

CLONING AND EXPRESSION OF GENETICALLY ENGINEERED SYNTHETIC ANALGESIC PEPTIDE MOLECULES IN YEAST CELLS AND THERAPEUTIC APPLICATION.

¹Dr.J.Venkateshwara Rao

ABSTRACT--*The healthier and comfortable life style of age old people and as well as handicapped persons are key indicators for success mile stone achievements in health sector. But in spite of latest equipments and gadgets the old age people and other handicapped persons are compromising on dignity of their life and leading a painful worried life with various ailments impacting their social status and conditions especially pain relaed problems like cancer, osteoporosis, spinal injuries artificial transplantation of limbs or organs,. These are all associated with heavy and chronic pain conditions. Severe pain is the most serious phobic condition for any patient who is undergoing surgical treatments. Even though the modern drugs are giving some analgesic effects, but they are showing more side effects and as well as most of the time they are fatal to organ or patients. In order to minimize the gap we would like to find out more suitable and effective natural novel analgesic peptide molecules by employing genetic engineering methods.Synthetic Analgesic peptide genes were prepared by Gene Cloning Methods. Site directed mutagenesis and codon optimization methods were adopted for development of recombinant synthetic genes. Their suitability of expression are verified insilico methods using various computational bioinformatics Tools like Vector 2.0 and Accelrys DS Modeller.The developed synthetic genes were cloned in suitable vectors and Expressed in Yeast Cells. Clones were purified and tested for their effectiveness of analgesic activities on mice models using hot plate assay method and Tail Immersion Methods.Our Synthetic genetically engineered built products were given prominent results when compared with standard analgesic molecules (55-70% effiency) that are available in market.Therefore, the challenge is encountered by these small synthetic recombinant genetically engineered molecules which competed with commercial well known chemical drugs, which have lot of side effects.*

Key words-- Analgesic Peptides, Codon Optimization, Site Directed Mutagenesis, Over expression, Synthetic Genes, Yeast Cells;

I. INTRODUCTION

In most of the pain handling cases there is an urgent demand for developing an ultimate novel molecule which can put stop for pain related conditions. In advance countries itself the epidemiological data suggest that nearly 40 percent of patients are suffering with hardship of pain and no satisfactory medicines are available to relieve form these chronic pains without side effects.

¹Asst.Professor, Dept of Zoology, University College of Science, Osmania University, Hyderabad,venbio@gmail.com, 7702355472.

Now a days pain management is getting a noble wide circulation to public because of increased life styles and affordability of treatments by individuals. In this condition contemporary medicines has to meet the challenges of classical drugs and deliver much more better analgesic attributes than ever before. Future medicine is looking forward for drugs to treat all kind of ailments without pain and it is also to achieve hallmark of services to their patients by health care industry. Advancements in both Genetic Engineering and Pharmacological Science for quite some time made it us possible to explore for more potent neuropeptides which play a significant role in understanding transmission of pain impulses and their signal transduction cascades. These neuropeptides which are synthesized in laboratory are working to match with targeted cell membrane receptors in specific locations in a well defined manner. Of course all these endogenous compounds have unfortunately a biological barrier to cross and reach the receptor locations and also have vulnerabilities towards enzyme degradations by host defence machinery mechanisms¹.

The above characteristics have drawn a line of boundaries to use them as drug molecules. However because of development of in-silico methods to study the peptide and receptor relationships, now a days it is possible to develop a drug molecule as quickly as possible by employing very little resources and man power. By doing an advance research work it can facilitate to make a better understanding of these small neuropeptide molecules that play a big role in majority of physiological processes including pain, analgesia, appetite and in several other inflammatory processes. In today's world there is huge demand for designing and usage of these drug molecules which challenge the chemical drugs. These naturally occurring opioid based neuro peptide molecules are very flexible for mutations and for showing enhanced activities.

The alkaloid based drug molecules works like induced fit models but whereas opioid neuro peptide molecules works like "Zipper" fashion based mechanisms which favours for optimization of the processes and use them as effective therapeutic molecules². The current millenium challenge is to promote such small peptide molecule which mimic chemical drugs molecules but works better than them.

