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INHIBITORY ACTIVITY AND PROTEOLYTIC STABILITY IN HUMAN SERUM OF PEPTIDE INHIBITORS OF THE VEGF-A_{165} / NRP-1 COMPLEX

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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, pancreatic, prostate, colon cancer or brain tumors (e.g. gliomas) [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 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 a significant achievement in this field was the identification (by screening a mutant phage library) 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].

In our efforts along this track, 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**).

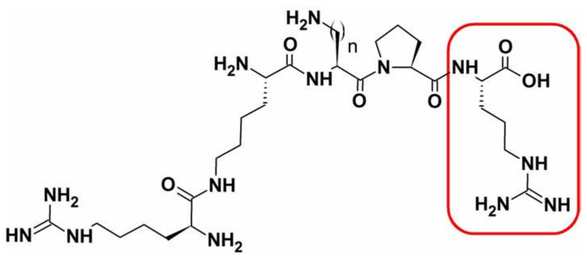
Detailed stability studies of these compounds in human plasma have shown that the first cleavage site is a detachment of the C-terminal arginine by carboxypeptidases, resulting in a loss of activity since arginine is a key element in the interaction between these inhibitors and NRP-1 [4].

PROJECT ASSUMPTIONS AND RESULTS

THE AIM OF OUR STUDY was to obtain more stable (and active) analogs of these branched peptidomimetics (**1** and **2**). To achieve this, we decided to substitute the C-terminal arginine with its mimetics (Table 1 and 2).

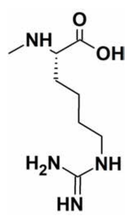
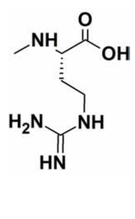
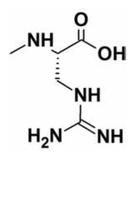
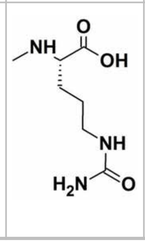
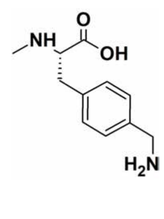
Table 1. Structure of the parent compounds and arginine mimetics used in our study.

PARENT COMPOUNDS



Dap[2] (n = 1) or Dab[2] (n=2)

ARGININE MIMETICS

Har	Agb	Agp	Cit	Phe(4-CH_2-NH_2)
				

All compounds were synthesized using the standard SPPS-Fmoc/tBu protocol.

INTERACTION MODEL FOR COMPOUNDS 1 AND 2

In our previous work, based on molecular dynamics, we put forward an interaction model for compounds **1** and **2** [4]. According to this model:

- the branched peptidomimetics adopt more than one binding pose at the NRP-1 binding cleft, with two poses (BP1 and BP2) being dominant and in mutual equilibrium (Fig. 1);
- 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;
- 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.

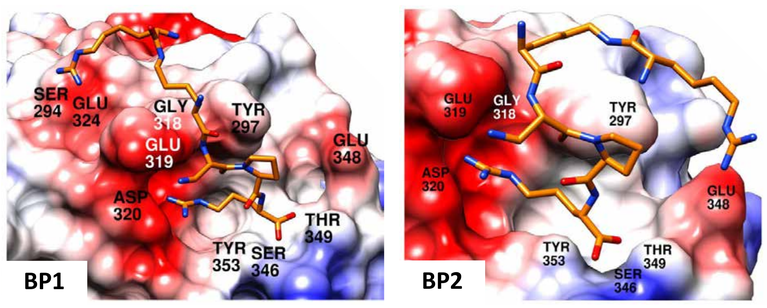


Figure 1. Two dominant binding poses found in molecular dynamics simulations for Lys(Har)-Dap-Pro-Arg as complexes with b1 domain of NRP-1. The NRP-1 receptor is depicted as an electrostatic color-coded surface (red: negative charges, white: neutral, blue: positive). Colors of ligands are orange, red and blue for carbon, oxygen and nitrogen, respectively. The peptidomimetics were modelled in the NRP-1 binding site (PDB: 2ORZ) using AutoDock4 (10.1002/jcc.21256).

DESIGN PRINCIPLES FOR PEPTIDES 3-12

With our desire to replace the C-terminal Arg, we took the described our previous model (Fig. 1) as a starting point for designing new peptides. We analyzed several possible modifications to the C-terminus:

- **Har[4]** - according to the crystal structure 5IJR [5], Har can be accommodated in the cleft although with some displacement of the binding mode compared to Arg is present in the cleft;
- **Agb[4]** or **Agp[4]** - we envisaged that some shortening in Xaa[4]-side chain should still be tolerated inside the binding cleft, given that the Lys(Har)[1] and Dap/Dab[2] side-chains are long and flexible and so that they should still be able to reach their interaction partners (Fig. 1);
- **Cit[4]** - for a kind of an acid-test to the importance of guanidine-Asp320 interactions: according to our modelling, Cit[4] side chain should enable forming one H-bond to Asp320 (but contrary to other analogues, without charge-assistance);
- **Phe(4-CH_2-NH_2)[4]** - to verify that the C-terminal residue with an aromatic ring could gain some affinity due to forming aromatic interactions with Tyr297.

INHIBITORY EFFECT OF OBTAINED PEPTIDOMIMETICS ON VEGF-A_{165} BINDING TO NRP-1

The inhibitory activity of the obtained compounds on VEGF-A_{165} binding to the NRP-1 was evaluated *in vitro* by modified competitive ELISA test following the previously described protocol [6].

