2. One would have expected that increased adhesion would reduce migration, however, the nature of cell origin may determine specific cellular responses.

I am very sorry I paste the wrong title and thanks very much for your patient explanation. We have revised the related content on page 12 and cited these two articles as reference 29 and 30.

Minor Essential Revisions

In the text, Figure 7 should be now Figure 8, Figure 8 should be now Figure 9, Figure 9A,B should be Figure 10A,B and Figure 10 should be Figure 11.

We have revised all the mistakes as shown in page 9 and 10. Thanks.

Please, check again orthographic and typographic errors
In their work entitled "Molecular Mechanisms of Action and Potential Biomarkers of Growth Inhibition of Dasatinib (BMS-354825) On Hepatocellular Carcinoma Cells.", the authors analyze the mode of action on 9 different HCC cell lines. They compare the effects measured by different assays with an analysis of major signalling pathways. After their study of the effects of different therapeutic compounds and their combinations (Chang et al., Anticancer Drugs, 2013, 24(3):251-9), this study tries to elucidate the mechanism if one of the used compounds, dasatinib.

The study appears to be well conducted; the data are reported according to the scientific standards. Especially in the light of the need for an improved therapy against HCC, the data presented here are of value for this research field.

The minor essential revisions I had asked for have been answered satisfactorily. The additions the authors did as a response to the other reviewer, especially figures 9 and 10, improve the value of the manuscript.

Thanks very much for your commence.

Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.
Declaration of competing interests:
I declare that I have no competing interests.
Reviewer’s report
Title: Molecular Mechanisms of Action and Potential Biomarkers of Growth Inhibition of Dasatinib (BMS-354825) On Hepatocellular Carcinoma Cells
Version: 1 Date: 6 February 2013
Reviewer: Jorge Martin-Perez

Reviewer’s report:
This manuscript intends to evaluate the effect of Dasatinib, a SFKs tyrosine kinase inhibitor, as a potential therapeutic agent in hepatocellular carcinoma (HCC). To this end, authors tested the effect of Dasatinib on 9 different HCC cell lines. This is an interesting approach. As in other solid tumors, not all cell lines that have activated Src (pSrc416) respond to Dasatinib treatment in terms of growth inhibition.

Major Compulsory Revisions:
In M&M section author indicated: “For dasatinib inhibition study, serum-starved cells were treated with various concentrations of dasatinib for 24 h prior to the addition of 20% FBS stimulation, and then were collected for western blotting analysis”. Could you please show that this treatment does not affect cellular viability?

Answer: according to your suggestion, we studied the cell viability by MTS assay (page 5, paragraph 4 and page 8, paragraph 2). We showed that treatment with various concentration of dasatinib in medium with serum free, 1% and 10% FBS, didn’t affect cell viability.

According to Figure 1, only Li-7, PLC/PRF/6 and Sk-Hep1 appear to be sensitive to this compound. Nevertheless, it is surprising that authors established correlation between sensitivity and the levels of p-Src416, t-Src or t-EGFR.

Answer: in our opinion, the term of sensitivity is not only used in sensitive cell lines. All the cell lines have their response to a certain drug, some are more sensitive while others are less sensitive. In order to avoid unnecessary confusion we replaced it by growth inhibition.

Dasatinib is considered as an inhibitor of SFKs tyrosine kinase activity, therefore
it would be more appropriate to set correlations between specific activity of Src, that is, the ratio of p-Src416/t-Src, similarly of p-EGFR/t-EGFR (Figure 2). There are not, as far as we know that Dasatinib regulates Srrc expression. If so, it is difficult to understand that authors conclude (page 7): “sensitivity of HCC cells to Dasatinib was associated with over expression of Src protein, low expression of EGFR and low expression percentage of p-Src in t-Src”. Furthermore, there is not quantitation of the results presented in the Western blots and, as indicated above, p-Src/Src, p-EGFR/EGFR, pFak/Fak, p-Stat3/Stat3, pAkt/Akt, pMapk42-44/Mapk42-44 are needed to properly determine the alteration of the state of activation of these molecules upon Dasatinib treatment (Figure 3).

Answer: according to your suggestion, we revised Fig. 2 and related content in main manuscript. We found that in gefitinib (EGFR TKI) resistant cell lines except Huh-7 the low specific activity of Src was significantly associated with high sensitivity to dasatinib. Interestingly, in all the 6 dasatinib resistant cell lines the lower ratio of p-Src/t-Src was significantly correlated with higher resistant to dasatinib. This suggested that there was different mechanism of action of dasatinib between sensitive and resistant HCC cell lines and need further research to investigate. No any correlation was shown between dasatinib sensitivity and p-EGFR/t-EGFR.

We also agree that dasatinib does not regulate Src expression. We did not mention that dasatinib influenced Src expression. Our results just showed there was correlation between the IC50 value of dasatinib and baseline expression level of t-Src. The mechanism of this correlation was unknown. Maybe our description on page 7 is not very clear, so we deleted it and re-summarized the conclusion in the section of discussion. We revised Fig.1 and put the quantitative analysis as Fig.1D. As far as we know TKI does not regulate total protein expression, we first screening the phosphorylated proteins in this study. We will determine the alteration of the state of activation of molecules that we interested in our future study.

