Author's response to reviews

Title: The siRNA targeted to mdr1b in vivo sensitizes murine lymphosarcoma to chemotherapy

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Author's response to reviews: see over
Dear, Editor in chief,

we’d like to tender our thanks to the reviewers for thorough analysis of the manuscript. According to the valuable remarks and recommendations of the reviewers we carefully revised the manuscript, improved the Introduction, successively and more accurately represented the obtained data, restructured the Discussion and added clarifications, renewed references and inserted missing citations. In the manuscript introduced corrections are marked by red.

A point-by-point response is attached below:

**Author's response to Referee № 1**

**Title:** The siRNA targeted to mdr1b and mdr1a mRNAs in vivo sensitizes murine lymphosarcoma to chemotherapy

**Revision:** 1 Date: 16 March 2010

**Author's response:**

1. “As far as siRNA to mdr1b gene affected mRNAs of both genes (mdr1a and mdr1b) it would be better to change paper’s title and mention mdr1a in the title. Similar corrections must be done in the abstract and all other parts of the paper.”

   The manuscript title was changed and special corrections were performed throughout the text.

2. “It is reasonable to write more (probably in the introduction) about mdr la and mdr lb proteins and their role in multidrug resistance of mouse tumors. It is necessary to use contemporary names for ABC transporters (or at least mention them). For example an official symbol of the gene mdr1b is now Abcb1b.”

   Relevant data were introduced into the text, see page 2-3.

3. “The names of all genes must be written in Italics.”

   The text was carefully examined for correct format of gene names. It is worth noting that in word combinations with siRNAs (i.e. mdr1b/1a and bcl-2 siRNA) the names of the genes are written in regular format.

4. “Almost all data presented in the paper are sound. The only exception is the data in Fig. 1B. Why the quantity of mdr1a mRNA increased under influence of 200 nM of siRNA? Was this increase revealed in several experiments? What this means? The answers on these questions have to be discussed in the paper.”

   The clarification was introduced into the manuscript, see page 10.

5. “Some references have to be renewed. In particular, references 2,3,4 and citations of the papers where the results of the application of siRNAs targeted to MDR genes are presented.”

   The references were renewed.
**Author's response to Referee № 2**

**Title:** The siRNA targeted to mdr1b and mdr1a mRNAs in vivo sensitizes murine lymphosarcoma to chemotherapy

**Revision:** 1  **Date:** 16 March 2010

**Author's response:**

1. “Authors could discuss a little more on the negative results shown by siRNA bcl-2 gene transfection especially considering that P-gp, encoded by the mdr1 gene, is playing a role in apoptosis and drug resistance.”

   We examined a role and contribution of Pgp in apoptosis and drug resistance and critically discussed the obtained results according with the literature data, see Discussion, see page 15-16.


   The reference (Mazzanti R., et al., 1990) was added into the manuscript, see page 7, ref. 22.

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**Author's response to Referee № 3**

**Title:** The siRNA targeted to mdr1b and mdr1a mRNAs in vivo sensitizes murine lymphosarcoma to chemotherapy

**Revision:** 1  **Date:** 16 March 2010

**Author's response:**

1. “Page 9: The authors state that 'the mdr1b siRNA has an appropriate binding site in mdr1a mRNA', thus explaining the somewhat unexpected knockdown of mdr1a. What do they mean by 'appropriate'? Good enough for acting as an siRNA (despite 3 mismatches), or is it rather a miRNA effect?”.

   Comments on a mechanism of action of mdr1b/1a siRNA were introduced into the text, see pages 14–15, paragraph 3.

   “In fact, at certain concentrations the semi-specific mdr1a knockdown is even more profound than the specific mdr1b knockdown (see Fig. 1B). More problematic in this context is the fact that the 'semi-specificity' of the mdr1b siRNA (as shown here by the parallel mdr1a knockdown) could actually also suggest some non-specific effects of the siRNA. To exclude this possibility, the authors need to perform some in vitro experiments using another specific siRNA to firmly exclude non-specific effects of their siRNA sequence”.

