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Bioorganic & Medicinal Chemistry Letters 14 (2004) 841–845
Bioorganic & Medicinal Chemistry Letters

Kringle 5 peptide–albumin conjugates with anti-migratory activity

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Received 15 October 2003; accepted 4 December 2003

Abstract—Three peptide fragments of the kringle 5 region of plasminogen and their respective N- and C-terminus maleimido derivatives conjugated to Cys34 of human serum albumin were evaluated in vitro using a human umbilical vein endothelial cell (HUVEC) migration assay and a human plasma stability assay. The N-terminus maleimido derivative of the 64 to 74 segment of kringle 5 conjugated to human serum albumin possessed remarkable anti-migratory activity.

1. Introduction

Plasminogen is comprised of five kringle subunits each of which exhibit anti-angiogenic activity either alone or as combinations of one another.[1–4] Of the five kringle units of plasminogen, the kringle 5 (K5) unit shown in Figure 1, has been reported to possess the most potent anti-migratory[5] and anti-proliferative[2,3] activity in vitro and in vivo and thus constituting a potent agent in limiting cancer metastasis and tumor growth.[6–8]

The critical residues of K5 responsible for activity have yet to be fully elucidated.[9,10] The N- and C-capped derivatives of fragments **B** and **C** on Figure 1 were reported to possess picomolar anti-migratory activity in vitro[9] and were considered as templates for the selective bioconjugation to Cys34 of Human Serum Albumin (HSA). We have demonstrated that the concept of bioconjugating peptides to albumin can prolong their presence in plasma by protecting them against enzymatic degradation and reducing elimination through the kidney while retaining most of their desired biological activity.[11–13] Furthermore, HSA has been shown to play a useful role in carrying anticancer agents through the blood stream thus enhancing their in vivo profiles.[14–16]

Encouragingly, a number of studies have been done on large molecules including a recombinant HSA-angiostatin K1-3 fusion protein through in vivo gene transfer,[17] human interferon gamma-K5 fusion protein,[18] a product known as K4.5 which arises from the in vivo degradation of plasminogen,[6,19] as well as the plasminogen fragments angiostatins K1-4 and K1-3.[6] All have demonstrated in vivo efficacy thus supporting our initiative.

Fragment **A** was the longest peptide in our study (Arg51 to Tyr74) where Cys63 was replaced with alanine. Fragment **A** was generated by combining the sections

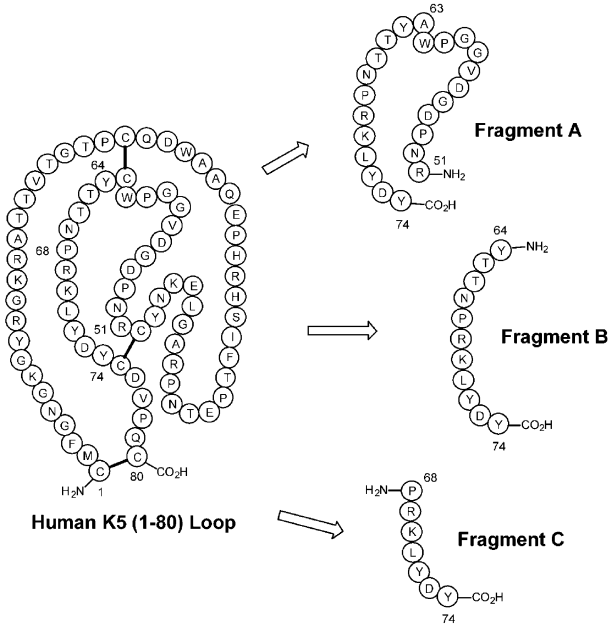

Figure 1. Structure of K5 and three fragments with potential anti-angiogenic activity.

