PHARMACOLOGICAL STUDIES OF CHIMERIC OPIOID PEPTIDE

DISSERTATION

SUBMITTED FOR THE PARTIAL FULFILMENT OF DEGREE OF

Master of Science

(Biotechnology)

by

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Candidate's Declaration

I, hereby declare that the work presented in this thesis entitled, **"Pharmacological Studies of Chimeric Opioid Peptide"**, in partial fulfilment of the requirement for the award of the degree of Masters of science in Biotechnology, Department of Biotechnology and Environmental Sciences (DBTES), Thapar University, Patiala, is an authentic record of my work during the period of six months from January, 2013 to June 2013, under the guidance of Dr. Santosh Pasha, Scientist-G, CSIR-Institute of Genomics and Integrative Biology, Delhi and Dr. Abhijit Ganguli, Associate Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree.

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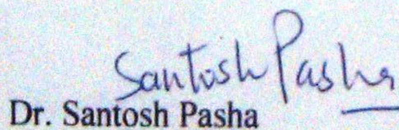
CERTIFICATE

This is to certify that Mr. Simranjeet Singh Bedi, student of M.Sc. Biotechnology, Thapar University, Patiala has successfully completed his dissertation project at Institute of Genomics and Integrative Biology (CSIR) entitled **"Pharmacological studies of chimeric opioid peptide"** under my guidance.

The purpose of his project has been fulfilled and he is now well acquainted with all the techniques of Solid Phase Peptide Synthesis and has gained proficient knowledge for *in vivo* analgesic activity (rat model) and *in vitro* animal cell culture for quantification of receptor expression by FACS studies.

During the project he has come across as a very hard working, diligent and enthusiastic team member. He has very clear understanding of concepts and is efficient for group scientific discussions and always come up with rationale ideas.

I wish him success for his future endeavours.



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Date: 28-06-13

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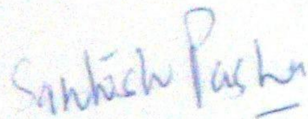

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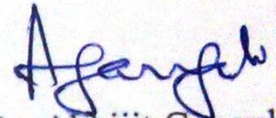

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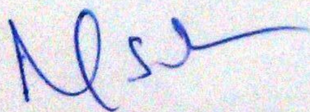

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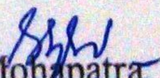

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With the blessings of Mata Sahib Kaur Ji, dedicated to my loving Grandparents

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Date: ***Simranjeet Singh Bedi***

Place:

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ABSTRACT

Opioid peptides are known since ages for their analgesic activity. In the present study, a chimeric opioid peptide was synthesized using Solid Phase Peptide Synthesis. The analgesic effect of the peptide was investigated by tail flick latency assay on male Wistar rats and positive results were observed with the doses of 20, 40 and 60 mg/Kg weight of rat. Pre-treatment with NBI attenuated the analgesia significantly, indicating the involvement of κ-opioid receptors. In tolerance studies, Rats were administered with the chimeric peptide twice daily at a difference of 12 hours and six days data suggested no tolerance development whereas the standard peptide indicated a decrease in analgesia after the day 3 treatment. Further, FACS was carried out using κ opioid receptor expressing transfected CHO cell lines, and the data obtained indicated reappearance of receptors after internalization on binding to the chimeric peptide.

KEY WORDS: Opiods, Antinociception, Tolerance, Solid phase peptide synthesis

Abbreviations:

(i) Amino Acids

Amino acid	Abbreviation	Symbol
Alanine	Ala	A
Arginine	Arg	R
Aspargine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

(ii) Reagents and solvents

BOC	Tertiary butoxycarbonyl
BSA	Bovine serum albumin
DCC	N, N’- Dicyclohexylcarbodiimide
DIPCI	N, N’ –Diisopropyl carbodiimide
DIPEA	N, N’ –Diisopropyl ethylamine
DMEM	Dulbecco's Modified Eagle Medium
DMF	N, N-DimethylFormamide,
EDT	Ethane di-thiol
FBS	Fetal bovine serum
Fmoc	9-Fluorenylmethoxy carbonyl
G418	Geneticin
HOBt	N-hydroxybenzotriazole
PBS	Phosphate buffer saline
SDS	Sodium Dodecyl sulfate
TIS	Tri isopropyl-silane

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1. INTRODUCTION

1.1 Pain

Pain as defined by the International Association for the Study of Pain (IASP) is an unpleasant sensory and emotional experience associated with actual or potential damage, or described in terms of such damage. Pain can come from any part of the body viz. skin, muscle, ligaments, joints, bones, injured tissue, nerves, internal organs or a combination of these types of pain. Pain signals normally travel from the affected part of the body along thousands of specialised nerve fibres, through the spinal cord, to the brain. However, in some cases (for example, pain after a stroke), damage to the brain or to the spinal cord itself can start the pain sensation. The primary purpose of pain is to serve as a warning to the body of impending damage. In normal healthy individuals, primary afferents relay noxious sensory information (e.g. thermal, mechanical and chemical stimuli) from peripheral tissues to the dorsal horn of the spinal cord. Here, these noxious stimuli are transduced into electrical signals and processed, and are subsequently relayed to the brain where they are perceived as painful.

1.2 Natural Pain Killers- *Opiates*

"Among the remedies which it has pleased Almighty God to give to man to relieve his sufferings, none is so universal and so efficacious as opium." - Thomas Sydenham, 1680

Swedish botanist and Father of taxonomy, Carolus Linnaeus in his book, *Genera Plantarum* first classified the opium poppy plant as *Papaver somniferum* meaning 'sleep inducing'. The milky fluid extracted from the seed capsule of the plant is highly narcotic after drying. It finds mention in the historic medical writings of Theophrastus (3rd century B.C.) and has been in use since the ancient times for anaesthesia, analgesia and for treating diarrhoea as well as sleeping disorders. The natural alkaloids found in the resin of *Papaver somniferum* such as morphine, codeine and thebaine etc. which are responsible for the narcotic, pain-killing and ecstatic properties of the plant are known as *Opiates*. The plant contains more than 20 such alkaloids which are

Fig.1. *Papaver somniferum*

extracted from its different parts. The pain relieving effect of opium and pure morphine has been used against severe pain. Alternative medicine systems such as Unani and Ayurveda are known to use Opiates as potent medicines in optimized dosages. The analgesic effect of opiates is due to decreased perception of pain, decreased reaction to pain as well as increased pain tolerance. The alkaloids bind to the receptors (opioid receptors) present in central and peripheral nervous system and gastrointestinal tract and produce the effects such as anti-nociception and euphoria.

1.3 Opioids

The term 'opiates' is now increasingly being replaced by the term 'opioids'. Whereas the term 'opiate' includes only the compounds naturally occurring in the plant *Papaver somniferum*, the term 'opioids' include all the compounds whether natural or synthetic, producing morphine like effects by binding to the opioid receptors. The opioids may be fully natural such as morphine and codeine etc. (opiates), semi synthetic such as hydromorphone and oxycodone created from natural opioids, fully synthetic such as fentanyl and dextropropoxyphene etc. and endogenous opioids such as endorphins and enkephalins etc. produced naturally in the body.

1.4 Tolerance by opioids

Although referred to as 'Magic drugs' since ages, a very selective and careful use of opioids is advised by the World Health Organization. This is because of the potential and often adverse side effects of opioids such as tolerance, dependence, constipation and hyperalgesia. Among all these, tolerance is one of the most commonly encountered adverse side effects and is the focus of the present study. Tolerance may be defined as a pharmacological phenomenon whereby, chronic exposure to the drug diminishes its analgesic effect and creates the need for a higher dose to maintain the effect. The dose response curve in opioid-tolerant animals shifts towards the right relative to that for opioid-naive animals (Fig.2).

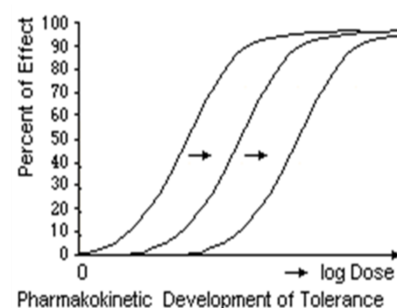


Fig.2 Rightward shift in opioid dose response curve in opioid-tolerant relative to opioid-naive

2. REVIEW OF LITERATURE

Endogenous opioids, receptors for these opioids, mode of action of opioids as analgesics and the tolerance development for opioids during chronic exposure have been an area of intensive research and the following presents a brief outline of the major works carried out so far.

2.1 Endogenous Opioids System: Opioids Receptors and Endogenous Opioids Peptides

2.1.1 Discovery of the Endogenous Opioid system

German chemist Friedrich Sertuner in 1805 isolated morphine from opium in 1805 (Dhawan *et al*., 1996). He named the compound after the name of Greek God of sleep and dreams, '*Morpheus*'. But it was more than a century later that the complex structure of morphine was confirmed through total synthesis by Marshall D Gates, Jr. in 1952 (Gates, 1953). Consistent with the 'receptor theory' established by Paul Ehrlich: *'Corpora non agunt nisi fixata'* ('substances do not act unless bound'), Beckett and Casy in 1954 suggested the existence of pharmacologically relevant receptors that morphine and other opioids bind to show their effect (Beckett and Casy, 1954). In 1965, Portoghese further suggested the existence of different types of opioids receptors for different opioids (Portoghese, 1965). To prove the existence of multiple types of opioid receptors, Goldstein in 1971 proposed to use radiolabelled compounds and in 1973, three different independently but simultaneously showed the existence of different types of receptors for different types of opioids (Pert and Snyder, 1973; Simon *et al*, 1973; Terenius, 1973). In 1976, Martin and co-workers provided first convincing evidence for the hypothesis suggested by Portoghese in 1965 and with the help of their behavioural and neurophysiological observations they proposed the existence of three types of opioids receptors. These receptors were named after the drugs used in the studies: mu (μ, for morphine, which induces analgesia, miosis, bradycardia, hypothermia, indifference to environmental stimuli), kappa (κ, for ketocyclazocine, which induces miosis, general sedation, depression of flexor reflexes) and sigma (σ, for SKF 10,047 or N-allylnormetazocine, which induces mydriasis, increased respiration, tachycardia, and delirium). Kosterlitz and coworkers proposed that a fourth type of opioid receptor, named delta (δ, for deferens), and is present in mouse vas deferens (Lord *et al*., 1977). But later, σ receptors were found to be non-opioid receptors. Thus, it became clear that there are three types of pharmacologically defined opioids receptors: μ, δ and κ. This was further confirmed

by the binding and functional studies by Reisine & Bell (1993), Kieffer (1995) and Satoh & Miami (1995). Exogenous opioids were found to bind with these receptors but a very valid point was put up that as these receptors were present in the body, there must be some endogenous opioids binding to them (Reisine, 1993). In 1975, Hughes *et al* found an endogenous opioid like substance from the rat brain, which was named "enkephalin". Later it was shown that this "endogenous opioid like substance" consist of two penta-peptides with two amino acid sequence (Hughes, 1975): Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu which were named as Methionine–enkephalin (met-enkephalin) and Leucine-enkephalin (Leu-enkephalin) followed by dynorphin A (Cox *et al.*, 1975) and β-endorphin (Li, 1976). Since these peptides were structurally different from the alkaloid opiates, they were referred to as *opioids* to include all non-peptides and peptides with opiate-like activity. All of these endogenous peptides share a common N-terminal tetrapeptide sequence (Tyr-Gly-Gly-Phe) but they differ in their C-terminal sequences and also in their preferential interaction with different Opioid receptors types. Based on this observation Goldstein proposed the common N-terminal sequence as the **'message'** sequence required for activation of Opioid receptors and the unique C-terminal sequences as **'address'** sequence which provide the required affinity for a particular Opioid receptor type (Chavkin, 1981). Later further research led to the discovery of similar endogenous peptides like endorphins (Li, 1976), and endomorphins (Zadina, 1997).

2.1.2 The Endogenous Opioid Peptides

The four major classes (Harrison, 1998) of mammalian peptides are

(a) Enkephalins

(b) Dynorphins

(c) Endorphins

(d) Endomorphins

The polypeptide precursor of the enkephalins, dynorphins and endorphins are proenkephalins-A, proenkephalins-B and proopiomelanocortin respectively. However the polypeptide precursor for recently discovered endomorphins has not been reported so far (Harrison, 1998).

2.1.3 The Endogenous Opioid Receptors

All the opioid receptor types belong to the Gi/Go-coupled superfamily of receptors (GPCRs) and are composed of a single polypeptide chain. The main feature of these proteins is the presence of seven trans-membrane sequences containing hydrophobic amino acid side-chains permitting the integration of the macromolecule into the lipid bilayer of the cell membrane by hydrophobic lipid protein interactions (Corbett *et al*., 1993). All of the opioid receptor types possess the same general structure of an extracellular N-terminal region containing N-glycosylation recognition sites, the seven trans-membrane domains taking part in the formation of the binding pocket, and intracellular C-terminal tail structure with phosphorylation and G-protein interacting sites. The connecting portions of the polypeptide chain form three extracellular (facing outside) and three intracellular (located whithin the cytoplasma) loops (Devi *et al*., 2001).

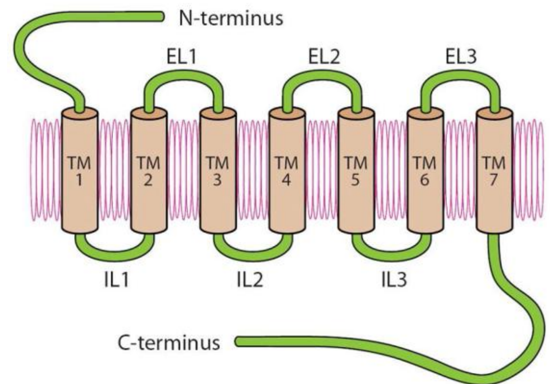


Fig. 3. Opioid receptor structure. EL- Extracellular loop; TM- Transmembrane

In 2000, the Committee on Receptor Nomenclature and Drug Classification of the International Union of Pharmacology (NC-IUPHAR, http:/www.iuphar.org) adopted the terms MOP, DOP, KOP to indicate *mu*-, *delta*-, *kappa*- opioid peptide receptors, respectively. However, in the present work, the terms μ, δ and κ will be used for the receptors

The three types are briefly explained in the following:

1) μ Receptor: The receptor is characterized by its high affinity for Morphine. It is the major receptor mediating action of morphine and its congeners. Endogenous ligands for μ receptor are Endomorphins-1 and Endomorphin-2, found in mammalian brain, produce biological effects ascribed to this receptor. Other opioids peptides like -endorphins, Enkephalins and Dynorphins bind to μ receptor with lower affinity. Two subtypes of μ receptors have been proposed:

a. μ1: Has higher affinity for morphine, mediates supraspinal analgesia and is selectively blocked by naloxone.
b. μ2: Has lower affinity for morphine, mediates spinal analgesia, respiratory depression and constipating action.

