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SUMMARY OF THE THESIS

In the present thesis, we attempted to explore the structural characteristics of two immunostimulating tetrapeptides: rigin[41] (Gly-Gln-Pro-Arg) and tuftsin[3] (Thr-Lys-Pro-Arg) and a few designed synthetic analogs and derivatives. The two pharmacologically important immunomodulators have been suggested to bind to their own specific receptors and induce distinct biological responses related to diverse immunological functions.[39,45-48] While tuftsin molecule has been extensively investigated for its structure-activity relationships, the research work on rigin has received relatively much limited attention. The existing literature, briefly reviewed in Chapter 1, indicate that there is only one instance: *c*(Thr-Lys-Pro-Arg-Gly) which displayed significant bio-activity than the native tuftsin.[97] The fact that [β-Ala^3]tuftsin analog also showed significant biological activity (~ 70%), suggests that the Pro residue in tuftsin can be substituted suitably to retain substantial biological activity.[64] Therefore, to develop more efficacious analog of these immunomodulators, it is important to establish its preferred solution conformation, likely to be recognized by their specific protein receptors. In the absence of any X-ray crystallographic information, the determination of three-dimensional structure of rigin, preferably in different solvent conditions, may be a prerequisite for structure based design. Here, it may be highlighted that the structure of receptor bound conformer of a bioactive peptide is often very close to the solution conformation(s) however, some times, the conformation of the molecule bound to its receptor may be distinct.

The Chapter 2 describes the chemical synthesis, purification, characterization and conformational analysis of a few designed synthetic analogs and derivatives of rigin and tuftsin. have been described. Different racemization free strategies of peptide bond formation have been employed. In a few cases, phosphonium coupling reagents: BOP and PyBOP, have been used where the conventional coupling methods have failed to give desired products.[312] The peptides were purified by silica-gel column chromatography, MPLC and/or HPLC methods and characterized by their melting points, R_f values, $[\alpha]_D^{25}$, ESI-MS. Both, the purity and identity of the peptides were ascertained by 1D and 2D ^{1}H NMR spectroscopy.

The Chapter 2 also provides a brief account of the theoretical methods *i.e.*, high-temperature MD simulations, under *implicit* solvent conditions using the representative dielectric constants of dimethylsulfoxide ($\varepsilon = 45$) and water $\varepsilon = 80$). The molecular modeling programs like TINKER and InsightII have been selectively utilized. To substantiate the theoretical results, the experimental techniques: 1D- and 2D ^{1}H-^{1}H NMR and/or CD spectroscopy have been simultaneously employed to establish the preferred solution conformations.

One of the principal goals of our investigation was to execute systematic MD simulations of rigin in *implicit* solvents and compare its conformational characteristics acquired experimentally under different environmental conditions. Previously, employing high temperature quenched MD simulations under the influence of distance dependent dielectric ($\varepsilon = r_{ij}$), we elucidated the conformational preferences of rigin as a tightly folded *distorted* type III β-turn structure stabilized by a salt-bridge *i.e.*, Gly $H_3N^+ \cdots COO^-$ Arg interaction. The study from this laboratory also established a synergy between the high-temperature MD simulations in *implicit* DMSO and 1D- and 2D ^1H NMR derived parameters acquired in DMSO-d_6 solution.[124] In Chapter 3 we further investigated the conformational features of rigin *via* high temperature MD simulations in *implicit* water and the experimentally derived 1D- and 2D ^1H NMR parameters acquired in aqueous PBS supported the preferred molecular structure. The combined results allowed us to propose that the preferred solution conformation of the tetrapeptide rigin may be a type VII β-turn structure accommodated across the Gln-Pro segment, not stabilized by an intramolecular interaction. However, the CD data observed in aqueous PBS and non-aqueous organic solvents indicated its strong solvent dependence. On the other hand, available literature indicated that the preferred solution conformation(s) of tuftsin could be an ensemble of β-turn and/or γ-turn structures, stabilized by a strong intramolecular hydrogen bond. In this Chapter we also emphasized that the CD curves of rigin and tuftsin, acquired under similar solvent conditions, did not allow us to extract structural information related to the specific turn types.

The conformational analysis of retro-rigin: Arg-Pro-Gln-Gly and [β-Ala^3]tuftsin analog are described in Chapter 4. The results of theoretical conformational analysis *via* MD simulations in *implicit* DMSO, in combination with experimental 1D- and 2D ^1H NMR derived parameters acquired in DMSO-d_6, revealed that retro-rigin tends to adopt a folded conformation, stabilized by a *classical* 4→1 intramolecular hydrogen bond *i.e.*, Arg C=O···H-N Gly interaction, and a salt-bridge involving the terminal residues *i.e.*, Arg $H_3N^+ \cdots COO^-$ Gly interaction. The preferred torsion angles: $\psi_{Arg} \sim 150°$, $\phi_{Pro} \sim -65°$, $\psi_{Pro} \sim 0.0°$, $\phi_{Gln} \sim -135°$, $\psi_{Gln} \sim 0.0°$, $\phi_{Gly} \sim 180°$ may represent an ensembles of solution conformation(s). Moreover, a comparative CD analysis of retro-rigin and rigin provide further support that retro-rigin favours relatively more folded structures as compared to rigin, under similar experimental conditions.

