SYNTHESIS, STRUCTURE-ACTIVITY RELATIONSHIPS AND BIOLOGICAL EVALUATION OF NOVEL HEDGEHOG-GLI SIGNALLING PATHWAY AGONISTS

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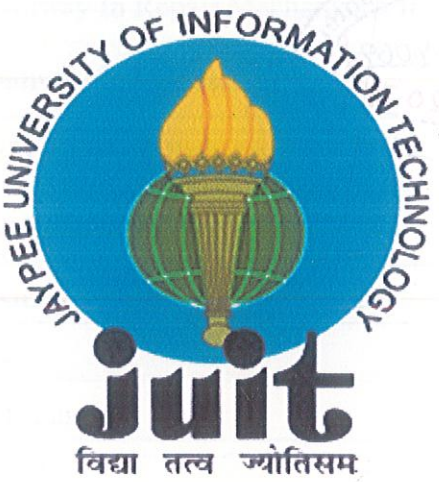
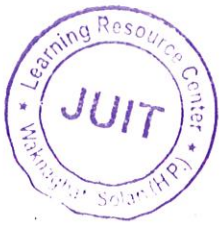
UNDER THE SUPERVISION OF **DR. NEERAJ MAHINDROO**





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Submitted in partial fulfillment of the Degree of

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DEPARTMENT OF PHARMACY
JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY
WAKNAGHAT

CONTENTS

CERTIFICATE

This is to certify that the thesis entitled "SYNTHESIS, STRUCTURE-ACTIVITY RELATIONSHIPS AND BIOLOGICAL EVALUATION OF NOVEL HEDGEHOG-GLI SIGNALLING PATHWAY AGONISTS", submitted by **Sana** (081776) and **Gautam Kumar** (081780) in partial fulfillment of the requirement for the award of the degree of Bachelor of Pharmacy to the Jaypee University of Information Technology, Waknaghat is a record of bonafide research work carried out by her under my supervision and guidance. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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Sana

Gautam Kumar Gautam

Date 26-05-2012

SUMMARY

Hedgehog-Gli signalling pathway has been shown to regulate stem cells and is a fundamental regulator of organogenesis in developing embryos and maintains tissue integrity in mature organisms. Smoothened agonists have been proposed as potential therapeutics for ischemia, neuronal injury/degeneration, wound repair, and retinal damage, where they could reactivate or stimulate repair mechanisms in situations in which normal regenerative capacity is compromised.

Literature review shows that tissue damage or brain injury is able to up regulate the production of Hh ligands and activates its signalling as a part of tissue repair process. These observations prompted an exploration to activate the Gli mediated transcription which could stimulate neurogenesis and helps in recovery from neuro degnerative diseases like Parkinson's and Alzheimers. This poses a challenge in the drug discovery process against this particular target.

Our study included design, synthesis and structure-activity relationship studies based on lead compound NMDA 225(Mahindroo*et al.* unpublished data) as hedgehog-Gli pathway agonists. Nineteen compounds were synthesized by coupling different amines and carboxylic acids and biological evaluation of four compounds was done on isolated mice neurons by evaluating ability to modulate mRNA expression of downstream genes. One of the compounds, **4**, showed Hh-Gli pathway agonist activity similar to purmorphamine, a known Hh-Gli pathway agonist. Further screening of synthesized compounds and confirmation of the activity of compounds **4** is being carried out.

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LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

Hh-Hedgehog

Shh-Sonic Hedgehog

Ihh-Indian Hedgehog

Dhh-Desert Hedgehog

PNS-Peripheral Nervous System

CNS-Central Nervous System

SAR-Structure Activity Relationship

Ptch-Patched

GNPs-Granule Neuron Precursors

Smo- Smoothend

CGNP-Cerebellar Granuler Neuron Precursors

Ci- CubitusInterrutus

HSC-Hedgehog Signalling Complex

Fu-Fused

Sufu-Suppressor of Fused

Gsk-Glycogen Synthase Kinase

NMR-Non Magnetic Resonance

TLC-Thin Layer Chromatography

PREP TLC- Preparative Thin Layer Chromatography

TEA-Tri Ethyl Amine

DIPEA- Di isoporpyl ethyl amine

DMF-Di Methyl Formamide

HBTU-O-Benzotriazole-*N,N,N,N*-tetramethyluroniumhexaflurophosphate

GLOSSARY

- **Paracrine signaling**: It is a mode of signaling whereby one cell (the sending cell) produces the signal (e.g. Hh ligand), but a different cell (the receiving cell) binds to and responds to it.
- **Patched (PTCH)**: The Hh receptor, a 12-transmembrane protein found at the cell surface or in primary cilia that binds to Hh to initiate ligand-dependent signaling.
- **Smoothened (SMO)**: A seven-transmembrane GPCR-like protein that transmits the Hh signal upon stimulation, leading to activation of the Gli transcription factors.
- **Hedgehog (Hh)**: The 25kD secreted ligand of the Hedgehog signaling pathway. There are three Hedgehog proteins in mammals, Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh), which all initiate signaling by binding their receptor, Patched.
- **Hh pathway inhibitor (HPI)**: Any drug, antibody, protein applied to cells or animals to inhibit Hh signaling.
- **Glioma-associated oncogene (Gli)**: A zinc finger transcription factor that mediates transcriptional responses to Hh signaling.

1. INTRODUCTION

The hedgehog (Hh) pathway is one of the pathways responsible for controlling the numbersand types of cells formed during development in species ranging from *Drosophila* to humans. The most well-known Hh-pathway ligand, sonic hedgehog (Shh), has been shown, in ever-expanding ways, to participate in central nervous system (CNS) development. Another ligand, desert hedgehog (Dhh), is essential for the proper formation of the peripheral nervous system (PNS).[1]Hhsignaling is also involved in homeostasis and stem cell proliferation in adult tissues. Furthermore, aberrant activation of the Hh pathway has been linked to the development of multiple human cancers.

Currently, the mammalian Hh signaling pathway is incompletely understood and may harbor some differences and/or additional pathway components. This thesis highlights recent information about the ways in which the Hh pathway is involved in degenerative neural disorders. We report synthesis and structure-activity relationship (SAR) studies of novel Hh-Gli pathway agonists and their biological evaluation in isolated neurons.

2. REVIEW OF THE LITERATURE

2.1 HEDGEHOG GLI SIGNALLING PATHWAY IN EMBRYONIC DEVELOPMENT

In a growing embryo, cells develop differently in the head or tail end of the embryo, the left or right, and other positions. The hedgehog signaling pathway gives cells information that they need to make the embryo develop properly. Different parts of the embryo have different concentrations of hedgehog signaling proteins.

Studies in both vertebrates and invertebrates have identified proteins of the Hedgehog (Hh)family of secreted signaling molecules as key organizers of tissue patterning. Initially discovered in *Drosophila* in 1992, Hh family members are observed in animals with body plans as diverse as those of mammals, insects and echinoderms. In humans three related Hh genes have been identified: Sonic, Indian and Desert hedgehog (Shh, Ihh and Dhh).

The developmental processes regulated by the *Drosophila* and vertebrate Hh signaling pathways appear to be remarkably conserved.[1]During development, the Hh/Shh cascade is extensively used during embryogenesis to trigger a plethora of effects in a variety of tissues. These effects range from anterior-posterior or dorsal-ventral patterning, cell fate determination to tissue outgrowth, suggesting that Hh signaling acts in a context-dependent fashion. The Hh/Shh cascade induces the expression of transcription factors and signaling molecules that are essential for animal development.[2]

Hedgehog signaling regulates cell differentiation and organ formation during embryonic development in animals.

