Author's response to reviews

Title: OSU-03012 sensitizes breast cancers to lapatinib-induced cell killing: a role for Nck1 but not Nck2

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Version: 2 Date: 13 March 2013

Author's response to reviews: see over
03/12/2013

Manuscript Revision, BMC Cancer

Dear Editorial Board,

We hereby submit for publication our revised manuscript entitled “OSU-03012 sensitizes breast cancers to lapatinib-induced cell killing: a role for Nck1 but not Nck2” authored by Mr. Winston Norvell West, Ms. Aileen Garcia-Vargas, Dr. Charles Edward Chalfant and Dr. Margaret Amy Park to BMC Cancer. This manuscript is a revision of manuscript number: 5274919458759764. This manuscript builds upon recent findings by Dr. Paul Dent and colleagues, and describes our recent findings that OSU-0312 and lapatinib synergize to induce an ER stress-mediated cell death via PERK-mediated signaling in both an ER positive and an ER negative breast cancer cell line. Additionally, we present the novel finding that eIF2-α phosphorylation is a central event in the cell death induced by this combination. Finally, we build on work done by others and show that eIF2-α phosphorylation is controlled in part by the adaptor protein Nck1 as a part of the Nck/eIF2/PP1 complex, but not its isoform Nck2. We have answered all the reviewers’ excellent comments (see below) and the manuscript is now much improved.

Sincerely,

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Version: 2 Date: 13 March 2013

Author’s Response to Reviewers: (see over)
Reviewer's report

Title: OSU-03012 sensitizes breast cancers to lapatinib-induced cell killing: a role for Nck1 but not Nck2

Version: 1 Date: 6 February 2013
Reviewer: Chang-Fang Chiu

Reviewer's report:
In this submitted manuscript, estrogen receptor (ER) positive and ER negative breast cancer cells were genetically manipulated to up- or down-regulate either eIF2-alpha or its phospho-mutant, Nck1, Nck2, then treated with OSU-03012, lapatinib or the combination and assayed for cytotoxicity/cytostaticity using clonogenic assays. Based on the data presented, it is concluded that OSU-03012 and lapatinib act synergistically to induce cell death via the down-regulation of Nck1/PP1 and the subsequent dissociation of this complex from eIF2-a, which likely leads to a PP1-mediated enhancement of eIF2-a phosphorylation at serine51, a central event in the induction of cell death by OSU-03012/lapatinib.

Major Compulsory Revision:

1. The author concludes that eIF2-a phosphorylation is a central event in the synergistic cytotoxicity/cytostaticity induced by the combination therapy of OSU-03012 and lapatinib. However, in Fig.3C, the p-eIF2-a levels in drug treated vector or S51A over-expression cells are not significantly different, but the decrease of colony formation induced by OSU/lapatinib treatment is significantly attenuated in S51A over-expression cells (Fig. 4D). Whether it involves ER transmembrane proteins other than PERK- eIF2-a, i.e. IRE1 and ATF6, needs further clarification. Experiments using siPERK, siIRE1-a and siATF6 may be one of the possible approaches.

Reply 1: We would like to thank the reviewer for their help in critiquing our work and making it ready for publication. With respect to Figure 3C, it is possible that ectopic expression of a phospho-mutant of eIF2-α induces an increase in the ER stress response (ie. phosphorylation of endogenous eIF2-α), which is why we observe an increase in basal phosphorylation of this protein when the phospho-mutant is overexpressed.

We have performed siRNA-mediated knockdown of PERK, IRE1a and ATF6 followed by survival assays. As shown in Figure 2, reduced PERK expression and, to a much lesser extent, reduced IRE-1a expression enhanced survival in MDA-MB-231 breast cancer cells when treated with OSU-03012/lapatinib in combination. Decreased ATF6 expression slightly decreased basal survival, but did not increase cell survival when treated with the combination. These data suggest that PERK (and to a much lesser extent IRE-1a) but not ATF6 are important mediators of OSU/lapatinib-induced cytotoxicity/cytostaticity. We therefore chose to focus on PERK-specific effects (ie. eIF2a phosphorylation) for the remainder of the manuscript.