To meet the above challenge it requires the methodology of identification of target receptors, validation, selection of potent effective drugs, modelling and selection of reactive peptide molecules, biosynthesis, structural validation, structure and activity relationships, conformation biology, molecular dynamics, cloning, expression and testing on animal models.

Even though Peptide Chemists have developed small molecules like Biphalin, Tachykinin but due high production costs and unstability of these molecules they not successfully completing clinical trials. This gap has created an opportunity for rDNA technologists to develop novel therapeutic analgesic peptide molecules which are small in size, high in stability and robust in action and with less side effects.

Hence Biotechnology products using rDNA Technology will give us an ample of opportunity to prepare and produce these small peptide molecules on large scale of production for industrial applications. Hence the current work emphasizes on production of these novel analgesic neuro peptide molecules designed by protein engineering method and expressed in Eukaryotic organisms like Yeast Cells for high level production.

II. MATERIAL AND METHODS

Recombinant Plasmids : The selected mutant sequences of all the peptides were codon optimized using DNA 2.0 software for maximum expression in Yeast cells (*Saccharomyces cerevisiae*). Codon optimization was carried out by keeping above 15% threshold and as per the codon usage frequency of standard Yeast tables. Secretary Signals in Yeast is alpha F with amino acid sequences of MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDL EGDF DVAVLFPNSTNNGLLFINTTIAASIAAKEEGVSLEKR were used. As wild sequences showed very little activity and many points were identified for mutagenesis, instead of using conventional site directed mutagenesis methods engineered synthetic genes were designed and purchased from Gene Art, Germany. These analgesic peptides were named as: JVY1, JVY2, JVY3, JVY4, and JVY5. For better expression the following vectors were used: Initially peptides JVY1- JVY5 were cloned in bacterial expression vector pET22b and transformed in BL21 cells for expression. Cloning Map and Sequence: Peptide-I (Endomorphin-2): AA Sequence: YGGFMGGGGYPFFGGGGYGGFL. Codon optimization Seq:ACGGTGGTTT TATGGGT GGTGGTGGTTATCCATTCTTTGGTGGTG GTGGTTATGGTGGTTTTCTG. Cloning Align ment: Hind III - E.CoRIAlpha F..... NheI—peptide 1.... kpnI-- NotI..... BamHI.

Basic JVY1 Sequence: (JVY1 to JVY5 are muteins):

AAGCTTGAATTCATGAGATTTCCATCTATTTTTACTGCTGTTTTGTTTGCTGCTTCTTCTGCTTTG GCTGCTCCAGTTAATACTACTACTGAAGATGAACTGCTCAAATCCAGCTGAAGCTGTTATTGGTT ATTCTGATTTGGAAGGTGATTTTGATGTTGCTGTTTTGCCATTTTCTAATTCTACTAATAATGGTTTG TTGTTTATTAATACTACTATTGCTTCTATTGCTGCTAAAGAAGAAGGTGTTTCTTTGGAAAAAAGAG CTAGCTACGGTGGTTTTATGGGTGGTGGTGGTTATCCATTCTTTGGTGGTGGTGGTTATGGTGGTTT TCTGTAATAGGGTACCAGATCTGCGGCCGCGGATCC . Standard recombinant DNA techniques were performed as described by Sambrook et al., (2001). Isolations were carried out by using Qiagen kits as per the manufactu -rers' instructions.

III. TRANSFORMATION

Synthetic genes were provided in plasmids. These plasmids were transformed in Top10 cells by CaCl₂ transformation. Transformants were maintained on slants and glycerol stocks for further usage. **Plasmid Isolation:** Plasmid cultures were grown in sensitive antibiotic medium and plasmid was isolated by Qiagen Kit. **Restriction Digestion and Elution:** Plasmids were digested using EcoR I and Not I **Ligation:** The purified fragments are ligated in to pET-22b vector and pCTON2 vectors using T4 DNA ligase. **Transformation:** The ligation mix was used in transformation of E.coli as per Sam brook & Maniatis et.al., 2001. **Screening of Recombinant Clones:** Overgrown colonies were inoculate were used for isolation of plasmids. **PCR and Amplification:** The clones were amplified by using forward and reverse primers. **Expression in Yeast and Purification:** Clones were expressed in yeast and partially purified by ammonium sulphate and Gel Chromatography methods.