- **Skin**: During the development of a normal hair follicle, Shh is expressed in the thickening embryonic epithelial layer, and its receptor Patched (PTCH) is expressed in the underlying dermal layer. The binding of Shh to PTCH leads to epidermal proliferation and in vagination to form a hair follicle.[3]
- **Cerebellum**: During normal development, a large pool of granule neuron precursors (GNPs) is generated on the surface of the developing cerebellum. PTCH restrains proliferation of GNPs until Shh is secreted by Purkinje neurons. The binding of Shh to PTCH relieves the repression of target gene activation and results in the proliferation of GNPs.[3]

- **Pancreas**: Down regulation of Hh expression is required to initiate mammalian pancreatic development. However, after the pancreas has fully developed, Hh signaling appears to have a different function. Ihh and Dhh are expressed in adult pancreatic β-cells and appear to be involved in the regulation of insulin expression.[3]

- **Gut**: The gastrointestinal tract develops from the embryonic gut tube, which is composed of two different germ layers: endoderm, which differentiates into the epithelial lining, and mesoderm. Folding of the primitive tube into the complex adult organ depends on Hh signaling between these two germ layers.[3]

2.2 HEDGEHOG PATHWAY IN REPAIR MECHANISMS IN ADULTS

Once development is complete, the expression of Hh ligands, Ptc, Smo, and Gli1 declines to low levels in normal healthy tissues, at least in rodents. More than a decade ago, there was speculation among developmental biologists that pathways such as the Hh pathway could be reactivated in adult tissue to promote recovery from damage. Possibly, supplying additional Hh protein could rebuild functional tissue by stimulating endogenous stem cells to proliferate and differentiate, similar to what happens in the embryo.Shh stimulates the survival and proliferation of neural precursor cells and plays a role in cell survival. An intravenous Hh agonist at doses that up regulate spinal cord Gli1 transcription also increases the population of neural precursor cells after spinal cord injury in adult rats.[4]

Hh signaling modulates tissue remodeling by controlling the fate of Hh-responsive cells. Healthy adult livers exhibit little Hh activity. However, cells involved in adult liver repair, including myofibroblasts and progenitors, are capable of producing and responding to Hh ligands.[5]

The participation of the Hh pathway in motor neuron differentiation in the developing spinal cord has been studied in detail.[6]

2.3 HEDGEHOG PATHWAY IN DISEASES AND REPAIR

2.3.1 Neurodegenerative Diseases

The importance of the Hh pathway in embryonic neural development has been well documented, but the role of Hh signaling in the adult brain is less well understood. In the adult rodent CNS and PNS, Hh ligands and their receptors, Ptc and Smo, are present, although the magnitude of Hh signaling seems to be lower and more restricted spatially.[7]

2.3.2 Acute Brain Injury[8]

The adult mammalian brain responds to injury by activating a program of cell proliferation during which many oligo dendrocyte precursors, microglia, and some astrocytes proliferate. Another common response to brain injury is the induction of reactive gliosis, a process where by dormant astrocytes undergo morphological changes and alter their transcriptional profiles. It has been reported that Shh is produced in reactive as trocytes after injury to the cerebral cortex and participates in regulating the proliferation of Olig2-expressing (Olig2) cells after brain injury.[8]

Therefore tissue damage is able to up regulate the production of Hh ligands and activate Hh signaling as part of the tissue-repair process. These observations prompted an exploration of the potential for stimulating Hh signaling to promote functional recovery in models of neurodegenerative disease.[8]

2.3.3 Parkinson's disease

Parkinson's disease is a progressive neurological disorder characterized by the loss of dopaminergic neurons in the sub stantianiagra. Shh is known to be involved in the differentiation of dopaminergic neurons during embryonic development.[9]

2.3.4 Peripheral Neuropathy

Shh is primarily important for CNS development, while the morpho genic effects of Dhh are seen in the PNS. Treatment with Hh protein has been shown to be protective and to enhance regeneration in PNS injury models. Diabetes mellitus is a chronic metabolic disease accompanied, in a significant number of cases, by peripheral neuropathies (pain and loss of sensation as the main symptoms). Again, treatment with Hh protein after the onset of symptoms significantly improved nerve-conduction velocity in diabetic rats. A marked improvement in nerve blood flow after treatment was also reported.[10]Hedgehog proteins may have therapeutic potential for the treatment of diabetic neuropathy.[10]

2.3.5 Ischemia

Improved blood flow and accelerated recovery of function are also seen in ischemic hind limbs following treatment with Shh.[11]Blood flow in the nerve decreases in diabetes—in rodents and in humans—leaving the nerve ischemic. Shh protein shows significant positive effects on nerve recovery from injury or disease in the nerve crush model. The

apparent mechanism of action underlying these effects is the ability of Hh to stimulate the production of neuro trophic and angiogenic factors in nerve perineurial cells.

2.3.6Maintenanceof Blood Vessels in the Heart

The investigators found that completely blocking hedgehog signaling in the heart of adult mice caused many small coronary blood vessels to disappear, leaving heart muscle short of oxygen and leading to heart failure. In mice with experimentally induced heart attacks, mildly inhibiting hedgehog signaling led to a worsening of their heart conditions.[12]

2.3.7Hair follicle cycling

In the skin, sonic hedgehog (Shh) is required for hair follicle morphogenesis during embryogenesis and for regulating follicular growth and cycling in the adult Hh-agonist can stimulate the transition from the resting (telogen) to the growth (anagen) stage of the hair cycle in adult mouse skin. Hh-agonist induced hair growth caused no detectable differences in epidermal proliferation, differentiation, or in the endogenous Hh-signaling pathway as measured by Gli1, Shh, Ptc1, and Gli2 gene expression when compared with a normal hair cycle.[13]

2.3.8 Hedgehog pathway: Role in cancer

Hh signaling is tightly controlled during cellular proliferation, differentiation, and embryonic morphogenesis. Alterations in the hedgehog pathway have been linked to basal-cell carcinomas, medulloblastoma, small-cell lung and gastrointestinal tract cancer. Thus, drugs targeting the hedgehog pathway maybe useful in the treatment of these cancers.[14]

2.3.9Other Diseases and Repair

Sonic hedgehog signaling plays an essential role during embryonic salivary gland epithelial branching morphogenesis.[15]It is also emerging targets for osteoporosis.[16]Glucocorticoids(GC) exposure can affect brain maturation when administered during the development phase that occurs after preterm birth. Shh enhances expression of the glucocorticoid-metabolizing enzyme 11β-HSD2, reducing the hormone's inhibitory effects on CGNP proliferation Shh enhances expression of the glucocorticoid-metabolizing enzyme 11β-HSD2, reducing the hormone's inhibitory effects on cerebellar granular neuron precursors(CGNP) proliferation.[17]

2.4 REGULATION OF HEDGEHOG PATHWAY

Transduction of the Hh signal to the cytoplasm utilizes an unusual mechanism involving consecutive repressive interactions between Hh and its receptor components, Patched (Ptch)and Smoothened (Smo). Several cytoplasmic proteins involved in Hh signal transduction are known in *Drosophila*, but mammalian homologs are known only for the Cubitusinterruptus(Ci), transcription factor (Gli(1-3)) and for the Ci/Gli-associated protein, Suppressor of Fused (Su(fu)).

Gli proteins

The three Gli proteins are zinc-finger transcription factors of >1000 amino acids that encode both activator and repressor functions. The fly Gli homologous protein, Cubitusinterruptus (Ci), is also both an activator and a repressor. In the absence of Hh signaling, Gli1 is transcriptionally silent but Gli2 and Gli3 can be expressed. In the presence of Hh ligands and activation of the trans membrane protein Smoothened (Smo), the Gli code is changed: Gli1 is activated transcriptionally, possibly by preexisting Gli2 or Gli3; Gli2 becomes an activator; and Gli3 is no longer cleaved.

