Dedicated to my beloved parents

Md. Abdul Hannan Chowdhury

and

Mrs. Khudeza Khanam Chowdhury

for selflessly backing me up throughout.

DECLARATION

I hereby declare that the thesis "**Application of Indirubin 3′-monoxime and Vanillin for increasing insulin sensitivity of adipocytes and reducing inflammation in macrophages by targeting A_{2A}AR and TLR4 signaling pathways"** being submitted to Department of Molecular Biology and Biotechnology, Tezpur University, Tezpur, Assam in partial fulfillment for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology, has previously not formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition

Date: (**Saynaz Akhter Choudhary)**

Place: Tezpur Department of Molecular Biology and Biotechnology

School of Sciences, Tezpur University

TEZPUR UNIVERSITY

CERTIFICATE OF SUPERVISOR

This is to certify that the thesis entitled "**Application of Indirubin 3′-monoxime and Vanillin for increasing insulin sensitivity of adipocytes and reducing inflammation in macrophages by targeting A_{2A}AR and TLR4 signaling pathways"** submitted to the School of Sciences, Tezpur University in requirement of partial fulfillment for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology is a record of research work carried out by **Ms. Saynaz Akhter Choudhary** under my supervision and guidance.

All help received by her from various sources have been duly acknowledged. No part of this thesis has been submitted elsewhere for award of any other degree.

Date:

Place:

(Dr. Suman Dasgupta, Ph.D.)
Designation: Assistant Professor
School: School of Sciences
Department: Molecular Biology and Biotechnology,
Tezpur University, Tezpur 784028

ACKNOWLEDGEMENTS

First and foremost, I bow in front of the Almighty and thank Him for providing me vigour to make a humble contribution to the society through my research work.

I shall forever remain indebted to my Ph.D. Supervisor, Dr. Suman Dasgupta, for believing in me and patiently supervising me throughout my Ph.D journey. His hard work, discipline, dedication and honesty to research have pushed me to carry out my experiments passionately throughout my Ph.D. tenure. I feel very fortunate as well it was a great privilege and honour to work under his able guidance.

I offer my gratitude to Prof M. K Choudhri, and Prof V. K Jain, the former and present Vice Chancellor of Tezpur University, respectively for providing me the opportunity to work in this esteemed university. I always feel fortunate to be a part of this university which has a very good environment for research among the students.

I sincerely thank Department of Biotechnology (DBT), and Department of Science and Technology (DST), New Delhi for providing me the financial support for carrying out my research work.

I deeply acknowledge the Heads, Department of Molecular Biology and Biotechnology, Tezpur University for extending all possible facilities for carrying out my research work.

I am also thankful to my Doctoral Committee members- Prof. Ashish K. Mukherjee, Prof. Robin Doley, and Dr. Venkata Satish Kumar Mattaparthi; all the members of the Departmental Research Committee and all the faculty members of Department of Molecular Biology and Biotechnology for their valuable suggestions and inspiration throughout the course of this study.

I thank all the faculty members, non-teaching staff and Ph.D. students of the MBBT department for directly or indirectly helping and supporting me.

I take immense happiness in acknowledging my lab members Sayani Mazumder, Archana Sinha and Dipanjan Banerjee, my close friends and well-wishers- Mohsin Khan, Saranga Nath, Lavina Sarma, Raktima Bhuyan, Mayuri Borah, Pinki Rani Deuri, Manisha Upadhyay, Mrigyanka Chakrabarty, Chayanika Boruah, Pushpanjali Halder,

Airy Sanjeev, Manoj Sharma, Upasana Khanikar, DR. Debabrata Phukon and Gitartha Darshan Baruah, for their constant support, encouragement and selfless love which strengthened me throughout. I also thank the projects students for assisting the research through their honest efforts.

