TITLE OF Ph.D. THESIS

"Designing, Syntheses and Characterization of Chimeric Peptides of Opioids and Anti-opioids and their Biological Activities"

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Abstract

Opioids are morphine like compounds and work as analgesics in the body on interaction with the opioids receptors by inhibiting transmission of pain stimulus to the central nervous system (CNS). These opioid receptors together with opioid peptides form the Endogenous Opioid System. Opioids inhibit transmission of pain stimulus to CNS by two well established actions on neurons viz; presynaptic and postsynaptic inhibition of pain transmission. However, the prolonged use of opioids also induces certain undesirable side effects due to which the efficacy of opioids as analgesics is minimized. The work in the thesis has been divided into two parts:

Part A: Opioids Induced Side Effects: Tolerance, Cross-tolerance and Physical dependence, and

Part B: Nanoparticles as delivery system to enhance the bioavailability of the opioid peptide.

Part A describes about the side effects induced by the prolonged use of opioids like tolerance, cross-tolerance and physical dependence. The classical approach proposed to account for these side effects includes opioid receptor desensitization, down regulation and internalization and brings about changes at the opioid receptor level in the body. However, the inability of these classical approaches to completely explain all aspects of tolerance and physical dependence has led to an alternative model called the 'Anti-opioid model for tolerance and physical dependence'. Repeated administration of opioids activates 'anti-opioid system' which then releases anti-opioid peptides bind anti-opioid receptors, to neutralize or compensate the effects of opioids in order to maintain body's equilibrium or homeostasis. There is now considerable evidence to indicate that these anti-opioid peptides play an important role in the development of tolerance and physical dependence in the body. It is thus interesting to understand how anti-opioid system interacts with the opioid system in the body. In the present work an attempt has been made to design such a peptide based on the concept of overlapping sequences of Met-enkephalin (YGGFM-opioid moiety) and FMRFa (anti-opioid moiety) present in endogenously occurring chimeric peptide Met-enkephalin-Arg^6-Phe^7 (MERF) and 'anti-opioid model' hypothesis. The peptide was designated as Chimeric peptide-YFa (YGGMFMKKKFMRFa) was joined the two sequences by three lysine residues to obtain distinct activity of both the sequences. YFa was synthesized by solid phase peptide synthesis, desalted by gel-filtration chromatography, analyzed and purified by reverse phase HPLC and characterized by MALDI and protein/peptide sequencer. The present study examines the tolerance and cross-tolerance effects of YFa (80 mg/kg) in relation with the standard opioid morphine (20 mg/kg) and then compared the same effects with the structurally and pharmacologically similar peptide DynA(1–13) (80 mg/kg), by measuring their analgesic effects after pretreatment with either YFa or morphine or DynA(1–13) by tail-flick test in mice. YFa did not induce tolerance and cross-tolerance effects to its analgesic action on day 5 after pretreatment with either YFa or morphine for 4 days. However, pretreatment with YFa for 4 days led to the development of cross-tolerance to the analgesic effects of morphine and also 4 days of pretreatment of morphine resulted in the expression of tolerance to its own analgesic effects. Similar expression of tolerance and cross-tolerance were also observed when YFa was compared with the κ opioid receptor agonist peptide DynA(1-13). Cross-tolerance effects between YFa and DynA(1-13) analgesia were also not observed on day 5. Interestingly, when YFa and DynA(1-13) were tested for their analgesic effects for 5 days, reduction in analgesia on day 3 was observed in case of DynA(1-13) whereas YFa maintained its analgesia for 5 days. This is due to the presence of anti-opioid sequence in YFa which may be acting as a putative antagonist to anti-opioid receptors or indirectly stimulating the opioid receptors for the observed analgesia. Thus, chimeric peptide YFa may serve as a useful probe to understand pain modulation and expression of tolerance and cross-tolerance behavior with other opioids.

Part B describes about the role of nanoparticle based delivery system for the delivery of opioid peptide. Generally, peptides are prone to degradation by proteases and peptidases present in the body and thus limit their bioavailability. Over other approaches, application of

polymeric nanoparticles as drug delivery system appears to be a better method of efficient drug targeting. Nanoparticles (NPs) are solid colloidal particles consisting of macromolecular compounds having 1 to 1,000 nm scale size range and have number of advantages, including high stability in vivo, long-term payload release, and the capability of permeating through small capillaries and into cellular tissues.