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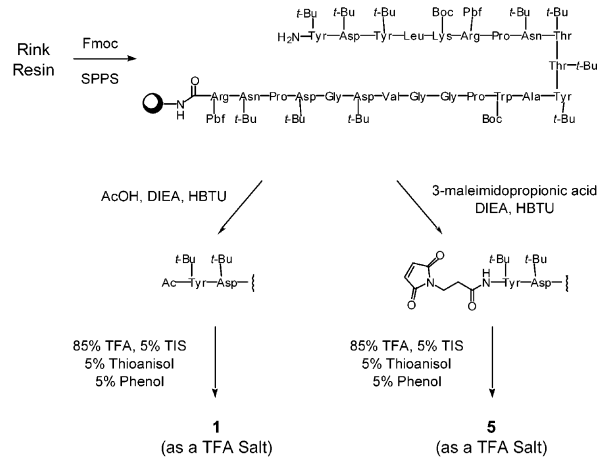
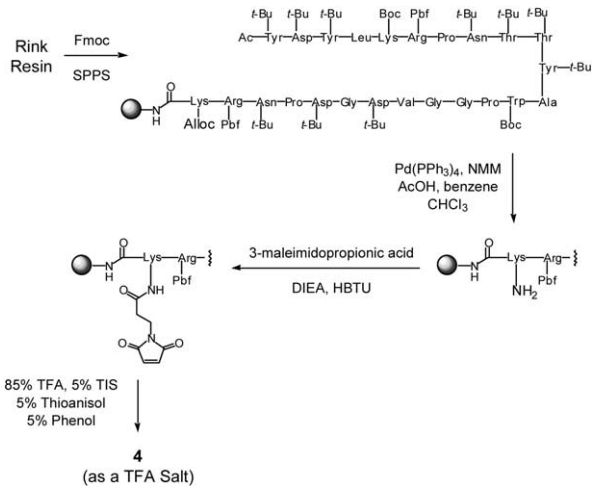
doi:10.1016/j.bmcl.2003.12.025



Scheme 1. Synthesis of native and N-terminus maleimido peptide derivatives.



Scheme 2. Synthesis of C-terminus maleimido peptide derivatives.

ranging from Arg51 to Trp62 (not shown), which was demonstrated to be active in vitro[9] with the sequence from Tyr64 to Tyr74. Hence, fragments **A**, **B** and **C** were postulated to contain the core features responsible for the anti-migratory activity of the K5 unit of plasminogen.

Herein we report the in vitro evaluation of three peptide fragments of K5 and their respective N- and C-terminus maleimido derivatives as HSA conjugates using human umbilical vein endothelial cell (HUVEC) migration and human plasma stability assays.

2. Chemistry

The synthetic targets **1** to **9** are displayed on Figure 2. **1** incorporates fragment **A** with its respective C- and N-terminus maleimido derivatives **4** and **5**. **2** contains fragment **B** with its respective C- and N-terminus maleimido derivatives **6** and **7** and **3** contains fragment **C** with its respective C- and N-terminus maleimido derivatives **8** and **9**. It is to be noted that the three C-terminus derivatives contain an added lysine to accommodate the maleimidopropionyl group, all derivatives were in the form of C-terminus amides and **1**, **2**, **3**, **4**, **6** and **8** were capped as N-terminus acetamides.

The nine peptides were prepared using standard solid-phase peptide synthesis protocol.[20] Scheme 1 displays the synthesis of native peptide **1** and the N-terminus maleimido derivative **5**. The native peptides **1**, **2** and **3** were synthesized by introducing acetic acid within the protocol as the capping step. Alternatively, to obtain the N-terminus maleimido derivatives **5**, **7** and **9**, 3-maleimidopropionic acid was used in the capping sequence in the same manner as acetic acid.

1, **2**, **3**, **7** and **9** were synthesized using the protocol in Scheme 1 while **4**, **6** and **8** were prepared using the protocol in Scheme 2 where a Nε alloc lysine was introduced as the C-terminus amino acid during the synthesis. The alloc was removed using palladium(0)[11,21–23] selectively freeing up the Nε amino group to accommodate the 3-maleimidopropionic acid, which was attached in the usual manner.

The crude peptides were removed from the resin using a trifluoroacetic acid (85%), triethylsilane (5%) and phenol (5%) cocktail and purified by reverse phase HPLC.[24] The synthetic results are displayed on Table 1.

The results for **2** and **3** were identical to those found in the literature.[9]

3. Results

The maleimido derivatives were added to 25% HSA and used immediately in the assays.[25] The conjugates and the free unbound compounds in solution were analyzed by LC/MS (electrospray).[10,12] The results are displayed on Table 2.