I wholeheartedly acknowledge my seniors- Dr. Yaseer Bashir, Dr. Anindhya Sundar Das, Dr. Anandita Basu, Dr. Kamalakshi Deka, Dr. Archana Deka, Dr. Simran Kaur and Dr. Bhaskarjyoti Gogoi, for inspiring and encouraging whenever I needed words of experience to keep going on.

I would like to thank all

Words are not there to express the endless sacrifice and hard work that my parents did for me to reach where I am. I owe my whole life and this degree to my beloved parents and my brothers. As I am ending the journey of Doctorate degree, I hope my father's departed soul is proud as his only dream was to see me as doctorate degree holder.

Finally, I would like to thank all the people whose direct and indirect support has helped me complete my research work.

Saynaz Akhter Choudhary

LIST OF TABLES

Table No.	Table Captions	Page No.
	CHAPTER- II	
2.3	Secretion source and effects of some key adipokines	14
2.6	Types of adenosine receptors and their physiological effects	20
2.11.1	Natural products (N) to derived natural products (ND) ratio for treating various medical disorders	27
2.11.2	Percentage of natural products (N) and derived natural products (ND) and their effects	28
2.12.2	Physicochemical characteristics of indirubin-3'-monoxime	32
2.13	Physicochemical characteristics of Vanillin	33
	CHAPTER- III	
3.1	List of primers and oligos used in this study	58-59
	CHAPTER- IV	
4.1	List of compounds (142 in number) used in present investigation.	68-69
	CHAPTER- VI	
6.1	Vanillin structure and developed its various analogs	113

LIST OF FIGURES

Figure No.	Figure Captions	Page No.
	CHAPTER-I	
1.1	A schematic representation of the overview of obesity and related complications	2
1.4(A)	Chemical structure of Indirubin-3`-monoxime	6
1.4(B)	Chemical structure of Vanillin	6
	CHAPTER-II	
2.3	A schematic representation of obesity mediated adipokine disregulation and related health disorders.	15
2.4	A schematic representation of mechanism of macrophage polarization and inflammation.	17
2.5	A schematic representation depicting the process of inflammation.	18
2.6	Detailed representation of Adenosine signalling pathway activation and dowmstream signalling	20
2.7.4	Schematic representation of LPS-TLR4 signaling pathway	25
	CHAPTER-IV	
4.1	Images representing chemical structure of indirubin- 3'-monoxime (I3M), vanillin (VNL), and cytisine (CYT), with their interactions with A_{2A}AR using ligplot..	69
4.2	Analysis of 2-NBDG uptake by 3T3-L1 adipocytes in response to Insulin or Ins + FFA in presence or absence of indirubin-3'-monoxime (I3M), or vanillin (VNL), or cytisine (CYT), viability analysis using MTT method in response to indicated concentrations of I3M, VNL, and CYT treated 3T3-L1 adipocytes, dose dependent effect of I3M or VNL on 2-NBDG uptake by 3T3-L1 adipocytes and Effect of adenosine deaminase (ADA), or SCH 58261 (SCH) on the I3M mediated attenuation of lipid-induced impairment of 2-NBDG uptake in 3T3-L1 adipocytes.	70-71
4.3	Expression analysis of pY-IRS1 and pAkt (S473 and T308) abundance in 3T3-L1 adipocytes in response to insulin or Ins + FFA in absence or presence of I3M and VNL without or with SCH 58261 (SCH) and representative immunofluorescence images showing Glut4 abundance and localization in indicated incubations of L6 cells.	72
4.4	Expression analysis pERK1/2 (T202/Y204), pp38 (T180/Y182) and pCREB (S133) abundance in 3T3-L1 adipocytes incubated without or with I3M in absence or presence of SCH 58261 (SCH).	73
4.5	Abundance of A_{2A}AR gene expression in control siRNA or A_{2A}AR siRNA transfected 3T3-L1 cells by RT-qPCR analysis, expression analysis showing pCREB (S133) abundance in control siRNA (siCon) and A_{2A}AR siRNA (siA_{2A}AR) transfected 3T3-L1 adipocytes incubated without or with I3M , VNL and [3H]NECA binding at A_{2A}AR and the effect of I3M or CGS 21680 on adenylyl cyclase activity/cAMP level via A_{2B}AR	74