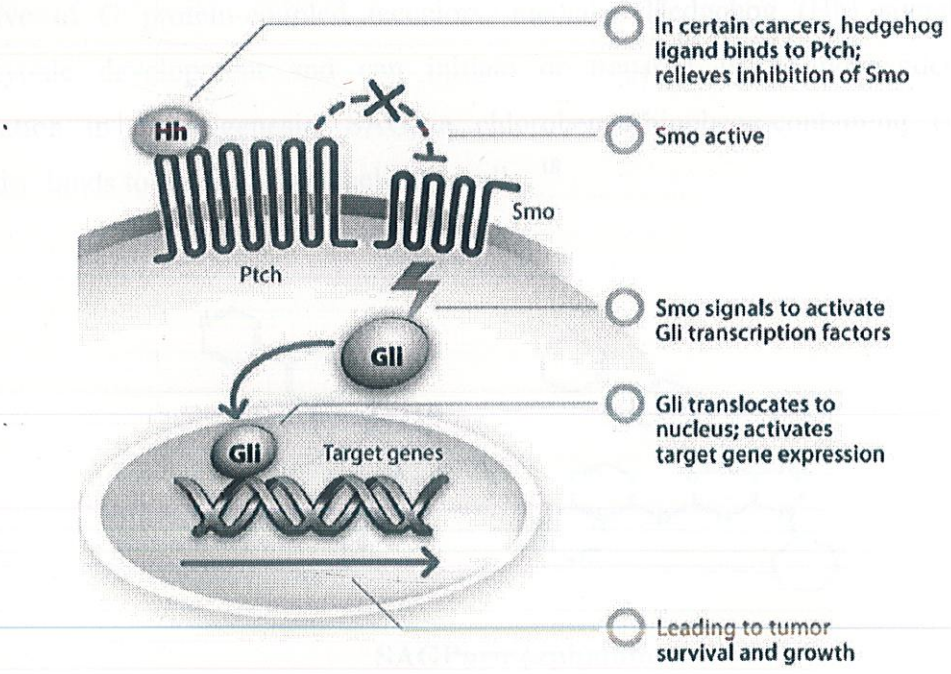


Figure 1: Hedgehog pathway activators and inhibitors

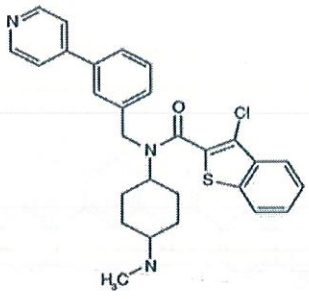
Hedgehog signaling is initiated by the binding of Hh ligand to Ptch which is a 12-transmembrane protein receptor. In the absence of Hh, Ptch acts to prevent high expression and activity of Smo. Downstream of Smo is a multi-protein complex known as

the Hedgehog signaling complex (HSC), which comprises the transcription factor Cubitusinterruptus (Ci), the serine kinase Fused (Fu), the kinesin-like molecule Costal 2 (Cos2) and Suppressor of fused (Sufu).When extracellular Hh is present, it binds to and inhibits Ptch, allowing Smo to accumulate and inhibit the proteolytic cleavage of the Ci protein (Gli in humans). This process most likely involves the direct interaction of Smo and Cos2 where the steps leading to Ci protein proteolysis are disrupted.Cos2 also binds to protein kinase A (PKA), protein kinase CK1 (formerly casein kinase 1) and glycogen synthase kinase 3 (GSK3), which are other kinases that are implicated in the Hh signaling pathway.[1]

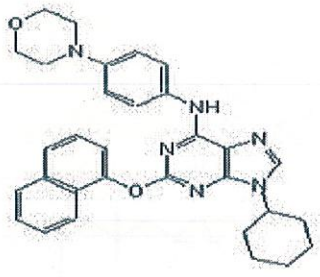
2.5 Small molecule modulators of Hedgehog pathway

2.5.1 Shh pathway agonists

SAG: SAG activates Gli-mediated transcription in a variety of cell types by binding to Smo. It competes directly with cyclopamine–Smo interactions. SAG is particularly attractive because of its potency to regulate Hh pathway at the concentration of ~3.0 nM, as well as its unique modularity feature for diversity-oriented synthesis. Smo, a distant relative of G protein-coupled receptors, mediates Hedgehog (Hh) signaling during embryonic development and can initiate or transmit ligand-independent pathway activation in tumorigenesis. SAG, a chlorobenzothiophene-containing Hh pathway agonist, binds to the Smo heptahelical bundle. [18]



SAG



Purmorphamine

Figure 2: a) Structure of SAG b) Structure of Purmorphamine

Purmorphamine: Purmorphamine is a synthetic Shh pathway agonist that activates Gli-mediated transcription in a variety of cell types an dbinds Smo and competes directly with cyclopamine– Smo interactions.[19]

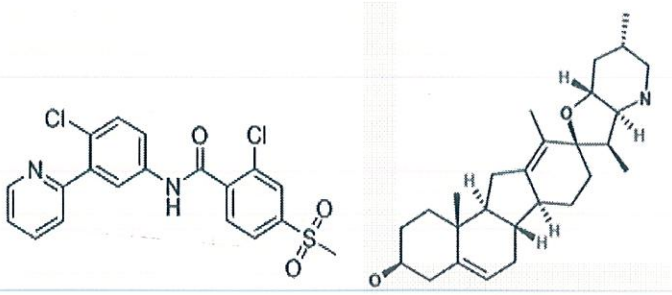
Glucocorticoids as Smoothened agonists: Halcinonide, fluticasone, clobetasol, and fluocinonide activate Hh signalling. These drugs demonstrated an ability to bind to Smo, promote Smo internalization, activate Gli, and stimulate the proliferation of primary neuronal precursor cells alone and synergistically in the presence of Shhprotein.[20]

Hedgehog protein: Shh encodes a secreted peptide, which is expressed in notochord and floor plate cells and can induce appropriate ventral cell types in the basal forebrain and spinal cord. Shh is sufficient to induce dopaminergic and other neuronal phenotypes in chick mesencephalic explants *in vitro*.[21]Shh is a general ventralizing signal in the CNS, the specific response being determined by the receiving cells. These results suggest that Shh may have utility in the induction of clinically important cell types.

2.5.2 Small Molecule Inhibitors of the Hh-Gli Pathway[22]

The aberrant activation of Hh-Glisignaling in several cancers has made it an attractive target for anticancer drug discovery. Drugs that impede hedgehog signaling are being tested against several of these cancers, including basal cell carcinoma, prostate cancer, pancreatic cancer, colorectal cancer, and medulloblastoma.

