N-linked Glycosylation Improves Bivalirudin Stability and Retains Efficacy in vitro

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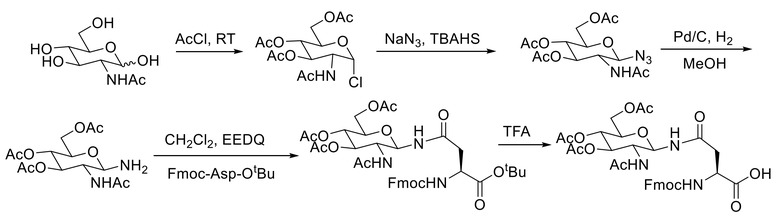
Abstract

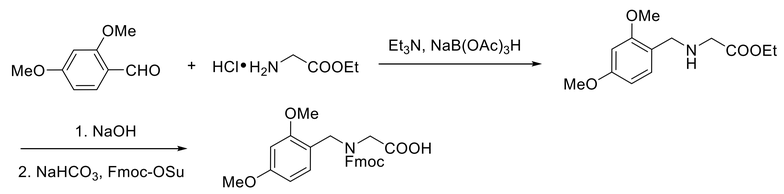
This project aimed to increase the stability of bivalirudin (a peptide thrombin-inhibitor) via N-linked glycosylation of the Asn-9 residue and verify that the glycopeptide had equivalent inhibitory effects. N-linked glycosylation of bivalirudin was achieved by synthesis of a glycoamino acid building block, Fmoc-Asn(GlcNAc)-OH. A stability test determined that glycobivalirudin was indeed more stable, and a SensoLyte 520 Thrombin Inhibitor Assay determined that glycobivalirudin was an equally potent thrombin inhibitor. These results indicate that the glycosylated drug is more stable *in vitro* while retaining its inhibitory properties. Future projects will explore the efficacy and stability of glycobivalirudin in blood serum or animals. Future research areas may examine the monosaccharide glycosylation method on other peptides and other glycosylation methods, such as conjugation to a dextran scaffold, for bivalirudin.

Introduction

Bivalirudin is an FDA approved direct thrombin inhibitor (DTI) used to prevent blood clotting during invasive cardiovascular procedures.[1] In addition to instability due to proteolytic cleavage, deamidation of bivalirudin's Asp-9 residue causes two major impurities (α and β-Asp), which occur in storage, manufacturing, and administration of the drug and render the drug ineffective.[2] (Figure 1) N-linked glycosylation, a post-translational modification process in natural proteins, predominantly modifies Asn residues, stabilizing them from deamidation reactions. Thus, N-linked GlcNAc was chemically installed onto the Asn-9 residue of Bivalirudin, and the glycopeptide was tested for its stability and inhibitory effects. Two other peptides containing isoAsp and Asp at the ninth residue were also synthesized to be used as HPLC standards for bivalirudin degradation.



Scheme 1. Synthesis of GlcNAc-Asn



Scheme 2. Synthesis of Dmb protected glycine

Methods

- GlcNAc-Asn was obtained in gram scale from a five-step chemical synthesis (Scheme 1)
- Modified glycine was obtained in gram scale via a two-step synthesis (Scheme 2)
- Modified amino acids were characterized by NMR according to reported procedures.[3-4]
- Peptides were synthesized using the modified amino acids and Solid Phase Peptide Synthesis, with results confirmed by ESI-MS and HPLC.
- The stability test was performed by dissolving the peptides in water and storing in a 25 C water bath for approximately 3 weeks. Afterwards the samples were removed and tested for purity with HPLC
- The efficacy of the peptides were tested using a SensoLyte 520 Thrombin Activity Assay, using various concentrations of the peptides. The assay includes thrombin and a fluorescent thrombin substrate; a fluorescence detector determined thrombin activity.

Results

- The modified glycine successfully prevented the aspartimide side reaction to help us obtain target peptides (compound 3 and 4, Figure 2).
- Four target peptides were successfully synthesized (Figure
  - isoAsp and Asp as standards to determine bivalirudin degradation (compound 3 and 4, Figure 2).
  - Bivalirudin (compound 1) and glycobivalirudin (compound 2) for stability and efficacy tests
- Stability test shows glycobivalirudin retained a higher percent purity after 3 weeks
- Efficacy test indicates that both peptides exhibit similar thrombin inhibition, and glycobivalirudin may be slightly more potent at lower concentrations, especially 1000 nM

