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Search for Peptidomimetic Inhibitors of the VEGF-A_{165} / NRP-1 Complex with Modification of the *C*-Terminal Arginine

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Introduction

Neuropilin-1 (NRP-1) is a cell surface receptor involved in a wide variety of signaling pathways, including physiological and pathological processes of angiogenesis. Its overexpression is associated with tumor aggressiveness and metastasis, which is observed, inter alia, in breast, colon cancer or brain tumors [1]. One of the most important ligands of NRP-1 and the main mediators of angiogenesis is vascular endothelial growth factor A_{165} (VEGF-A_{165}), which acts as a pro-angiogenic factor by interacting with the b1 domain of NRP-1. Compounds that block this interaction are potential inhibitors of the VEGF-A_{165}/NRP-1 complex that may find application in the diagnosis and therapy of cancer. One of significant achievements in this field was the identification of the heptapeptide Ala-Thr-Trp-Leu-Pro-Pro-Arg (A7R), which selectively inhibits the binding of VEGF-A_{165} to NRP-1 and reduces angiogenesis and breast cancer growth *in vivo* [2,3]. Based on the structure of the A7R *C*-terminal tetrapeptide, we designed stronger inhibitors, in particular: Lys(Har)-Dap-Pro-Arg (**1**) and Lys(Har)-Dab-Pro-Arg (**2**). However, detailed stability studies of these compounds in human plasma (*in vitro*) have shown that the first cleavage site is a detachment of the *C*-terminal arginine by carboxypeptidases, resulting in a loss of activity since arginine is a key element in the interaction between these inhibitors and NRP-1 [3,4].

Results and Discussion

The aim of the present study was to obtain more stable (and active) analogs of these branched peptidomimetics (**1** and **2**). For this purpose, we decided to replace *C*-terminal arginine with its mimetics. In our previous work [4], based on molecular dynamics, we put forward an interaction model for compounds **1** and **2**. According to this model: (i) the branched peptidomimetics adopt more than one binding pose at the NRP-1 binding cleft, with two poses (Figure 1, BP1 and BP2) being dominant

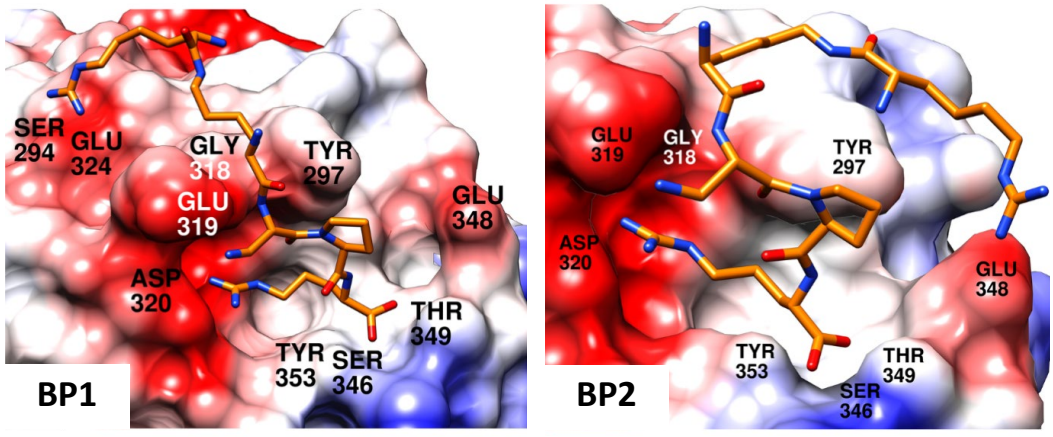

Fig. 1. Two dominant binding poses (PB1 and PB2) found in molecular dynamics simulations for Lys(Har)-Dap-Pro-Arg as complexes with b1 domain of NRP-1. The NRP-1 receptor is depicted as an electrostatic color-coded surface (red: negative charges, white: neutral, blue: positive). Colors of ligands are orange, red and blue for carbon, oxygen and nitrogen, respectively.

and in mutual equilibrium; (ii) the peptidomimetics insert their *C*-terminal Arg residue in the shallow cleft at the protein surface and form several interactions, including H-bonds to Asp320, Ser346, Thr349 (Figure 1); (iii) the middle and *N*-terminal parts of the peptidomimetic retain some residual mobility and switch between positioning BP1 and BP2, forming several interactions in each, including H-bonds to Gly318, Glu319, Glu324, Ser294, Tyr297, Glu348.