Cyclopamine, a naturally occurring alkaloid, was the first Smo antagonist to be reported. It has been shown to inhibit Hh signaling and to induce the remission of medulloblastoma in a transgenic mouse model. Smo inhibitor GDC-0449 developed by Genentech, was recently approved by FDA for advanced basal cell carcinoma.[23]Acyclopamine derivative IPI-926 is also under clinical development. Another Smo agonist NVP-LDE225, is also in clinical trials.[24]



GDC-0449 Cyclopamine

Figure 3: a) Structure of GDC-0499 **b) Structure of Cyclopamine**

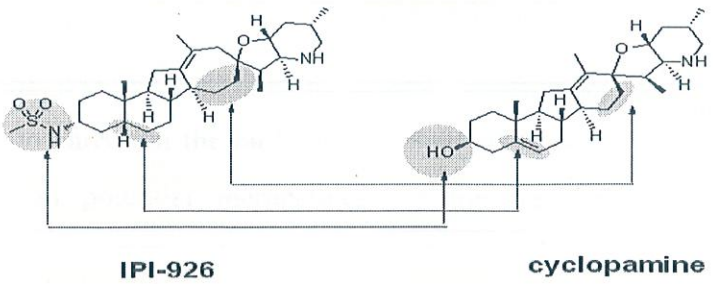


Figure 4: Structural relationship between IPI-926 and cyclopamine

2.5.3 Gli-mediated transcription inhibitors:

Novel inhibitors of Gli1-mediated transcription acting downstream in the Hh-Gli pathway, GANT 61[25], and NMDA 298-1,[26] have also been reported as potential anticancer agents were reported.

3. AIMS OF THE STUDY

The main goal of this project was to design, synthesize, study structure-activity relationships, and to carry out the biological evaluation of novel Hedgehog Glisignaling pathway agonists as potential therapeutics for the treatment of neurodegenerative disorders.

The Hh-Gli pathway agonist**NMDA225**was selected as lead molecule based on preliminary studies carried out by Mahindroo *et al.* (unpublished data) during their drug discovery project for Gli-mediated transcription inhibitors.[26,28] Building on NMDA225 we aimed to design and synthesize analogs in an effort to reveal the structure-activity relationships and evaluate the compounds in the relevant *in vitro* models using isolated adult rat neurons.

4. METHODOLOGY

4.1 Chemistry

All chemicals and reagents used in current study were of laboratory grade. The reactions were monitored by thin layer chromatography (TLC) on silica GF254 plates. The ^{1}H NMR spectra were recorded at with BRUKER NMR, 500MHz spectrometer using tetramethylsilane as the internal reference, with CDCl3 as solvent at Ambedkar Centre for Biomedical Research, Delhi University, The chemical shifts were reported in parts per million(ppm).

General Procedure[26]

Mixture of appropriate carboxylic acid (1eq), HBTU (2.5eq) and TEA (3eq) in DMF was prepared. The above mixture was allowed to stand for 30 minutes at rt. Desired amine (2eq) was added to mixture and was stirred for 2 hrs at rt. Workup was done by adding water and extracted with ethyl acetate followed by successive washings with water and brine. Separated organic layer was dried over anhydrous sodium sulphate. Solvent was removed *in vacuo* using rotary evaporator.

The crude mixture was purified using **column chromatography**. The procedure is as follows:

Column was packed with Silica gel (60-120 mesh) using wet method and compound was eluted with 10-15% ethyl acetate hexane. Those fractions that contained the product were taken and solvent was removed from them using rotary evaporator to obtain the purified product.

4.1 General Reaction[26]

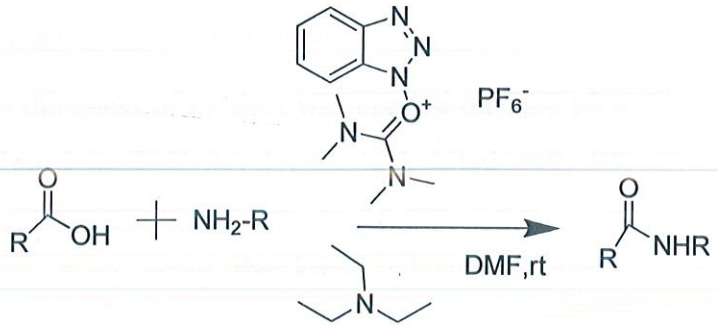
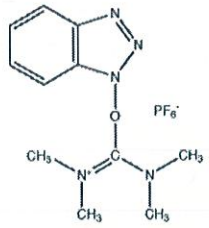


Figure 5: General Reaction of Amide coupling

Reagent and conditions: HBTU, TEA, DMF, rt

Mechanism of Reaction[27]



HBTU ($C_{11}H_{16}F_6N_5OP$)

O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluorophosphate

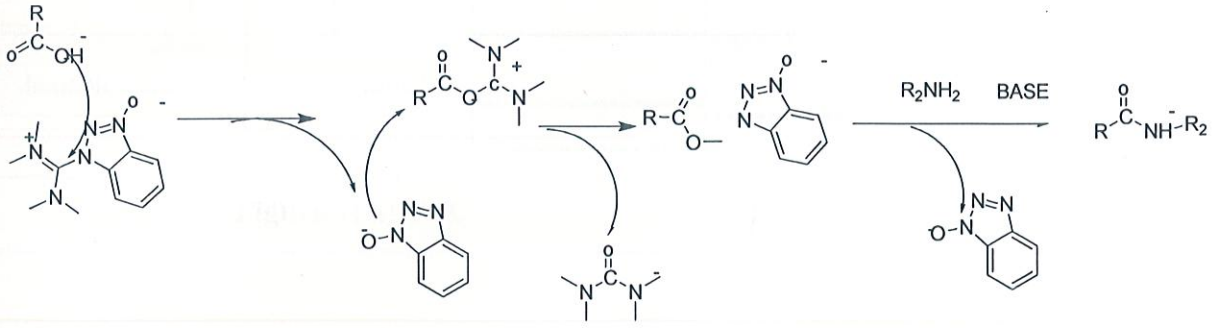


Figure 6: Mechanism of reaction of HBTU coupling

PREPARARTIVE TLC (PREP TLC): Two compounds (**SN005** and **GK005**) were purified using PREP TLC as follows:

a) Thin layer chromatography(TLC) was examined in various solvent systems. The optimal solvent system obtained for the separation was 10 % ethyl acetate in hexane for SN005 and 10 % ethyl acetate in hexane for GK005.

b) PREP TLC plates (15X20) of 2mm thickness were prepared using silica GF254 with 2% CaSO4 as binder in Stahl's applicator.

c) Band of 2mm thickness of product was applied on TLC plate (2 cm from below). The plate was developed in TLC developing tank filled with 100 ml of solvent until the solvent front reaches within one inch of the top. After the solvent was dried multiple development was done using the same solvent chamber to get band of desired concentration

d) Plate was removed and allowed to dry. It was then visualized using UV light and bands were marked lightly using pencil.

e) Bands were scraped using edged knife and was then filtered in glass funnel. Solvent was removed using rotary evaporation and purified product was obtained.

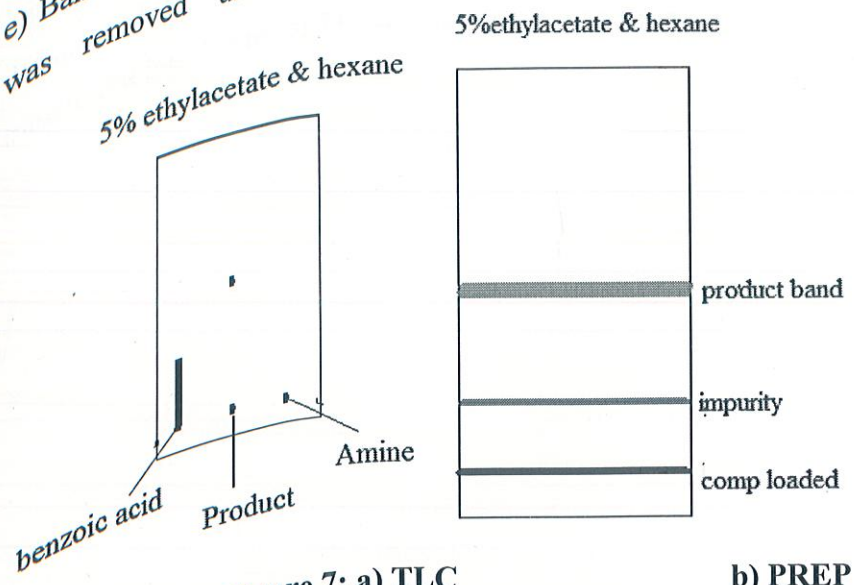


Figure 7: a) TLC **b) PREP TLC**

BIOLOGICAL EVALUATION:

Compounds were tested for their activity on culture of adult rat neurons. The neurons were isolated as follows:

Buffers and Materials: **1.** HEPES BUFFER SALINE(HBS) contains 10mM HEPES(N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid), 145mM NaCl, 22mM KCl, 5mM glucose. 1000 ml of HBS was prepared and pH was adjusted to 7.3.

