

Design, synthesis and pharmacological evaluation of novel INSL3 antagonists

Fazel Shabanpoor^{1, 2*}, R. Anthony Hughes³, Frances Separovic², John D. Wade¹

¹ Howard Florey Institute, ² School of Chemistry, ³ Department of Pharmacology, University of Melbourne, Victoria 3010, Australia
E-mail: fazel.shabanpoor@florey.edu.au

Introduction

Insulin-like peptide 3 (INSL3) is a circulating peptide hormone belonging to the relaxin-insulin superfamily of peptides, which comprises: insulin; relaxins -1, 2, 3; INSL3, INSL4, INSL5, INSL6, and INSL7 and insulin-like growth factor I and II [1]. INSL3 consists of two chains (A and B) connected via two inter- and one intra-chain disulphide bond [2]. INSL3 has been shown to play important roles in the reproductive system, including promotion of germ cell maturation, and the growth of the gubernaculum resulting in trans-abdominal migration of testes into the scrotum [3]. Recent structure-activity studies in our laboratory have led to identification of cyclic peptide mimetics of the INSL3 B-chain that act as INSL3 antagonists [4,5]. The most potent of these peptides has been shown to compete with the binding of ¹²⁵I-INSL3 to its G-protein coupled receptor RXFP2 also known as LGR8 expressed in HEK293 cells ($pK_i = 6.68 \pm 0.08$, $n = 3$). We have further demonstrated that some of these peptides antagonise cAMP mediated signalling in these cells (i.e. they act as INSL3 antagonists). For one of these peptides, we have shown that it can decrease testicular weight in rats when administered locally *in vivo*, consistent with an ability to increase apoptosis of spermatozoa [4]. In the present study we have designed and synthesised a wide range of INSL3 mimetics in an attempt to identify novel low molecular weight mimetics of INSL3 with higher affinity for RXFP2 which can be used as lead compounds to develop clinically useful fertility control drugs.

Results and Discussion

An *in silico* structure-based drug designing approach was used to design INSL3 B-chain analogues. Four different series of analogues were synthesised on solid phase using microwave power (A: disulfide constrained, B: lactam constrained, C: helix-inducing N-caps, D: helicogenic residues). The cyclization of disulfide constrained analogs was carried out in 0.1 M NH_4CO_3 (1mg/3ml) using dipyrindyl disulfide (DPDS, 5 eq). The lactam bond was formed between the side-chains of Lys or Dab and Glu or Asp using four different cyclization strategies. Use of either HBTU or DIC with HOAt as coupling reagent failed to form amide bond between the side chains of the residues mentioned above and, thus, the desired cyclic peptide could not be obtained. However, use of PyBOP in combination with HOAt did give the cyclic peptide, although the reaction rate was slow taking more than 24 h for the reaction to go to completion (Fig1.A). Finally, HBTU was used as coupling reagent in the cyclization reaction that was carried out using microwave power (25 W, 75 °C). After 10 min the reaction mixture

was monitored by HPLC which showed the absence of starting linear peptide indicating the completion of reaction (Fig.1.B).

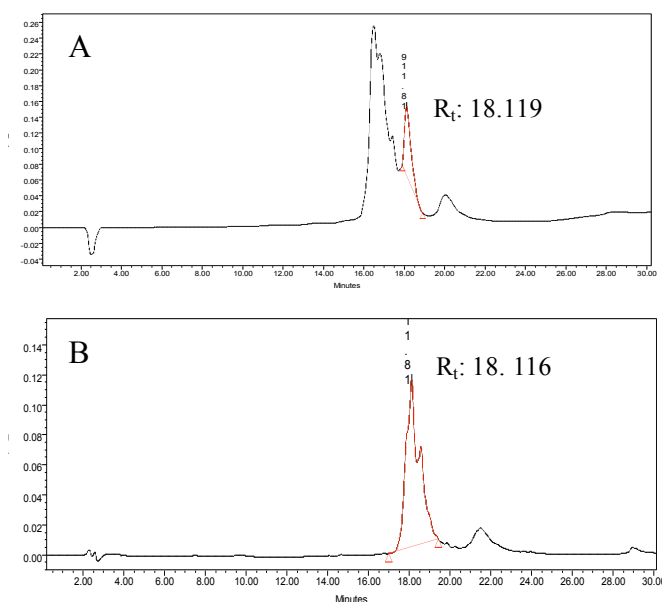


Fig.1.A: HPLC chromatogram of lactam bond formation between the side chains of Lys and Asp using PyBOP and HOAt after 24 h. B: Lactam bond formation using HBTU as coupling reagent using microwave irradiation (75 °C).

The secondary structure of each synthetic INSL3 B-chain mimetics was determined using circular dichroism (CD) spectroscopy. All the peptides showed very low level of helicity in phosphate buffered saline (PBS) at pH 7.4 but the level of helicity significantly increased upon addition of 20% TFE which is known to increase helicity by providing a low dielectric environment that favors the formation of intra-helix hydrogen bonds [6].

Since neither disulfide nor lactam constraints managed to stabilize INSL3 B-chain, we decided to graft the key receptor binding residues which are located in the helical region of INSL3 B-chain on an alternative scaffold such as β -hairpin (Fig.2) which has previously been used to mimic the α -helix moiety [7].

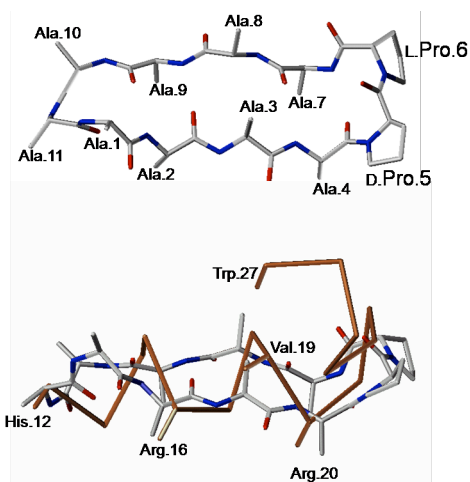


Fig.2. β -hairpin with D-Proline-L-Proline template (top); overlap of the INSL3 B-chain backbone onto the design β -hairpin showing the similar positioning of the binding residues both on the β -hairpin scaffold and helical region of the B-chain (bottom).

The β -hairpin mimetics were synthesised on histidine pre-loaded chlorotrityl resin using a CEM Liberty microwave peptide synthesizer. The peptides were cleaved from the resin using 2% TFA and the crude linear peptides were dissolved in DMF (5mg/ml) and then cyclized by using HBTU (3 eq) and DIEA (3% v/v) and stirring at room temperature. RP-HPLC analysis showed that the cyclization reaction was completed in less than five minutes as there was no linear peptide remained. The secondary structure of these compounds remains to be determined.

Our future direction is to determine the receptor binding affinity of these analogs which dictate our future direction toward further refining and possibly designing next generation of INSL3 mimetics.

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