2) κ Receptor: is defined by its high affinity for ketocyclazocine and Dynorphin A. Norbinaltorphimine is a selective κ -antagonist. Two subtypes of κ receptors κ1 and κ3 are functionally important. Analgesia caused by κ agonist is primarily spinal (κ 1) or supraspinal (κ 3)

3) δ Receptor: has high affinity for leu/met Enkephalins which are its endogenous ligands. The δ mediated analgesia is mainly spinal (δ receptors present in dorsal horn of spinal cord). Naltrindole is a selective δ antagonist.

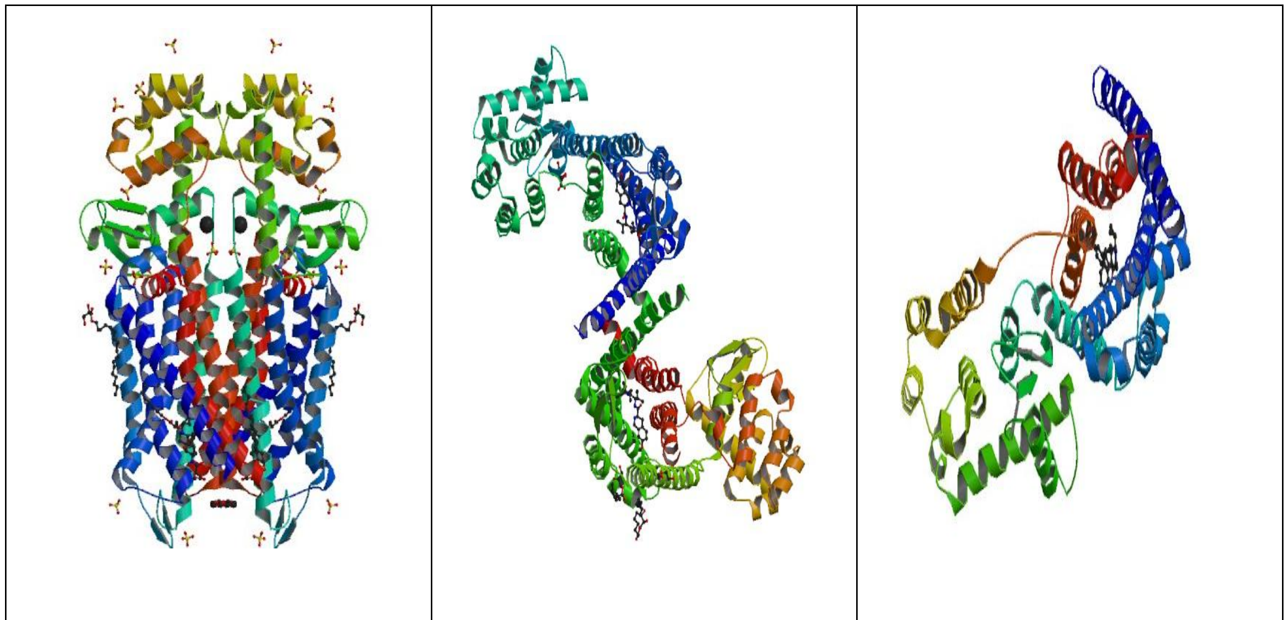


Fig. 4. Structures of μ, κ and δ opioid receptors bound to morphine, JDTic and naltrindole respectively (Manglik *et al*., 2012; Wu *et al*., 2012; Granier *et al*., 2012)

2.2 Opioids and antinociception

Opioids work as analgesics in the body by inhibiting neurotransmission of pain stimulus to the central nervous system: brain and spinal cord (Muller, 1997). The following section explains neurotransmission of pain followed by discussion of mechanism of action of opioids as analgesics.

2.2.1 Neurotransmission of Pain Stimulus

Pain stimulus has been extensively reviewed by Hodgkin (1952), Lefkowitz (1992) and Voet (1990). Noxious stimulus like pain is transmitted to central nervous system i.e. brain and spinal cord through specialized cells called neurons (Gringauz, 1997). These cells have large cell body called Soma containing most of the cytoplasm and organelles. Neurons have two fibers like extension:

(a) ***Dendrites*** are short numerous extensively branched processes and are specialized in receiving stimuli from either peripheral parts of the body or another neuron.

(b) ***Axon*** is a single process extending from the cell body and conducts the signals/stimulus of pain etc. received by the dendrites to the next neurons.

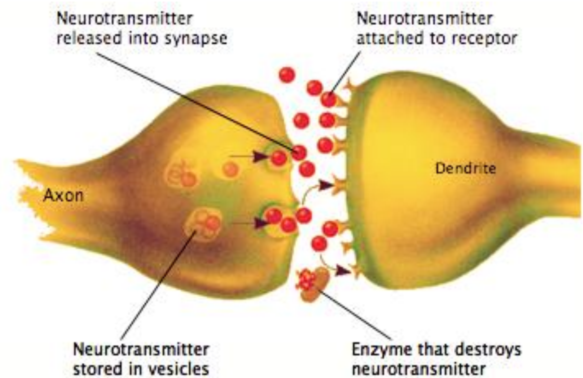


Fig. 5. Transmission of stimulus takes place by release of certain neurotransmitters into synaptic cleft

This conduction of stimuli from one neuron to the next occurs at places called ‘synapses’. A synapse is the space between axon tip of one neuron and the cell body of the next neuron and controls communication between neurons. The cell membrane forming the surface of neurons maintains an electrical potential (called “resting potential”) and is electrically excitable. Pain (noxious) stimulus from peripheral parts of the body causes “excitation” of pre-synaptic neurons. The noxious stimulus causes a temporary change in the charge across the pre-synaptic neuron’s cell membrane termed as ‘depolarization’. This depolarization of the pre-synaptic neuronal cell membrane triggers release of excitatory neurotransmitters like acetylcholine, substance P, glutamate etc. which are present in vesicles near the synaptic knobs of the pre-synaptic neuron, into the synaptic cleft. The neurotransmitters then move across the synaptic cleft and bind to the receptors present on postsynaptic neuron’s cell membrane which then undergoes depolarization. This depolarization of post synaptic neuron then, in turn triggers the release of excitatory neurotransmitters. Thus the pain stimulus is transmitted from one neuron to next neuron in the form of depolarization which on reaching the central nervous system is interpreted as pain sensation. Under the normal condition when the neuron is not ‘excited’ by a stimulus/ signal the cell membrane of pre-synaptic neuron is said to at **Resting Potential.**

This resting potential is the membrane potential across the pre-synaptic neuron's cell membrane and it exists due to charge difference across the membrane. This charge difference is due to the presence of negatively charged species inside the neuron and excess positive charge in the fluid matrix surrounding the neuron. The ions involved in setting up this potential difference across the membrane are sodium (Na^+) and potassium (K^+) ions. This potential difference across the membrane is generated because neuron's cell membrane is permeable to the outward diffusion of K but not the inward diffusion of Na. The neuron cell membrane also has enzyme like Na-K ATPase which transport Na out of the neuron and pump K into the neuron cytoplasm.

The non permeability of neuron cell membrane to inward Na ions movement means that once Na are pumped out they are not allowed to go back into neuron's cytoplasm, but instead there is a build up of Na ion concentration (concentration gradient) outside the neuron cell membrane. Thus the neuron is depolarized and the cytoplasm of neuron is negative with respect to outside of neuron. The value of this resting potential is about -70 mV.

When pain stimulus reaches the pre-synaptic neuron, it excites the neuron by temporarily changing the neuron cell membrane's permeability to Na^+ and K^+ ions. This is brought about by opening of Na^+ channels which allows Na^+ ions to flow into cytoplasm of neuron.

This sudden influx of Na ions alters the potential difference across the cell membrane of pre-synaptic neuron (from -70mV to +40 mV) by creating a build up of positive charge inside the neuron thereby making the cytoplasm of neuron positively charged with respect to fluid matrix outside the neuron.

This process of alteration of potential difference of the pre-synaptic neuron from its resting potential value of -70mV to +40mV is called as depolarization. This depolarization of pre-synaptic neuron, in turn, triggers opening of Ca channels present in neuron cell membrane thereby causing an influx of Ca from fluid matrix into neuron's cytoplasm. This influx stimulates the vesicle containing neurotransmitters present in the synaptic 'knobs' to fuse with the neuronal cell membrane and release the neurotransmitters into the synaptic cleft. The neurotransmitters move across the synaptic cleft to bind to specific neurotransmitters receptors on the cell membrane of next neuron i.e. postsynaptic neuron .The binding of the neurotransmitters to its receptors on the post synaptic neuron, in turn,

triggers opening of Na channels of this neuron thereby causing depolarization of post synaptic neuron. Thus pain stimulus is transmitted from one neuron to next in the form of depolarization which on reaching the central nervous system is then interpreted as "pain sensation".

2.3 Mechanism of opioid action as analgesic

Opioids have two well established action on neurons by which these inhibit neurotransmission of pain stimulus to the central nervous system as discussed here:

(1)**Pre-synaptic inhibition of pain stimulus**

In pre-synaptic inhibition of pain stimulus, opioids first bind to opioid receptors present on membrane of pre-synaptic neuron cell (Akil, 1998). This binding then leads to the closing of Ca^{2+} channels of on the pre-synaptic neuron (Macdonald, 1986; Tsunoo, 1986; Schroeder, 1991; Seward, 1991). When pain stimulus excites the pre-synaptic neuron, it gets depolarized. However in the presence of opioids, this depolarization can no longer open Ca^{+} ion channels. This then reduces or stops the entry of Ca into cytoplasm of pre-synaptic neuron which is essential for the release of neurotransmitters from the pre-synaptic as explained earlier. Thus the release of neurotransmitters like substance P[41] (Mudge, 1979), acetylcholine (Mulder, 1984) and glutamate (Gannon, 1992) in to the synaptic cleft is inhibited. Since no neurotransmitters now binds to receptors present on post synaptic neuron cell membrane, the post synaptic neuron does not undergo depolarization or excitation thereby the transmission of pain stimulus is inhibited.

(2)**Post synaptic inhibition of pain stimulus**

Opioid receptors are also present on membrane of postsynaptic neuron. When opioid bind to opioid receptor present on the post synaptic neuron, this binding causes closing of Na ion channels and opening of K ion channels (Alreja, 1993; Loose, 1990). This then triggers k ion to flow from post synaptic neuron's cytoplasm into fluid matrix outside thereby causing the cytoplasm of postsynaptic neuron to have a potential more negative than the resting potential (Alreja, 1993; Loose, 1990).

This process which is called 'hyperpolarisation' of membrane of postsynaptic neuron reduces the postsynaptic neuron's ability to undergo depolarization and hence inhibits the transmission of pain stimulus to the next neuron (Alreja, 1993; Loose, 1990). The mechanism

by which opioids regulate the closing of Ca ion channels (on pre-synaptic neuron) and Na ion channel (on post-synaptic neuron) as well as opening of K ion channels (on post synaptic neuronal cell membrane) can be described as follows:

Opioid ligands bind to opioid receptors (which are G- protein coupled receptors (Loose, 1990) present on neuronal cell membrane, both pre-synaptic as well as postsynaptic neuron, which transduces this signal into the neuronal cell. G-proteins are mainly of two types: G_s (G-stimulatory proteins) and G_i (G-inhibitory proteins). Opioid receptors are coupled to G-inhibitory proteins (Harrison, 1998).

When no ligand is bound to opioid receptor, G_i protein exists in neuronal cell membrane as a hetero-trimer composed of three subunits-α, β and γ (Harrison, 1998). At rest opioid receptor is not bound with G_i protein but when opioid ligand binds to the opioid receptor it causes a conformational change in receptor and receptor binds with G_i protein. This coupling results in separation of α subunit from βγ subunits. Then α subunit binds and inhibits adenylyl cyclase (Hyman, 1996; Sharma, 1975) thus preventing the formation of 3', 5' cylic AMP (cAMP) from ATP (Kurose, 1983). cAMP activates enzyme protein kinase A (PKA) which opens Ca^{2+} ion and Na^{+} channels remain closed. It has recently been reported that G_i- proteins can directly open K^{+} channels (Huang, 1995) without involving cAMP dependent enzymes like PKA etc.

As mentioned earlier, opioids are used primarily as analgesics to give relief from pain. However, the prolonged use of opioids also induces certain undesirable side effects such as tolerance and physical dependence in the body whereby the efficiency of these opioids as analgesics is decreased.

2.4 Opioid Induced Tolerance

Out of many negative side effects caused by opioids, the one having the highest pharmacological significance is tolerance. Tolerance may be defined as a phenomenon in which an increased amount of drug is required to produce the same level of drug effect after repeated use of the drug. Development of tolerance involves complex biochemical procedures at the cellular and sub-cellular level. Though many mechanisms have been proposed for the same but due to conflicting reports, no mechanism is yet universally accepted.

Activation of opioid receptors induces changes in intracellular second messenger system by inhibition of adenylyl cyclase via G_i protein. Activation of opioid receptors also inhibits voltage-

dependent Ca^{2+} channels and activates inwardly rectifying K^+ channels (Law *et al.*, 2000). On activation of opioids receptors, GPCR kinases (GRK) are recruited to plasma membrane and specifically phosphorylate the receptors associated to the opioids ligands (Williams *et al*., 2001; Liu *et al*., 2001 and Hausdorff *et al*., 1990). This phosphorylation weakens the association with G proteins and increases the binding affinity to a group of proteins known as "arrestins". This adds to uncoupling effect and causes the delinking of receptor from the G protein. This is referred to as "desensitization" (Williams *et al*., 2001; Liu *et al*., 2001; Lohse *et al*., 1990). This means that even when the opioid ligand binds to opioid receptor, it no longer couples to G_i protein and hence results of loss of ability of opioids to inhibit the formation of cAMP and thus no analgesia is caused.

Binding of activated and phosphorylated receptors with arrestins also induces receptor internalization resulting in fewer available receptors at the cell surface for activation by opioids. This adds to the reduction of the agonist signalling and thus causes opioids tolerance (Williams *et al*., 2001; Koch *et al*., 1998).

