Author’s response to reviews

Title: Results of multicenter double-blind placebo-controlled phase II clinical trial of Panagen preparation to evaluate its leukostimulatory activity and formation of the adaptive immune response in patients with stage II-IV breast cancer

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Author’s response to reviews: see over
Dear Editors,

We were pleased to know our work was evaluated positively and potentially acceptable for publication in BMC Cancer. We had it corrected and so provide both the revised version of the text and figures (changes highlighted yellow), as well as point-by-point response to the comments raised by the reviewers. As you will notice, we agree with most if not all the points raised, and would like to thank the reviewers for identifying critical areas that needed clarification and modification. Please note we have introduced minor changes into the title of the paper, following the advice of the native English speaker.

Sincerely yours,

Dr. Sergey S. Bogachev,

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**Reviewer:** norikazu masuda
This paper seems to contain the new aspects of immune related strategy to improve the outcome of patients with breast cancer. Some readers might be able to get some investigational information from this article which has been filled up enough details of experimental results. There is no additional comments to success in acceptance.

**Reviewer:** laura bracci
The manuscript describes the results of a multicenter, double-blind placebo controlled phase II clinical study in which breast cancer patients were treated with chemotherapy and a dsDNA-based preparation (Panagen). The results are interesting, but the clinical relevance of the data provided is not clearly addressed. The manuscript is clearly written, although lengthy. There are too many additional figures and text which make the reading cumbersome and hide the main message of the study. Some figures and figure legends are not exhaustive enough.

**Major Revisions**
1. Cytotoxicity of patients’ T cells has been assayed against the adenocarcinoma cell line MCF-7 and cytokine production by patients’ PBMCs has been measured either ex-vivo or following mitogenic stimulation. These are immunological readouts of immune competence but do not support the conclusion of the induction of anticancer immune responses by Panagen treatment (as stated in page 3, line 19-20). In my opinion, the induction of tumor specific immune-responses by Panagen treatment needs to be addressed.

2. The authors state: “This medication can also boost individual anticancer adaptive immunity resulting from the activation of dendritic cells by the circulating tumor cell debris.” (Page 3 lines 21-23). This sentence in mostly speculative as it is not supported by data within the study submitted for publication. It probably refers to previous data collected in mouse models and in vitro human settings but has not been demonstrated in the current study. As a matter of fact, the authors write that from the analysis of dendritic cells they failed to uncover pronounced different trends between the placebo and the Panagen groups (page 20, lines 18-21).

We agree with your comment and appropriately modified the conclusions in the Abstract. To comprehensively analyze the data from MTT assay using MCF-7 cells as a target, HLA typing of both MCF-7 and patient material would be necessary. We could not perform such typing for technical reasons. Therefore, our MTT tests only served to evaluate the anticancer competence of innate immunity cells, rather than specific cytotoxic T-cell anticancer activity. Macrophages and NK cells represent the cells of innate anticancer immunity. They were isolated from patients’ blood using ficoll-urografin gradient centrifugation, and were present within total mononuclear cell population. No attempt to evaluate specific cytotoxic T-cell activity was made again due to technical limitations. Nonetheless, CD8+ perforin+ T cells were consistently present in the patient blood samples, suggestive of the development of personalized adaptive immune response.
Maintenance of the original activity level of innate immunity cells, development of adaptive cytotoxic immune response and significantly reduced frequency of cancer relapse 3 years following the therapy – these observations clearly point to the anticancer activity of Panagen. Thus, we changed the wording across the text to "adaptive immune response driven by CD8+ perforin+ T-cells". Anticancer activity of Panagen is thus discussed as showing protection of innate anticancer immunity, induction of adaptive immunity (in all likelihood, anticancer immune response) and significant decrease in disease relapse during the follow-up study.

3. I do not see the clinical relevance of the cytokine profiles observed in Panagen vs placebo groups. It should be better supported or better clarified.

We moved the images illustrating cytokine data from supplementary into the main body of the paper, worked to describe the results more clearly and merged two parts of the text together. Your work (Schiavoni et al, Cancer Res 2011) clearly demonstrates that cyclophosphamide stimulates PBMCs to secrete type I IFN, and that anticancer activity of cyclophosphamide is further enhanced when it is combined with IFNα.

Our cytokine profiling experiments failed to show significant increase in IFNα secretion. We nevertheless looked back and revised our clinical experiments on healthy participants. We realized that Panagen does boost IFNα secretion, but the choice of sampling timepoint was wrong. IFNα secretion peaked on day 7 after stimulation of PBMCs, whereas in the present clinical study, IFNα secretion was measured 24 hrs post stimulus, as was recommended in the manual to cytokine profiling kit (which actually was optimal for most of the cytokines, data not shown). This observation nicely complements the results from clinical trial, therefore the therapy may be argued to stimulate development of anticancer adaptive immune response.
Figure. Time-course analysis of IFNα production by healthy donor PBMCs, when stimulated with dsDNA (solid blue line, circles), Poly I:Poly C (red dashed line, squares) or mitogens (green dashed line, diamonds). Values obtained by subtraction of spontaneous from induced IFNα levels are shown.