Table 1: Strains and Vectors used in the Study

Name	Description	Vector used
Yeast		
EBY 100	GAL promoter and His selection marker	pCTNO2

IV. BIOLOGICAL ASSAYS IN ANIMAL MODELS

Animal Studies:

Animal studies were carried out after ethical clearance.. Initially planned for two types analgesic studies: 1. Hot plat method: After treatment mice were kept on hot plate at 55°C and checked for tolerance time. 2. Tail immersion method. After treatment mice tail was immersed in hot water at 55°C and checked for tolerance time.

V. RESULTS

1. Plasmid Construction and Analysis on Gel:

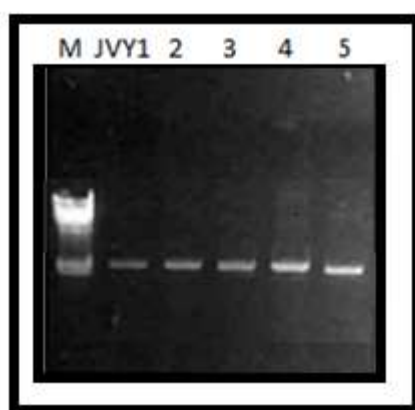


Figure 1: Agarose Gel Electrophoresis for JY1 to JY5 Plasmids. M: Marker; 1-5: JY1 to JY5.

2. Screening of Recombinant Clones:

Overnight grown colonies were inoculated for isolation of plasmid. Subsequent digestion of the plasmids with EcoRI and Not I revealed the presence of specific sizes corresponding to insert sizes used for ligation.

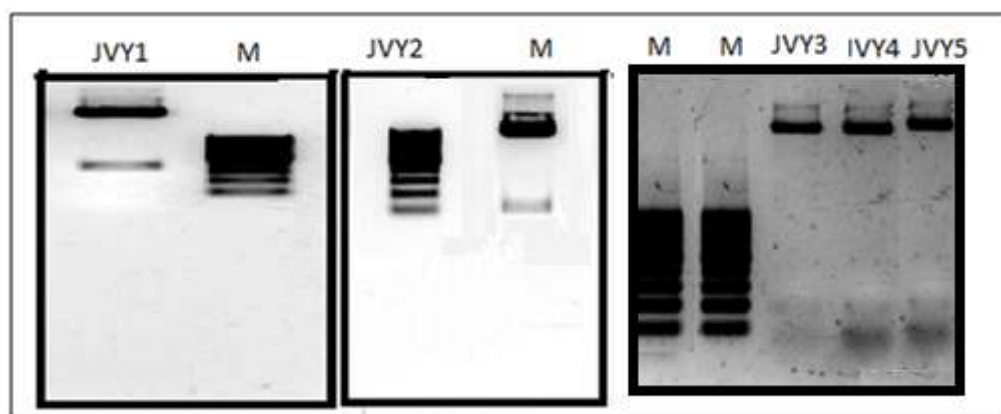


Figure 2: JY1 to JY5 Plasmids using Agarose Gel Electrophoresis. The Gene Fragments were separated by Eco RI and Not I Enzymes.