The overall results of theoretical (*via* MD simulations in *implicit* DMSO) as well as experimental (*via* 1D- and 2D ^1H NMR parameters acquired in DMSO-d_6) conformational

analysis performed on [β-Ala^3]tuftsin analog indicated that the torsion angles: $\psi_{Thr} \sim 180°$, $\phi_{Lys} \sim -90°$, $\psi_{Lys} \sim -70°$, $\phi_{\beta\text{-}Ala} \sim 175°$, $\theta \sim 55°$, $\psi_{\beta\text{-}Ala} \sim -95°$, $\phi_{Arg} \sim -100°$ may characterize the significantly folded solution conformation(s). However, the folded molecular topology may not be stabilized by any intramolecular interaction. The analysis of CD curves of [β-Ala^3]tuftsin in the solvents of varying polarities showed its strong solvent dependence nevertheless, in polar structure promoting organic solvent: TMP, the analog predominantly preferred an ordered non-random structure.

Extensive literature on structure-function relationship of tuftsin indicated that each chemical entity in tuftsin molecule is critically important for its biological functions.[39,45-48] Therefore, the design strategy we adopted for synthesizing the analogs of tuftsin and rigin involved incorporation of a long hydrophobic tail at the C-terminus. The two analogs of tuftsin: Thr-Lys-Pro-Arg-Pda (T1) & Thr-Lys-Pro-Arg-β-Ala-Pda (T2) and two analogs of rigin: Gly-Gln-Pro-Arg-Pda (R1) & Gly-Gln-Pro-Arg-β-Ala-Pda (R2) have been synthesized. The details of chemical synthesis of these analogs have been described in Chapter 2 and the conformational studies using CD spectroscopic technique, are reported in Chapter 5. Noteworthy, while emphasizing the potential therapeutic usefulness of tuftsin, this molecule was covalently modified by linking a palmitoyl residue at the C-terminus *via* ethylenediamine as a linker. This particular analog in fact, facilitated its incorporation into liposomes, for targeting to specific cells bearing tuftsin receptors. In our design strategy of tuftsin and rigin analogs, the hydrophobic chemical entities like $-(CH_2)_{14}-CH_3$ (T1 or R1) and $-\beta\text{-Ala}-(CH_2)_{14}-CH_3$ (T2 or R2) of were incorporated at the C-terminus. Unlike ethylenediamine, the β-Ala amino acid is considered to be a non-toxic and metabolizable non-proteinogenic residue, widely distributed in animal and plant kingdoms.

The CD spectra of native tuftsin and rigin in aqueous PBS showed the appearance of a weak negative shoulder at ~ 234 nm, attributed to a small proportion of non-random ordered structure(s). An overall comparison of CD curves of native tuftsin and T1 in the solvents of varying polarities: aqueous PBS, TFE and TMP, suggest that the long hydrophobic tail can influence the structural characteristics of tuftsin particularly, in polar organic environment and promotes and/or stabilize the secondary structural features. Although, the observed CD spectral patters of T1 in three different solvents compare well with those of T2 nevertheless, the presence of β-Ala residue further promotes the preferred solution conformation of the tetrapeptide.

The analyses of CD spectral features of native rigin and R1 indicate that hydrophobic moiety exerts significant influence on the preferred solution conformation, presumably by promoting the ordered secondary structures particularly in organic solvents studied. Also, a comparison of CD spectra of R1 and R2 in the three solvents, suggests that the presence of β-Ala moiety does not influence and/or alter the preferred solution conformation of native rigin and the stabilizing effect is more pronounced in structure promoting organic solvents.

The results of analysis of different cytokines: TNF-α, IL-6, IL1α and IL1β, indicative of macrophage activation, suggests that an unsubstituted non-proteinogenic β-Ala residue may be an acceptable replacement for the L-proline residue in the design strategy of these two immunomodulators. The conformational analysis of retro-rigin *i.e.*, its strong preference for the folded β-turn structures, in conjunction with its ability to induce cytokine release (similar to the those reported for retro-tuftsin[99]), suggests that β-turn topology may also be worth considering in future design plan for these two immunomodulators. The covalent incorporation of a long hydrophobic tail *i.e.*, Pda moiety, at the C-terminus of tuftsin and rigin, appears to be an attractive approach for selective inductions of specific cytokines and therefore, merit further consideration for designing improved bioactive derivatives of these two inmmunomodulators.

One of the major objectives of the present investigation has been to synthesize novel analogs/derivatives of the two structurally and functionally related immunomodulator: tuftsin and rigin and examine their conformational preferences employing theoretical as well as experimental methods. We believe that in the absence of any X-ray crystallographic analysis, the available conformational models may provide means of initiating the design of newer analogs and derivatives of tuftsin and rigin capable of exhibiting significant immunomodulating properties.