Author's response to reviews

Title: Novel anti-HER2 monoclonal antibodies: synergy and antagonism with tumor necrosis factor-alpha

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Author's response to reviews: see over
Marinette Lacson
On behalf of Dr. Valerie Speirs,

Dear Editor,

Enclosed please find our manuscript by Ceran et al. entitled "Novel anti-HER2 monoclonal antibodies: synergy and antagonism with tumor necrosis factor-\(\alpha\)" that has been revised according to your requests and referee comments. We highlighted all changes made when revising the manuscript and correctly formatted it.

For research carried out on animals, a sentence has been inserted in Methods section to include relevant information with regard to animal ethics.

With regard to comments by Reviewer Thomas Hughes, we thank him for his appreciation of our manuscript. He had a request for some minor but essential revisions to improve manuscript clarity:

1) Introduction, 2nd para. The authors comment that Trastuzumab is “quite efficient” as a treatment – could the authors be more specific about what they mean by this? Similarly, the authors say that acquired resistance is “frequent” – could the authors comment how frequent?

We addressed these two critics in "Introduction" section by providing more specific information.

2) Methods. I was surprised that the authors fixed cells before the flow-cytometry assays. Why was this, and would the assays work on live (unfixed) cells?

We did not fix cells intentionally. We just followed a working protocol available in the lab for flow cytometry. Although we did not test directly, we assume that our antibodies would also work with unfixed cells since they display biological activity when added onto adherent cells in cell culture plates, as described in the manuscript in many experiments.

I was also surprised by the use of only a very short incubation allowing cells to attach before additional of test compounds (antibodies/TNF) in cell growth assays. Are the authors confident that cells had actually attached in this period, or is there a possibility that some of the influences they detect relate to inhibition of attachment, rather than influences on growth? Do they get the same results if longer (over-night?) periods are allowed for attachment?

For these studies, we followed the protocol described in the initial testing of mouse anti-HER2 antibody 4D5 (Lewis et al., 1993) in order to compare our antibodies with the humanized form of this antibody which became Trastuzumab. We included this reference to methods section. We did not carry out experiments after overnight culture prior to antibody treatment. However, we believe that the effects with observed is due on growth inhibition, rather than attachment inhibition. We compared the effects of nonspecific IgG, Trastuzumab and our five anti-Her2 antibodies on cell growth at 3 and 6 days respectively. At day 3, 5 of these antibodies did not inhibit cell growth. Trastuzumab (at doses between 0.63 \(\mu\)g/ml and 10 \(\mu\)g/ml) and BH6 (at 5 \(\mu\)g/ml and 10 \(\mu\)g/ml) caused a partial inhibition which become more pronounced at day 6. If the effect were related to the inhibition of attachment, we would have seen an immediate inhibition detectable at day 3. Based on these observations, we believe it is unlikely that our observed effects relate to inhibition of attachment.

3) The legends for Fig 6 and Additional File 3 suggest that things are described in other
files/figures. Do the authors mean this – the files/figures contain more data rather than anything in the way of description.

This refers to experimental conditions. We did not want to repeat the protocol description in order to save space. We are sorry that this created some confusion. We revised the legends of these figures to include the experimental conditions.

With regard to comments by Reviewer Cagatay Günes, we also thank him for his appreciation of the work and minor essential requests to improve the manuscript quality. We thank him for his appreciation of the work and his minor essential requests to improve the manuscript quality.

1) Minor essential Revisions: The authors demonstrate convincingly the generation and the specificity of the antibodies. Two of these antibodies (BH1 and BH6) also show some inhibitory effects on cell proliferation if used alone. The studies in combination with TNF-α are rather inconclusive. In the light of the Trastuzumab/Pertuzumab synergistic effects, it would be interesting to test the combination of BH1 and BH6 with these antibodies.

We already tested combinations of Trastuzumab with BH1, BH6, BH2 and BH5 at 2.5 ug/ml and 5 ug/ml doses on SK-BR-3 cells. There was a small increase in cytotoxicity in combination experiments, but we prefer not to include these preliminary data in the manuscript. Firstly, because we have already an unusually high number of figures (8 figures and 5 supplementary figures) on the paper. We are worried that the focus of the article will be lost with additional data. Secondly, we feel we should perform a complete synergy analysis between our antibodies, Trastuzumab and Pertuzumab with under a separate project.

2) Discretionary Revisions:

a) p.5., lines 13-14. The sentence: “Thus, almost...treatment” is not necessary and can be omitted.

We deleted this sentence.

b) Legend to Fig. 5. In Figure 5A, it is indicated that antibody concentration is in the range of 0 to 10μg/ml whereas in the legend to Figure 5A it is given in the range of 0 to 5μg/ml. This inaccuracy should be corrected.

Sorry for this mistake. It was corrected in the figure legend.

We hope that we have answered all the issues adequately and that the manuscript is now acceptable for publication.

Sincerely,

Mehmet Ozturk, PhD