2. It has been widely accepted (in detail: Biochem Pharmacol, 2013, 85:653; Can Res 2012, 72:1321; Ann N.Y. Acad Sci 1271 (2012) 20-32) that, while proliferating in sub-optimal microenvironment (hypoxia, hypoglycemia, etc), chronic ER stress and permanently increased levels of GRP78 expression provide a survival advantage to tumor cells. This phenotype sets tumor cells apart from most normal cells. It shouldn’t mean that cancers cells are more resistant
to ER stress. In contrast, the already engaged ER stress response system in cancer cells provides promising druggable targets. One important approach is development of pharmacological ER stress aggravators (ERSAs). OSU-03012 is one of the ERSAs that may pharmacologically aggravate the already engaged ER stress in cancer cell, which can be used to “overload” this engaged system and thus push the cell from pro-survival state to its pro-apoptotic module. I would suggest integrate this information into the discussion, such as p.11 the last paragraph.

**Reply 2:** The reviewer raises an excellent point. We have changed this paragraph to integrate this information into the manuscript. The new paragraph reads: “ER stress aggravators (ERSAs) are a relatively recent addition to our arsenal of therapeutic agents for the treatment of cancer. There are multiple reports that ER stress factors are upregulated in many types of cancer suggesting that these pathways are ones to which cancers may become addicted and therefore represent good targets for treatment. OSU-03012 represents one ERSA which may be used to enhance ER stress pathways in cancer cells. This may activate a response in which the cancer cell shifts from using ER stress signaling as a pro-survival mechanism to a pro-apoptotic one. Our findings demonstrate that eIF2-α phosphorylation is a major event in the cell death pathways induced during treatment with OSU-03012/lapatinib. Furthermore, the question whether other molecules that induce ER stress will also enhance lapatinib-induced cell killing should be pursued in light of these studies.” (see p. 13, highlighted)

3. In the Results and Discussion (p.12, 2nd paragraph), the author mentioned “However, autophage ...... whereas in breast cancer cells the role of autophage seems to be more protective.” However, the cited references describe quite different study models, which is not necessarily the mechanism of sensitization of breast cancer cells to lapatinib-induced cell killing by OSU-03012. Looking into the expression of autophagy related proteins, such as conversion of LC3-I to LC3-II and altered expression of p62, will be more direct evidences to support the conclusion.

**Reply 3:** We agree and thank you for your valuable critique. To address this criticism, we performed a time-course experiment using OSU/lapatinib in combination to determine the effect of OSU/lapatinib on autophagy induction (see the following figure).

![LC3 and Actin Western Blot](image)

These data suggest that autophagy is indeed induced as an early response to OSU-03012/lapatinib treatment, but the induction of LC3B (lower band) levels off/drops at the 24 h time point. These data do provide some evidence that OSU-03012/lapatinib treatment may induce a protective form of autophagy early, then decrease the autophagic response at later time points (e.g. after ER stress has been induced and after the cell death process is initiated). However, the effect of autophagy inhibition on OSU-03012/lapatinib mediated cell death/survival has yet to be determined for breast cancer cells and currently lies outside of the scope of these studies. We have therefore removed the statements referencing autophagy in the results/discussion. Instead, we plan to make these studies part of a follow-up manuscript.
Discretionary Revision:
In p.13, paragraph 2, line 2: The decrease in both clonogenic capacity and eIF2-a phosphorylation in MDA-MB-231 cells after OSU-03012 and lapatinib combination treatment was “rescued” by the ectopic expression of Nck1 (see Figure 4C), but not by ectopically expressing Nck2. To my understanding, it should be: The increase in eIF2-a phosphorylation and decrease in clonogenic capacity in MDA-MB-231 cells after OSU-03012 and lapatinib combination treatment was “rescued” by the ectopic expression of Nck1 (see Figure 4C), but not by ectopically expressing Nck2.

Reply: Thank you for identifying this typographical error, you are absolutely correct. We have changed the manuscript appropriately.