After internalization, the receptors may have two fates (Whistler *et al*., 1998):

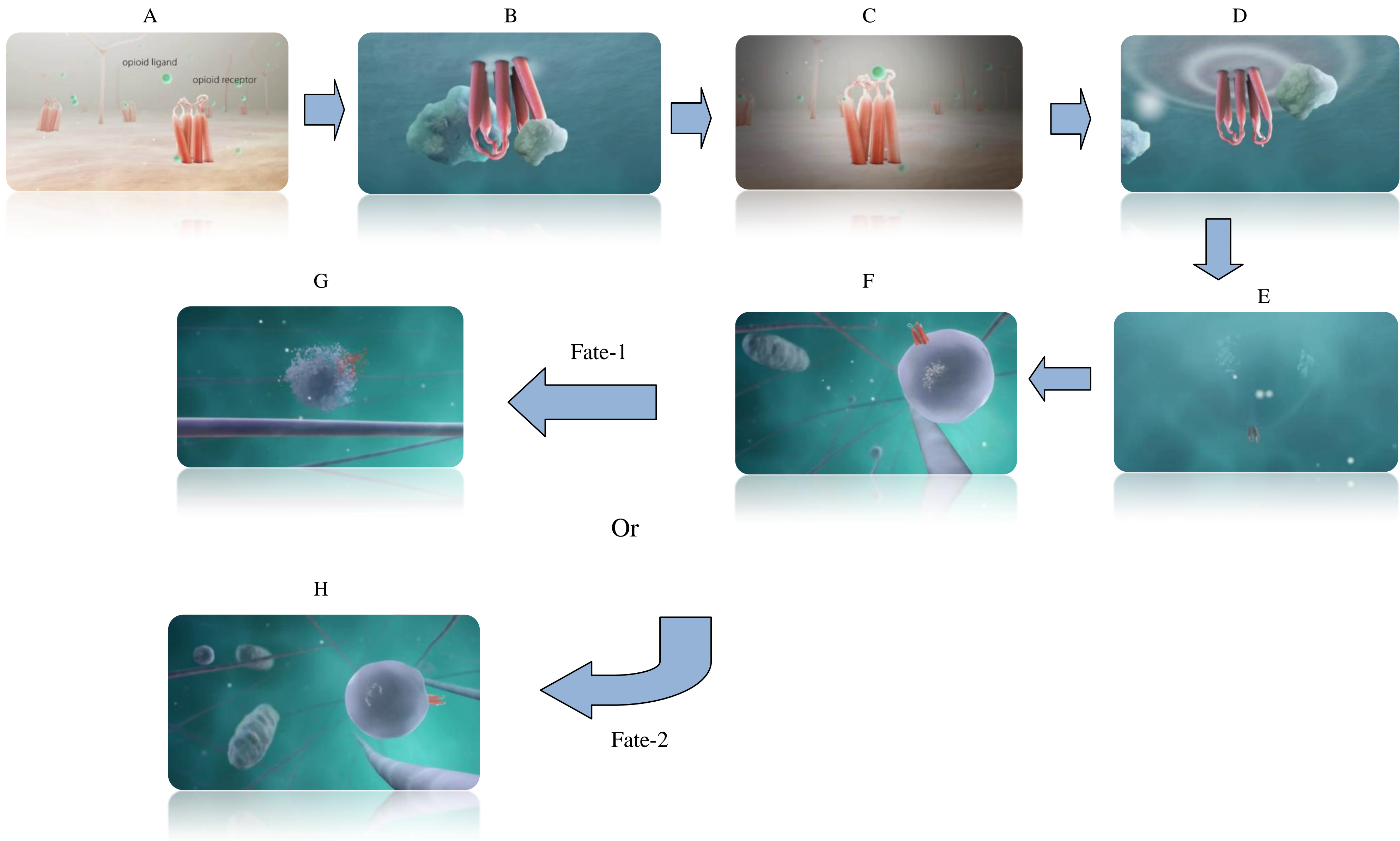
1) They can be dephosphorylated and then cycled back to the plasma membrane as competent receptors

2) They can be degraded by proteases in the lysosomes

Dephosphorylation and subsequent recycling of receptors result in of same number of receptors available initially to the opioid ligands. But in the cases where receptors undergo the alternate fate, there is an availability of lesser number of receptors to the subsequent exposure of the opioids and thus there is a need for more molecules (more amounts) to be given to have the same effect. This leads to the development of tolerance to the opioids.

2.5 Factors determining the fate of internalized receptors

In HEK293 cells expressing opioid receptors, the opioids [D-Ala2, N-Me-Phe4, Gly5-ol]-enkephalin (DAMGO), met-enkephalin, etorphine, or methadone, but not morphine, induce receptor desensitization (Whistler *et al*., 1998 and Alvarez *et al*., 2002). In hippocampal neurons expressing native opioid receptors, chronic treatment with DAMGO, methadone, or morphine induce opioid tolerance (Bushell *et al*., 2002). Two studies show that some receptor desensitization could be induced by morphine (Bohn *et al*., 2000 and Borgland *et al*., 2003).



Process of Internalization of opioid receptors. **A**- Opioid receptors (O.R.) present on cell membrane; **B**- G protein coupled to O.R.; **C**- opioid ligand attachment to receptor; **D**- G protein uncoupling; **E**- Internalization initiation; **F**- Receptor internalized; **G**- Receptor degradation; **H**- Receptor recycled *(courtesy, Dennis Wei, University of Toronto, Canada)*

However, it is generally agreed that morphine is much less potent at inducing receptor desensitization than opioids with high intrinsic efficacy such as DAMGO, fentanyl, and etorphine (Cox and Crowder, 2004). Intrinsic efficacy is a conceptual parameter that relates the number of receptors occupied to the magnitude of the receptor-mediated response. To generate a given effect, it is necessary to occupy a number of receptors out of the total population, the so called "fractional receptor occupancy" (Chavkin and Goldstein, 1982; Mercadante, 1999).

The number of receptors that need to be occupied to create an analgesic effect is believed to be inversely proportional to the intrinsic activity; in other words, the larger the number of unoccupied receptors (receptor reserve) that exist when a drug achieves analgesia, the greater the intrinsic efficacy of the drug (Chavkin and Goldstein, 1984; Duttaroy and Yoburn, 1995; Ivarsson and Neil, 1989; Sosnowski and Yaksh, 1990). Thus, agonist efficacy seems to be an important factor in determining opioid receptor desensitization. Similarly, opioids with high intrinsic efficacy induce a rapid receptor internalization, whereas in most studies morphine does not induce receptor internalization (Kieffer and Evans, 2002; Cox and Crowder, 2004 and Zang *et al*., 1998) and in two studies induced a slower and less complete receptor internalization than opioids with high intrinsic efficacy (Bushell *et al*., 2002 and Borgland *et al*., 2003). This inefficiency of morphine in inducing receptor desensitization and internalization may be attributable to its inability to induce receptor phosphorylation by GRK after receptor activation (Zang *et al*., 1998). Thus, the ligand-receptor complex, rather than the receptor itself, may determine the active conformations of opioid receptors and induction of distinct cellular responses by individual ligands (Zaki *et al*., 2000 and Keith *et al*., 1998). Therefore, it is very important to observe that the effect of any peptide on the fate of internalized receptor, though can be predicted, but the actual effect can only be known by experimentation.

2.6 Anti opioid Peptide model

According to this model there is an anti-opioid system within the body which works parallel to the endogenous opioid system. Repeated administration of opioids in the body activates this anti-opioid system which then tries to neutralize or reduce the opioid effects in order to maintain body's equilibrium or homeostasis. This model postulates that brain synthesizes and secretes certain peptides called the 'anti-opioid peptides'. These anti-opioid peptide bind to specific receptor to produce the anti opioid effects to attenuate or weaken opioid induced analgesia and are also responsible for the development of tolerance and physical dependence (Rothman, 1992).

Initially when opioids are administered in the body, these bind to opioid receptors to produce analgesia. Opioid receptor stimulation also activates the anti-opioid system to release the anti-opioid peptides which upon binding to anti-opioid receptors attenuate opioid induced analgesia. As more opioid is administered, the anti-opioid system tries to reduce the effect of the opioid drug by secreting more amounts of anti-opioid peptides in the body to maintain the homeostasis. When these anti-opioid peptides can completely neutralize the analgesia produced by the opioid drug, tolerance development to opioid induced analgesia is observed, and a higher dose is then required to achieve the original analgesic response. When the administration with opioid drug is suddenly discontinued, the endogenous opioid system is no longer stimulated, but the anti-opioid system remains activated and thus continues to secrete anti-opioid peptides. The presence of this relative excess of these anti-opioid peptides in the body is responsible for the withdrawal symptoms indicating the development of physical dependence. Neuropeptides shown to have anti-opioid activity in behavioral studies in rodents include vasopressin, oxytocin, nociceptin, neuropeptide FF (NPFF), and cholecystokinin (CCK).

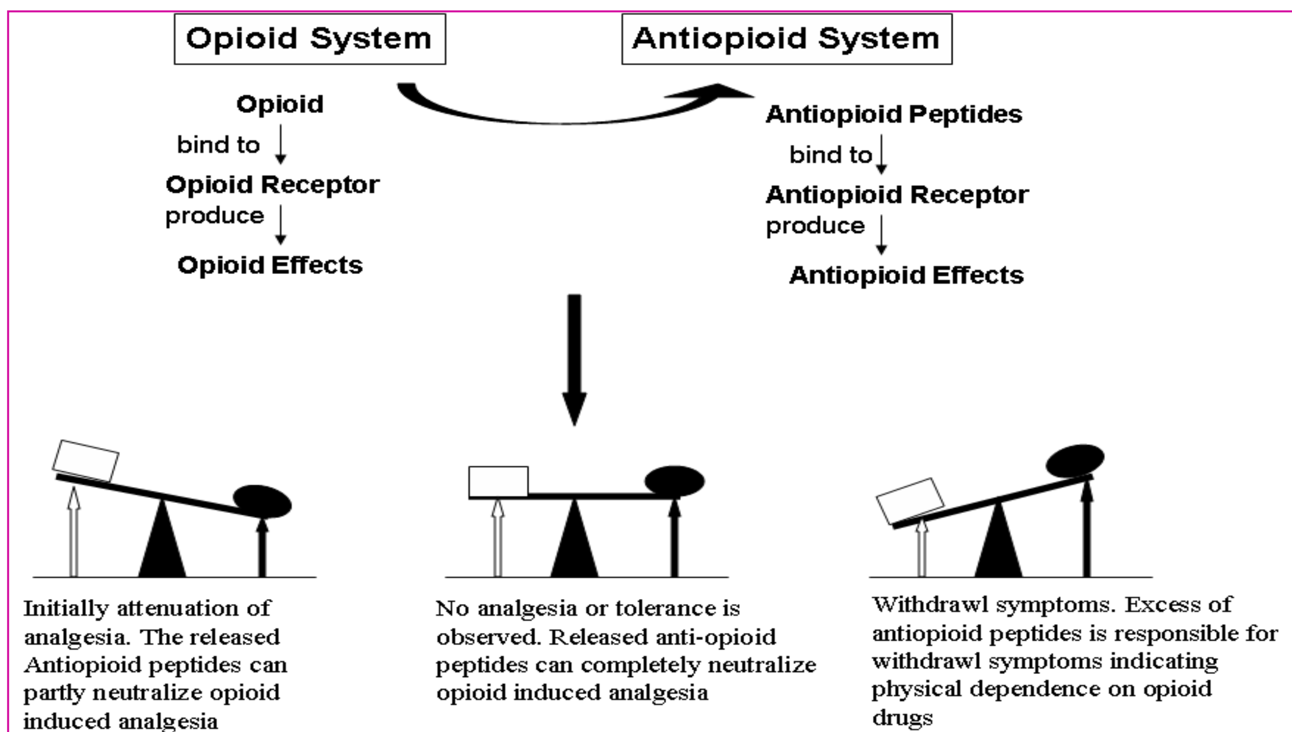


Fig. 6. A flow diagram depicting Opioid Antiopioid system

2.7 Endogenous Chimera MERF and novel peptide design

Rossier at al. (1980) reported the existence of an endogenous opioid peptide with sequence Tyr-Gly-Gly-Phe-Met-Arg-Phe (Met-enkephalin-Arg6-Phe7 or MERF) in human, mouse and bovine brain (Rossier *et al.*, 1980). The first five amino acids (Tyr-Gly-Gly-Phe-Met) of this sequence correspond to the sequence of an endogenous opioid peptide Met-enkephalin and the last four amino acids (Phe-Met-Arg-Phe) correspond to an anti-opioid peptide FMRF found endogenously. Therefore, the peptide reported by Rossier *et al* is a *chimeric opioid peptide* with overlapping sequences of an opioid and an anti opioid.

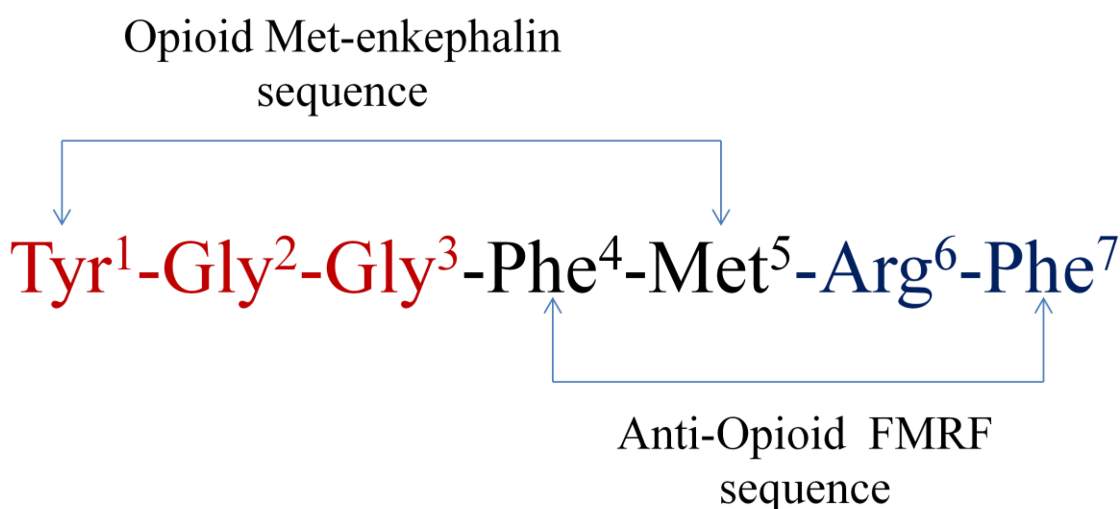


Fig. 7. Amino acid sequence of MERF showing its overlapping chimeric structure

Anti-opioid hypothesis states that chronic use of opioids triggers the release of anti-opioid peptides like NPFF, FMRFamide etc. These peptides bind to the specific anti-opioid receptors and weaken the opioid analgesia and hence cause the development of opioid tolerance in the body. Thus in accordance to anti-opioid hypothesis, if the anti-opioid receptors can be blocked by a suitably designed peptide which can act as an antagonist for the anti-opioid receptors, then this shall lead to the attenuation in development of tolerance.