8-HSA conjugate was submitted to Edman degradation N-terminal sequencing.[26] Following the 34th cycle, MALDI-TOF mass analysis[27] of the sample extract showed the presence of an entity with M*r* 1647, identified as **10** (Fig. 3), which was found to be absent following the randomly chosen 31th and 39th cycles. **10** was independently synthesized to confirm its molecular

Table 1. Compounds obtained by solid-phase synthesis

Compd	Yield[a] (%)	M*r*[b]		Purity (HPLC) (%)
		Expected	Measured[c]	
1	17	2695.3	2696.6	>99
2	9	1473.7	1473.9	97.1
3	10	994.5	994.6	95.9
4	9	2974.4	2976.1	98.2
5	10	2806.0	2805.9	97.8
6	6	1752.9	1753.7	98.6
7	17	1582.7	1583.1	96.7
8	8	1273.7	1273.9	>99
9	9	1103.5	1103.7	95.1

[a] TFA salt.
[b] M*r* of free base.
[c] Average value from multiple charged species by ES/MS.

weight (M*r* 1646.7).[28] This result confirmed the selective bioconjugation of **8** to Cys34 of HSA.

We evaluated the ability of native peptides and HSA conjugates to inhibit cell migration of HUVEC cells in a modified Boyden chamber assay.[5,7,29] HUVEC cells were exposed to the peptides or HSA conjugates in the presence of basic Fibroblast Growth Factor (bFGF, 25 ng/mL) for 4 h. The number of migrating cells in each treatment group was counted using light microscopy.

Angiostatin K1-3 inhibited the bFGF-induced migration of HUVEC cells in a dose-dependent manner (Fig. 4). The effect of angiostatin K1-3 in our assay correlates well with data published by other groups.[7]

Peptides **1**, **2**, **3** and their HSA conjugates were tested in the same assay. Results presented on Figure 5, demonstrated that peptides **2** and **3** were very active while peptide **1** was not.

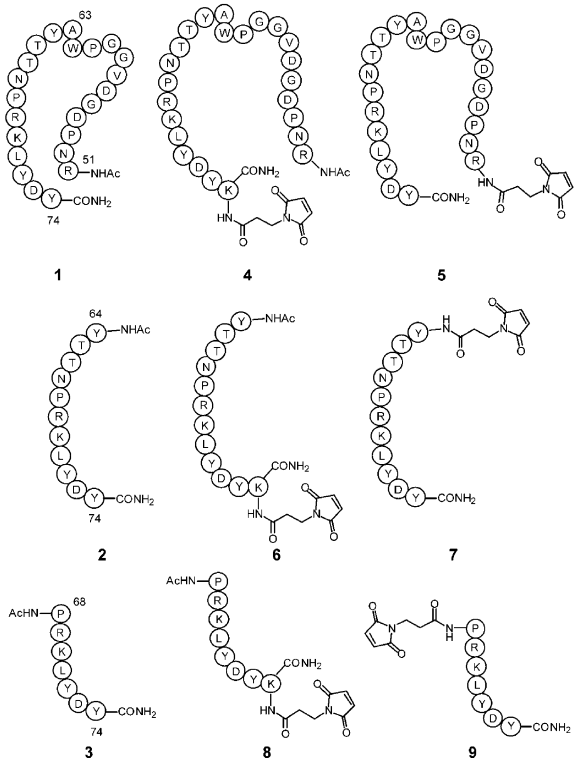


Figure 2. Structures of peptides and maleimido derivatives used within this study.

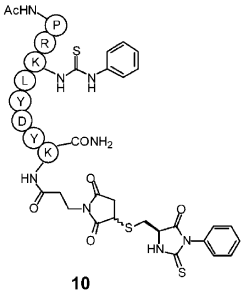


Figure 3. Structure of residue obtained from Edman degradation of **8**-HSA after 34 cycles.

The general trend is that HSA anchoring of the maleimidopropionamide at the N-terminus on all three peptides **1**, **2** and **3**, had beneficial effects as seen by the responses of **5**-HSA, **7**-HSA and **9**-HSA. **7**-HSA was the most potent in inhibiting cell migration in our assay. An intriguing effect was noticed with derivatives **5**-HSA and **7**-HSA, which showed more in vitro activity than their respective parent peptides **1** and **2**. There is no explanation at this time for this phenomenon, only the speculation that the peptide can adopt a more favorable conformation once bound to the albumin molecule resulting in enhanced bioactivity. The best results with a C-terminus derivative was with **8**-HSA conjugate of the shortest peptide **3** derived from fragment **C**.

