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Conformational Changes in Testis Specific Homing Peptide in Polar and Apolar Environment

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For therapeutic agents to exert their pharmacological effects, need to cross the biological membranes and enter into the systemic circulation to reach the site of action. A 16 amino acid long testis specific THP3 peptide identified using phage display approach was synthesized by solid phase peptide synthesis, purified using RP-HPLC and characterized by mass spectrometry (MALDI). The CD spectra of THP-3 peptide obtained in water and apolar solvents like trifluroethanol and hexafluroisopropanol. The peptide in water adopted mostly beta and coiled structures. Addition of apolar solvents led to significant changes in CD spectra of peptide due to decrease in dielectric constants. These changes in conformations of testis specific homing peptides may be play a role in molecular interaction of peptide and plasma membrane of the testicular cells.

Key words: Blood-testis barrier, Peptide, CD spectra, Conformation, Ellipticity.

To safeguard specific organ function, the body must maintain compartments with a controlled environment and gradients of different solutes and ions. To sustain these gradients, organs have layers of cells forming barriers between the environment and the organ tissue (Tscheik *et al.,* 2013). Endothelial cells lining blood vessels help forming the blood-brain barrier (BBB) or the blood-retina barrier (BRB). Blood-testis barrier (BTB) is formed by the Sertoli cells, while the Schwann cells electrically seal the myelin sheaths of neurons. The common function of these barrier-forming cells is to regulate the transport of water, nutrients, ions, and other solute molecules, in order to maintain the organ-specific environment in the steady-state. In addition, efflux transporters facilitate the removal of noxious agents and metabolic end products from the organ tissue. However, these functions may impede the uptake of many drugs affecting their bioavailability in specific compartments. Novel approaches to improve targeted drug delivery have been introduced lately. The identification of therapeutic agents has been sometimes compromised by their biological behavior following administration. Therapeutically effective drug needs to possess favorable characteristics to cross the biological membrane barriers into the systemic circulation and reach the site of action (Salama *et al.,* 2006). The major pathway for absorption or transport of a drug depends on its physicochemical characteristics as well as the membrane features. In general, lipophilic drugs cross the biological membrane transcellularly while hydrophilic drugs cross the membrane paracellularly. In order to ameliorate drug absorption via the transcellular pathway, the physicochemical features of the drug have to be manipulated (lipophilicity, pKa, conformation, H-bond characteristics, etc.) or the membrane

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characteristics have to be altered (Salama *et al.,* 2006).

The blood-testis barrier (BTB) in mammals refers to the specialized junction structures found between Sertoli cells in the seminiferous epithelium located closely to the basement membrane (Dym and Fawcett 1970; Setchell B 1980; Mruk and Cheng 2004). Unlike blood-brain and blood-retina barriers which are composed of TJ between the capillary endothelial cells, the BTB is constituted by coexisting TJ, actin-based adherens junctions (AJ) and intermediate-filament-based desmosome-like junctions. The BTB also divides the seminiferous epithelium into the basal and the apical compartment and is crucial for spermatogenesis by limiting the access of hormones, electrolytes, nutrients and other biological substances to the developing germ cells from the systemic circulation (Dym and Fawcett 1970; Setchell B 1980; Cheng and Mruk 2002). It also forms an immunological barrier to segregate post meiotic germ cell antigens from the host immune system, creating a unique environment for germ cell development (Mruk and Cheng 2004; Bart *et al.,* 2002; Griswold *et al.,* 1998). However, this barrier also creates a natural obstacle for delivering substances across the BTB, including contraceptives. If a delivery vehicle can be developed to reversibly and transiently disrupt the BTB, it will become a valuable tool to target contraceptives behind the BTB to disrupt spermatogenesis. In this study, a testis specific homing peptide was synthesized, characterized and conformation study for the same was undertaken.

MATERIALS AND METHODS

All the solvents and chemicals used were of analytical grade. 9-fluronyl methoxycarbonyl (Fmoc) amino acids derivatives, 1-[Bis(dimethylamino) methylene]- 1H-Benzotriazolium hexafluorophosphate(1-)3-oxide (HBTU), m-cresol, 1-hydroxy-benzotriazole (HOBt), Rink Resin (0.42 mmol/g loading efficiency) were purchased from Novabiochem (Germany). Thioanisole and Ethanedithiol were purchased from Sigma-Aldrich, USA. Piperidine, N, N-dimethylformamide (DMF), diisopropylethylamine (DIPEA), Trifluroethanol (TFE), 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP), trifluoro-acetic acid (TFA) and 1, 3-diisopropylcarbodiimide (DIC) were purchased from SD fine chemicals, India.