3. Gene Amplification by PCR:

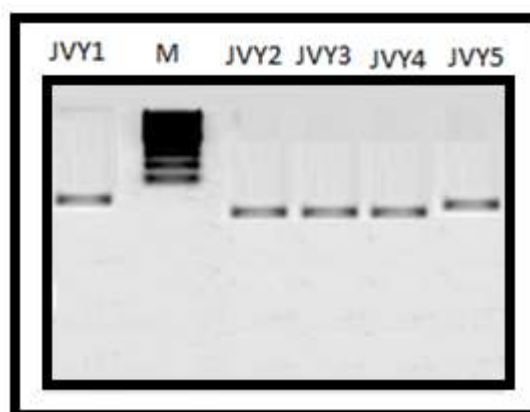


Figure 3: From Left to Right, First well with JY1, 2nd Well with Marker 100 bp, Third well with JY2, 4th Well with JY3, 5th well with JY4 and 6th Well with JY5 Gene amplified products.

VI. ANIMAL OBSERVATION

Table 2: Bioactivity of Recombinant Analgesic Peptides

A. Tail Immersion Methods.

Analgesic Peptides with Acetone Precipitated Analgesic Peptides							
Mice No	+ Ve Control Fentanyl Group	Group I JY1	Group II JY2	Group III JY3	Group IV JY4	Group V JY5	-Ve control Group 4
Tail Immersion Test Response of Mice in Seconds (Triplicate Exp Avg taken)							
M1	61	45	48	46	47	43	9
M3	62	59	58	54	55	56	11
M4	65	54	52	53	52	55	8
M5	58	49	54	54	52	48	10

M6	63	52	49	52	49	51	9
M7	66	58	53	49	56	57	7
M8	64	58	52	51	57	58	9

B. HOT Plate Method:

Analgesic Peptides with Acetone Precipitated Analgesic Peptides							
Mice No	+ Ve Control Fentanyl Group	Group I JYV1	Group II JYV2	Group III JYV3	Group IV JYV4	Group V JYV5	-Ve control Group 4
Tail Immersion Test Response of Mice in Seconds (Triplicates with Average)							
M1	9	6	7	8	7	6	4
M3	8	6	5	6	5	7	3
M4	8	7	6	7	5	6	3
M5	9	8	7	8	6	7	4
M6	8	6	6	6	7	5	3
M7	9	7	8	7	6	7	3
M8	8	6	7	6	6	6	4

VII. DISCUSSION

Endomorphin molecules were selected and their sequences were codon optimized using DNA 2.0 software for maximum expressions in yeast cells Codon optimization studies were carried out by using codon usage frequency tables of Yeast by using 15% threshold values.

Secretory signal of alpha F with amino acid sequences of MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLFPFSNSTNN GLLFINTTIASIAAKEEGVSLEKR was used for expression in yeast cells. As wild sequences revealed little activity in yeast cells, many points were identified for compatibility with yeast genome by doing site directed mutagenesis in silico methods and engineered synthetic genes were procured from Gene Art Company from Germany. Synthetic genes were transported by Gene Art company in plasmids. These plasmids were then transformed into Top10 cells by CaCl₂ transformation. These transformants were maintained on slants for long term storage purpose.

Plasmid cultures were grown in antibiotic sensitive medium and plasmids were isolated by Qiagen Kit. Plasmids were digested using EcoR I and Not I restriction enzymes. The purified fragments are ligated in to pCTON2 vectors using T4 DNA ligase. The ligation mix was used in transformation to yeast as per Sam brook & Maniatis et.al., 2001.

Overgrown colonies were inoculate were used for isolation of plasmids. The clones were amplified by using forward and reverse primers. These analgesic peptides were named as: JYV1, JYV2, JYV3, JYV4, and JYV5. For better expression . Initially peptides JYV1- JYV5 were cloned in yeast expression vector pCTON2 and transformed

in Yeast cells EBY 100 for expression. JYV 1-5 were induced with 100mM IPTG, after expression, partially purified acetone precipitation methods. These partially purified peptide molecule are used as oral drug molecules and fed to mice for experimental analysis and conducting of animal studies or bioassays. Animal studies were carried out after ethical committee clearance. The OU ethical committee has approved the animal studies for all five recombinant opioid peptides molecules which were used after dialysis or partial peptide purification. The mice were brought from NIN (National Institute of Nutrition), Hyderabad and minimum number of mice were utilized for studies (7 x 7). And no harm is done during experimental procedures as per guidelines. The mice were divided into seven groups each with 7 groups of mice. One is negative control group maintained by oral feed with un induced culture supernatant, one positive control group given 1 picogram subcutaneously and remaining 5 groups were tested for JYV 1-5 were induced with 100mM IPTG, after expression, partially purified by salt and acetone precipitation.