Therefore, the opioid and the anti opioid sequences of the above peptide if segregated and linked with three lysine residues result in a single molecule capable of binding to opioid receptors through its N-terminus and to block the anti opioid receptors through its C-terminus simultaneously. The three lysine residues provide the required positive charge to the molecule to make it capable of cell penetration.

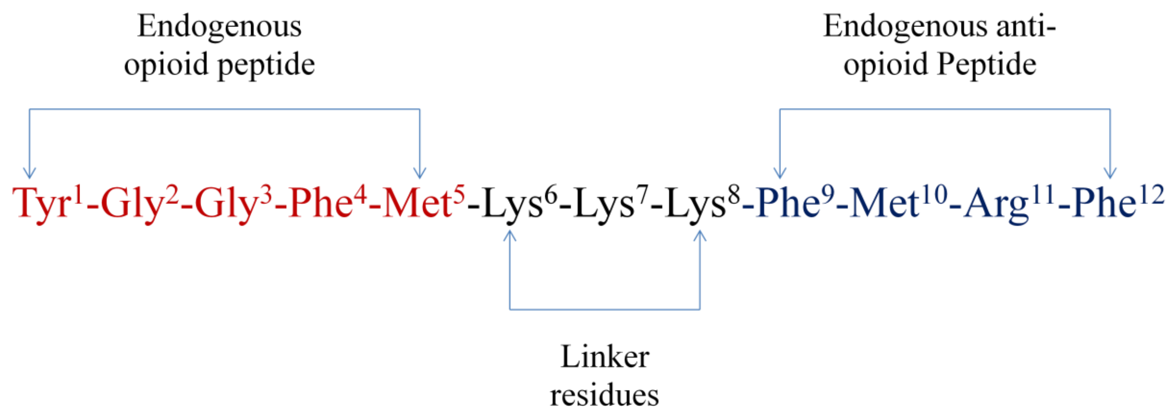


Fig. 8. Structure of peptide 2, with segregated opioid and anti-opioid sequences

From the aforementioned discussion, it is imminent that opioid peptides continue to be extensively important as an intervention of pain. In this study, two peptides were synthesised, one as a standard molecule (found endogenously in our body) and the other being the test molecule. The pharmacological properties of the test molecules were investigated

3. OBJECTIVES

After the rigorous study of the literature present on the subject of analgesic opioid peptides and after the designing of the chimeric peptide mentioned in section 2.7, the following objectives of the present work were planned and executed:

Objective I:

To synthesize the two opioid peptides: Peptide 1 (A Standard molecule required in chronic studies of test molecule) and Peptide 2 (the test molecule)

Objective II:

Pharmacological study of Peptide 2 including its antinociceptive behaviour, antagonist pretreatment study and tolerance development study of the molecule. Tolerance development studies further consist of chronic treatment of peptide on male Wistar Rats and FACS studies using transfected cell line.

4. MATERIALS AND METHODS

All the chemicals and reagents used in the process were of highest quality. HPLC grade solvents obtained from Merck Millipore, Billerica, USA were used during the synthesis.

4.1 Peptide Synthesis

Both the peptides, i.e. peptide 1 (standard peptide) and peptide 2 (Test peptide) were synthesised using solid phase peptide synthesis (Merrifield, 1959). Batch wise technique was adopted during the process using ACT-90 Peptide Synthesizer (Advanced ChemTech, Louisville, KY, U.S.A.).

4.1.1 Resin Swelling and attachment of the Amino Acids

2 g Rink Amide MBHA resin (Novabiochem®, EMD Millopore, Billerica, MA, U.S.A.), used as the solid support for the synthesis, was swelled in DMF (Sigma-Aldrich, St. Louis, U.S.A.) for 1 hour in the synthesis flask. To remove the Fmoc protection group from the resin, it was treated with 20% piperidine in DMF (4 cycles, 5 minutes each). Resin was then given ten washings with DMF to remove all the piperidine. Further, 1st Fmoc protected amino acid, (Novabiochem®, EMD Millopore, Billerica, MA, U.S.A.) in the sequence was added along with DIPCI and HOBt (Coupling step). Shaking at this step was carried out for about 3 hours and was followed by ten cycles of washings by DMF to remove excess amino acid.

The quantities of amino acid, DIPCDI and HOBt to be added were calculated as follows:

Rink amide (MBHA) Resin loading = 0.56 mM/g

Amount of resin taken = 2 g

Total substitution = 0.56×2 = 1.12 mM

$$\text{Amount of amino acid (in g)} = \frac{\text{Mol wt. of the a.a.(gm)} \times \text{Total substitution} \times \text{No. of equivalents}}{1000}$$

$$\text{Amount of HoBt (in g)} = \frac{\text{Mol wt. of the HoBt (gm)} \times \text{Total substitution} \times \text{No. of equivalents}}{1000}$$

Amount of DIPCI = Mol Wt of the DIPCI x Total substitution x No. of equivalents (in µl)

This was followed by deprotection cycle in which Fmoc protection of 1st amino acid was removed using 20% piperidine in DMF (4 cycles, 10 minutes each).

Similarly, couplings of all the amino acids in the sequence were done followed by subsequent deprotection cycles to obtain the desired peptides.

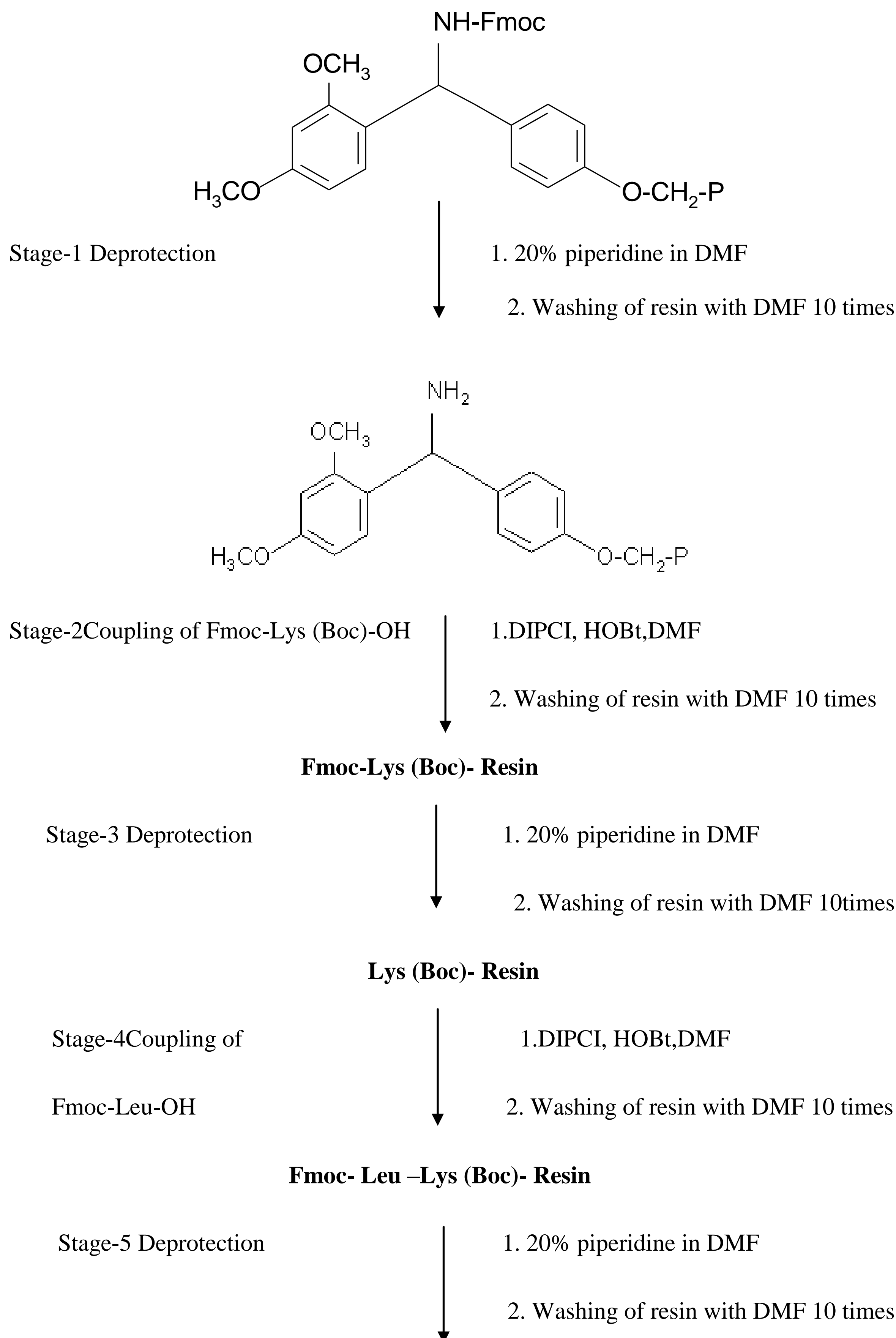
4.1.2 Kaiser Test

To ensure if the coupling or the deprotection has taken place, Kaiser test (Wellings and Atherton, 1997) was performed after each coupling or deprotection. A little amount of resin, thoroughly washed with DMF, was taken and was transferred to a fusion tube. Then, 0.5g Ninhydrin in 10 ml of ethanol, 80 g phenol in 20ml of ethanol and 0.4 ml of 0.0001 M aqueous solution of potassium cyanide in 20ml of dry distilled pyridine were added to the fusion tube in the ratio 2:1:1. Blue beads indicated free amino groups showing depreotection and colourless beads indicated absence of free amino groups showing coupling.

4.1.3 Cleavage of Amino acid from resin

Resin was taken in a conical flask and cleavage cocktail containing 95% TFA, EDT, Phenol and TIS was added. The flask was closed and left to stand at room temperature with intermittent swirling for sometimes. The resin was removed by filtration under reduced pressure and was washed twice with clean TFA solution. The filtrate was further concentrated under rota-vapour by removing TFA. Then concentrated peptide was washed twice with ether. Most of the ether was evaporated to achieve a good precipitation of the crude peptide.

Flow diagram for the Synthesis of Peptide -I



Leu –Lys (Boc) - Resin

Stage-6 Coupling of of Fmoc-Lys(Boc)-OH ↓ 1.DIPCI, HOBt, DMF

2.Washing of resin with DMF10 times

Fmoc-Lys(Boc)- Leu –Lys (Boc) - Resin

Stage-7 Deprotection ↓ 1. 20% piperidine in DMF

2. Washing of resin with DMF10 times

Lys(Boc)- Leu –Lys (Boc) - Resin

Stage-8 Coupling of ↓ 1. DIPCI, HOBt,DMF

Fmoc-Pro-OH 2.Washing of resin with DMF 10 times

Fmoc-Pro -Lys (Boc) - Leu –Lys (Boc) - Resin

Stage-9 Deprotection ↓ 1. 20% piperidine in DMF

2.Washing of resin with DMF 10 times

Pro - Lys (Boc) - Leu –Lys (Boc) - Resin

Stage-10 Coupling of ↓ 1.DIPCI, HOBt, DMF

Fmoc-Arg(Pbf)-OH 2.Washing of resin with DMF10times

Fmoc-Arg(Pbf)- Pro - Lys (Boc) - Leu –Lys (Boc) - Resin

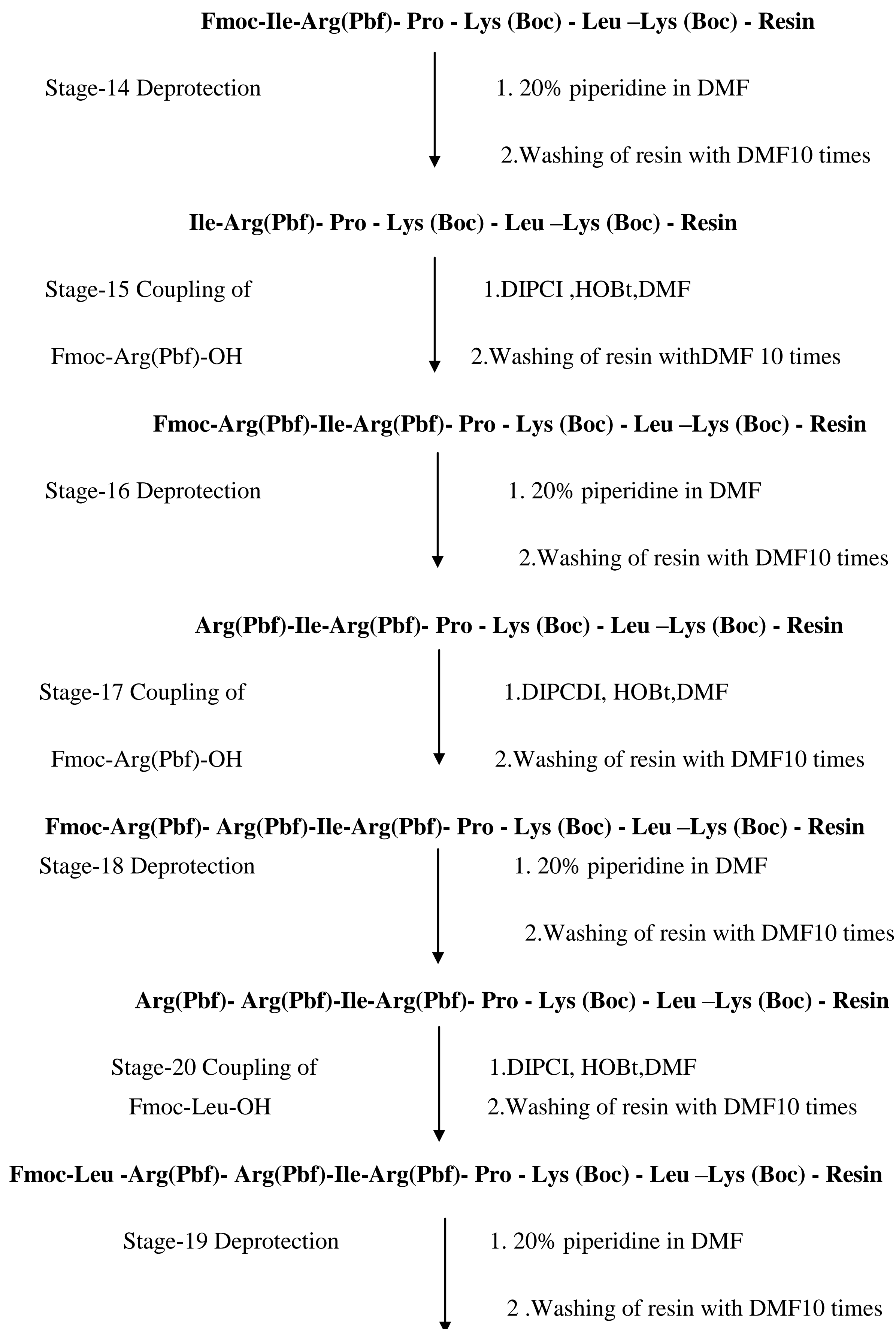
Stage-12 Deprotection ↓ 1. 20% piperidine in DMF

