Author's response to reviews

Title: The anticancer activity of lytic peptides is inhibited by heparan sulfate on the surface of the tumor cells

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

Thank you for the review of our manuscripts entitled: “The anticancer activity of lytic peptides is inhibited by heparan sulfate on the surface of the tumor cells” and giving us the opportunity to submit a revised version. We find the criticism from the reviewers constructive and have revised the manuscript according to their suggestions as described below.

Response to comments from Ivarne Tersariol:

1. It is reported in the literature that 30mM chlorate partly inhibits GAGs sulfation (see ref. 53). In our experiments the medium contained sulfate which make the inhibition of sulfation less efficient. We did not increase the chlorate concentration above 30 mM because high concentrations are known to affect the cell viability. The reason why chlorate only had a moderate effect upon the cytotoxic activity of the Lfcin and KW5 peptides may be due to a) only partial inhibition of the sulfation; and/or b) the involvement of other anionic membrane components for the cytotoxic activity of the CAPs, as discussed at page 18 in the revised manuscript.

2. We have tested our lytic peptides against several different cancer cell lines and found that the cells are killed via a lytic mode of action. This has been studied by confocal microscopy an electronmicroscopy. In addition, we previously reported that the cytotoxic activity by lactoferricin was not inhibited by pan-caspase inhibitors excluding that the cells are killed by apoptosis (see ref 8). Some of this information is now included in the “Introduction” (page 3, last paragraph).

3. We do agree that the interaction between soluble heparin and the cationic sector/fase of the lytic peptides could also inhibit interactions between the peptides and other negatively charged lipids in the cell membrane. This experiment indirectly shows that our peptides interact stronger with heparin-like molecules than with CS. The stronger affinity to heparin-like molecules versus CS has recently been confirmed in our lab by affinity-chromatography and the results from these studies will be published in a separate paper. Doing
an experiments with soluble CS and heparin with the pgsA-745 and CHO-K1 cells will therefore not give any further information.

4. We agree that there may be other differences between the pgsA-745 and the CHO-K1 cells than just the lack of GAG since it is known that HS is involved in growth factor-induced signaling. We found that the cells did not need growth factor signal transduction to maintain cell survival for 24 h. Since the cells were treated by the peptides for 24 h or less the experiments were performed in serum-free medium. Hence the role of HS as a co-factor for growth factors is not very relevant in this experiment. However, we have modified the sentence the reviewer refers to, by replacing: “clearly demonstrate” to “clearly indicate” (page 16, line 4).

Our lytic peptides (CAPs) are designed to irreversibly destabilize the cytoplasmic membrane whereas cell penetrating peptides (CPP’s), which are not used in our study, are designed to penetrate without destabilizing the cytoplasmic membrane. We have included a section in the “Introduction” that describe the mechanism of action of lytic peptides in more detail (CAP’s) (page 3, last paragraph).

5. See comments above regarding use of serum-free medium. By performing extensive structure-relationship studies on lactoferrin and lactoferricin analogs we have been able to identify structural parameters that are critical for lytic activity against cancer cells. These structural parameters have been optimized in de novo designed peptides. Hence the KW5 peptide is more active and kills cancer cells more effectively and rapidly than lactoferricin. This is the reason why the incubation time was much shorter for the KW5 peptide than for lactoferricin. A paragraph is now included in “Methods” explaining why we used different incubation periods for the two peptides (page 7, second section, line 9).

6. We have now included the result of the statistical calculations from the values presented in Table 2 in the Result section (page 13, last part of first section).

7. We have included the number of cells in Fig. 4.

8. We agree that this is an important experiment. However, as described in the manuscript; despite that the experiment was repeated several times, the results were inconsistence. It is well known that heparitinase is difficult to work with in cell cultures (personal communication. Prof. Masaki Yanagishita, Tokyo Medical and Dental University).

9. We disagree with the reviewer that Fig 7 is irrelevant. Size of the GAG chains/PGs may be of importance since that could influence on the number of peptide interaction sites. Moreover, GAG interaction sites further away from the cytoplasmic membrane may hinder the peptide more efficiently. It has also been been reported that lactoferrin binds weaker to small GAGs than to larger GAG’s (see ref 68), indicating that it is important to investigate whether size could explain the differences in IC\textsubscript{50} values between the two cell lines. Since no other reviewers did comment on this figure, and one of the other reviewers
even wants to discuss the structure of the GAGs in more detail, we suggest keeping the figure in the revised manuscript.

10. See comments above. Our peptides target the cytoplasmic membrane and therefore intracellular trafficking is not relevant for the present study.

**Response to comments from George Tzanakakis**

**Major Comments**

1. More detailed information regarding the general mechanism of action by CAPs has been included in the “Introduction” (page 3, last section).

2. and 4. We agree that the sulfation pattern of the GAG chains may be relevant for the interaction of the peptides with the GAG chains. This possibility is now mentioned in the “Discussion” and appropriate references have been added (page 18, first section and refs 69-72).

3. We agree it is an important experiment. However, as described in the manuscript; despite that the experiment was repeated several times the results were inconsistency (see also comment 8, Tersariol’s report).

