Review of: "Designing a Novel Functional Peptide With Dual Antimicrobial and Anti-inflammatory Activities via in Silico Methods"

Davor Juretić

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Overall, the authors initiated an interesting approach to constructing artificial multifunctional peptides but failed to provide convincing and coherent arguments that their method is quantitative and reproducible.

The first word in the title (Designing) is misleading. The authors did not design anything. There is nothing about design in their Materials and Methods section named "Design of Functional Peptides ... ". The words "predicted and selected" would be more appropriate. However, the authors did not construct their own prediction algorithm connected to the design procedure. They employed several user-friendly web servers developed by other scientists to predict peptides' functional properties. In our experience, these servers are not always available, and their accuracy was not thoroughly tested on peptides outside training and testing sets used by the algorithm's developers. It was an uncritical usage by Shin et al. (2022), devoid of recognizing each algorithm's weak and strong points and devoid of feedback from their experimental results on the performance, accuracy, and quality of these theoretical tools. Perhaps, it was unavoidable because the authors restricted their testing and validation procedure to only two peptides claimed to be designed. Two points are not enough to get any statistics about prediction quality.

Worse, the first peptide, named Ak-N', was not designed or predicted. It is the N-terminal part (20 residues) of mature spider toxin peptide c32159 identified from homology analysis with oxotoxins. The "rational design "consisted of an arbitrary choice of 20-residue long fragment from a mature peptide which is predicted to have less-than-optimal activity by six out of eight servers giving a numerical score or probability as the output. The choice was among five out of 29 peptides with identical lenghth (20 AA) because these five peptides were selected as the "most powerful" by some unexplained general scoring method. The second peptide, named Ak-N'm, was obtained through an ill-defined workflow, selection, residue substitution, and scoring procedure that had nothing to do with design. It heavily leaned on the multiple usages of the AntiInflam server, probably due to the output results with two other algorithms for the anti-inflammatory activity (AIPpred and PreAIP), which predicted better such activity for peptides outside the group of five selected ones. The authors should have mentioned that they used a slow SVM prediction procedure by the AntiInflam, but they did not mention it, as if they wished to make it more difficult for other researchers to repeat their findings.

Supplementary Table 2 indicates that improved anti-inflammatory activity is, as a rule, associated with higher toxicity for red blood cells. The Ak-N'm peptide is the rare exception to that rule. It is possible that the authors' "rational design

"method consisted in looking for rare peptides for which it was feasible to increase anti-inflammatory and decrease hemolytic activity simultaneously. If so, the authors should have mentioned and discussed it instead of insisting that their goal was to design a dual functionality peptide: anti-inflammatory and antimicrobial. If that was the initial goal, the results do not support that it was reached, as we discussed below.

The experimental part of the Shin et al. paper and its description also raises some concerns.

1. It is well known that peptide activity strongly depends on whether it is amidated or not at its C-terminal. The authors did not include this crucial information in the Materials and Methods section describing peptide synthesis.
2. The hemolytic activity of peptides is under-estimated when the dense suspension of red blood cells is used (4%) as the authors did.
3. Figure 2 panels A to E are scrambled to such an extent that none of them corresponds to Figure 2 legend "explaining "which panels correspond to certain bacterial species.
4. Regardless of which Figure 2 panel illustrates the inhibition of E. coli growth, there is no case of equal inhibition (MIC) by Ak-N' and Ak-N'm. Thus, the authors overestimated the antibacterial activity of the Ak-N'm, the sole novel peptide they describe as designed.
5. Awkward abbreviations for peptides first confused the authors. Their words "the N-terminus of c32159 and its modified sequence were presented and named Ak-N' Ak-N', respectively "do not distinguish between Ak-N' and Ak-N'm. Both are named Ak-N'.
6. The authors used an unusual method to oversell their paper in the Discussion chapter. They repeated twice the sentence: "The utilization of such multifunctional peptides can be beneficial as they can target pathogens and host immunity simultaneously. "

Readers would like to know how much the functionality was improved by authors who repeatedly asserted that they devised a method to improve functionality. Lesser toxicity to mammalian cells is a desirable goal if modifications to antimicrobial peptides do not produce significantly lower antibacterial potency. In rare cases, that goal has been achieved. The authors did not mention any design procedures that simultaneously lowered toxicity and increased bacteriostatic or maintained bacteriostatic and bactericidal activity. On the contrary, their presented MIC results (Table 3) testify that the Ak-N'm peptide is about 4 to 40 times weaker bacteriostatic than the natural Ak-N'm peptide (if we exclude the previously mentioned suspect case of E. coli). Antifungal and antiviral activity is also predicted for both peptides, but the authors did not examine changes in these functionalities after three amino acid substitutions were introduced in Ak-N' to construct Ak-N'm. They got disappointing and hardly mentioned result that the anti-cancer activity is significantly weaker for the Ak-N'm.

While it is clear that some functionalities are mostly lost during the "design procedure" (antimicrobial and anti-cancer), it is less clear what is the value of the gained anti-inflammatory activity. The comparison with some known anti-inflammatory peptides would be helpful in the same experiments as a control for possible advantages of Ak-N'm. Since the authors did not perform such a comparison, it isn't easy to rate Ak-N'm among other anti-inflammatory peptides.

The final paragraph in the Discussion chapter implies that the therapeutic index (TI) for Ak-N'm is better than the TI for the "parent "peptide Ak-N'. Unfortunately, the authors did not go high enough in peptide concentrations to calculate the TI. Hence, we do not have the evidence needed to conclude that the loss of antimicrobial activity of Ak-N'm is compensated by an increase in TI.