The partially purified peptide molecules were taken in water bottle and fed to mice. Mice were observed for body temperature, body weight, water and food intake. The body weight was found to be 24 ± 1 gm (range) on zero dose. The individual mice food intake was observed and it was found to be 4 ± 0.5 gm and individual mice water intake was found to be 4 ± 0.5 ml per day

We planned for two types analgesic studies: 1. Hot plate method: After treatment mice were kept on hot plate at 55°C and checked for tolerance time. 2. Tail immersion method. After treatment mice tail was immersed in hot water at 55°C and checked for tolerance time.

Analgesic studies were conducted by two well known methods: 1. Hot plate Method: Mice were kept on hot plate at 55°C after treatment and checked for tolerance time for triplicates. Average time was taken. In the second method, Tail immersion method tail is immersed in hot water bath at 55°C after treatment and checked for tolerance time. For hot plate method the group JYV3 has exhibited maximum activity followed by group JYV1 and then JYV2 and JYV5 and last by JYV4. It shows that JYV3 is the more tolerable analgesic peptide composition than other peptide molecule. In tail immersion method the group JYV1 has exhibited maximum activity followed by group JYV5 and then JYV2 and JYV3 and last by JYV4. It shows that JYV3 is the more tolerable analgesic peptide composition than other peptide molecule. Overall JYV1 has exhibited first position in first test and second position in second test tests and exhibited stable activity by the experimented animal models.

VIII. CONCLUSION

Our final goal is to see these engineered peptide molecules how they can compete with market products like Fentanyl compounds. In our observation, the synthetic peptides in crude form itself are giving a firm competition to formulated compound molecules. The group JYV1 is exhibiting nearly 55-70% performance levels when compared to commercial product which is a standard analgesic molecule. Hence, in future, it gives an opportunity for us to develop more potent molecules which can give stiff competition to existing analgesic molecules so that patients can get relieved from all kinds of side effects and ailments.^{5,6,7}

IX. ACKNOWLEDGMENT

The author specially acknowledges for Department of Biotechnology, Govt of India as the work was carried out by the financial assistance given under DBT-RGYI grant.

REFERENCES

1. Perez de Vega MJ, Ferrer-Montiel A, González-Mniaz R Recent progress in non-opioid analgesic peptides. Arch Biochem Biophys. 2018, 660:36-52.
2. Arkady M Kotin¹, Maksim O Emelyanov¹ and Oleg A Kotin² “Low-molecular synthetic peptides with non-narcotic type of analgesia: comparative study and mechanism of analgesic activity” Molecular Pain Volume 15: 1–13.
3. Crescenzi, Orlando & Guerrini, Remo & Picone, Delia & Salvadori, Severo & Tancredi, Teodorico & Temussi, Piero. (2000). Solution structure of nocistatin, a new peptide analgesic. Biopolymers. 53. 257-64.
4. Amodeo, Pietro & Guerrini, Remo & Picone, Delia & Salvadori et.al., (2002). Solution structure of nociceptin peptides. Journal of peptide science : an official publication of the European Peptide Society. 8. 497-509.
5. Janecka, A & Perlikowska, Renata & Gach, Katarzyna & Wyrebska, Anna & Fichna, J. (2010). Development of Opioid Peptide Analogs for Pain Relief. Current pharmaceutical design. 16. 1126-35.
6. Dray A, Perkins MN. Bradykinin and inflammatory pain. Trends in Neuroscience 1993; 16: 99–104.
7. Saladin PM, Zhang BD, Reichert JM. Current trends in the clinical development of peptide therapeutics. IDrugs.. 2009;12:779–784.