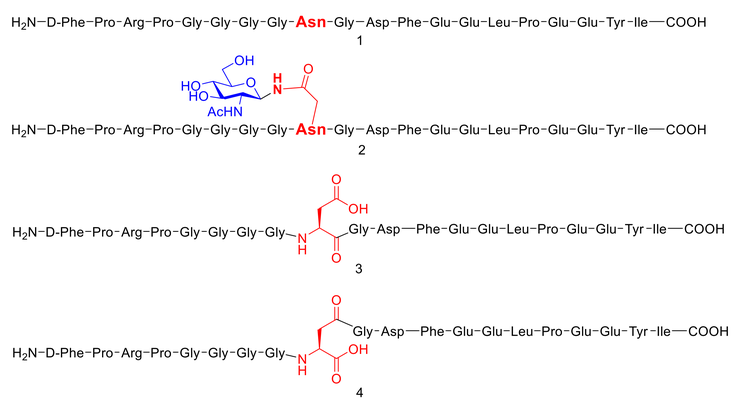


Figure 2. Synthesized peptides for stability test

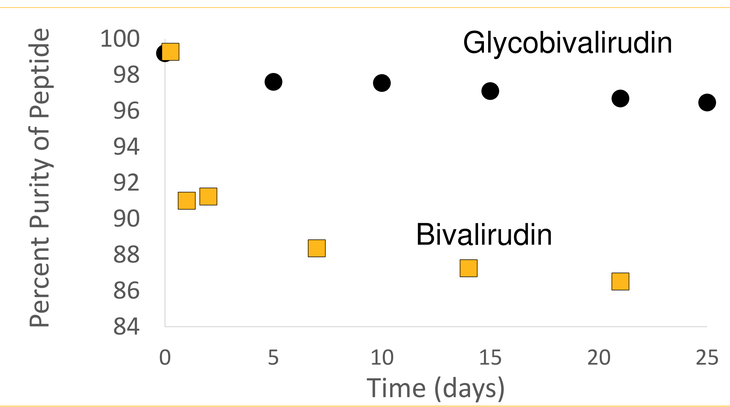


Figure 3. The results of the stability test on HPLC. Glycobivalirudin retained a higher purity over time when stored in a 25 C water bath.

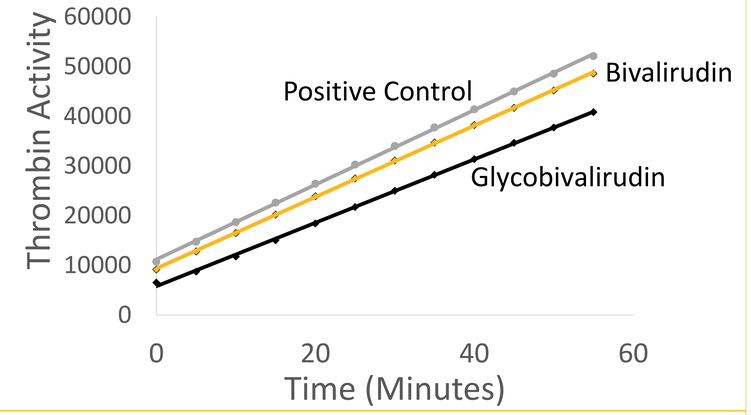


Figure 4. The results of the thrombin-activity assay using 1000 nM for both peptides. While both peptides inhibit thrombin, the glycopeptide was slightly more potent at this concentration.

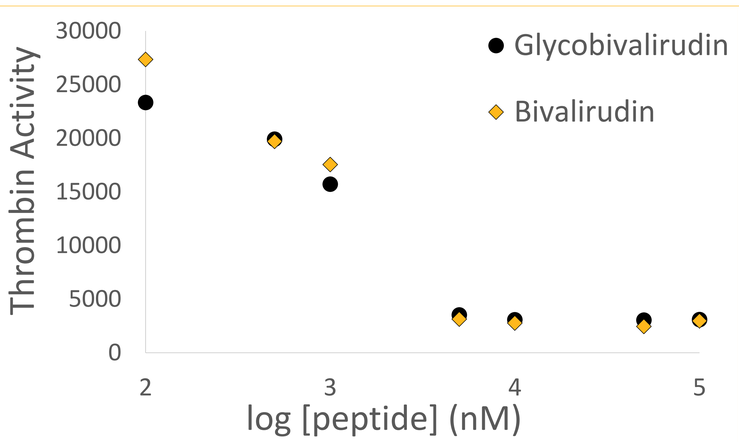


Figure 5. The results of the thrombin-activity assay using 100 nM to .1 mM concentrations of each peptide. Both peptides inhibited thrombin completely from 5000 nM and above, and both showed similar inhibition at the lower concentrations.

Discussion

- Stability test can be improved by using human blood serum. This test would accelerate peptide degradation and provide an indication of the glycopeptide's stability in blood
- Thrombin-Activity test can be improved by testing more concentrations between 100 and 5000 nM
- Other glycosylation methods can be compared to determine optimal glycosylation method

Conclusions

- Glycoamino acid and modified glycine amino acid were successfully synthesized and incorporated into bivalirudin analogs
- Glycobivalirudin, bivalirudin, and two other related peptides successfully synthesized
- The glycopeptide showed improved stability and equivalent thrombin-inhibiting efficacy compared to the original drug
- Further work can determine if the glycosylation method improves stability while retaining efficacy in other small peptide drugs

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References

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(3) *Bioorg. Med. Chem. Lett.*, **2011**, *21*, 4973–4975.
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