With our desire to replace the *C*-terminal Arg, we took the described above model as a starting point for designing new compounds. We analyzed several possible modifications to the *C*-terminus and confronted them with our previous model through molecular docking. Our speculation was that it should be possible to shorten the arginine side chain to Agb (2-amino-4-guanidino-butyric acid) or even Agp (2-amino-3-guanidino-propionic acid). Given that Lys(Har)[1] and Dap/Dab[2] side-chains are long and flexible, it was envisaged that some shortening in Xaa[4] should be tolerated. The first and second residue should still be able to reach their interaction partners (Figure 1). And shorter Xaa[4] should be still able to interact with Asp320 and Ser346/Thr349 (Figure 2A and 2B). We contemplated also extending the Xaa[4] residue to Har (homoarginine). According to crystal structure 5IJR [5], Har can be accommodated in the cleft although with some displacement of the binding mode compared to if Arg is present in the cleft. We supposed however that interactions of Lys(Har)[1] and Dap/Dab[2] would allow retaining significant affinity. Furthermore, it was interesting to see if the *C*-terminal residue with an aromatic ring could gain some affinity due to forming aromatic interactions with Tyr297 (Figure 2D). Finally, a kind of an acid-test to the importance of guanidine-Asp320 interactions was provided by a Cit[4]-analogue, which is able to form one H-bond without charge-assistance (Figure 2C). This analogue was quite decently scored by docking.

To evaluate the accuracy of the design assumptions the inhibitory activity of the obtained compounds on VEGF-A_{165} binding to the NRP-1 was estimated *in vitro* by modified competitive Enzyme-Linked Immunosorbent Assay (ELISA), following the previously described protocol [6]. Obtained results revealed that extension of Arg[4] side chain (**1** and **2**) by introducing one methylene