The human plasma stability analysis was performed on the three native peptides and the six corresponding HSA conjugates in order to assess the ability of albumin to protect these peptides from enzymatic degradation. The assay consisted of adding the peptides or the pre-formed

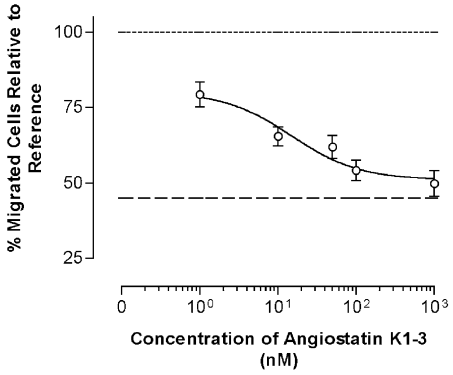


Figure 4. Inhibition of HUVEC migration in the presence of angiostatin (K1-3) control.

Table 2. Analysis of HSA conjugates in solution

Conjugate	M*r*		Unbound maleimide (%)
	Predicted	Measured[a]	
4-HSA	69,422	69,415	0.22
5-HSA	69,253	69,247	1.6
6-HSA	68,225	68,233	0.50
7-HSA	68,055	68,072	1.9
8-HSA	67,722	67,718	0.18
9-HSA	67,551	67,548	1.1

[a] Using the measured molecular weight of an albumin standard analyzed by LC/MS prior to conjugate sample analysis.

Table 3. Summary of anti-migratory and stability results

Compd	Anti-migratory activity	Human plasma stability
1	−	+
4-HSA	−	+ + +
5-HSA	+ +	+ + +
2	+	−
6-HSA	+	+ + +
7-HSA	+ + +	+ + +
3	+ +	− −
8-HSA	+ +	+ + +
9-HSA	+	+ + +

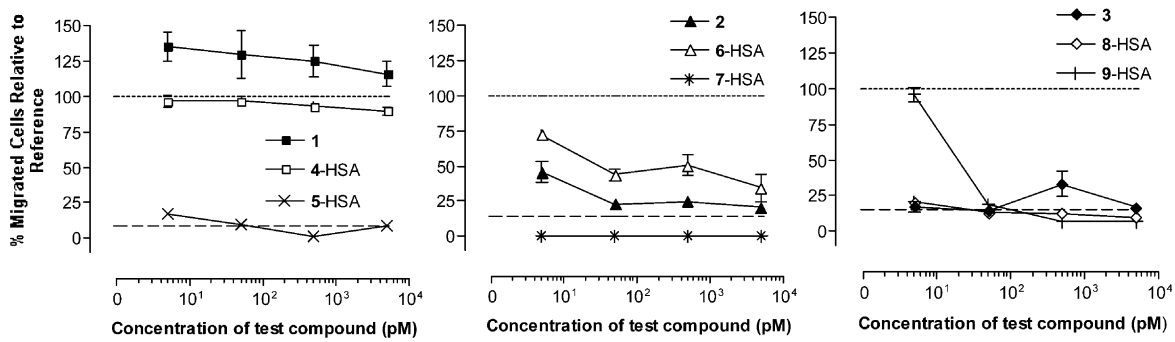


Figure 5. Inhibition HUVEC migration by K5 peptides and HSA conjugates. (- - -) with bFGF, (—) without bFGF.

