Author's response to reviews

Title: Synthetic Lethal Screen Identifies NF-kappaB as a Target for Combination Therapy with Topotecan for patients with Neuroblastoma

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Author's response to reviews: see over
Re: Synthetic Lethal Screen Identifies NF-kappaB as a Target for Combination Therapy with Topotecan for patients with Neuroblastoma (MS: 2118454452580874)

Dear Dr Danrolf de Jesus,

Thank you for your correspondence of November 17, 2011 communicating comments from two expert reviewers. We are pleased with the positive reviews of our manuscript and helpful comments. We have found that the reviews have allowed us to improve the manuscript considerably. We have carefully considered these critiques and made significant changes to our manuscript. We hope that the revised manuscript is satisfactory to the reviewers and now suitable for publication in BMC Cancer.

Specific responses to the points raised by the reviewers are as follows: Our responses are in the courier bold font for easier visualization.

Reviewer 1:

We thank the reviewer for the positive comments.

Reviewer 2:

Given this paper is based on RNAi screen results, evidence for siRNA inhibition of target gene expression (by immunoblot) needs to be shown at least for the top nine hits that correlates with timing of siRNA-enhanced topotecan growth inhibition.

We thank the reviewer for pointing out the importance of showing evidence for inhibition of target gene expression by our siRNA hits. We repeated the siRNA experiments and performed quantitative RT-PCR on the 5 key genes that target NF-κB pathway (BIRC4, NFKB1, RIPK1, TNFRSF8 and TNFRSF25) using Taqman assays. The results showed that the siRNAs can effectively knockdown
target gene expression by more than 50% comparing to the controls. The result is incorporated in Figure 1A.

The methods mention that siRNA was added for 24 hrs followed by 72 hrs incubation with topotecan, but exact time that measurement for enhanced effects was assessed is not written nor depicted in a figure or legend; Therefore it is not clear whether all siRNA’s were assessed for topotecan synergy at same time point or when maximal target gene knockdown occurred. This is critical to understanding functional relevance of enhancer gene knockdown in relation to potentiation of topotecan-induced cell growth arrest.

The reviewer makes an excellent point and we would like to clarify the exact time of measurement by cell titer glo proliferation assay is 96 hours post siRNA transfection. Because the entire siRNA screening involves 418 genes, with 4 siRNA sequences being used, we have used the same time point to assess for synergy for all of the genes. We have chosen to knock down the genes for 24 hrs prior to drug addition to allow maximal target gene knockdown in the presence of the drug. We have modified the corresponding section in the Methods to make this clear.

For Figure 2B, the Actin band at t=0 is much less intense than the other time points and gets darker with treatment time, suggesting unequal protein loading in lanes. Does the one actin blot represent protein loading for all blots tested, or just one? This would affect conclusions made about phospho-p65/relA that may be decreased in lane one due to decreased protein loading. Also, a faint line for phosphorylated IKB-alpha is evident at 6 and 24 hours post topotecan treatment. Phosphorylation of IKB-alpha precedes it’s degradation, yet in this experiment, phospho IKB-alpha is present at the same time and after total IKB protein is decreased to almost undetectable, begging the question of NFKB activation causing total IKB protein degradation through phosphorylation of IKB-alpha. Repeating the experiment with a stronger phospho IKB-alpha Ab and showing equal protein loading in all lanes for all blots used would support the conclusions that NFKB is activated.

We agree with the reviewer’s comments and have repeated this experiment. The new Western blot (now as Figure 1C) clearly showed an even loading of the protein for each lane. In addition, this blot showed degradation of total IκB-α protein, with phosphorylation of IκB-α before its degradation and an increase of phospho p65/RelA with topotecan treatment over time. Therefore this western blot clearly demonstrated that NF-κB pathway was activated by topotecan. We hope the quality of this western will be satisfactory to the reviewer now.

For Figure 1C, evidence for target gene knockdown at protein level should be shown at the time where significant differences in siRNA treated cells are noted on the graph.

We thank the reviewer again for emphasizing the importance of demonstrating knockdown of the target genes by siRNAs. We repeated the siRNA knockdown experiment of NFKB1 and have
included an additional western blot (now Figure 1E) demonstrating an effective knockdown of NFKB1 protein at 72 hrs for the siRNA used in Figure 1C (now Figure 1D). We have also modified the manuscript accordingly.

Although bortezomib enhances topotecan induced growth inhibition and apoptosis, the statement that this is specifically through NFKB inhibition by bortezomib needs to be shown experimentally in NB cell lines used in these studies before such conclusive statements can be made (last sentence; paragraph 1;page 13 and last sentence; paragraph 1;page 14). As mentioned in the discussion, bortezomib is a proteasome inhibitor and can inhibit cancer growth by affecting multiple pathways. In other NB models, bortezomib potency does not inhibit NFKB at all but rather decreases pro-apoptotic proteins like Bcl-2 or Mcl-1 or increases anti-apoptotic Bcl-2 family members (Hagenbuchner, J., et. al., Jnl. Bio.Chem., 2010, Luo, P., et. al., Pharmazie 2010, and Naumann, I., et. al., Clin Can Res 2011).