THP-3 peptide, (NH_2-SGRYVPDYHWQHGGGC-$CONH_2$) was identified after four rounds of *in-vivo* biopanning using Ph.D.-12™ Phage Display Library Kit (New England Biolabs, USA). Peptide synthesis

THP3 peptide was synthesized on the Rink-amide resin using standard solid phase Fmoc-Chemistry. First amino acid (6 equimole) was coupled by symmetric anhydride method using DIPC (3 equimole) as coupling agents and DMAP (0.1 equimole of loading efficiency of resin) was added to catalyze the coupling reaction. Subsequent amino acids were coupled using HBTU and HOBt activation after N-deprotection of anchoring amino acid using 20% piperidine in DMF (v/v). N-deprotection and coupling were checked by Kaiser Test (Kaiser *et al.,* 1970).

Deprotection and cleavage of peptide from resin

After completion of the desired sequence synthesis, peptide (THP-3) was cleaved from rink amide resin using TFA ,thioanisole, ethanedithiol, water and m-cresol in the ratio of 82.5: 5: 2.5: 5:5 and peptide was precipitated in chilled dry diethyl ether.

RP-HPLC Purification

The peptide was analyzed on analytical column (Phenomenex, Luna, 5µ, C18, 150 x 4.6 mm) and purified on semi preparative reversed phase column (UltroPac column TSK, 10µ, C18, 300 x 7.8 mm) using HPLC (Shimadzu, Japan). Binary gradients of water and acetonitrile having 0.1 % TFA (v/v) were employed for analysis as well as purification with 1.0 ml/minute flow rate and absorption (in aufs) was monitored at 214 and 280 nm using photo diode array detector (PDA).

MALDI -Mass Spectrometry:

The peptide peak-fraction in HPLC was concentrated in speed-vac concentrator and analyzed for expected mass of the peptide using MALDI spectrometry.

Circular Dichroism (CD) Spectroscopy

CD spectra of THP-3 peptide solutions (0.1 mg/mL concentration) were recorded in wavelengh range of 190-250 nm using CD spectropolarimeter (J-810 Model, JASCO, Japan) with rectangular quartz cell of 0.1cm pathlength cell. The CD machine was calibrated using d-10 campho sulphonic acid (60mg/100ml water). The

CD spectra were expressed in molar ellipticity, [è], in degcm^{2}dmol^{-1} calculated using the equation [è] = 100ø/c.l, where ø is observed ellipticity (millidegree), c is the peptide concentration (mole/liter) and l is the pathlength (cm). Data analysis and acquisition were performed using inbuilt spectra manager software. An average of four scans was recorded with scanning speed of 100 nm/minute and response time was 1 second. The CD spectra were recorded in water, TFE and HFIP to ascertain the conformation acquired by the peptide in polar and apolar environment.

RESULTS

Testis specific peptide THP-3, NH_2-SGRYVPDYHWQHGGGC-$CONH_2$ was identified by employing four rounds of *in-vivo* biopanning in Swiss albino mice using 12-mer Phage display library. Using standard solid phase Fmoc chemistry, THP-3 peptide was synthesized. The coupling of each amino acid was monitored using Kaiser test wherein primary amine group reacts with ninhydrin results in blue coloration. The purification analysis of peptide was carried out by RP-HPLC. The purified peptide was collected by fractionation of major peak corresponding to retention time of 29-30 min in 40 min linear gradient (Figure-1).The characterization of peptide was carried out by mass spectrometry as shown in Figure-2 that resulted in mass of 1830.90 Da (expected mass 1817.5 Da). CD spectra recorded to elucidate the conformation of THP-3 peptide in water (polar) and membrane mimicking apolar environment using solvents like TFE and HFIP are shown in Figure 3 and 4. The CD Spectra of the peptide (0.1mg/ml) in water (black color line) and increasing concentration of TFE, 50% (red color line) and 99% (blue color line) are shown in figure 3. CD spectrum in water shows a dominant negative minimum at 196 nm with a cross over to have positive band around 225 nm. Addition of TFE (decrease in dielectric constant) concomitantly decreased the 195 nm band and a progressive red shift in minima upto 205 nm was seen. Further it was interesting to observe the inception of a negative shoulder at 218 nm along with 205nm band indicating increase in ordered confirmations in peptide as expected and was further corroborated in quantitative estimates of secondary structures (table 1a) obtained using spectra manager software. The additions of HFIP also have similar changes in the CD spectrum. Figure 4 shows decrease in 195 nm band with progressive red shift to 202 nm. Quantitative analysis also revealed inception of ordered structures with incremental addition of HFIP (Table 1b)