Level of interest: An article whose findings are important to those with closely related research interests

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: OSU-03012 sensitizes breast cancers to lapatinib-induced cell killing: a role for Nck1 but not Nck2

Version: 1 Date: 27 January 2013
Reviewer: Ching-Shih Chen

Reviewer's report:
In this study, the authors demonstrated the synergistic interaction between lapatinib and OSU-03012 in suppressing the proliferation of MDA-MB-231 and BT474 cells, and attributed this synergistic effect to the ability of this therapeutic combination to enhance ER stress by downregulating Nck1 expression, thereby facilitating eIF2-alpha phosphorylation, in part, by promoting its dissociation from complexes with Nck1 and PP1. Overall, this is a well-prepared manuscript with solid mechanistic data to support the conclusion. However, a few minor issues warrant attention.

1. Fig. 1A-D depict the effects of OSU-03012, lapatinib, and the drug combination on cell death and colony formation in both cell lines at a single concentration, i.e., 2 microM. The authors should present these results at other concentrations, as they have reported in the isobologram analysis in Table 1, to show the general effect of this drug combination.

Reply 1: We would like to thank the reviewer for their extremely helpful critique of our manuscript. We have performed additional survival studies using 1 uM, 2 uM and 3 uM OSU and lapatinib in combination (see Figure 1A (new)) in BT474 and MDA-MB-231 cell lines. These results highlight the differences between the BT474 and MDA-MB-231 cell lines mentioned by Dr. Chen in comment 2. Specifically, BT474 cells demonstrate sensitivity to OSU-02012 and lapatinib as single agents at a lower concentration (2 uM) than MDA-MB-231 cells (3 uM) which may explain the higher CI values demonstrated by this cell line. Additionally, MDA-MB-231 cells show an enhanced sensitivity to the combination treatment at all time points. We have also integrated these findings into the results/discussion section.

2. In the clonogenic assay, the synergistic effect of the drug combination is particularly striking in MDA-MB-231 cells relative to BT474 cells (Fig. 1C and D), which warrants elaboration.

Reply 2: We agree with this assessment. Hence, we have added language in the discussion section (please see p 10 and 11 highlighted). The section now reads: “Interestingly, OSU-03012 and lapatinib combination therapy was more effective against MDA-MB-231 cells than BT474 cells. Therefore, our findings argue that targeting ER stress proteins may increase the efficacy of traditional therapies for metastatic breast cancers [11-13] since the BT474 cell line is much less invasive than the triple negative MDA-MB-231 cell line [25, 26]. Specifically, we found a greater decrease in cell viability and a lower CI value for synergy between OSU-03012 and lapatinib in the triple negative cell line MDA-MB-231 (harvested from the metastatic pleural ascites) than in ErbB2-amplified BT474 cell line (harvested from a primary site). These findings provide support for the hypothesis that OSU-03012 and lapatinib in combination may be more effective against metastatic breast cancers than non-metastatic breast cancers. This study is therefore in line with the recent studies by Sanz-Pamplona et. al., which showed that upregulation of GRP94, an ER stress protein, is an effective marker for brain metastases of
breast cancers [27], and others [28], which showed that other ER stress markers are upregulated during suspension conditions.

Our data demonstrating that MDA-MB-231 cells are more sensitive to the combination of OSU-03012/lapatinib are also in general agreement with the findings in Fig. 7B, that PP1 associates significantly less with eIF2-α after OSU/lapatinib treatment in MDA-MB-231 cells than in the BT474 cell line. While PTEN, Raf, and Akt levels and mutation status appear to be similar in both MDA-MB-231 and BT474 cells [29-31], BT474 cells express a constitutively active form of PI3KCA (K111N), in addition to overexpressing ErbB2 [32]. It may be that upregulation of the PI3K/Akt pathway represents a potential pathway of resistance for cell lines treated with OSU-03012/lapatinib in combination. Therefore, inhibitors of the PI3K pathway should be combined with OSU-03012/lapatinib in future studies.”

3. The Western blot analysis in Fig. 4A, lower panel (i.e., effect on Nck2 expression) should be repeated to make sure that the loading controls are even among different samples.

Reply 3: This is an excellent point, and we have repeated the western blot to show our loading controls are even (see new Figure 5A).

Level of interest: An article of outstanding merit and interest 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: None to declare