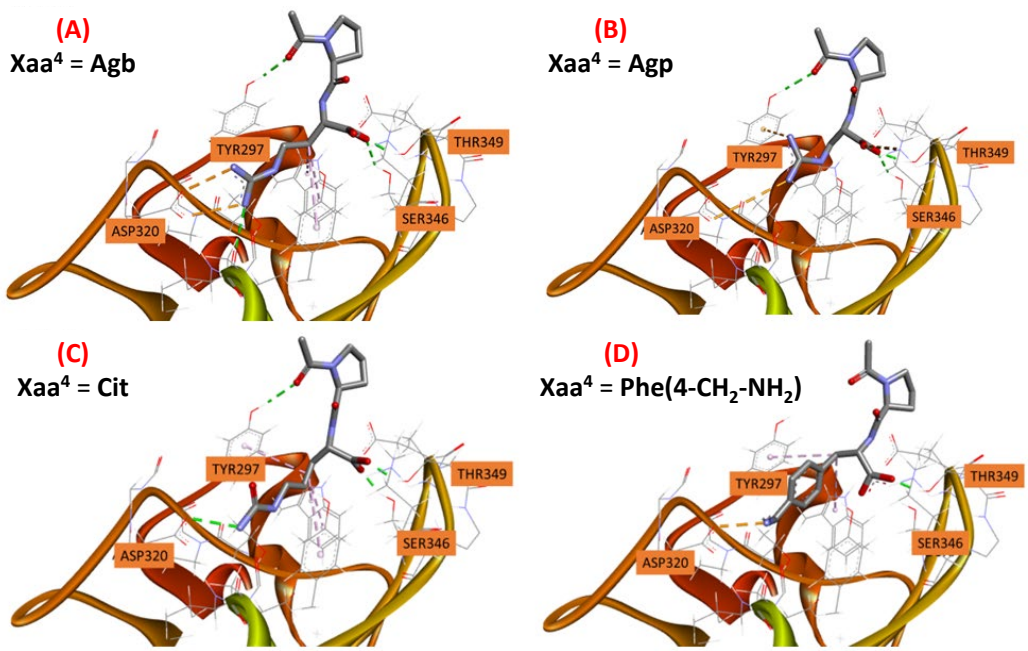

Fig. 2. Predicted binding poses for the C-terminal part of the analogues with (A): Agb[4], (B): Agp[4], (C): Cit[4] and (D): Phe(4-CH_2NH_2)[4]. Protein atoms shown as lines. Ligand atoms shown as sticks (only Pro[3]-Xaa[4] part). Only selected residues of the NRP-1 binding cleft shown. Hydrogen display in the ligands suppressed. Dotted lines represent interactions (green – H-bond, orange – electrostatic, pink – hydrophobic).

Table 1. Inhibitory effect of obtained peptidomimetics on VEGF-A_{165} binding to NRP-1.

No	*Structure*	*IC_{50} [μM] ± SD*
A7R	Ala-Thr-Trp-Leu-Pro-Pro-Arg	11.3 ± 3.2
1	Lys(Har)-Dap-Pro-Arg	8.4 ± 1.6
2	Lys(Har)-Dab-Pro-Arg	4.7 ± 0.6
3	Lys(Har)-Dap-Pro-Har	29.2 ± 1.8
4	Lys(Har)-Dab-Pro-Har	14.3 ± 2.3
5	Lys(Har)-Dap-Pro-Agb	87.5 ± 10.3
6	Lys(Har)-Dab-Pro-Agb	105.7 ± 17.4
7	Lys(Har)-Dap-Pro-Agp	147.6 ± 45.9
8	Lys(Har)-Dab-Pro-Agp	164.7 ± 21.1
9	Lys(Har)-Dap-Pro-Cit	169.7 ± 55.6
10	Lys(Har)-Dab-Pro-Cit	193.3 ± 20.6
11	Lys(Har)-Dap-Pro-Phe(4-CH_2-NH_2)	22.8 ± 5.9
12	Lys(Har)-Dab-Pro-Phe(4-CH_2-NH_2)	19.8 ± 1.8

group to obtain Har^4 (**3** and **4**, respectively), gives only a slight decrease in inhibitory activity as compared to the parent compounds. However, contrary to our speculation, shortening the Arg^4 side chain (**1** and **2**) to Agb (- 1 x CH_2, **5** and **6**) or further to Agp (- 2 x CH_2, **7** and **8**) leads to a significant reduction in the inhibition of VEGF-A_{165} binding to NRP-1. Similarly, replacement of the guanidine group (Arg^4, in **1** and **2**) by the urea group (Cit^4, **9** and **10**), therefore limiting the interactions (with Asp320), in the shallow binding cleft, to only one H-bond without charge-assistance (salt bridge) significantly reduces the inhibitory activity. Interestingly, the replacement of the alkyl side chain of Arg^4 by the aromatic ring of Phe(4-CH_2-NH_2) with a simultaneous change of the guanidine to an amino group (**1** and **2** *vs.* **11** and **12**, respectively), resulted in a slight decrease in activity, comparable to that for Har.

Conclusions

In conclusions, our experimental results showed that:

- position of the guanidinium group (which strongly depends on the length of the methylene chain) in relation to Asp320 has a crucial effect on the inhibitory activity that changes as follows:
  Arg (3x CH_2) ≈ Har (4x CH_2) > Agb (2x CH_2) > Agp (1x CH_2);
- limited number of the interaction between the side chain of *C*-terminal residue and Asp320 strongly reduces inhibitory activity
  Arg (guanidine group) >> Cit (urea group)
- replacement of ionic interaction with aromatic interaction (inside the binding cleft) allows to maintain inhibitory activity at a similar level
  Arg (guanidine group) ≈ Phe(4-CH_2-NH_2) (aromatic ring).

Unfortunately, the synthesized analogues are all weaker inhibitors of the VEGF-A_{165}/NRP-1 complex than expected, based on the modelling in the design stage. One of the possible reasons for

this disagreement might be that the design was based on static dockings without accounting for flexibility of the protein binding cleft and the possibility of dual/multiple binding modes. However, further studies using the molecular dynamics are warranted to understand and rationalize the obtained experimental data and to guide future designs.

Acknowledgments

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