2.Washing of resin with DMF 10 times

Arg(Pbf)- Pro - Lys (Boc) - Leu –Lys (Boc) - Resin

Stage-13 Coupling of Fmoc-Ile-OH ↓ 1. DIPCI ,HOBt,DMF

2.Washing of resin with DMF10 times


Fmoc-Ile-Arg(Pbf)- Pro - Lys (Boc) - Leu –Lys (Boc) - Resin
Stage-14 Deprotection
1. 20% piperidine in DMF
2.Washing of resin with DMF10 times
Ile-Arg(Pbf)- Pro - Lys (Boc) - Leu –Lys (Boc) - Resin
Stage-15 Coupling of
Fmoc-Arg(Pbf)-OH
1.DIPCI ,HOBt,DMF
2.Washing of resin withDMF 10 times
Fmoc-Arg(Pbf)-Ile-Arg(Pbf)- Pro - Lys (Boc) - Leu –Lys (Boc) - Resin
Stage-16 Deprotection
1. 20% piperidine in DMF
2.Washing of resin with DMF10 times
Arg(Pbf)-Ile-Arg(Pbf)- Pro - Lys (Boc) - Leu –Lys (Boc) - Resin
Stage-17 Coupling of
Fmoc-Arg(Pbf)-OH
1.DIPCDI, HOBt,DMF
2.Washing of resin with DMF10 times
Fmoc-Arg(Pbf)- Arg(Pbf)-Ile-Arg(Pbf)- Pro - Lys (Boc) - Leu –Lys (Boc) - Resin
Stage-18 Deprotection
1. 20% piperidine in DMF
2.Washing of resin with DMF10 times
Arg(Pbf)- Arg(Pbf)-Ile-Arg(Pbf)- Pro - Lys (Boc) - Leu –Lys (Boc) - Resin
Stage-20 Coupling of
Fmoc-Leu-OH
1.DIPCI, HOBt,DMF
2.Washing of resin with DMF10 times
Fmoc-Leu -Arg(Pbf)- Arg(Pbf)-Ile-Arg(Pbf)- Pro - Lys (Boc) - Leu –Lys (Boc) - Resin
Stage-19 Deprotection
1. 20% piperidine in DMF
2 .Washing of resin with DMF10 times

Leu -Arg(Pbf)- Arg(Pbf)-Ile-Arg(Pbf)- Pro - Lys (Boc) - Leu –Lys (Boc) - Resin

Stage-20 Coupling of Fmoc-Phe-OH ↓ 1.DIPCI, HOBt,DMF

2.Washing of resin with DMF10 times

Fmoc-Phe- Leu -Arg(Pbf)- Arg(Pbf)-Ile-Arg(Pbf)- Pro - Lys (Boc) - Leu –Lys (Boc) Resin

Stage-21 Deprotection ↓ 1. 20% piperidine in DMF

2.Washing of resin with DMF10 times

Phe- Leu -Arg(Pbf)- Arg(Pbf)-Ile-Arg(Pbf)- Pro - Lys (Boc) - Leu –Lys (Boc)-Resin

Stage-22 Coupling of Fmoc-Gly-OH ↓ 1.DIPCI, HOBt,DMF

2.Washing of resin with DMF 10 times

Fmoc-Gly Phe- Leu -Arg(Pbf)- Arg(Pbf)-Ile-Arg(Pbf)- Pro - Lys (Boc) - Leu –Lys (Boc)-Resin

Stage-23 Deprotection ↓ 1. 20% piperidine in DMF

2.Washing of resin with DMF10 times

Gly-Phe- Leu -Arg(Pbf)- Arg(Pbf)-Ile-Arg(Pbf)- Pro - Lys (Boc) - Leu –Lys (Boc)-Resin

Stage-24 Coupling of Fmoc-Gly-OH ↓ 1.DIPCI, HOBt,DMF

2.Washing of resin with DMF10 times

Fmoc-Gly -Gly-Phe- Leu -Arg(Pbf)- Arg(Pbf)-Ile-Arg(Pbf)- Pro - Lys (Boc) - Leu –Lys (Boc)-Resin

Stage-25 Deprotection ↓ 1. 20% piperidine in DMF

2.Washing of resin with DMF10 times

Gly -Gly-Phe- Leu -Arg(Pbf)- Arg(Pbf)-Ile-Arg(Pbf)- Pro - Lys (Boc) - Leu –Lys (Boc)-Resin

Stage-26 Coupling of Fmoc-Tyr(tBu)-OH

1.DIPCI, HOBt,DMF

2.Washing of resin with DMF 10 times

Fmoc-Tyr(tBu)-Gly -Gly-Phe- Leu -Arg(Pbf)- Arg(Pbf)-Ile-Arg(Pbf)- Pro - Lys (Boc) - Leu –Lys (Boc)-Resin

Stage-27 Deprotection

1. 20% piperidine in DMF

2.Washing of resin with DMF10 times

Tyr(tBu)-Gly -Gly-Phe- Leu -Arg(Pbf)- Arg(Pbf)-Ile-Arg(Pbf)- Pro - Lys (Boc) - Leu –Lys (Boc)-Resin