Although there are various techniques for the preparation nanoparticle based delivery system but in recent years, self assembly of proteins/peptides/drug candidates with natural or synthetic polyelectrolyte to form Polyelectrolyte Complexes (PECs) on a colloidal level generating optically homogeneous and stable nano-dispersions has drawn great interest. Furthermore, these do not require sonication and organic solvents during preparation, thereby minimizing possible damage to drug candidates during PEC formation in comparison to other procedures. Such type of complex formation depends on many factors including coulombic interactions, hydrophobicity of the polymer–molecule pair, and the conformational features of the polymer. The size of nanoparticulate/PEC species is critical for cell binding and internalization and Zeta potential is a surrogate marker for the colloidal stability of PECs/NPs. Both of these factors are important for an efficient nanoparticulate delivery system.

In the present study we have employed polyelectrolyte complexes based nanoparticles formation strategy to develop a nanoparticle delivery system of chimeric peptide-YFa of Met-enkephalin and FMRFa to prove its enhanced efficacy in comparison to the bare peptide. Preliminary studies were performed to understand the Polyelectrolyte complexes (PECs) nanoparticles formation between polyanionic polymer of sodium salt of polyacrylic acid (PAA) and cationic model peptides containing varying number of lysine residues. These peptides and peptide YFa were synthesized by solid phase method, desalted by gel-filtration chromatography, analyzed and purified by reverse phase HPLC and characterized by MALDI and protein/peptide sequencer. Nanoparticles were formed on self assembling of cationic peptides with PAA at different charge ratios ($Z_{+/-}$). The morphology of these kinds of nanoparticles is mainly governed by the composition of the complexes, which can be expressed as $Z_{+/-}$ i.e., the ratio of positively charged units of cationic peptides to the concentration of anionic units of the polymers present in the system. In the present study, at lower $Z_{+/-}$, the particles were elongated in shape but adopted spherical shape of 75-100 nm in diameter at higher $Z_{+/-}$ values, in atomic force microscopy (AFM). This study proposes a way that the polyelectrolyte nanoparticles containing cationic peptides obtained by this methodology can form such type of nanoparticles with other biologically active cationic peptides for their use in biological applications.

On the basis of nanoparticles formation by PAA and cationic peptides we investigated the performance of polyelectrolyte complexes based nanoparticles in improving the antinociceptive (analgesic) activity of cationic chimeric peptide-YFa at lower dose. Size of the nanoparticles decreases and zeta potential increases with concomitant increase in $Z_{+/-}$. The nanoparticles at $Z_{+/-}$ 12 are spherical with 70±7 nm diameter in AFM and displayed positive surface charge and similar sizes (83±8 nm) by Zetasizer. The nanoparticles of $Z_{+/-}$12 are used in this study. Entrapment efficiency of approximately 90% of the peptide in nanoparticles was determined by reverse phase HPLC. Cytotoxicity by MTT assay on three different mammalian cell lines (liver, neuronal and kidney) revealed lower toxicity of nanoparticles. Hematological parameters were also not affected by nanoparticles compared to normal counts of water treated control group. Nanoparticles containing 10 mg/kg YFa produced increased antinociception, ~36 %, in tail-flick latency test in mice, whereas the neat peptide at the same concentration did not show any antinociception activity. All the test samples were administered intraperitoneally (IP). This enhancement in activity is attributed to the nanoparticle associated protection of peptide from proteolytic degradation. *In vitro* peptide release study in plasma determined by reverse phase HPLC also supported the antinociception profile of nanoparticles. YFa resembles endogenously occurring opioid peptide the dynorphins therefore it may also show similar pharmacological action like dynorphins through peripheral mechanisms. In fact, the early onset of antinociceptive effect in just 5 min and localization of fluorescent nanoparticles in lungs by administration of nanoparticles favors for the peripheral opioid receptors stimulation by YFa and then subsequent action through central mechanisms. Thus, our results suggest of a potential nanoparticle delivery system for cationic peptide drug candidates for improving their stability and bioavailability.