   We gave a more clear explanation on selection of mdr1b/1a siRNA sequence, see page 9.
2. “*In vitro effects on cell viability: dose-response curves should be shown to support the authors' conclusions. Those should also include data for cyclophosphamid and embichin since they are used in the later in vivo experiments*”.

The figure was introduced into the text, see pages 11, and 18 (figure legend).

As for cyclophosphamide and embichin, some clarifying details were introduced into the text, see page 15.

“It is also relevant to note that the knockdown of mdr1b leads to an only very partial reversal of the cellular resistance. Based on the authors' previous work, the reversal of the resistant phenotype upon mdr1 knockdown could be expected and is not really surprising. Consequently, one could actually wonder why the increase in sensitivity (as determined by LD50 values) is not more profound. The authors should comment on that”.

Based on the design of RLS_{40}, a complete reversal of its chemosensitivity to the level of parental RLS line should not be expected. RLS_{40} cells were obtained by a long-term cultivation in the presence of gradually increasing vinblastine concentration. After this, a positive selection resulted in surviving of a Pgp+ cell clone. After siRNA treatment a negative selection was performed: the cells with effectively downregulated Pgp died, while those in which the Pgp level did not decrease, survived thus forming another cell population. Probably, a single application of siRNA is not sufficient for a total reversal of MDR phenotype in RLS_{40} cells. In our opinion, to restore the Pgp expression level and vinblastine IC_{50} to the level of RLS cell, a multiple siRNA treatment is required due to the negative selection. Furthermore, it is well known that the abnormality in gene expression arose in the process of neoplastic transformation, and chemotherapeutic treatment is unmanageable and drug resistance could never be reversed to initial normal status. However, the observed inhibition of tumor growth showed that even a single application of mdr1b/1a siRNA is reasonable.

3. “*The in vivo data on the increase of antitumor effects after mdr1b knockdown are appealing. It is imperative, however, that the authors actually demonstrate reduced mdr1b levels in the tumors. This will exclude non-specific siRNA effects*”.

The corresponding data were introduced into the text, see pages 11, and 13. The reviewer can find below the primary data on RT-PCR analysis from *in vivo* experiment. This figure will be introduced into the text, if the reviewer considers it necessary.
Figure 1. Silencing of mdr1b and mdr1a genes in lymphosarcoma RLS40 by siRNAs. RT-PCR analysis (8% PAGE) of mdr1b mRNA level (A) and mdr1a mRNA level (B) in the RLS40 ascites transfected in vivo with mdr1b/1a siRNA (100 nM) 48 h after transfection. (C) The expression levels of mdr1b and mdr1a genes in the tumor cells transfected with mdr1b/1a siRNA. The expression levels of mdr1b and mdr1a genes in the tumor cells transfected with luc siRNA (specific gene product/β-actin = 100%) were used as controls.

4. The seeming absence of a contribution of bcl-2 to the cellular resistance could also be based on the rather poor knockdown of bcl-2. Perhaps 50% bcl-2 downregulation is simply not sufficient to see an effect? Please comment.

Comments were included, see page 16.

5. Comparing the effects shown in Fig. 3, one gets the impression that ex vivo transfection is less efficient than in vivo transfection (i.p. treatment, Fig. 3C). The authors should comment on that.

Comments are introduced into the text, see page 15.

Other issues / Minor Essential Revisions:

1. The beginnings of the first two sentences of the abstract are redundant: One of the … - One of the …

The paragraph was improved, see page 2.

2. Page 3, 2nd paragraph: The characteristics of and differences between the cell lines RLS and RLS40 are somewhat unclear from this paragraph. The description on page 13 is clearer. 'apoptosis-induced' should probably read 'apoptosis-inducing'.

The paragraph was improved, see page 3. “Apoptosis-induced' was corrected for 'apoptosis-inducing'.

3. Methods, 2nd paragraph: The procedure of the initial tumor cell injection is not described. The description only starts after the development of the i.p. tumors. Also, the composition or the vendor of the lymphocyte separation medium should be stated. Finally, the statement that cells were cultivated for 1 month is unclear since later descriptions (Results
section) rather give the impression that cells were i.m. injected early after isolation. The whole description of the procedure needs some clarification.

