Author's response to reviews

Title: Immunotherapy of hepatocellular carcinoma with small double-stranded RNA

Authors:

Tatyana O Kabilova (kabilova@nibo.ch.nsc.ru)
Larisa V Kovtonyuk (lkovtonyuk@mail.ru)
Evgeniy V Zonov (zoman89@gmail.com)
Elena I Ryabchikova (lenryab@nibo.ch.nsc.ru)
Nelly A Popova (nelly@bionet.nsc.ru)
Valeriy P Nikolin (nikolin@bionet.nsc.ru)
Vasily I Kaledin (kaledin@bionet.nsc.ru)
Marina A Zenkova (marzen@nibo.ch.nsc.ru)
Valentin V Vlassov (vvv@nibo.ch.nsc.ru)
Elena L Chernolovskaya (elena_ch@nibo.ch.nsc.ru)

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Author's response to reviews: see over
Dear Włodzimierz Krzyzosiak,

Thank you for careful study of our manuscript and for your very useful remarks and comments. We revised the manuscript and let us respond your questions and comments.

Minor Essential Revisions:

**Question 1:** The invention and initial characterization of immunostimulatory RNA that was analyzed in this study was described in the previous paper by Kabilova et al. 2012, and the structure/sequence features of isRNA should be briefly presented also in this study in the Introduction section.

**Response 1:** Thank you for the important remark. We added some sentences about sequence/activity relationships of isRNAs under study in the Introduction section (P5 L2).

**Question 2:** It is also not clear which cellular sensors of RNA could be activated by the isRNA and what is the role of specific sequence motifs in this activation. The authors may wish to comment on possible mechanisms and pathways involved in the isRNA action in the Discussion section.

**Response 2:** Thanks for the remark; we added a discussion of possible mechanisms of isRNA action (P15 L4).

**Question 3:** The title of the manuscript is not precise (“22-nt double stranded RNA”) since isRNA is composed of 19 bp duplex and 3 nt overhangs at the 3’ ends.

**Response 3:** Thank you for the important remark. We changed the title of the manuscript (P1 L1).

**Question 4:** Abstract section, Results, first sentence: the phrase “here and after” may be omitted

**Response 4:** We removed the phrase “here and after” (P2 L13).

**Question 5:** Background section: RIG-I and PKR are only the 2 examples of cytosolic receptors of nucleic acids; the proper name of PKR is “dsRNA-dependent protein kinase” and not “dsRNA-dependent protein kinase R”.

**Response 5:** Thank you for the remark. We corrected the name of PKR (P4 L9) and added the other examples of cytosolic sensors (P4 L8).

**Question 6:** References 17 and 18 are not well chosen to represent publications describing activation of immune system by exogenous nucleic acids; the authors missed few recent

Response 6: Thank you for the important remark. We replaced the references 17 and 18 to more recent references (P18 L26).

Question 7: “The antitumor activity of TLR 3/7/8/9 agonists has been demonstrated “in” several tumor types …”
Response 7: We corrected the mistake on P4 L16.

Question 8: Methods section: there is no information regarding Poly I:C source and quality.
Response 8: Thanks for the remark; we added the information about Poly(I:C) in Methods section (P6 L3).

Question 9: Results section: the sequence of isRNA was described in Methods section; there is no need to repeat it.
Response 9: We removed the sequence of isRNA from Results section (P9 L5).

Question 10: Discussion section: The statement “The immunostimulatory properties of siRNAs depend on the presence of immunostimulating motifs in their structure…” should be rewritten.
Response 10: We rewrote the statement “The immunostimulatory properties of siRNAs depend on the presence of immunostimulating motifs in their structure…” (P13 L12).
Dear Steve Pascolo,

Thank you for careful study of our manuscript and for your very useful remarks and comments. We revised the manuscript and let us respond your questions and comments.

Major revisions:

**Question 1:** Interferon-alpha is detectable in serum after intra-venous injections (Figure 1). Is it also detectable after intra-peritoneal injection? Intra-peritoneal injections are used in the anti-cancer experiments (Figure 2)....

**Response 1:** Thank you for the important remark. We performed an experiment and presented data on the measurement of serum levels of IFN-α and IL-6 after intraperitoneal injections at different time-points.

**Question 2:** Figure 2: Mock treatment (Lipofectamine) increases tumor volume compared to untreated controls. Thus although isRNA treatment is significantly effective when compared to Mock, it is not when compared to control. Any explanation why Lipofectamine would increase tumor growth?

**Response 2:** Thank you for the interesting question. In the literature, there is evidence, that lipid-based carriers may mediate side effects, such as inflammation (Tseng et al., 2009), that may promote carcinogenesis at HCC diseases (Wang, & Chen, 2013; Seki et al., 2011). We discussed this point on P14 L12.

**Question 3:** Figure 2: Poly (I:C) that is efficacious in triggering interferon-alpha (Figure 1) is not providing control of tumor growth and is even boosting lung and liver metastasis (Figure 6). Any explanation for this unexpected result?

**Response 3:** Although poly(I:C)/Lipofectamine complex effectively induces IFN-α synthesis, it also leads to prolonged and efficient increase of pro-inflammatory cytokine IL-6 level. Development of inflammation may be the main reason of boosting lung and liver metastasis after treatment with poly(I:C). We discussed this point on P14 L12.

**Question 4:** Is interferon-alpha necessary for the anti-metastasis effect (repeat experiments using neutralisation of interferon-alpha or interferon-alpha receptor by antibodies or using KO mice).

**Response 4:** We cannot state that IFN-α is necessary for the anti-metastasis effect, however our previous results showed, that isRNA with the same sequence, but shorter on a nucleotide (19 b.p. long with 2-nt overhangs) has no antiproliferative as well as interferon-inducing activities in tumor cells and PBMC (Kabilova et al., 2012). We consider that the administration of large
amounts of antibodies in mice is not reasonable, because it can cause a variety of reactions, such as immunostimulation. Unfortunately, knockout mice are not available for us.

Minor revisions:

Question 1: "For" is missing page 4 "been demonstrated several tumor types"
Response 1: We corrected the mistake on P4 L16.

Question 2: Page 5, paragraph isRNA line 7: Is siRNA correct? Or it is meant isRNA?
Response 2: We replaced “siRNA” by “isRNA” (P5 L12).

Question 3: Page 12: Inhibition of primary tumor growth: p<0.01? In Figure 2, p<0.05
Response 3: We corrected the mistake on P2 L19 and on P13 L17.