**Minor comments**

1. We have rephrased the sentence according to the comment given by the reviewer (page 16, line 8).

2. This is very detailed information and is not very relevant for our discussion on CS versus HS.

3. The statistical significance has been checked and found correct.

4. IC50 values were not obtained in the experiments with lactoferrin and high levels of HS and CS. Consequently, standard deviation could not be calculated.

**Response to comments from Jorg Engel,**

We appreciate the overall positive comments from the reviewer. We agree that the effect of low molecular weight heparin on patients with advanced malignancy is very interesting. However, since the mechanism of action is still not known we do not see how this study could be directly related to our findings.

**Response to comments from Leticia V Costa Lutofu.**

1. By performing extensive structure-relationship studies on lactoferrin and lactoferrin-analogs we have been able to identify structural parameters that are critical for lytic activity against cancer cells. These structural parameters have been optimized in *de novo* designed peptides. Hence the KW5 peptide kills the cancer cells more effectively and rapidly than lactoferricin. This is the reason why the incubation time was much shorter for the KW5 peptide than for lactoferricin. We have explained why we use different incubation periods for the two peptides in “Methods” (page 7, last section, line 9). See also comments to Ivarne Tersariol.
2. Paired t-test was used in the statistical analysis in Fig. 2, 3, 4, and 6. This was done in order to control experimental variability. Since each experiment is independent of each other, there can be some inter-experimental variation. Each experiment included treated cells and control cells (Fig. 2 and 6). In order to explore the differences between the two groups, the result obtained from the treated cells and the control cells from each independent experiment were paired. A paired t-test will give the most correct answer regarding the mean difference between treated and control groups since the two groups is positively correlated in a paired t-test and sources of scatter will be corrected.

In figure 6 each independent experiment contained cells that were treated with only peptide (control group), peptide + 10 µg/ml HS, peptide + 100 µg/ml HS, peptide + 10 µg/ml CS and peptide + 100µg/ml CS. The values obtained for the control group (cells treated with only peptide) were paired with the values obtained from the cells treated with peptide and different concentration of HS and CS. We therefore find the use of a paired t-test appropriate.

In figure 3 and 4 we are not comparing two different treatments or two different cell lines. In figure 3a we are comparing the cytotoxic effect of LfcinB against a CHO cell line that expresses GAGs and its GAG deficient mutant pgsA-745. In each independent experiment the values obtained from the cytotoxic affect of LfcinB against the GAG expressing cell line was paired with the values obtained from LfcinB against the GAG deficient mutant cell line. In figure 3b we are comparing the cytotoxic effect of KW5 against a CHO cell line that expresses GAGs and its GAG deficient mutant pgsA-745 with the same experimental design as described for LfcinB. We therefore find the use of a paired t-test appropriate.

In figure 4 we have examined if there is a difference in the amount of [35S] sulfate incorporated into macromolecules at the cell surface of FEMX and HT-29 cells. Three independent experiments were performed for each of the two cell lines. In order to reduce experimental variability (such as reduced activity of the isotope) the two cell lines were treated as pair. In each independent experiment, the FEMX and HT-29 cells were radiolabeled at the same time, with the same stock of [35S]sulfate and treated in the exact same way. The results from each independent experiment were therefore analyzed by a paired t-test.

In Table 1 we chose to illustrate the difference in the cytotoxic activity against FEMX and HT-29 by using the ratio of the IC$_{50}$ value between HT-29 and FEMX. The ratio of the IC$_{50}$ value reveals the differences in cytotoxic activity between FEMX and HT-29 cells.

3. Page 15. We have discussed this issue in “Discussion” (page 18, first section)

Page 16. We agree that other factors than HS may affect the differences in cytotoxic activity against HS-expressing lymphoma cell lines and lymphoma
cell lines without HS. We have therefore replaced the word “established” with “support” in the “Discussion” (page 16, last line).

We have changed the dots to commas as requested by the reviewer.

Response to comments from David Phoenix

It is correct that our final conclusions is opposite to our hypothesis and we have now more clearly commented this inconsistency by rephrasing our hypothesis in the “Introduction” and more clearly stated that we obtained the opposite results, in the beginning of the “Discussion” (page 14, first section).

We have also given more specific information on the mode of action of CAPs in the “Introduction” (page 3, last section) and that there are differences in relative specificity for cancer cells and normal cells for different CAPs.

Response to comments from Oleg Chertov

Since serum inhibit the activity of CAPs they are not ideal for a systemic administration. Our earlier reported in vivo studies indicate that CAPs may have a potential for local treatment of solid tumors. We have addressed this point in the “Introduction” (page 3, line 10).

The two lactoferrin papers that are referred to are not relevant since they are describing activities induced by the protein lactoferrin and not the peptide fragment lactoferricin that is studied in the present work.

Our lytic peptides do not act via stereo-specific resceptors but via electrostatic interactions. Their mode of action has been described in more detail in the “Introduction” (page 3, last paragraph)

Minor essential revision.

P8, We have replaced “where” with “were”.

We hope that our revisions meet the requests from the reviewers so that the revised manuscript can be accepted for publication.

Sincerely Yours

Prof. Øystein Rekdal