Response to Reviewer-2's comments:

We would like to thank the reviewer for their helpful comments and suggestions. Also, we would like to thank the reviewer for acknowledging our lab's research work.

1. Combining synergic molecules with different MOAs within a nanoparticle is a major advance as a general concept and the authors' specific combination data should be more widely known. Title is needlessly obscure. Both drugs must be mentioned in title specifically.

   Response: We would like to thank you for this suggestion. Both the drug names, gefitinib and GSK461364A are now mentioned and the title has been changed accordingly.

2. A paragraph or two in the Introduction must be devoted to GSK461364A, giving basic pharmacology parameters, MOA or putative MOA, and an overview of polo-like kinases' role in normal cell cycling and in GB. The entire Introduction requires a rewrite.

   Response: The introduction has been modified with separate paragraphs explaining the role of EGFR in normal and GBM cells and the mechanism of action of gefitinib has been explained. Similarly, we have included a paragraph that focuses on polo like kinases and mechanism of GSK461364A.

3. Fig.1 must be corrected. Having an inhibition "T" arrow from an orange circle to the cell cycle circle is unacceptable. The details must be shown. Likewise unacceptable is the "dephosphorylation" arrow. By what means dephosphorylation ? Does gefitinib really dephosphorylate BAD or rather does gefitinib inhibit BAD phosphorylation ? The difference is not trivial. In other word much detail must be added to Fig. 1 for it to become meaningful.

   Response:

   - We have modified Figure 1 and tried our best to add some details for the mechanism of actions of gefitinib and GSK.
   - Gefitinib is a tyrsosine kinase inhibitor that inhibits the phosphorylation of tyrosines present on either sides of the transmembrane receptor. In addition to this, literature points out that gefitinib causes cell death by desphosphorylating BAD, specifically in serine 112 residue. As we know, desphosphorylated BAD binds to Bcl2 protein and triggers apoptosis. We have changed the diagram to gefitinib causing BAD

desphosphorylation, specifically in Serine-112 and triggering apoptosis, instead of gefitinib dephosphorylating pBAD.

The diagram was based on the following few references. Please find the screenshots of the articles and their references below.

J Neurooncol (2011) 105:507–522
DOI 10.1007/s11060-011-0632-3

LABORATORY INVESTIGATION - HUMAN/ANIMAL TISSUE

Gefitinib induces apoptosis in human glioma cells by targeting Bad phosphorylation

Cheng-Yi Chang · Chiung-Chyi Shen · Hong-Lin Su · Chun-Jung Chen

Received: 21 November 2010 / Accepted: 17 June 2011 / Published online: 9 July 2011
© Springer Science+Business Media, LLC. 2011

Abstract Gefitinib, a selective epidermal growth factor receptor tyrosine kinase inhibitor, is under clinical testing and use in cancer patients, including glioma. However, the molecular mechanisms involved in gefitinib-mediated anticancer effects against glioma remain largely uncharacterized. Gefitinib inhibits cell growth and induces apoptosis in human glioma cells. Gefitinib also induces death of H4 cells with characteristics of the intrinsic apoptotic pathway, including Bax mitochondrial translocation, mitochondrial outer membrane permeabilization, cytochrome c cytosolic release, and caspase-9/caspase-3 activation. The importance of Bax in mediating gefitinib-induced apoptosis was confirmed by the attenuation of apoptosis by Bax siRNA and Bax channel blocker. Gefitinib caused Bad dephosphorylation, particularly in serine-112, and increased its binding preference to Bcl-2 and Bcl-xL. The dephosphorylation of Bad in gefitinib-treated cells was accompanied by reduced intracellular cyclic AMP content and protein kinase A (PKA) activity. Adenylyl cyclase activator forskolin attenuated, but PKA inhibitor H89 augmented, gefitinib-induced Bad dephosphorylation. Bax mitochondrial translocation, caspase-9/caspase-3 activation, and viability loss. Intriguingly, a nonselective protein phosphatase inhibitor okadaic acid alleviated gefitinib-induced alterations, except Bad dephosphorylation. In parallel with the higher basal PKA

Author Manuscript

inhibit apoptosis. Normal MAPK-dependent serine 112 phosphorylation is rendered superfluous. Indeed, we have shown that inhibiting MAPK has little or no effect in tumor cells in which the pathway is not activated by mutation (data not shown). In systems terminology, the BAD protein acts as an "Or" logic gate or switch; phosphorylation at either site in response to either pathway is sufficient for its inactivation.