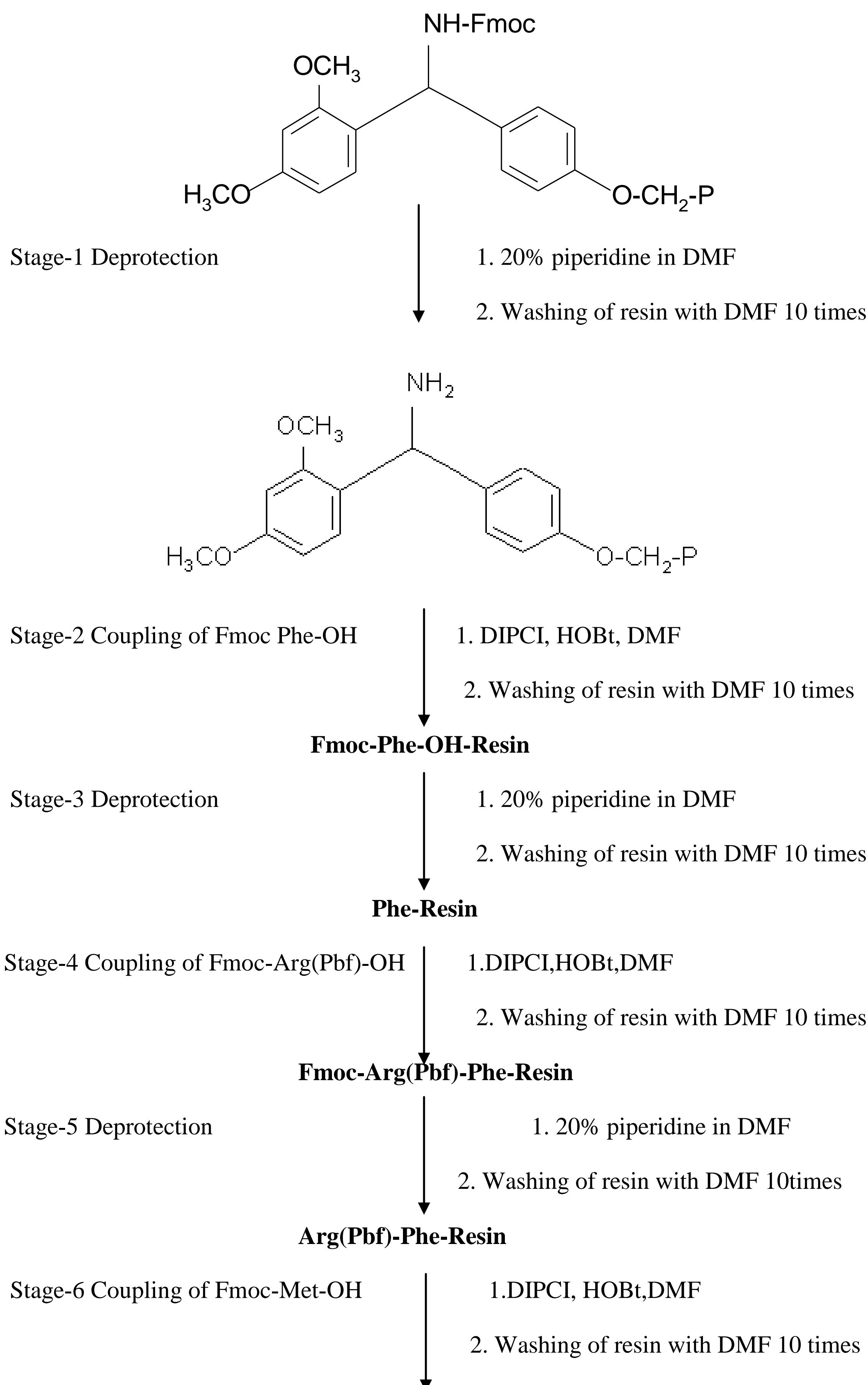
Stage-28 Final cleavage

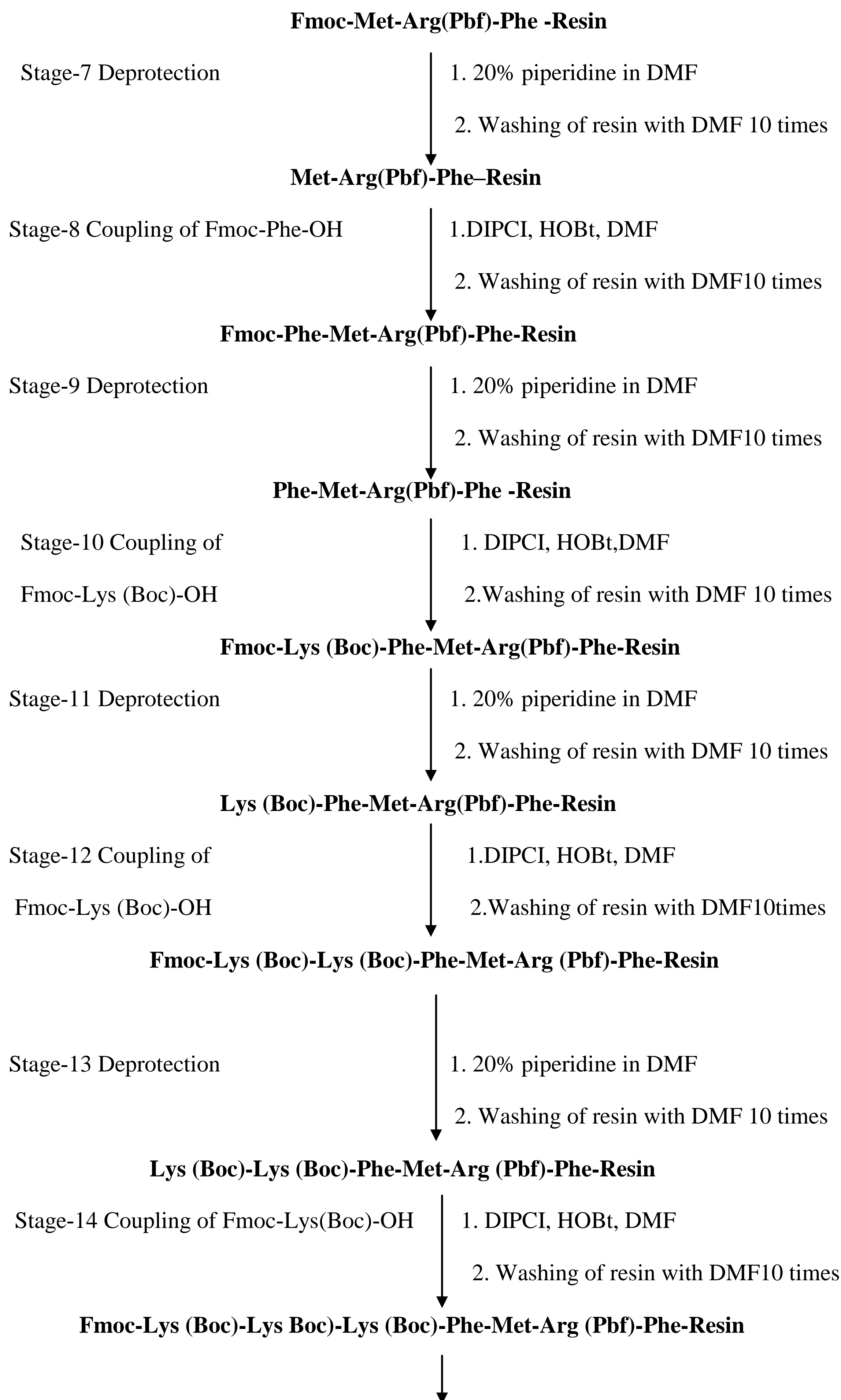
TFA,EDT, Phenol,TIS,

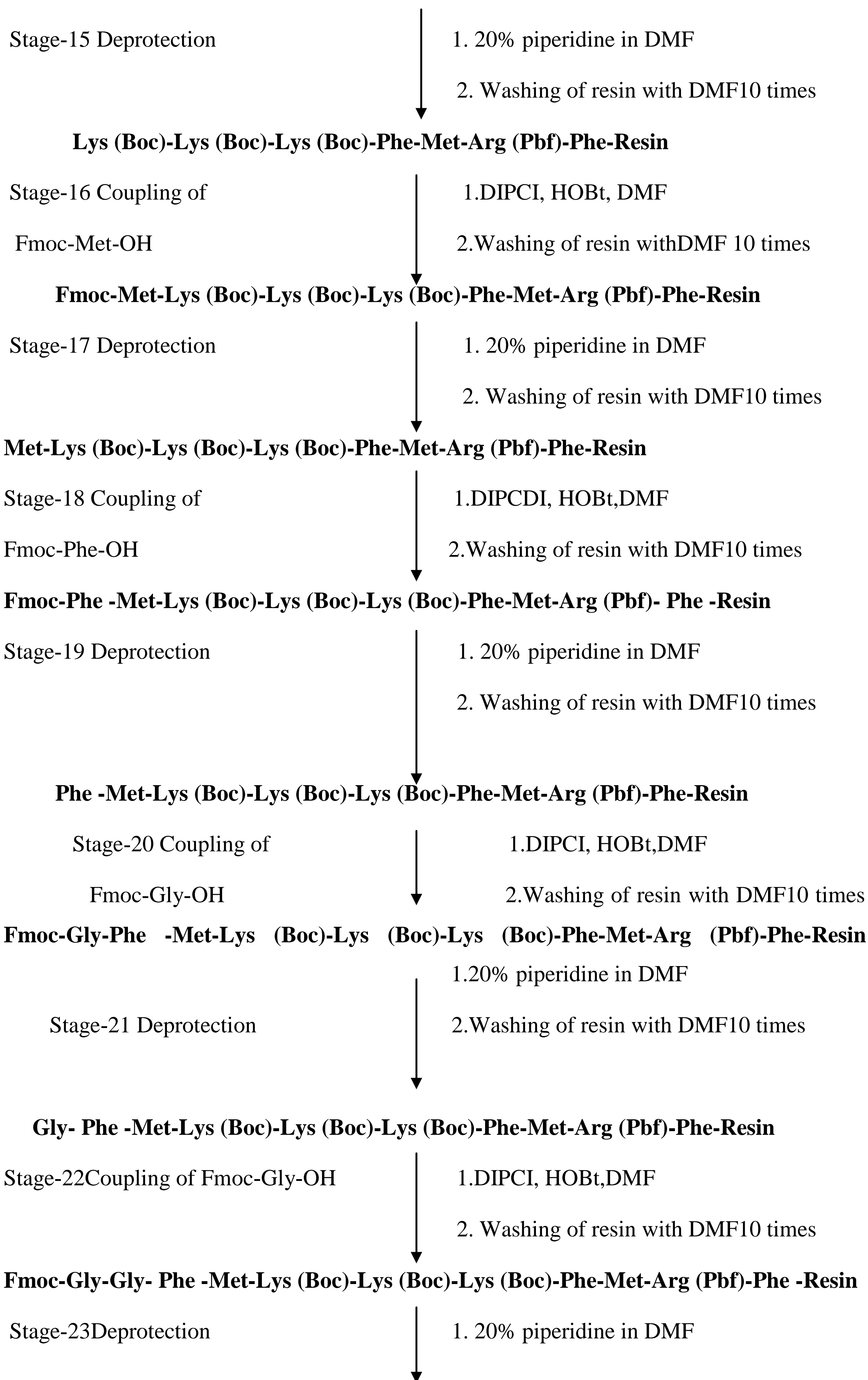
(90%,2.5%,2.5%,2.5%,2.5%)

NH_2-Tyr-Gly -Gly-Phe- Leu -Arg- Arg-Ile-Arg- Pro - Lys - Leu –Lys - $CONH_2$

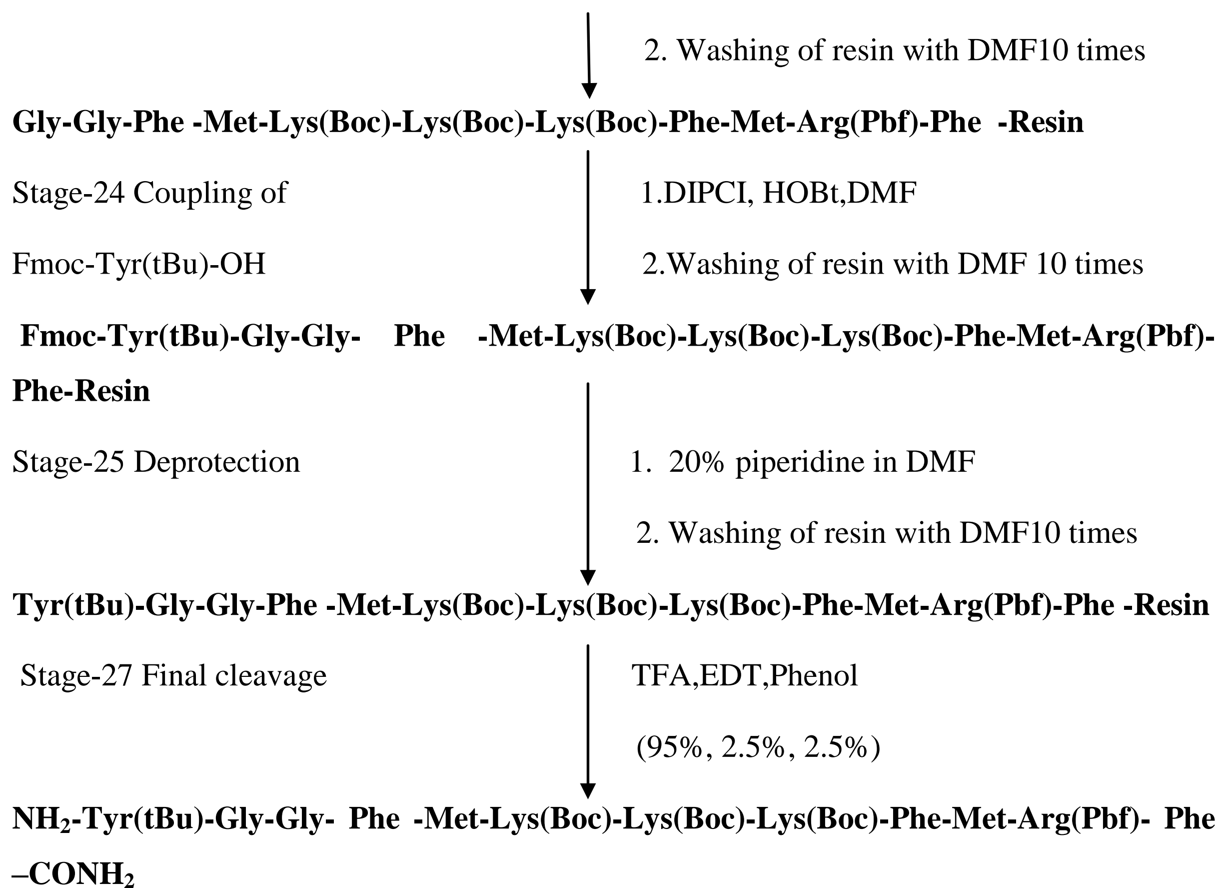
Flow diagram for Synthesis of Peptide -II




Fmoc-Met-Arg(Pbf)-Phe -Resin
Stage-7 Deprotection
1. 20% piperidine in DMF
2. Washing of resin with DMF 10 times
Met-Arg(Pbf)-Phe–Resin
Stage-8 Coupling of Fmoc-Phe-OH
1.DIPCI, HOBt, DMF
2. Washing of resin with DMF10 times
Fmoc-Phe-Met-Arg(Pbf)-Phe-Resin
Stage-9 Deprotection
1. 20% piperidine in DMF
2. Washing of resin with DMF10 times
Phe-Met-Arg(Pbf)-Phe -Resin
Stage-10 Coupling of
Fmoc-Lys (Boc)-OH
1. DIPCI, HOBt,DMF
2.Washing of resin with DMF 10 times
Fmoc-Lys (Boc)-Phe-Met-Arg(Pbf)-Phe-Resin
Stage-11 Deprotection
1. 20% piperidine in DMF
2. Washing of resin with DMF 10 times
Lys (Boc)-Phe-Met-Arg(Pbf)-Phe-Resin
Stage-12 Coupling of
Fmoc-Lys (Boc)-OH
1.DIPCI, HOBt, DMF
2.Washing of resin with DMF10times
Fmoc-Lys (Boc)-Lys (Boc)-Phe-Met-Arg (Pbf)-Phe-Resin
Stage-13 Deprotection
1. 20% piperidine in DMF
2. Washing of resin with DMF 10 times
Lys (Boc)-Lys (Boc)-Phe-Met-Arg (Pbf)-Phe-Resin
Stage-14 Coupling of Fmoc-Lys(Boc)-OH
1. DIPCI, HOBt, DMF
2. Washing of resin with DMF10 times
Fmoc-Lys (Boc)-Lys Boc)-Lys (Boc)-Phe-Met-Arg (Pbf)-Phe-Resin



Stage-15 Deprotection
1. 20% piperidine in DMF
2. Washing of resin with DMF10 times
Lys (Boc)-Lys (Boc)-Lys (Boc)-Phe-Met-Arg (Pbf)-Phe-Resin
Stage-16 Coupling of
Fmoc-Met-OH
1.DIPCI, HOBt, DMF
2.Washing of resin withDMF 10 times
Fmoc-Met-Lys (Boc)-Lys (Boc)-Lys (Boc)-Phe-Met-Arg (Pbf)-Phe-Resin
Stage-17 Deprotection
1. 20% piperidine in DMF
2. Washing of resin with DMF10 times
Met-Lys (Boc)-Lys (Boc)-Lys (Boc)-Phe-Met-Arg (Pbf)-Phe-Resin
Stage-18 Coupling of
Fmoc-Phe-OH
1.DIPCDI, HOBt,DMF
2.Washing of resin with DMF10 times
Fmoc-Phe -Met-Lys (Boc)-Lys (Boc)-Lys (Boc)-Phe-Met-Arg (Pbf)- Phe -Resin
Stage-19 Deprotection
1. 20% piperidine in DMF
2. Washing of resin with DMF10 times
Phe -Met-Lys (Boc)-Lys (Boc)-Lys (Boc)-Phe-Met-Arg (Pbf)-Phe-Resin
Stage-20 Coupling of
Fmoc-Gly-OH
1.DIPCI, HOBt,DMF
2.Washing of resin with DMF10 times
Fmoc-Gly-Phe -Met-Lys (Boc)-Lys (Boc)-Lys (Boc)-Phe-Met-Arg (Pbf)-Phe-Resin
1.20% piperidine in DMF
Stage-21 Deprotection
2.Washing of resin with DMF10 times
Gly- Phe -Met-Lys (Boc)-Lys (Boc)-Lys (Boc)-Phe-Met-Arg (Pbf)-Phe-Resin
Stage-22Coupling of Fmoc-Gly-OH
1.DIPCI, HOBt,DMF
2. Washing of resin with DMF10 times
Fmoc-Gly-Gly- Phe -Met-Lys (Boc)-Lys (Boc)-Lys (Boc)-Phe-Met-Arg (Pbf)-Phe -Resin
Stage-23Deprotection
1. 20% piperidine in DMF


2. Washing of resin with DMF10 times
Gly-Gly-Phe -Met-Lys(Boc)-Lys(Boc)-Lys(Boc)-Phe-Met-Arg(Pbf)-Phe -Resin
Stage-24 Coupling of
Fmoc-Tyr(tBu)-OH
1.DIPCI, HOBt,DMF
2.Washing of resin with DMF 10 times
Fmoc-Tyr(tBu)-Gly-Gly- Phe -Met-Lys(Boc)-Lys(Boc)-Lys(Boc)-Phe-Met-Arg(Pbf)-Phe-Resin
Stage-25 Deprotection
1. 20% piperidine in DMF
2. Washing of resin with DMF10 times
Tyr(tBu)-Gly-Gly-Phe -Met-Lys(Boc)-Lys(Boc)-Lys(Boc)-Phe-Met-Arg(Pbf)-Phe -Resin
Stage-27 Final cleavage
TFA,EDT,Phenol
(95%, 2.5%, 2.5%)
NH2-Tyr(tBu)-Gly-Gly- Phe -Met-Lys(Boc)-Lys(Boc)-Lys(Boc)-Phe-Met-Arg(Pbf)- Phe –CONH2

4.2 Purification and Characterization of Synthesized Peptides

4.2.1 Gel filtration

Dry Sephadex G-25 was swollen in 20% acetic acid overnight at room temperature and was then packed into chromatographic column. The cleaved compound was removed from resin by filtering the solution-mixture containing the desired compound through the sintered funnel. The filtrate was then processed by rotavapour and the final extract was pooled out in eppendorf tubes. It was dissolved in 20% acetic acid and loaded upon the surface of the column. 40-50 fractions each of 4ml were collected and their O.D. was observed spectrophotometrically at 220nm. Those fractions with high O.D. were pooled out in a flask and lyophilized. After this the purified desalted peptides were further checked for their homogeneity using reverse phase high performance liquid chromatography (RP-HPLC) technique.

4.2.2 High Performance Liquid Chromatography (HPLC)

The RP-HPLC analysis of peptides was performed on a Waters column (4.6×250 mm, 300 Å, 5-μm particle size) using reverse phase HPLC (Waters 600, USA) with linear gradients of water containing 0.05% TFA (gradient A) and acetonitrile containing 0.05% TFA (gradient B) at a flow rate of 0.8 ml/min and with 1% gradient B/min, starting at a concentration of 10% gradient B. Photo Diode Array detector was used.

4.2.3 Lyophilization

The fractions showing high O.D. (at 220nm) were collected in different lyophilization flasks and layered with ethanol. It was temporarily stored at -70°C or lyophilized directly. Powdered peptides were weighed and stored in vials at -70°C.

4.2.4 Mass Spectrometry

The synthesized peptides were characterized by LC-MS/MS (Quattro micro API, Waters). Parameters used were, Capillary voltage 30, Cone voltage 3.5, RF Lens 0.2, Dissolvation gas temperature 300ºC, Dissolvation gas pressure 600 liter/hour and Cone gas 50 liter/hour.

4.3 Pharmacological studies of the synthesized peptide

4.3.1 Assessment of the Analgesic behaviour: Tail Flick Latency Assay

To check for, and measure the analgesic activity of the synthesized compound, tail flick latency assay was used involving laboratory rats (Wistar strain). The tail flick latency assay or tail flick test uses analgesia meter giving heat to the rodent's tail to detect nociception. In normal rodents, the noxious heat sensation causes a prototypical movement of the tail via the withdrawal reflex. An investigator normally measures the time it takes for the reflex to be induced.

4.3.1.1 Animal Handling

Wistar strain of rats (male), 180-230 g, was housed 4 per cage, kept at 12-hr light/12 hr-dark cycle in the animal house of the institute and fed standard rat chow and water *ad libitum*. Animals were habituated to the animal room at a temperature of 22°C–25°C for a week before the experiment. The animals were handled according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of environment and forests (Animal Welfare Division), Government of India, and the institutional animal ethical committee, Institute of Genomics and Integrative Biology (Delhi, India). All rats were accustomed to the testing conditions for 5 days, and each rat was trained on the tail-flick apparatus before starting the actual experiment in order to minimize the stress induced by handling.