4.6	cAMP analysis and expression analysis of pCREB (S133) of 3T3-L1 adipocytes without or with VNL in absence or presence of SCH-58261, IL-10 promoter analysis, ChIP assay showing pCREB (S133) binding to IL-10 promoter and real time quantitative PCR analysis showing fold change of IL-10 mRNA expression in 3T3-L1 adipocytes.	75
4.7	Expression analysis of pNF-kB (S281) and pIkB-(S32) abundance in 3T3-L1 adipocytes , κB luciferase assay and real time quantitative PCR analysis showing fold change of MCP-1, IL-1β, IL-6 and TNF-α mRNA expression in 3T3-L1 adipocytes incubated without or with FFA in absence or presence of VNL or VNL+SCH-58261.	76-77

Figure No.	Figure Captions	Page No.
	CHAPTER-V	

5.1	Cellular image of $A_{2A}AR$ stable clone of CHO cells, RT-qPCR analysis of $A_{2A}AR$ gene expression in CHO cells, determination of EC_{50} value of I3M by measuring percentage activity of cAMP assay and representative curve with I3M competing for [^{3}H]NECA binding to $A_{2A}AR$.	85
5.2	Expression analysis showing pCREB (S133) abundance and analysis of 2-NBDG uptake by 3T3-L1 adipocytes in response to insulin (Ins) or Ins + FFA (palmitate) in absence or presence of CGS 21680 (CGS).	86
5.3	Expression analysis showing pCREB (S133) abundance in control siRNA (siCon) and $A_{2A}AR$ siRNA (si$A_{2A}AR$) transfected 3T3-L1 adipocytes, RT-PCR analysis showing pro-inflammatory (MCP-1 and iNOS) and anti-inflammatory (IL-10 and TGF-β) markers gene expressions, expression analysis showing pAkt (S473) abundance in control siRNA (siCon) and $A_{2A}AR$ siRNA (si$A_{2A}AR$) transfected 3T3-L1 adipocytes and analysis of 2-NBDG uptake by 3T3-L1 adipocytes transfected with control siRNA (siCon) and $A_{2A}AR$ siRNA (si$A_{2A}AR$).	87
5.4	Expression analysis showing abundance of pCREB (S133) level, IL-10 promoter-luciferase assay, real-time quantitative PCR analysis showing fold change of IL-10, IL-13, IL-4 and TGF-β mRNA level and anti-inflammatory cytokines (IL-10 and TGF-β) gene expressions in 3T3-L1 adipocytes.	88-89
5.5	RT-PCR analysis of IL-10 and TGF-β gene expressions in 3T3-L1 adipocytes transfected with scrambled plasmid or CMV500 A-CREB and effect of conditional media on 2-NBDG uptake in FFA-induced insulin-resistant L6 myotubes.	90
5.6	Expression analysis showing pNF-κB (S281) and pIκB-α (S32) abundance and RT-PCR analysis of MCP-1 and iNOS gene expressions in 3T3-L1 adipocytes treated with different concentrations of I3M.	90-91
5.7	κB luciferase assay in transfected 3T3-L1 adipocytes without or with FFA (palmitate) in absence or presence of I3M or I3M + SCH 58261 (SCH), expression analysis showing abundance of pan-phospho-PKC (βII Ser660) level, real-time quantitative PCR analysis showing fold change of MCP-1, TNF-α, IL-6 and IL-1β mRNA level in 3T3-L1 adipocytes and ELISA	92