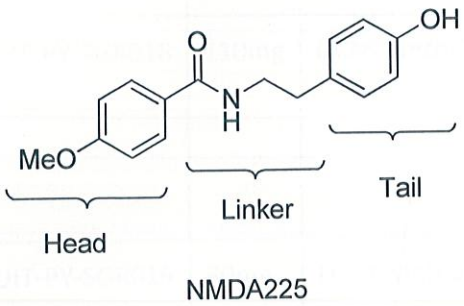
2. Hanck's Balanced Salt Solution contains 0.44mM potassium phosphate, 5,37mM potassium chloride, 0.34mM sodium phosphate dibasic, 136.89mM sodium chloride and 5.55mM D-Glucose.

Procedure- Rat was subjected to cervical dislocation and hippocampus and cerebellum was isolated and kept on petri dish containing HEPES buffer separately .After washing the cerebellum decand the solution and HEPES was added again to the same dish. Tissue was chopped and mixed with the buffer. After washing with the buffer it was transferred into 15ml sterile tube. 20.5 ml of papain solution was then added to the tube and mixed slowly with pipette. The tube was then kept for incubation for 15 min at r.t after which supernatant was removed. HBS was again added and tissue was immediately triturated

with 1 ml pipette. Then the tube was centrifuged at 2000 rpm for 10 min, solution was decand and 300µl of Trizol was added. After proper mixing with pipette, solution was transferred into 1.5ml DEPC tube for RNA extraction.

4. RESULTS AND DISCUSSIONS

The research efforts to design and synthesize Gli-mediated transcription inhibitors by Mahindroo *et al.*[26,28] also resulted in identification of NMDA225 as potential Hh-Gli pathway agonist.(Mahindroo et al unpublished data) We selected this molecule as our lead molecule for the present project as preliminary data for NMDA225 showed significant Hh-Gli agonist activity, drug-like characteristics and substantial scope for modification and optimization. The molecule can be divided into three parts; head, linker and tail (Figure 7).



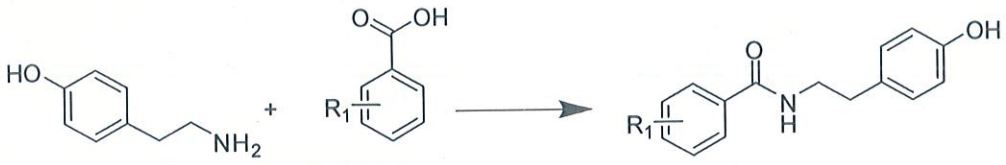
NMDA225

Figure 8: NMDA225 was selected as lead compound for the present studies based on preliminary studies.(Mahindroo et al unpublished data)

We decided to study the structure-activity relationships at each of the three parts by first starting with head part. Tyramine was coupled with different benzoic acids in presence to HBTU and DIPEA in DMF to synthesize compounds **1-5 (Table 1)**. All the compounds were obtained in good yield after purification by column chromatography.

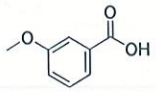
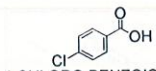
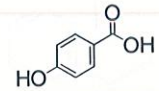
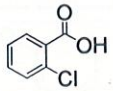
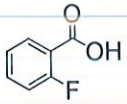
Next we to keep the head part constant and vary the tail part. Compounds **6-11 (Table 2)**, with benzoic acid as head part, and various aliphatic and aromatic amine as tail part were synthesized. Similarly, compounds **12-17 (Table 3)** were synthesized with indole-3-acetic acid contributing the head part of the molecule by coupling with amines using the general procedure. Compounds **18** and **19 (Table 4)** were synthesized from 4-hydroxy benzoic acid by coupling with cyclohexylamine and aniline, respectively.

Scheme 1: Amide coupling between tyramine and different benzoic acids

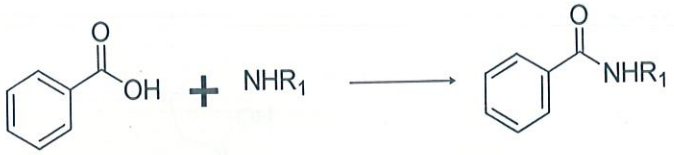


Reagents and conditions: HBTU, DIPEA, DMF, rt

Table 1

S.NO	BENZOIC ACID(R1)	PRODUCT	YIELD	TLC CONDITION	RF VALUE
1	 3-METHOXY BENZOIC ACID	JUIT-PY-SGR018	110mg	DCM:Methanol::98:2	0.5
2	 4-CHLORO BENZOIC ACID	JUIT-PY-SGR019	80mg	DCM:Methanol::98:2	0.5
3	 4-HYDROXY BENZOIC ACID	JUIT-PY-SGR020	125mg	DCM:Methanol::99:1	0.6
4	 2-CHLORO BENZOIC ACID	JUIT-PY-SGR021	70mg	DCM:Methanol::98:2	0.5
5	 2-fluoro BENZOIC ACID	JUIT-PY-SGR022	103mg	DCM:Methanol::99:1	0.6

Scheme 2: Amide coupling between benzoic acid and different amines



Reagents and conditions: HBTU, DIPEA, DMF, rt

Table 2: Compounds 6-11

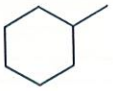
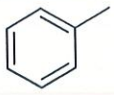
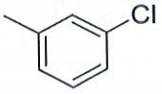
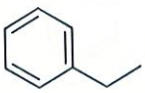
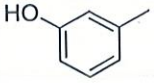
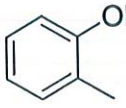
S.NO	AMINE(R1)	PRODUCT	YIELD	COLUMN CONDITION	RF VALUE
6		JUIT-PY-SNOO1	9%	Hexane:ethylacetate::9:1	0.5
7		JUIT-PY-SNOO3	11%	Hexane:ethylacetate::9:1	0.5
8		JUIT-PY-SNOO4	92%	Hexane:ethylacetate::9:1	0.6
9		JUIT-PY-SNOO5	58%	Hexane:ethylacetate::9:1	0.5
10		JUIT-PY-SNOO6	39%	Hexane:ethylacetate::9:1	0.6
11		JUIT-PY-SNOO7	90%	Hexane:ethylacetate::19:1	0.6

Table 3: INDOLE-3-ACETIC ACID derivatives (Compounds **12-17**)