4.3.1.2 Intra-peritoneal Injection:

To check the analgesic effect of the peptide formed, it was administered intraperitoneally into rats. For IP injection into the animal, following points must be taken care of:

a) Animal should be held properly - holding it from tail base, and then holding it from back (left hand) and scruff by massaging gently. If the animal defecate/urinate, it shows that it is under stress, thus may give improper results

Fig. 9. A rat being injected intraperitoneally

b) Hold animal in such a position as head down slightly and exposing peritoneum region with skin of that region tight.

c) Hold the syringe (in right hand) at 45° angle to the peritoneum (site of injection) in bevel up position.

d) Insert the needle about 90% (tuberculin syringe 2ml) in the left/right quadrant of peritoneum and pull back a little the syringe plunger.

e) If no blood/mucus/fluid comes out and only air comes out, push the plunger completely to give the required dose and immediately after that, take out the needle from animal.

4.3.1.3 Tail Flick Procedure:

Antinociceptive response was measured using an Indian-made tail-flick apparatus (Inco Pvt. Ltd., Ambala, India). Briefly, at the beginning of the study, the intensity of the heat stimulus in the tail-flick apparatus was adjusted to elicit a response by control or untreated rats in 4–6 sec. To minimize tail skin tissue damage, the cutoff time was set at 20 sec. Rats were given three baseline trials, each separated by 10 min, followed by intra-peritoneal injection of physiological saline and the peptide (in concentrations of 20 mg, 40 mg and 60 mg per Kg of rat's weight), and were tested 5, 15, and 30 min later. Inhibition of tail-flick response was expressed as the percentage of the maximum possible effect (%MPE), calculated as:

Percentage Maximum Possible Effect = $[(T_1-T_0)/(T_2-T_0)] \times 100$

where,

T_0 (seconds) = baseline

T_1 (seconds) = time taken to flick the tail after administration of dose

T_2 (seconds) = Maximum exposure (20 seconds)

Use of different concentrations helped in determining the optimal dose for further experiments.

4.3.2 Tolerance development studies: Effect of chronic treatment of peptide

Further, an experiment was conducted to assess the tolerance of Peptide 2. In that study, 3 groups of rats (n = 6, each group) were separately treated with saline, peptide 1 and peptide 2 per day given twice daily (with a difference of 12 hours) for 6 days. A concentration of 40 mg/Kg (found to be the optimal dose in tail flick latency assay; discussed later in the results section) was used in the experiment and rats were given a dose of 20 mg/Kg in the morning and then in the evening. The saline and peptide 1 treatments were taken as the negative and positive controls, respectively. Rats were given three baseline trials; each separated by 10 min, followed by intra-peritoneal injection of physiological saline and the peptides, and were tested 5, 15, and 30 min later.

4.3.3 Determination of receptor specificity: Antagonist pre-treatment study

Pharmacological study was performed using specific antagonists to determine which of the three opioid receptors - μ, κ and δ - mediated the observed antinociceptive action of chimeric opioid peptide. This was important because in the further study for tolerance development using cell culture, particular cells transfected to express that particular receptor were to be used.

Procedure

The optimum antagonist dose required to block a particular receptor was determined using a range of doses and were given 5 min prior to intra-peritoneal injection of optimised dose of the test peptide. Tail-flick latency was measured after 5, 15, 30, 45 and 60 min.

4.3.4 Reassessing the tolerance development *in vitro:* Using FACS studies

As the results of antagonist pre-treatment studies made it clear beyond doubt that the test peptide synthesised was μ opioid receptor specific (discussed later in the results section). hKOR-CHO cells which are Chinese Hamster Ovary cells transfected to over-express κ opioid receptors, were procured and were maintained and then Fluorescence Activated Cell Sorting Studies were performed to check the condition of receptors at different times of test peptide exposure.

4.3.4.1 Maintenance of recombinant hKOR-CHO cell line

hKOR-CHO cell line requires DMEM high Glucose medium for its growth, which was prepared as follows:

900 ml of autoclaved double distilled water filtered through 0.2µ filter was taken. Then 1.5 gm of $NaHCO_3$ and 16.4 gm of powdered media (Sigma-Aldrich, U.S.A.) was added. After this, 100 ml FBS was added. Finally, 10 ml of antibiotic-antimycotic solution was added into this. The media was then filtered using with 0.2µm filters. 5-7 ml of this media was kept overnight in incubator at 37°C and 5% CO_2 in a cell culture flask to check for any contamination.

Revival and Growth of hKOR-CHO cells

Vial of frozen cell line kept at -80 °c was taken out in ice bath and quickly transferred to 37°C water bath and heated for 1-2 minutes for thawing. The contents of the vial were then transferred to a 15ml Centrifuge tube, and respective media was added. The cell suspension was then centrifuged at 1000rpm for 3mins at 25°C. The supernatant was discarded and the cell pellet was added to a T-25cm^2 cell culture flask containing 5ml of DMEM high glucose media. It was kept at 37°c in an incubator for 24 hrs. During this time live cells adhere to the surface of the culture flask. The media was replaced with fresh media so that any traces of cryopreservant left will be removed. This process continued for 4-5 days before proceeding with the experimentation on these cells.

Splitting and plating of cells:

The media was aspirated off from the flask. Then 3ml of 1X PBS was added for washing of the cells and to inhibit the action of FBS. PBS was aspirated off. 500 µl of trypsin-EDTA solution was added and the flask incubated at 37^{0}C and 5% CO_2 for 2-3 minutes. 5 ml complete media was added to the flask, the contents were mixed well using pipette and taken out in a 15 ml centrifuged tube. The cell solution was centrifuged at 1000 rpm for 3 minutes at 25^{0}C, to pellet down the cells. The supernatant solution was discarded and the cell pellet re-suspended in the remaining media by tapping. 20 µl of this cell suspension was taken and transferred into another T-25 cm^2 flask

4.3.4.2 Fluorescence Activated Cell Sorting: Experiment

Cells were seeded onto 24-well plates at density of 4 X 10^4 per well and grown overnight. Cells after washing were treated with optimized dose 10 nM of agonist for various time intervals *viz.* 0min, 15mins and 30minutes in incomplete DMEM media (without FBS) at 37°C. Then the cells were trypisined and fixed with 4% paraformaldehyde followed by incubation with blocking reagent, 1% BSA in 1X PBS for 45 min. at room temperature. After blocking, cells will be incubated with kappa opioid receptor primary antibody, overnight at 4^0C, followed by incubation with secondary antibody for 45min. at room temperature. Finally cells were analyzed using FACS Callibur (BD Biosciences).

5. RESULTS AND DISCUSSION

Both the peptides were synthesized by solid phase synthesis and characterized by HPLC. Each successive step of synthesis was monitored by Kaiser Test used for confirmation of complete coupling and deprotection so as to ensure that all amino acids are perfectly attached through peptide bond in the desired sequence. The peptides were desalted by size exclusive column chromatography. Peptides were soluble in 20% acetic acid and were passed through G-25 column. They were characterised using Mass Spectrometry (Electro Spray Ionization-MS) and the following MS Spectra were obtained.

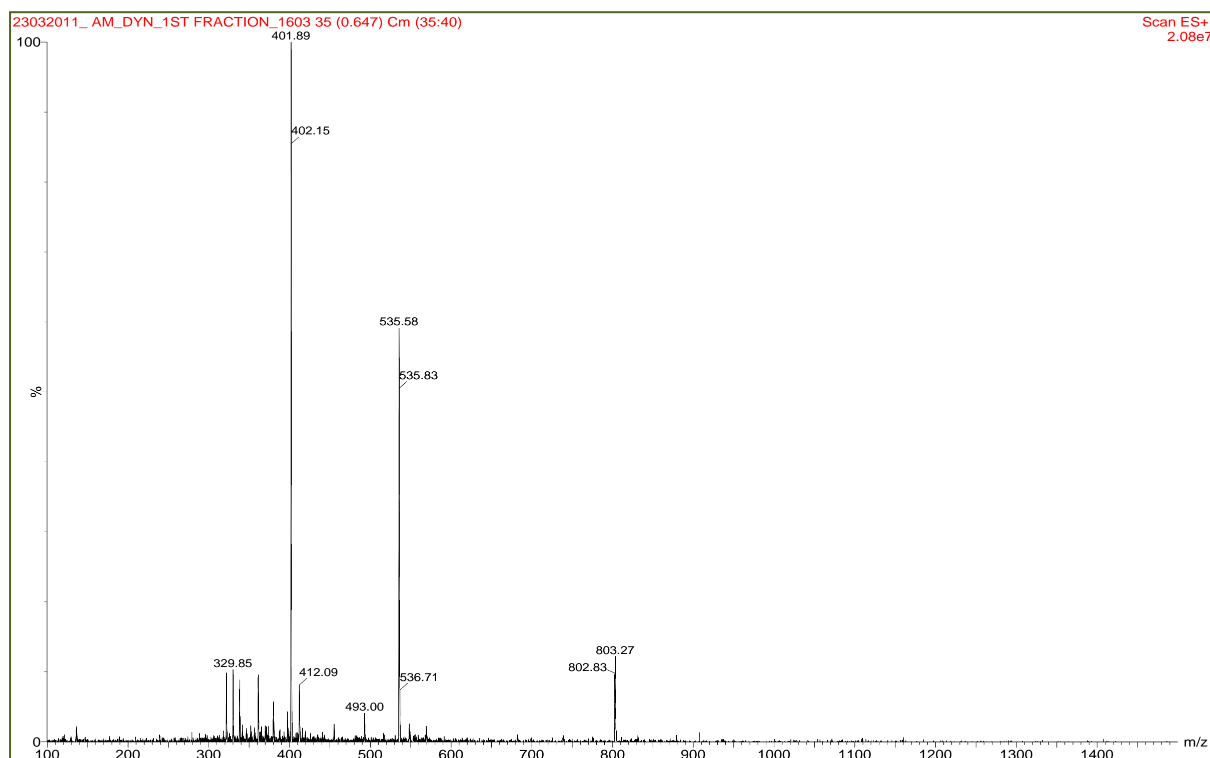


Fig. 10. Mass Spectrum - Peptide 1 (Standard Peptide)

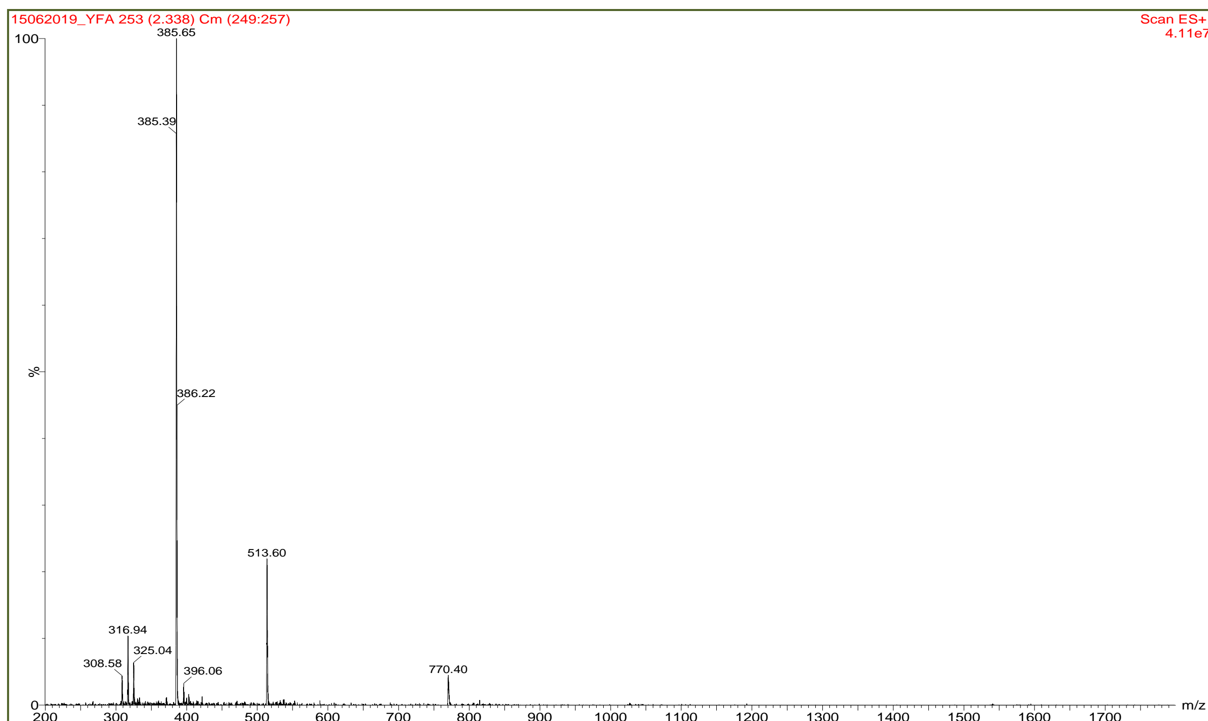


Fig. 11. Mass Spectrum of Peptide 2 (Test Peptide)

After characterization by mass Spectroscopy, the purity of the peptides was checked and measured using High Performance Liquid Chromatography (Waters Inc., U.S.A.). The following are the HPLC profiles of both the peptides.