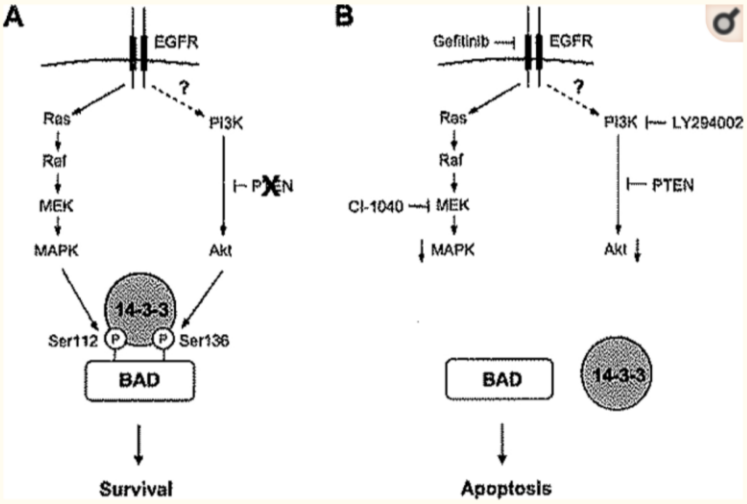


Figure 8

A model for role of BAD as an apoptotic switch in PTEN-deficient fumor cells

A: In PTEN-deficient tumor cells, activation of PI3K and Akt is EGFR independent. The BAD protein acts as a switch that integrates the antiapoptotic effects of the EGFR/MEK/MAPK and PI3K/Akt pathways.

B: Dephosphorylation of BAD abolishes BAD binding to 14-3-3 and is required for the synergistic induction of apoptosis in response to inhibition of both pathways.

It follows that inhibition of the activated Akt signaling pathway is not sufficient to activate apoptosis. When Akt is inhibited in response to PTEN induction, the cell relies on EGFR/MAPK signaling, the

Reference: She, Qing-Bai, et al. "The BAD protein integrates survival signaling by EGFR/MAPK and PI3K/Akt kinase pathways in PTEN-deficient tumor cells." *Cancer cell* 8.4 (2005): 287-297.

4. Line 41 on pg.1 must be corrected. "hostile effects" is unacceptable English use in this context

   Response: This word has been modified to toxic effects.

5. If the authors could organize their Introduction better, intelligibility would be served. Ideally a paragraph devoted to PLK, another paragraph to EGFR and gefitinib, and another to the very

important concept of simultaneously interfering with multiple non-parallel targets in the dysregulated cancer signaling pathways as more effective than sequential treatments. This latter requires its own paragraph and discussion with delineated example[s].

Response:

- The introduction has been modified with separate paragraphs for EGFR and gefitinib; PLK-1 and GSK461364A.
- More information has been added for the combination therapies that target signaling pathways with appropriate references in the introduction as well as in the discussion.

6. It would be preferable to show the graphic output of the Malvern Zetasizer 121 Nano ZS90.

Response: The graphical representation of the size and zeta potential recorded in the Zetasizer has been included in the supplementary information. Please refer to Supplementary Figure 3 in the updated manuscript.

7. Given the importance of nanoparticles' attributes to the authors' work, a separate paragraph explaining why nanoparticles might get better intracellular uptake than would ordinary soluble molecules would enhance this paper's value.

Response: Exclusive experiment on cellular uptake was demonstrated in our previous study (Figure 5). [Velpurisiva, Praveena, et al. "GSK461364A, a Polo-Like Kinase-1 Inhibitor Encapsulated in Polymeric Nanoparticles for the Treatment of Glioblastoma Multiforme (GBM)." *Bioengineering* 5.4 (2018): 83.].

In addition to this, we cited this article as a reference to the cellular uptake in the introduction section that describes the use of nanoparticles in drug delivery. We included some information in the discussion related to our study.

8. I would prefer to see "GSK461364A" used throughout the paper, not "GSK". GSK is the well-known abbreviation of a drug company.

Response: The term GSK has been replaced with GSK461364A across the paper.

9. The authors' lab work seems well done, appropriate to support their conclusions.

Response: Thank you for this encouraging feedback.