Reagents and conditions: HBTU, DIPEA, DMF, rt

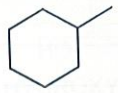
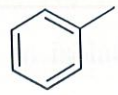
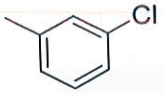
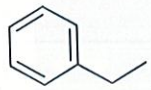
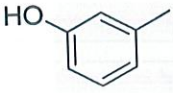
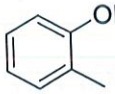
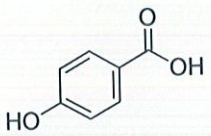
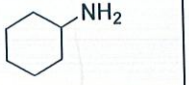
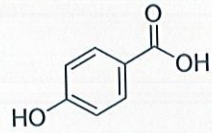
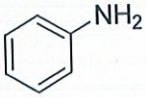
S.NO	AMINE(R2)	PRODUCT	YIELD	COLUMN CONDITION	RF VALUE
12		JUIT-PY-GK001	22%	Dichloromethane:Methanol:: 98:2	0.4
13		JUIT-PY-GK003	15%	Hexane: Ethyl acetate ::95:5	0.6
14		JUIT-PY-GK004	70%	Hexane: Ethyl acetate ::9:1	**0.3**
15		JUIT-PY-GK005	90%	Hexane: Ethyl acetate ::9:1	0.5
16		JUIT-PY-GK006	69%	Hexane: Ethyl acetate ::19:1	0.7
17		JUIT-PY-GK007	81%	Hexane: Ethyl acetate ::19:1	0.5

Table 4: 4-Hydrxybenzoic acid derivatives (Compounds **18-19**)

Reagents and conditions: HBTU, DIPEA, DMF, rt

Comp No.	BENZOIC ACID	AMINE	PRODUCT	TLC CONDITION	RF
18	 4-HYDROXY BENZOIC ACID	 CYCLO HEXYL AMINE	JUIT-PY-SGR012	Dichloromethane: Methanol::99:1	0.6
19	 4-HYDROXY BENZOIC ACID	 ANILINE	JUIT-PY-SGR013	Dichloromethane: Methanol::98:2	0.5

Compounds **1-4** were tested for their ability to induce mRNA expression of Gli1 and cyclin D1 in isolated neurons from adult rat cerebellum. Purmorphamine[19] was used a positive control and DMSO as solvent control. The concentration of DMSO was limited to 0.5% for each of the tested compounds.

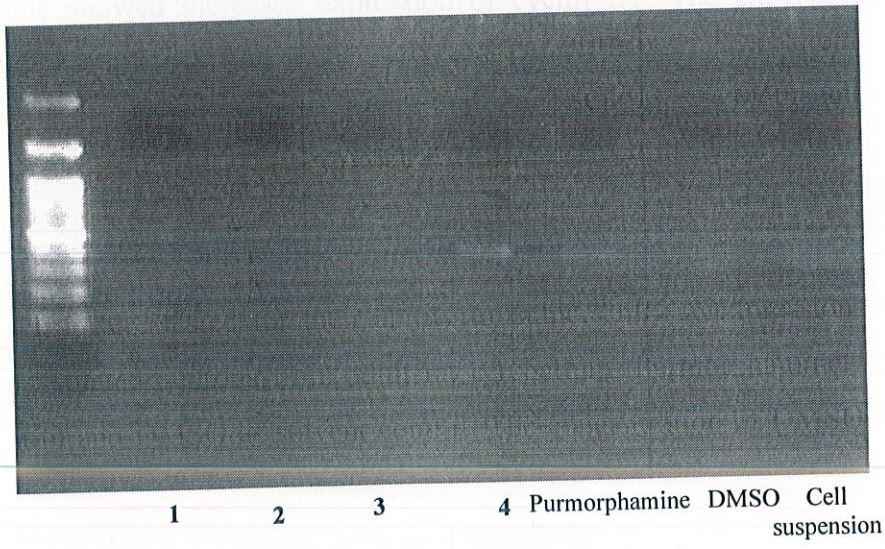


Figure9 : Gli1 mRNA expression in presence of 50 μM of test compounds (Compounds 1-4), purmorphamine, DMSO (Solvent control) and normal control.

Compound **4** showed increased expression of Gli1 mRNA as compared to purmorphamine, a known Smo agonist,[19] thus showing that it might be a potential lead for further follow up.

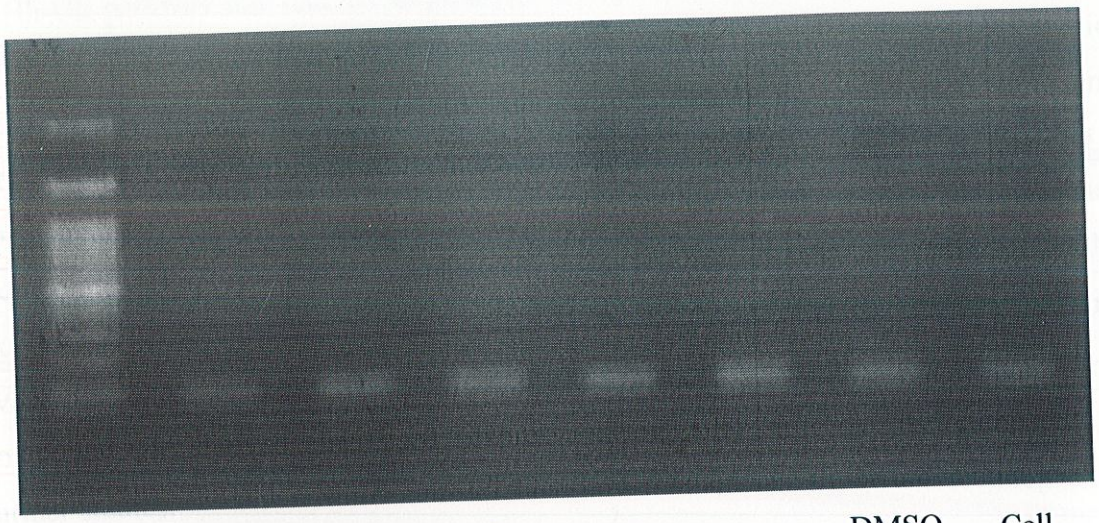


Figure 10: Cyclin D1 mRNA expression in presence of 50 μM of test compounds (Compounds 1-4), purmorphamine, DMSO (Solvent control) and normal control.

Cyclin D1 is responsible for cell proliferation and is downstream of Gli in Hh-Gli pathway. We also tested compounds **1-4** for ability to induce cyclin D1. Compound **4** and purmorphamine showed increased expression of cyclin D1. The experiment needs to repeated for further confirmation of the results and for statistical significance.

CONCLUSION

In this study, we report the design, synthesis and biological evaluation of novel agonists of Hh-Gli pathway that can act as potential therapeutics in neurodegenerative disorders. Nineteen analogues of lead molecule NMDA225, selected on basis of previous studies, were synthesized to study SAR at head and tail part. Four of the synthesized compounds were evaluated for ability to induce mRNA expression of Gli1 and cyclin D1, two of downstream effectors in Hh-Gli pathway, in isolated neurons from adult rat cerebellum. Compound 4(JUIT-PY-SGR021)enhanced expression of both Gli and cylin D1 mRNA expression, which was comparable to positive control purmorphamine. Further biological evaluation of the other synthesized compounds and confirmation of the results for the compound **4** is currently being carried out to decipher the SAR and select the lead for further studies.