In Figure 4 correlations need to be done for pSrc/Src, pAkt/Akt, pFak/Fak. Similar corrections are needed in Figure 5.

Answer: in Fig.4 and Fig.5, our purpose is to analyse the correlation amongst the inhibition of different activated proteins by dasatinib, the inhibition of activated protein is calculated by the following formula, for example: \( \frac{pS_{rc(D)}}{\beta-actin(D)} \), D for dasatinib treatment, C for control, other than directly compared the p-Src (D) with p-Akt (D) or p-FAK (D).
In Figure 6 only results for one of the Dasatinib-sensitive cell line (PLC-PRF/6) were shown, what happens with the others? Why authors did not make quantitation for p-Src/Src and pEGFR/EGFR? The image of pSrc appears to be the same as in Figure 3. It looks like pSrc416 signal increases up to 0.01 μM Dasatinib and then decreases. Is that correct? If so, how it can be explained?

Answer: the reason is that PLC/PRF/6 is the only dasatinib-sensitive cell line expressed both high levels of Src and EGFR. In order to know whether dasatinib will affect EGFR signaling pathway in this cell line, we tested the expression of p-EGFR1068. We added the quantitative analysis for p-Src/Src to Fig.6. We didn’t test the t-EGFR. We repeated this test three times and used the same image for both. Based on our analysis, the expression of p-Src slightly increased at 0.01μm, but it does not has significant difference (p>0.05) comparing with the control. The slight increase may be due to the weakness of signal, so that a small difference looks obvious.

In Figure 7, why different targets were measured in panel A versus panel B?
Answer: I am sorry. The p-EGFR should be replaced by p-FAK861 for PLC/PRF/6.

In Figures 8 and 9 there are not statistical analyses of the data.
Answers: we added the statistical analyses of the data in the manuscript.

In Figure 10, microscopy images of invasion assay were shown; quantification and statistical analyses are needed.
Answers: we added the quantification and statistical analyses in the manuscript.

In the third paragraph of the discussion authors reported: “Huh-7 expresses escalated levels of activated FAK576/577 and increases cell adhesion and migration after dasatinib treatment”. How authors explain that cell adhesion and migration increase at the same time?
Answer: cell adhesion and migration were tested separately and under different experimental environment. Briefly, we used cell adhesion assay to test the ability of cells adherent to ECM protein, the ability of cell migrating to chemo-attractant was studied using transwell assay. A previous study reported that prostaglandin E₂ (PGE2) promoted cell adhesion, migration and
invasion by mediating FAK/paxillin/Erk2 signal pathway in the same cell line [29]. Differently, expression the SrcDN mutation was able to alter the FAK/Src complex control of cell adhesion, spreading and migration in breast cancer cell line, which decreased the rate of migration and spreading, but increased size of peripherally localized adhesions [30]. The mechanism of dasatinib induced increases of cell adhesion, migration in Huh-7 cells requires further investigation.

Minor comments:
There are some typographic errors.
We corrected the typographic errors.

Conclusion:
Although this scientific document represents an interesting approach to define sensitivity of HCC cell lines to Dasatinib, the results need to be strengthened to establish useful conclusions for future therapeutic applications.

Level of interest: An article of importance in its field
Quality of written English: Needs some language corrections before being published
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests:
I declare that I have no competing interests
Reviewer's report

Title: Molecular Mechanisms of Action and Potential Biomarkers of Growth Inhibition of Dasatinib (BMS-354825) On Hepatocellular Carcinoma Cells

Version: 1 Date: 7 February 2013
Reviewer: Oliver Renner

Reviewer's report:

Report of Oliver Renner

In their work entitled "Molecular Mechanisms of Action and Potential Biomarkers of Growth Inhibition of Dasatinib (BMS-354825) On Hepatocellular Carcinoma Cells.", the authors analyze the mode of action on 9 different HCC cell lines. They compare the effects measured by different assays with an analysis of major signalling pathways. After their study of the effects of different therapeutic compounds and their combinations (Chang et al., Anticancer Drugs, 2013, 24(3):251-9), this study tries to elucidate the mechanism if one of the used compounds, dasatinib.

The study appears to be well conducted; the data are reported according to the scientific standards. Especially in the light of the need for an improved therapy against HCC, the data presented here are of value for this research field.

Minor Essential Revisions:
- Figure 1: Although it is mentioned in the result section, I would recommend indicating in the figure or the legend the unit of the y-axis (µg).
  Answer: we added the unit of the y-axis (µM) in the Fig.1.

- Figure 2 and 4: Besides the labelling of the y-axis, it is not clear how the values of the x-axis were calculated.
  Answer: we revised the labels of x-axis in both of the figures and added the explanation to the related legends.

Level of interest: An article of importance in its field

Quality of written English: Acceptable

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

Declaration of competing interests:
I declare that I have no competing interests.