DISCUSSION

Tissue specificity reduces the side effects and dosages of the drugs makes tissue specific drug delivery more promising than the systemic one. Cells have inherent property to internalize the biomolecules present around the cell surface. In this study the phage display library was used to identify the testis specific ligand/peptide sequence by *in-vivo* biopanning approach in mice. The peptide sequence was synthesized using Fmoc chemistry and its C terminal was maintained as Cys amide to help conjugation with drug/ reporter molecules for further studies. The peptide was eluted in about 80% ACN concentration with retention time 29.5 minute which indicates a strong hydrophobic interaction between hydrophobic groups of peptide and C18 column. A hump was appeared in chromatogram (figure 1) at about 30 minutes which might be due to partial dimerization

Table 1 a : Estimated percent % secondary structure of THP-3 peptide in water and different concentration of TFE

Secondary structure	Water	50% TFE	99% TFE
Helix (%)	0.0	0.0	0.0
Beta (%)	64.7	78.7	80.6
Turn (%)	8.3	0.0	0.0
Random (%)	26.9	21.3	19.4

Table 1 b. Estimated percent % secondary structure of THP-3 peptide in water and different concentrations of HFIP

Secondary structure	Water	50% TFE	99% TFE
Helix (%)	0.0	0.0	0.0
Beta (%)	64.7	72.9	81.2
Turn (%)	8.3	3.8	0.0
Random (%)	26.9	23.2	16.9

of peptide disulphide bonds between two molecules of peptide by spontaneous oxidation of thiol groups of cysteine present on C- terminal of the peptide. The molecular mass characterized by MALDI shows the unimolecular (largest peak) and a partial high molecular mass peak of 3659.08 Dalton which conforms to the calculated mass of dimer peptide (3634.0 Dalton)

The structure of ligand determines the possible interaction with target membrane. With membrane interaction, the ligand is structured due to conformation of the peptide backbone in physiological condition (Langelaan and Rainey 2010). There are numerous studies concerning the conformation that peptides adopt in membrane mimetic environments (Nanga *et al.* 2009). At the peptide-membrane interface, both amphipathic helices and turn structures are commonly formed in peptide ligands and both hydrophobic and electrostatic interactions can be responsible for membrane binding (Lucyk *et al.*2006; Langelaan and Rainey 2009; Coutant *et al.* 2007). In this study, THP-3 peptide in aqueous environment acquire mostly of beta structure (as quantitated by spectra manager software). In aqueous solution the peptide also has a considerable quantity of random coil, which might provide flexibility for adoption of any type of ordered structures in changed environments as happen in a biological system. The Near UV CD spectra provide information on the conformational environment of the aromatic side chains in the peptide backbone that have been used in following tertiary structure (functional structure) changes as a result of perturbations (environment change). Figure 5 shows near UV CD spectra of peptide in water and 99 % HFIP. The CD spectrum in water have a undefined minimum around 280 nm which become pronounced on addition of HFIP suggesting different conformational environment

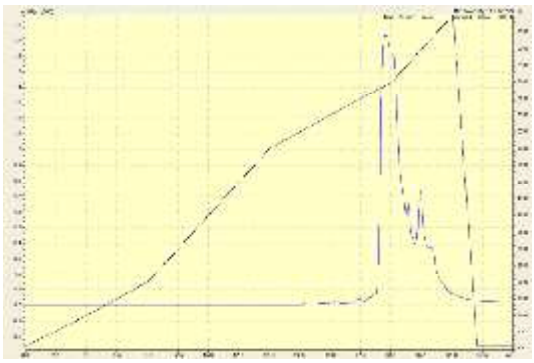

Fig. 1. Semi-preparative RP-HPLC of THP-3 peptide at 280 nm using C-18 column. Red encircled area represents desired peak

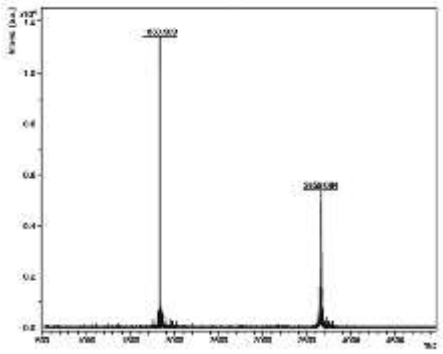

Fig. 2. Mass spectrum of HPLC purified THP-3 peptide showing charged fragments. Observed mass of desired peak: 1830.90 Expected mass: 1817.90. A fragment of mass 3659.08 represents the dimer of desired fragment