NMR SPECTRAS

Figure 11: NMR Spectra of compound no.12(JUIT-PY-GKOO1)

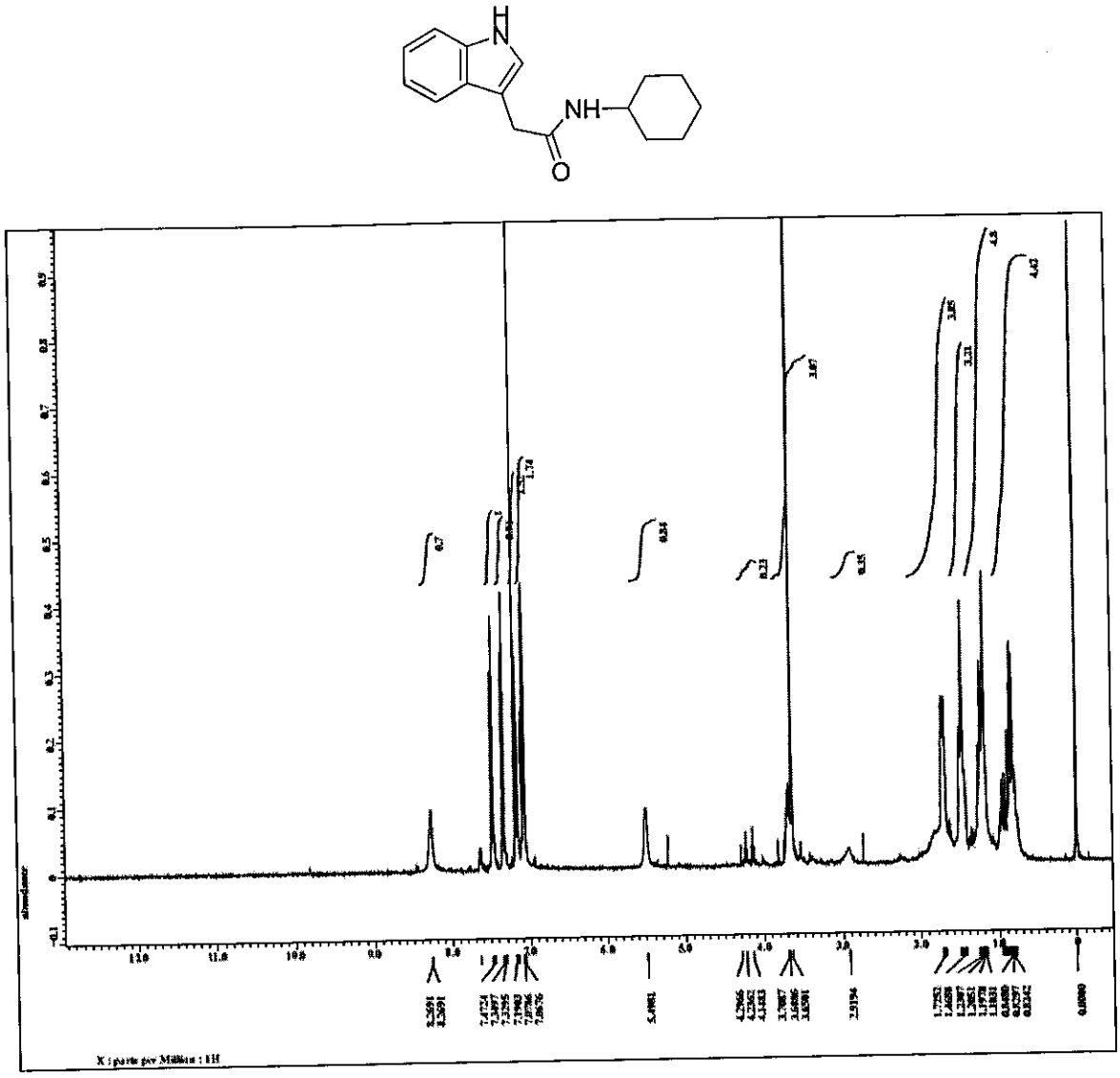
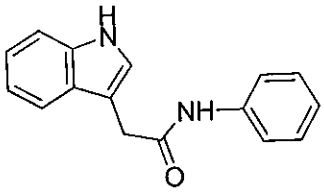


Figure 12: NMR Spectra of compound no.13(JUIT-PY-GK003)



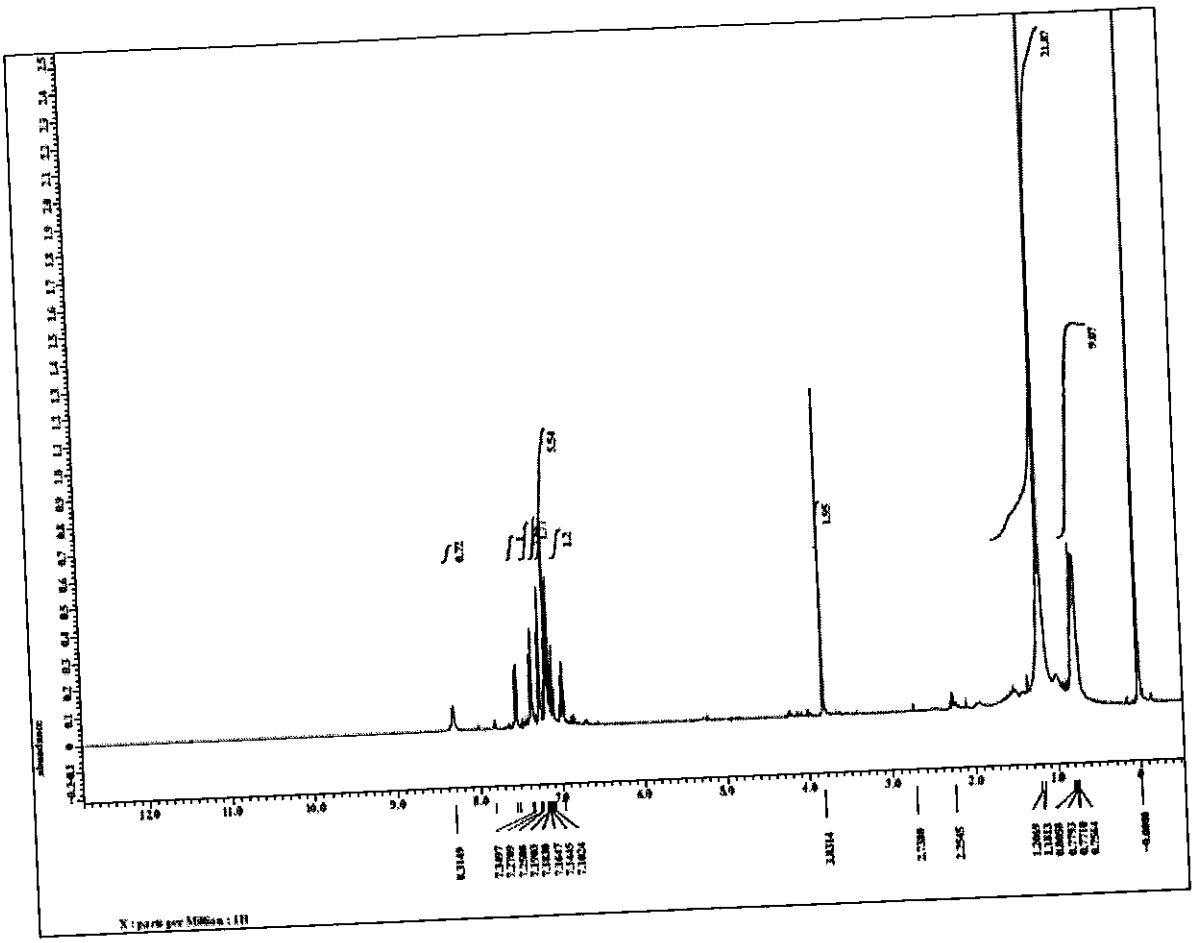
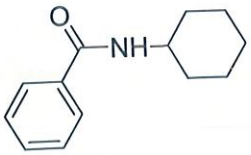


Figure 13: NMR spectra of compound no.6(JUIT-PY-SN001)