Table 2. 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**

Odd numbers refer to compounds with Dap at position 2, where even numbers refer to compounds with Dab at position 2.
IC_{50} values were calculated from three independent experiments, each performed in duplicate.

Obtained results revealed that:

- extension of Arg[4] side chain (**1** and **2** vs. **3** and **4,** respectively) gives only a slight decrease in inhibitory activity;
- 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;
- replacement of the guanidine group (Arg[4], in **1** and **2**) by the urea group (Cit[4], **9** and **10**) 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) coupled with the 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.

METABOLIC STABILITY

The *in vitro* metabolic stability of the most potent peptidomimetics (**3**-**4** and **11**-**12** in comparison to **1**-**2**) has been determined in human serum.

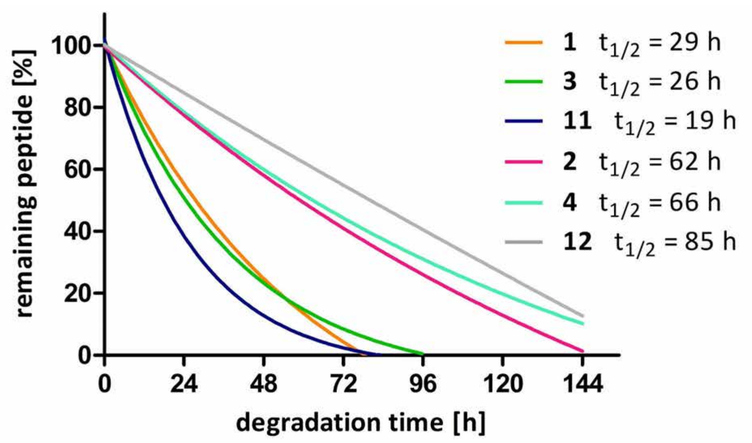


Figure 2. Comparison of the *in vitro* metabolic stability for **1**-**4** and **11**-**12**. For clarity of the figure, the markers on the curve and error bars have been removed.

Obtained results revealed that:

- all tested compounds degraded proteolytically at rates comparable to the parent compounds (**3**, **11** vs. **1** and **4**, **12** vs **2**), (Fig. 2);
- peptidomimetics containing Dab as the second residue are more stable as compared to those containing Dap, (Fig. 2);
- stability of compounds in the Dap[2] series changes as follows **1 > 3 > 11** while in the case of the Dab[2] series it changes in the opposite direction, i.e. **12 > 4 > 2**, (Fig. 2);
- all tested compounds were degraded in accordance with three independent and parallel metabolic pathways (Fig. 3).

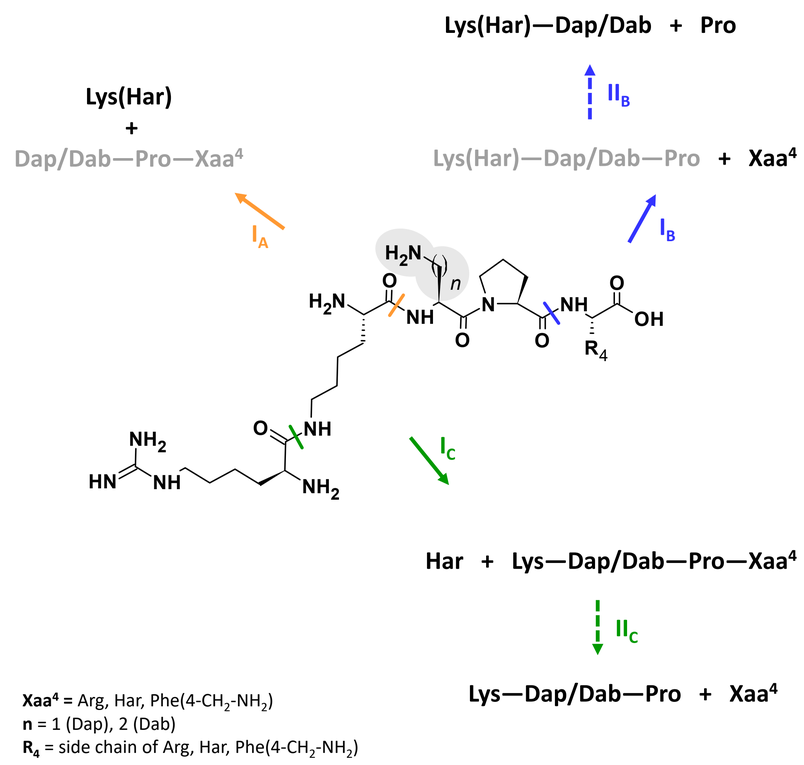


Figure 3. Probable degradation pathways and cleavage sites of **1**-**4** and of **11**-**12**. The general structure of these compounds is shown with marked locations of the first major enzymatic cleavage sites (for pathway I_A, I_B and I_C). Peptide sequences in grey have not been identified by mass spectrometry. II_B and II_C indicate further degradation pathways for Lys(Har)-Dap/Dab-Pro and Lys-Dap/Dab-Pro-Xaa peptides, respectively.

CONCLUSIONS

OUR STUDY SHOWED THAT:

- location 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)

- substitution of Arg[4] with its mimetics Har or Phe(4-CH_2-NH_2) does not significantly improve proteolytic stability compared to parent compounds. However, compounds with Dab[2] ($t_{1/2}$ = 62-85 h) are more stable than those with Dap[2] ($t_{1/2}$ = 19-29 h);
- proteolytic degradation occurs through the three independent and parallel pathways.

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