The reviewer makes an excellent point and we have investigated this further with a series of western blotting experiments. We hypothesized that bortezomib enhances topotecan induced growth inhibition and apoptosis through NF-κB inhibition. The immunoblots showed that bortezomib prevented degradation of total IκB-α protein (although phosphorylated) induced by topotecan, suggesting the NF-κB pathway is inhibited. We have incorporated this result in Figure 2E. We also agree with the reviewer that the synergistic effect of bortezomib to topotecan may involve other pathways rather than NF-κB pathway alone as suggested by the reference the reviewer included. We adjusted our discussion to reflect this point.

Table 1 should rank genes based on their “fold” change in cell growth (and p value if applicable) to denote the most significant enhancer genes.

We have presented the data in table 1 according to the names of the genes because in each screen three different concentrations of topotecan are used. We consider it a ‘hit’ when at least one dose shows synergy. We did not rank the actual ‘fold’ change because our criteria for a hit is for any gene, at any one dose of topotecan, that causes ≤0.8 fold cell growth compared to its own siRNA control. Therefore, ranking by fold changes was not that straightforward due to different screening conditions. It would have made the table very complicated or even confusing to readers if the actual fold changes were included.

The Methods state that “Combination Index was calculated using CompuSyn software” - A brief explanation of the CI calculation and how that determines/relates to synergy with reference to Ting-Chao Chou’s papers should be provided in the Methods section. Please denote if single agent IC50 determinations were also made using CompuSyn, or describe in Methods how IC50’s were determined otherwise. Please explain the rationale for why only two very close concentrations of one of the two drugs are used to test for synergy. For example, the NSC676914 synergy concentrations chosen: 1uM and 1.5uM in SH-SY5Y and 1uM and 2.5
uM in NB-1691, while lower than the calculated IC50, they hardly span a wide range of concentrations. A statistically clearer explanation of synergy methods and results (beyond the fact that the points were away from the diagonal additive line) would help support conclusions for Figure 2A.

We have included a brief explanation of the CI calculations in the manuscript in the methods section, with reference to the original paper. The doses used for the synergy experiments are specifically chosen to be sub-optimal doses lower than the IC50 of each individual agent.

NSC676914 inhibits NFkB but does not inhibit tumor cell proliferation in other cancer cell lines (breast, etc.). The results mention single agent IC50 results for NSC676914. It would support NFkB as a true target in NB to show the IC50 results in a graph figure, supporting it has single agent activity at low uM concentrations in NB not treated with topotecan.

We thank the reviewer for the comment. Although NSC676914 does not inhibit cell proliferation in other cancer cell lines, the inhibitor does have an effect on cell proliferation in neuroblastoma. Before we test for the drug combination, we first measure the IC50 of both drugs in 3 neuroblastoma cell lines (SK-N-AS, NB-1691 and SH-SY5Y) at 24 hrs, and confirmed that each individual drug causes inhibition of cell proliferation. In particular, the IC50s of NSC676914 are 7 µM, 4 µM and 2 µM for SK-N-AS, NB-1691 and SH-SY5Y respectively (graphs attached). Sub-IC50 doses of the drugs are then used in combination and combination index is measured according to Chou’s theorem (Reference 18 in the manuscript).

Figure 3 should include pictures from the control as well as bortezomib alone treatment arms and provide the time point in therapy that the picture was taken.
The pictures shown in Figure 3 were taken at two weeks post treatment. Mice from the control and bortezomib treated arms became morbid by the end of the treatment. Therefore, at the time point when the pictures were taken, mice from the control and bortezomib treated groups had all been euthanized and were not shown in the figure.

Discussion. References should be provided for comments stating Topotecan and Bortezomib dose limiting toxicities. Of note, in the Phase I Children’s Oncology Group study of Bortezomib in children with refractory solid tumors (ADVL0015), the dose limiting toxicity with Bortezomib was thrombocytopenia along with other hematologic grade 2-3 toxicities that were not dose limiting. Therefore, there are potential overlapping hematologic toxicities with bortezomib and topotecan that, while not precluding the combination, would need to be monitored (Blaney, SM, JCO 2004).

We thank the reviewer for pointing out the potential overlapping toxicities of the two drugs. Although both drugs may cause neutropenia and thrombocytopenia, both are reversible and noncumulative and should be well tolerated in patient. We have taken the reviewer’s point and rewritten the paragraph in the discussion section.

Reference 16 does not refer to NSC676914 at all but to the millennium compound, MLN120B, which it seems has been compared to NSC676914 in other literature, but is not the same compound.

We apologize for the error and have amended the reference accordingly.

There is no mention in Methods of the reagent NSC676914 or where it came from.

We apologize for the omission of the source of the reagent NSC676914 and have included it in the methods section.

A more detailed description of the Metacore website/software assessment for pathway analysis of RNAi data should be provided or at least referenced.

We agree to the reviewer and have incorporated a more detailed description of the Metacore software in the methods section.

There are more up to date references using topotecan in upfront NB therapy (JH Park, et al, JCO 2011).

We thank the reviewer for the suggestion and have incorporated the reference in the manuscript accordingly.
We appreciate the second reviewer’s thorough and helpful comments which helped us to improve our manuscript. We hope our revised manuscript is satisfactory to both reviewers and can be now acceptable for publication in BMC Cancer.

The authors have declared no conflict of interest.

Sincerely,

Javed Khan