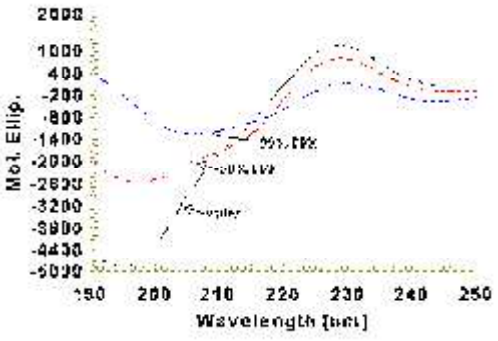

Fig. 3. Spectra of THP-3 peptide in polar and apolar solution :water, 50% TFE and 99 % TFE

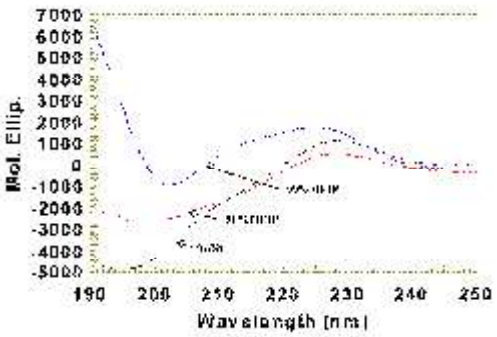

Fig. 4. Spectra of THP-3 peptide in polar and apolar solution :water, 50% HFIP and 99 % HFIP

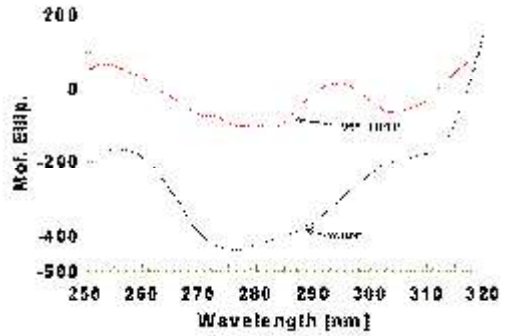

Fig. 5. Spectra of THP-3 peptide in near UV wavelength in polar and apolar solution :water, and 99 % HFIP

of the aromatic residues in peptide backbone.

The CD spectra of peptide were recorded in the increasing concentrations of TFE and HFIP to deduce the inherent propensity of the peptide for a particular secondary structure in apolar environment. Organic solvents like TFE and HFIP are widely used in such studies to create a membrane mimicking apolar environment and elucidate the conformations adopted by the peptides (Haney and Vogel, 2009). TFE is used as a solvent to induce ordered conformations in peptide/ protein backbone, particularly helical structures (Shiraki *et al.,* 1995; Yang *et al.,* 1995). Alcohols weaken non-local hydrophobic interactions and enhance local polar interactions (i.e., hydrogen bond) of proteins (Shiraki *et al.,* 1995). The effect of additional fluorine substitution on additional alkyl group in HFIP enhances its effect as a denaturant as well as a helix inducer in comparison to TFE.

When we added TFE and HFIP there was substantial increase in beta structure instead of alpha helix. Although, TFE and HFIP which are known helix inducers, there was no relative decrease in amount of random coil. We hypothesized that the THP-3 peptide exploits the random coil portion for binding and interaction with testicular cell membrane. This interaction might help in binding of the peptide to the plasma membrane of the testicular cells. The percentage of beta conformation increases in TFE and HFIP, it indicates that when peptide interacts with cellular membranes it will acquire a regular conformation and get stabilized. Even though the HFIP is strong helix inducer, only a small amount of helix (1.9%) was induced in 99% HFIP which may helps in interaction of peptide with testicular cells.

In conclusion, this testis specific peptide likely to be used for specific testicular delivery has shown to adopt ordered as well as random coil conformations in water. The presences of random coil conformation in a biological peptide are equally important to provide flexibility to the molecule to help acquire functional conformation in the changed environment. This was mimicked in present study using polar solvents that resulted different CD characteristics of same peptide suggesting different backbone conformation. Near-UV CD indicates different conformational environment as depicted in side chain CD of aromatic residues present in the peptide of the peptide. This study appears to establish and interactions backbone may be used 1) as a direct carrier of therapeutic agents which increase the concentration of the drugs in locality of the testis results in maximum bioavailability of the drugs, 2) as an intracellular drug delivery agent when ligated to a CPP and 3) as a vehicle of imaging agents.

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