4. The clinical data reported in additional file 8 (long-term follow-up analysis) should be enriched with the tumor stage of each patient at the time of enrolment to evaluate the clinical benefit of the treatment with respect to cancer severity.

We appreciate this very interesting and pertinent suggestion. The data reporting tumor stage are now incorporated in the Table and in the Figure, and are appropriately discussed in the text.

5. The authors disregard that similarly to Doxorubicin, in addition to depleting suppressive T lymphocyte subpopulations Cyclophosphamide has been shown to induce immunogenic cell death (Schiavoni et al, Cancer Res 2011). Although this information will not change the overall message of the study, it needs to be included in the discussion and properly cited as it would reinforce the data provided and reflect a more comprehensive review of the literature in the field (page 10, lines 23-25, page 11, lines 1-14).

It is very unfortunate that your great work was published when we were half the way through our clinical trial. Had we seen it before the protocol was compiled, better choice of many of the timepoints would have been made and matching with your data would be easier. Yet, we took your work into consideration when analyzing our results. If we move to phase III studies, the results from your work will serve as a blueprint for trial design so as to analyze the development of adaptive immune response in patients enrolled to the study. Appropriate discussion of your data is now part of the text.

6. The authors often refer to the results of a phase I clinical study but it’s not clear whether the data are published or unpublished (e.g., pag.7, line 23; page 9, lines 19-22; page 17, line 9). Please specify and if data are published please provide reference.

Unfortunately, no paper was published to summarize our phase I clinical trial data. Yet, our official report was approved by the Ministry of Healthcare of the Russian Federation. This is now clarified in the instances on page 7 and page 9, and removed from the page 17 altogether.

7. It is not clear to me whether the cell counts reported in Figure 2 are mean values. If yes, it should be better specified in the figure legend and the number of samples analyzed per time point should be indicated in the figure of in the figure legend.

Figure 2 shows median values in groups. This, as well as the number of samples in each point, is now specified in the figure legend.
**Minor Essential Revisions**

1. Introduction is too long. I would write it more concisely.

   We had the Introduction restructured and made it more concise. Information on the choice of methods has been entirely moved to the Results and Discussion.

2. The Materials and Methods section is largely incomplete. The DNA quantification methods should be briefly described besides referring to the paper (page 12, line 25). In the section “brief outline of phase II….etc”, the information on clinical trial approval and administrative issues should be separated from the information on patients’ recruitment and treatment in order to make the latter information easily accessible to the reader. The primary and secondary endpoints of the clinical study should be included in this section. There are too many details regarding the administrative and ethical compliances of the study. This part should be more concisely written.

   Following your suggestion, Materials and Methods part has been corrected. We now provide detailed protocol for quantification of nucleic acid content. Administrative information and other formalities have been moved into supplementary, whereas primary and secondary endpoints were moved from the supplementary into the main text.

3. References throughout Introduction section are missing.

   References have been carefully revised and appear correct.

4. Reference n.74 is wrong. The journal is Cancer Biol Ther. 2014 instead of Cell Death Dis according to PubMed database.

   Thank you for catching this mistake. The truth is that paper was bounced from CD&D and published in CBT, while our manuscript was in review.

5. In figure 2, I would report the chemotherapeutic regimens as headings instead of the capital numbers “I”, “II”, “III”

   The regimens are now shown the way you suggest. It does look better.

6. Too many references are in Russian language which makes it difficult to access data reported therein.

   We carefully revised the references. Most of the references in Russian language are somewhat redundant and additional to the English references. Only two references in Russian are non-redundant, Arzamastsev et al. and Spirin. The former reference reports our pre-clinical data, which was only published in the Russian journal. The issue with the latter reference is resolved as we provide detailed description of the method. One more reference, Rizhikova et al., has been introduced to accurately describe cytokine profiles in healthy donors.
7. Page 14, line 8: stage number is differently written: 2 vs III-IV.
Corrected.

8. Figure 5 is not clear. Why are two graphs in the Panagen group and one in the Placebo group? Please clarify by adding more information either in the figure legend or in the plot.
Panagen group is shown by two graphs to illustrate the fact that the data fall into two distinct subgroups, responders and non-responders. Further details are now provided in the figure legend.

9. The indication of the chemotherapeutic regimen (if FAC or AC) in Figure 6 and in figure legend are missing.
Corrected.

10. There are too many additional files and figures. I would recommend including some of the data provided as “additional” into the main figures by creating multi-panel figures.
We chose to move the information from additional files 7 and 8 into the main text. In the additional file 2, detailed description of the methods used is provided. Data from the additional files 2, 3 and 4 are in fact summarized in Figures 2 and 3. Those files only served to provide more detailed information to the readers. Additional files 5 and 6 focus on CD34+/45+ cells and activation of adaptive immunity. This is discussed in the main text, but we believe that a more detailed description of these data would render the paper too bulky.

**Discretionary Revisions**

1. Given the fact that it is phase II study, the outcome of combined Chemo+Panagen treatment vs Chemo alone in terms of clinical efficacy is particularly relevant and must be better supported. The data reported in additional file 8 (long-term follow-up analysis) should not be provided as supplementary material but rather included in the manuscript (for example as table).

Additional file 8 is now part of the main text.