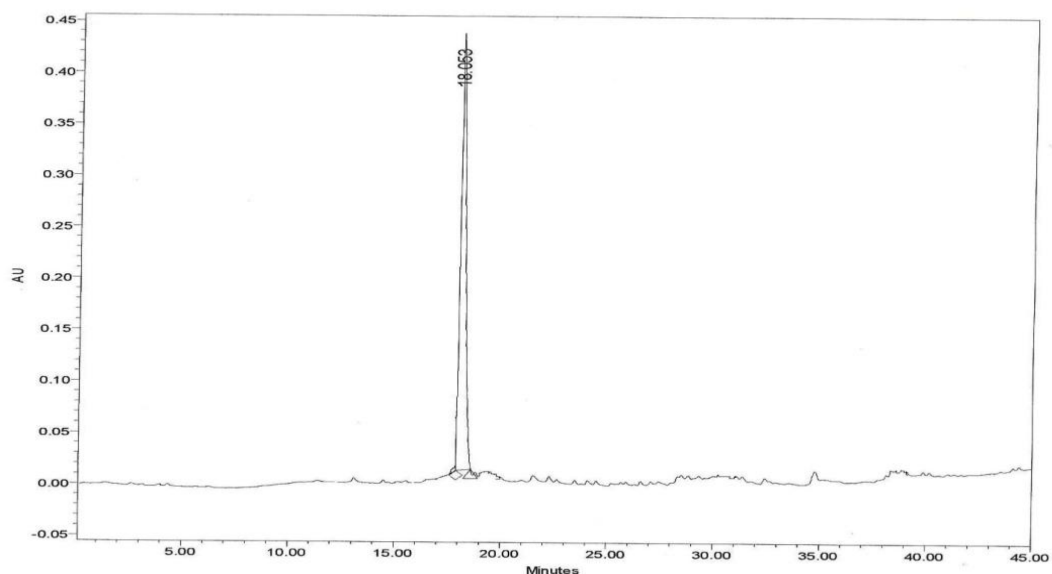


Fig. 12. HPLC chromatogram of Peptide 1 (Standard peptide)

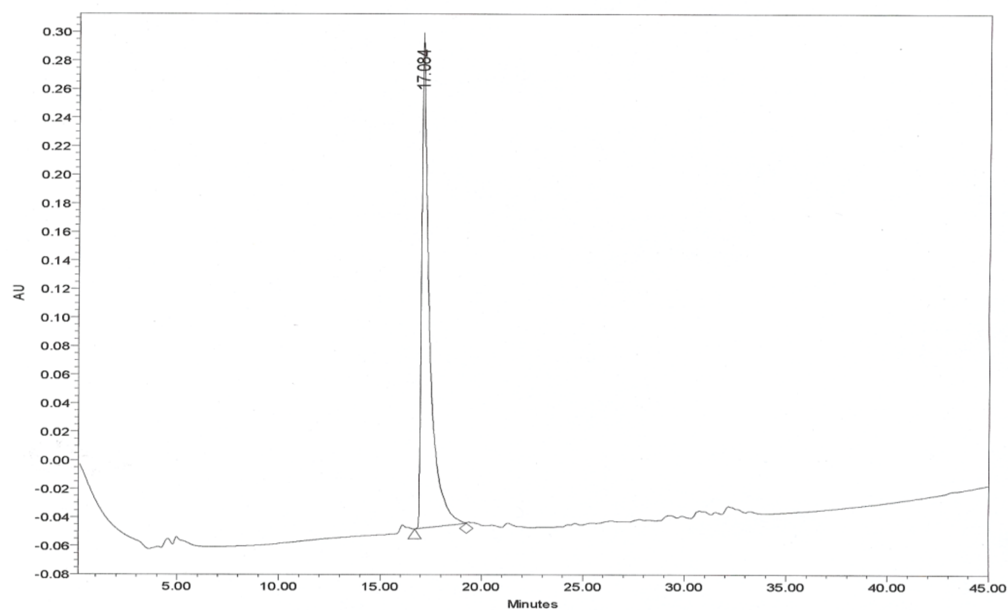


Fig. 13. HPLC chromatogram of Peptide 2 (Test Peptide)

Table. 1 Calculated, Observed molecular weight & % area in HPLC of peptides

S.No.	Name	Molecular weight (calculated)	Molecular weight (Observed)	HPLC(% area)
1	**Peptide**	**1604.00**	**1604.25**	**98.99 %**
2	**Peptide**	**1538.00**	**1538.41**	**96.43 %**

A single major peak of the synthesized peptide was observed. Along with this few minor peaks were also observed depicting impurities. Percentage area of the major peak was calculated.

The peptides synthesised were pure enough to be used for the cell culture and animal studies (Kumar *et al.*, 2011)

Pharmacological study of both the peptides:

1. Antinociceptive behaviour

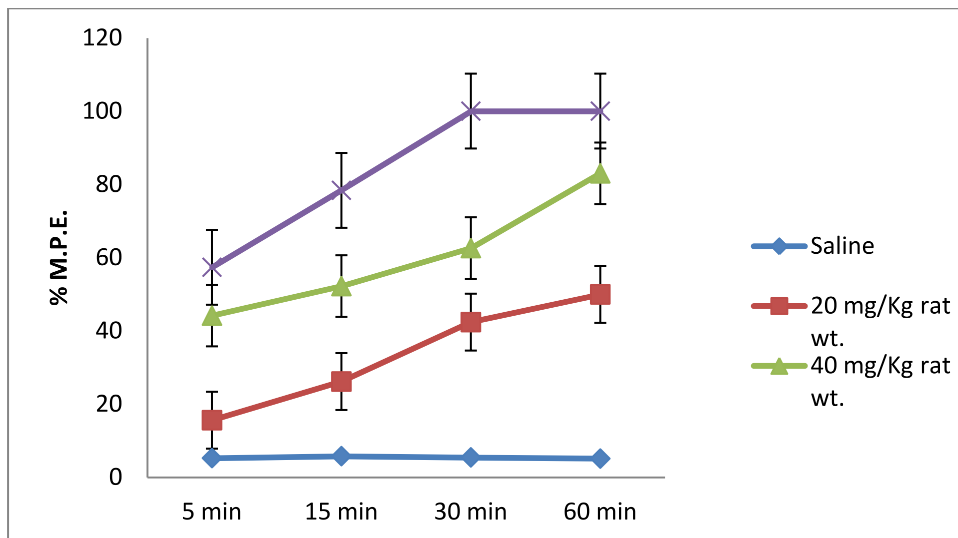


Fig. 14. Graph showing antinociceptive effect (M.P.E) observed in Rats; Error bar indicates standard deviation from mean on the basis of three replicate experiments

Tail flick latency assay data shows the extent of antinociception exhibited by the test peptide. A good amount of antinociception is seen and at dose of 40 mg/Kg of rat, we see a constant increase with time. Therefore, this set of dose was further used for antagonist pre-treatment studies.

2. Effect of chronic treatment of peptide

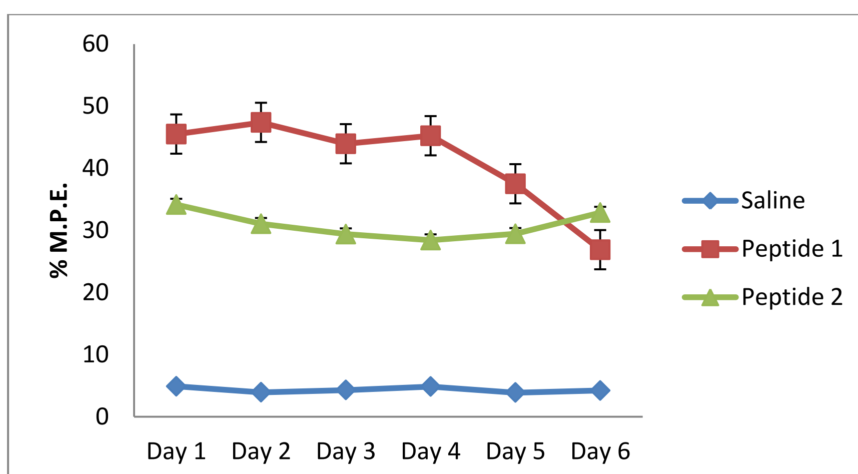


Fig. 15. Graph for average M.P.E. values obtained on 3 groups of rats (n=6); Error bar indicates standard deviation from mean on the basis of three replicate experiments

No considerable antinociception is seen during all six days with saline (0.9% NaCl), in line with it being a negative control for the experiment.

Peptide 1 (Positive control) shows a good antinociceptive action which decreases steeply after 3rd day, showing development of tolerance.

Peptide 2 (Test Molecule) shows good antinociceptive effect but unlike Peptide 1, shows no tolerance during six days. Instead, an increase in antinociceptive effect is seen on the 6th day.

2. Antagonist pretreatment study

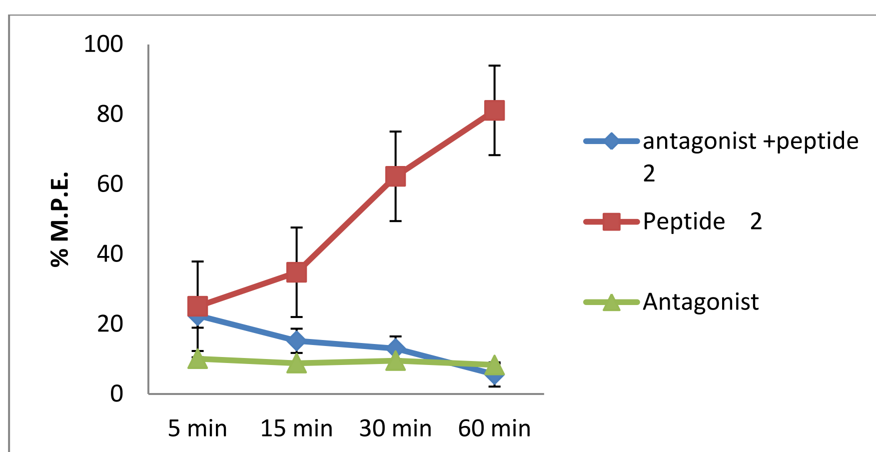


Fig.16. Graph showing M.P.E observed with antagonist pretreatment for κ receptor; Error bar indicates standard deviation from mean on the basis of three replicate experiments

The above data is for the set tested for Kappa receptor (antagonist is NBI - norbinalatorphimine). On injecting κ antagonist NBI and test peptide together, there is a significant decrease in antinociception (antagonist given 5 minutes prior to peptide injection). This clearly shows that antagonist had already bound the receptor for which the test peptide was specific. As NBI is a κ antagonist, it is evident that the test peptide is κ specific. Hence, for FACS studies, hKOR cell line, expressing high amount of κ receptors was used.

FACS Study

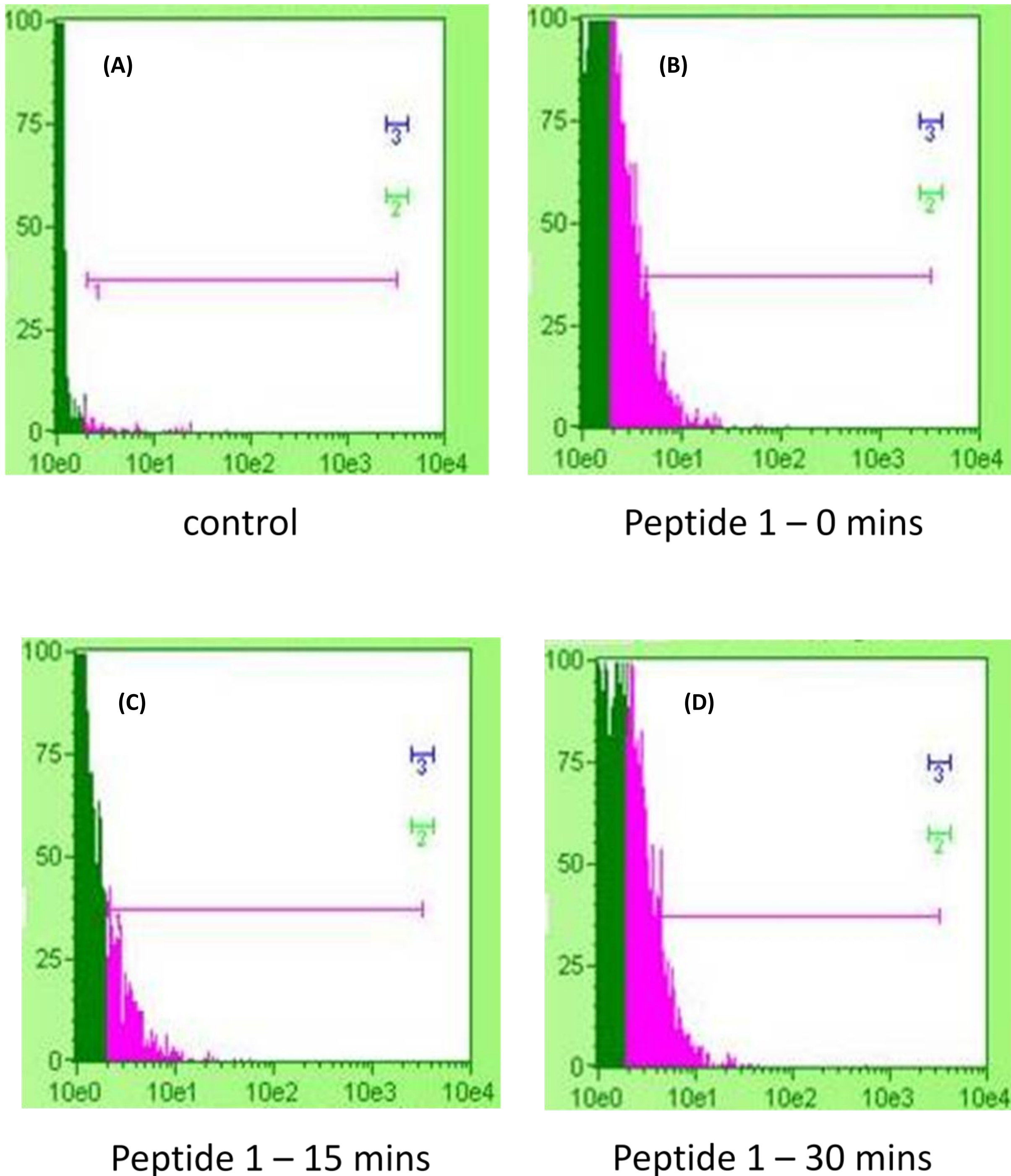


Fig.17. Fluorescent Activated Cell Sorting results showing different amounts of fluorescence

Fluorescent Associated Cell Sorting studies indicated the absence of fluorescence in control (A). At 0 minute (B), good amount of fluorescence was observed. In 15 minutes (C), the fluorescence decreased and further in 30 minutes (D) the fluorescence again increased. In 0 minute reading, almost all the receptors were free, therefore, a good amount of fluorescence

was observed and in 15 minute reading, it decreased as a result of binding of the test peptide molecules to it. But a sudden increase in fluorescence was seen in 30 minute reading which was almost equivalent to the amount of fluorescence observed in 15 min. This could be explained by the hypothesis given by Whistler *et al*. (1998) that the receptors after internalization on binding to opioid ligands can have two fates. Either they get degraded or they get revived and reappear on the surface. In the case of peptides where the receptors reappear, tend to cause no or less tolerance as the number of receptors available will not decrease on subsequent administration and thus same amount will be effective every time. Thus these FACS results suggest that the receptors reappeared after 30 minutes and thus this peptide possibly will cause no or at least less dependence.

CONCLUSION

The test peptide and the standard peptide synthesized came out to be more than 95% pure. The peptide 2 (test peptide) NH_2-Tyr-Gly-Gly-Phe-Met-Lys-Lys-Lys-Phe-Met-Arg-Phe–$CONH_2$ shows **good antinociception** in tail flick latency assay. **No tolerance development was observed in chronic studies on rats**. The FACS studies suggest that on binding of test peptide to the opioid receptors, more recycling of receptors takes place than the degradation thus making it, a good opioid analgesic. For confirming the FACS results showing reappearance of receptors, studies at mRNA level (RT-PCR) and protein expression level (Western Blot) are further desired.

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