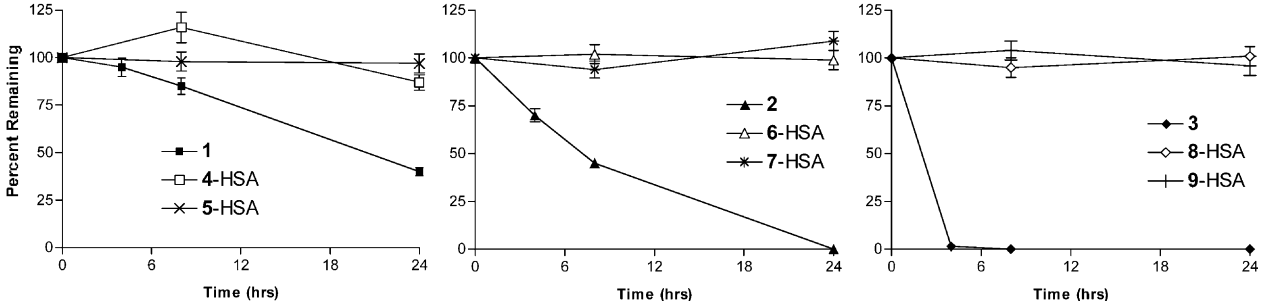


Figure 6. Stability of native peptides and HSA conjugates in human plasma.

conjugates (to a final concentration of 312 μM) directly to human plasma and monitoring either the abundance signal from the free peptide or the HSA conjugate by LC/MS over time.[11] The results are shown on Figure 6.

Peptides **2** and **3** were completely degraded after 24 h although the larger peptide **1** did show a prolonged stability profile compared to the other two. Nevertheless, in all cases the HSA conjugates were very stable in human plasma.

The in vitro results are summarized on Table 3 as a degree of anti-migratory activity and plasma stability.

Although **1**, derived from fragment **A** was proposed as a potentially active compound, it turned out to be inactive while its N-terminus HSA conjugate **5**-HSA was a very respectable inhibitor of cell migration. The same could be said of the relationship between **2** and **7**-HSA where the former was less active than the conjugate. These two pairs of peptides and conjugates brought to light the importance of the anchoring at the N-terminus in order to confer activity. Alternatively, the shorter peptide **3** and its C-terminus conjugate **8**-HSA displayed equivalent anti-migratory activity. In this case, the position of anchoring was less important. Based on these results, one could argue that the PRKLYDY peptide contains the essential segment needed for anti-migratory activity. Yet if a longer portion of the peptide segment is constructed, which includes Tyr64 (close proximity to Tyr72 and Tyr74 in X-ray structure of apo-$K5_{HPg}$)[30] within a rigidified structure, the activity can be further improved through N-terminus anchoring to a large protein such as albumin as demonstrated by conjugates **5**-HSA and **7**-HSA.

Finally, while the conjugation to HSA enhances the bioactivity of some of these peptides, it protects all of them from plasma degradation.

4. Conclusion

It is possible to build bioactive K5 peptide-HSA constructs by attaching their maleimido derivative to Cys34 using a simple structure activity relationship approach. One conjugate, **7**-HSA showed remarkable activity in our in vitro anti-migratory HUVEC assay. Furthermore, all of the HSA conjugates were demonstrated to be stable to hydrolysis in human plasma. These results were used to support further in vivo studies to be reported at a later date.

Acknowledgements

We would like to thank Marcos DiFalco at the Sheldon Biotechnology Institute (McGill University, Montreal Canada) for his assistance in the Edman N-terminal sequencing of albumin conjugates.

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24. Purification on a Phenomenex Luna 10 μ phenyl–hexyl 21×250 mm column with a 10–30% acetonitrile (0.045% TFA) gradient in water (0.045% TFA) over 60 min at 18 mL/min and analysis on a 5 μ 10×250 mm column using a 10–40% gradient at 0.5 mL/min over 20 min.
25. The conjugations were done by adding the test compounds **4–9** (625 μmol) to commercial 25% HSA (3.73 mmol, obtained from Alpha Therapeutics Corporation, Los Angeles, CA USA, which was a mixture of mercaptalbumin and capped thiol albumin 40:60 by LC/MS) at pH 7.0 and incubating at 37 °C for 2 h. Analytical methods are described in ref 11. The final ratios of conjugates/mercaptalbumin/cappped HSA were 20:20:60 by LC/MS.
26. 8-HSA was applied onto SDS-PAGE and subsequently electroblotted onto a PVDF membrane and the protein was revealed with coomassie blue. The protein band was then excised, washed and submitted to solid phase N-terminal Edman sequencing using an Applied Biosystems Procise 492 protein sequencer followed by MALDI-TOF analysis using a Voyager-DE linear biospectrometry workstation.
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