Corrections were introduced into the text, see pages 4, and 5.

4. mRNA analysis was only performed by semi-quantitative RT-PCR rather than qRT-PCR. While, given the functional data, this seems to be sufficient in this context, the authors should state that their data on knockdown efficacy may not be as precise as qRT-PCR data.

The authors introduced specification that RT-PCR is a semi-quantitative method, see pages 6 and 10. Although RT-PCR did not allow obtaining the precise data on the levels of gene expression, we considered that RT-PCR analysis was enough for primary estimation of siRNA potential, especially, in parallel with other methods, such as MTT test and Rhodamine 123 efflux assay.

5. Cell viability in vitro: The authors only performed experiments with vinblastine but more important would be cyclophosphamid which was employed in later in vivo experiments.

The corresponding comment was made above, see major revision no. 2 and page 15 in the manuscript.


Correction was done, see page 6.

7. ‘Intact cells’ should read ‘wildtype cells’. The authors’ intention is to state that cells were untransfected. However, it has to be assumed that cells, also after transfection, are still intact!

Correction in terminology was done, see pages.

8. Page 7, 6th line from bottom: control luciferase siRNA --or-- mdr1b siRNA. The word ‘and’ would refer to a double treatment.

Replacement was done, see page 8.

9. Page 8: It appears surprising that embichin was used, which was not mentioned previously nor included in the cell viability assay.

Explanation was introduced into the text, see page 15.

10. Page 9: By definition, Verapamil would be a --positive-- control in this context. Same paragraph: as long as no data from the wildtype cells are shown, the authors cannot state that ‘the control luciferase siRNA did not influence the Pgp activity…’

‘Negative’ was replaced by ‘positive’, see page 10. The data on the wild type cells were added to Figure 2.

11. Page 11, 2nd paragraph: again, the distinction between both cell lines is somewhat unclear. Do the authors mean that bcl-2 expression in RLS cells went down when the RLS40 cells were generated from the RLS cells? Why?
A particular property of tumor tissue is heterogeneity. Initially, the bcl-2 expression level in RLS tumor was high. During generation of RLS40 cells from RLS cells by the toxic pressure of low-dosed vinblastine, Pgp+ cells had the opportunity to survive and got the priority. The mechanism of survival and resistance of RLS40 cells was switched over from Bcl-2-mediated to Pgp-mediated as compared with the parental RLS line. Thus, the total level of mdr1a and mdr1b gene expression in the tumor population increased, and the total level of bcl-2 gene decreased.

12. Page 11, 3rd paragraph: '..: combined treatment including mdr1b siRNA and vinblastine...'. Was it really a combination? This reviewer got the impression that it was a subsequent treatment and not combined.

Replacements were done.

13. Page 11, average lifetime: Do the numbers show that the double treatment led to a decreased average lifetime? Why? Wouldn't an increase be expected?

We apologize! The days were transposed by unfortunate mistake. The lifetime of mice treated with mdr1b/1a siRNA and cyclophosphamide have increased! Corrections were done, see page 13.

14. The Discussion section (especially page 13, 2nd paragraph) should be shortened to avoid repetitions of information given previously (e.g., in the Results section).

The discussion section was restructured and improved.

15. Fig. 1B: What do the error bars represent? Multiple experiments?

The error bars represent the data on three independent experiments.

16. Fig. 2B: Error bars should appear in different colors or at least in grey scale, otherwise nobody can tell which error bar belongs to which data point.

The figure was improved.

17. Fig. 3A-C: The cryptic labelling, although correct, is confusing. Instead of giving numbers and letters, the authors should use more descriptive abbreviations.

Example in Fig. 3A:

Columns: - CP / + CP
Rows: wt / luc siRNA / mdr1b siRNA

The abbreviations were changed to become more understandable.

The authors state that they performed statistics, but in the figures no p-values and/or asterisks are shown and should be added.

The asterisks were added.