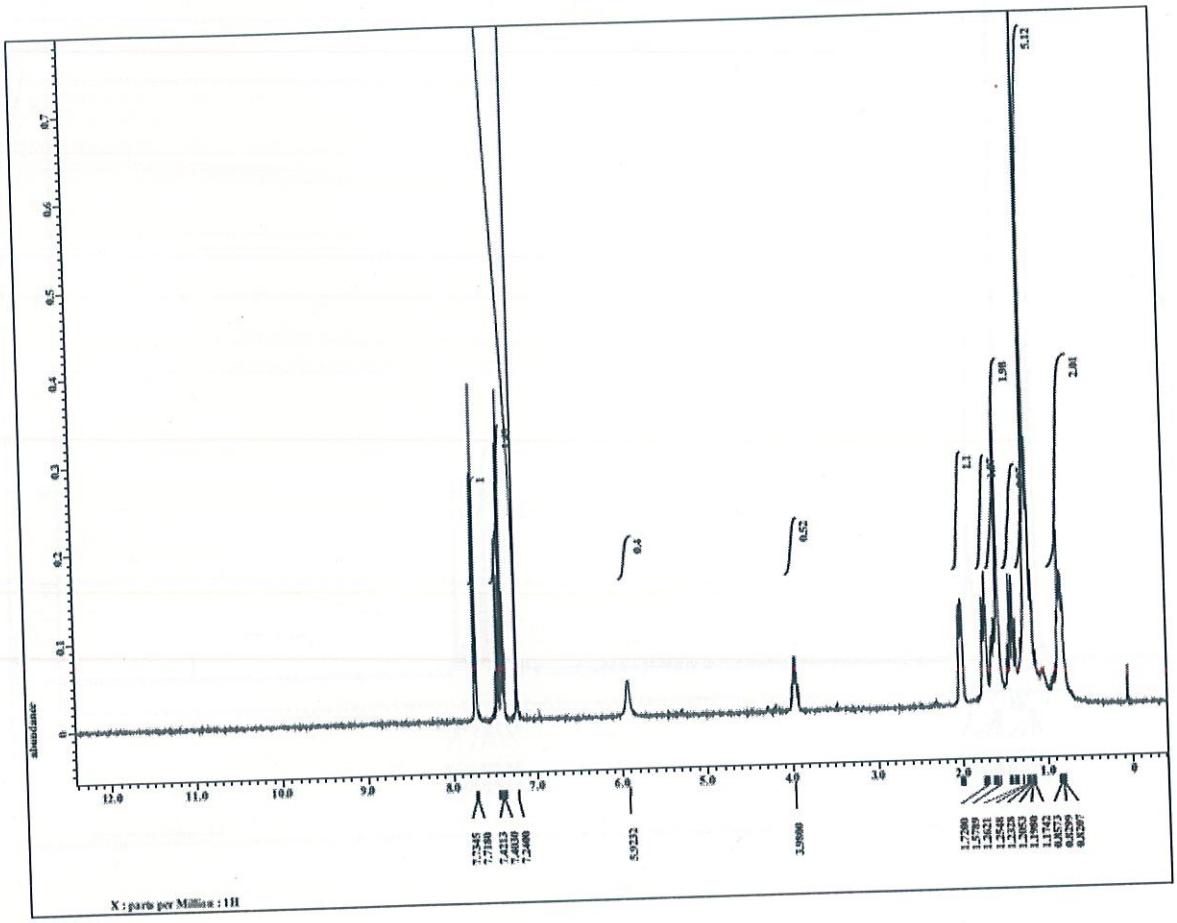
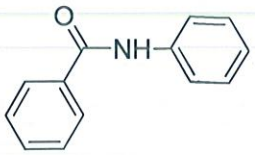
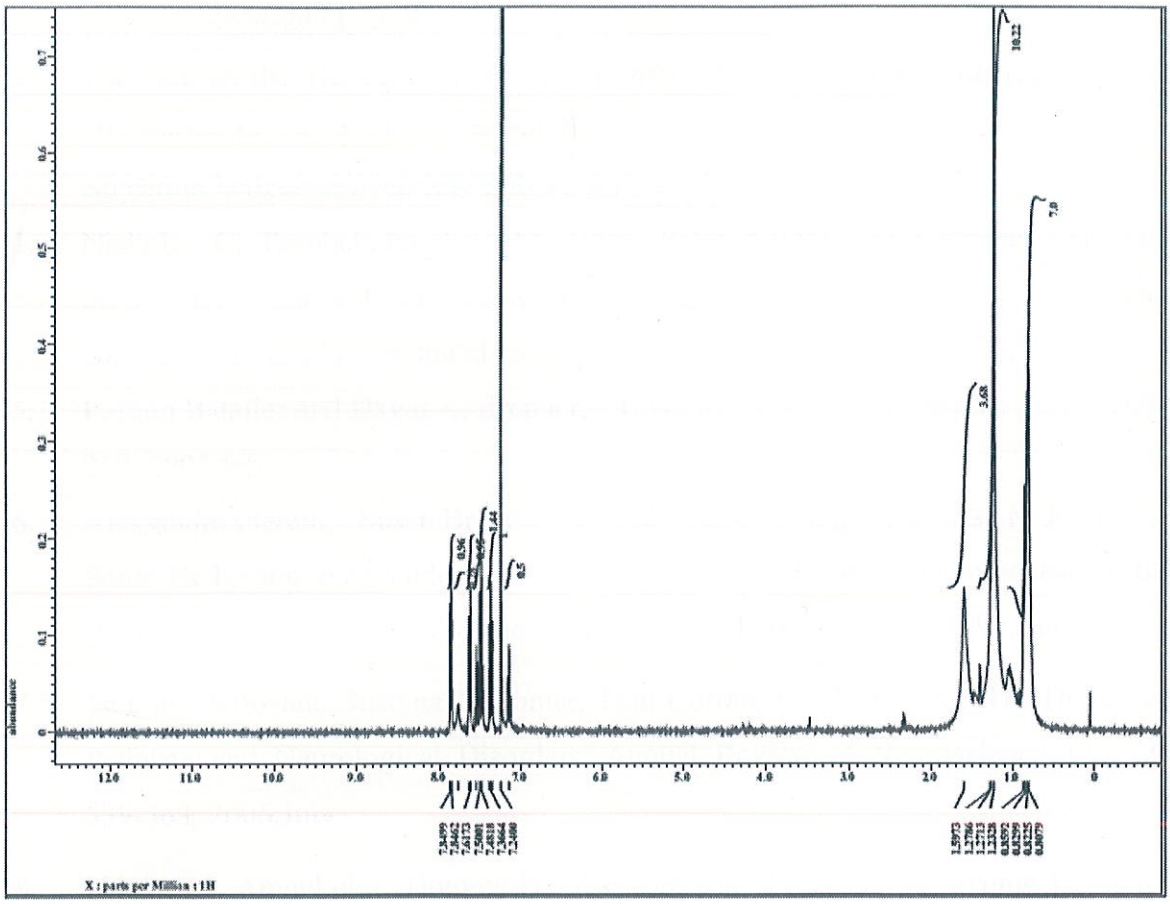


Figure 14: NMR spectra of compound no.7(JUIT-PY-SN003)





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BRIEF BIODATA OF STUDENTS

Sana is pursuing Bachelor of Pharmacy and will be completing her degree in June 2012. She if further interested in exploring different pathways in human system which are still unexplored but can be novel targets for unmet medical needs. Her future goals include establishing a career in research and education.

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Gautam is pursuing Bachelor of Pharmacy and will be completing his degree in June 2012from Jaypee University of Information Technology, Waknaghat. He is looking to pursue higher studies where he can refine his knowledge and management skills further. He would like to work as a project manager in an organization where he can prove his ability and learn new things.

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