	detecting IL-6 secretion level in cell culture supernatant of 3T3-L1 adipocytes.	
CHAPTER-VI		
6.1	Expression analysis showing abundance of pNF-κB (S536) levels and κB luciferase assay in THP1 macrophages in response to different TLR agonists and κB luciferase assay of THP1 macrophages incubated without or with LPS in absence or presence of vanillin or different VNL precursors or homologs.	101-102
6.2	Cell viability analysis of indicated concentrations of VNL for different time periods, measurement of κB luciferase activity in transfected THP-1 cells treated with LPS in absence or presence of indicated concentrations of VNL, immunofluorescence images showing pNF-κB (S536) level in THP-1 macrophages, expression analysis of pNF-κB (S536), ChIP assay showing pNF-κB binding to the IL-6 promoter in response to LPS.	103
6.3	Flow cytometric analysis of CD80 and CD206 levels in RAW264.7 macrophages and efficacy of VNL on NF-κB, AP-1, and IRF-3 reporter assays.	104
6.4	RT-PCR analysis showing the abundance of MCP-1, TNF-α, IL-1β, iNOS, IL-6, IFN-γ, IFN-α, IFN-β, CCL5, IL-1RN, TGF-β, IL-10 gene expressions and expression analysis showing the abundance of TNF-α, IFN-γ, and IL-10 protein expressions in the THP-1 macrophages under the indicated conditions.	105
6.5	Expression analysis showing the abundance of pNF-κB (S536), pIRAK4 (T345/S346), pIRAK1, pTAK1 (S412), pIKKα/β (S176), pJun (S73), pFos (S32), pERK1/2 (T202/Y204), pJNK (T183/Y185), pp38 (T180/Y182), and pIRF3 (S386) levels in THP-1 macrophages.	106
6.6	Expression analysis and RT-PCR images showing abundance of pNF-κB (S536) and IL-1β mRNA levels in THP-1 macrophages expressing wild-type or constitutively active forms of MyD88 (MyD88-WT or $MyD88_{L265P}$), or IRAK4 (IRAK4-S or IRAK4-L), or IRAK1 (IRAK1-WT or $IRAK1_{F196S}$), or TAK1 (TAK1-WT or TAK1-$CA_{\Delta 22\ N\text{-terminal}}$ amino acids), or IKKβ (IKK2-WT or $IKK2_{S177E,\ S181E}$), and measurement of κB luciferase activity in the constitutively activated MyD88, or IRAK4, or IRAK1, or TAK1, or IKKβ expressing THP-1 cells transfected with κB reporter plasmid.	107
6.7	Expression analysis showing abundance of pNF-κB (S536) levels in THP-1 macrophages expressing wild-type or constitutively active form of MyD88 (MyD88-WT or MyD88L265P), or IRAK4 short isoform or long isoform (IRAK4-S or IRAK4-L), or wild-type IKK2 or constitutively active form of IKK2 (IKK2-WT or $IKK2_{S177E,\ S181E}$) and measurement of κB luciferase activity in THP-1 cells cotransfected with kB luciferase plasmid along with either MyD88-WT or MyD88L265P plasmid or IRAK4-S or IRAK4-L plasmid, or IKK2-WT or $IKK2_{S177E,\ S181E}$ plasmid.	108-109
6.8	Immunoprecipitation analysis of the indicated tagged antibodies followed by the immunoblotting with tagged or target antibodies showing protein-protein interactions in THP-1 macrophages transfected with TLR4-YFP, MD-2-FLAG, and CD14-Myc; or TLR4-YFP and TIRAP-HA; or TIRAP-	

	HA and MyD88-YFP; or MyD88-YFP and IRAK4-FLAG; or IRAK4-FLAG; or IRAK1-FLAG and TRAF6-Myc plasmids.	
6.9	Expression analysis showing abundance of pNF-κB (S536) levels; and RT-PCR analysis showing IL-1β gene expression in PANC-1 and MIAPaCa-2 cells.	110
6.10	SPR analysis showing representative sensorgrams obtained from flowing of the indicated concentrations of VNL over the IRAK4 immobilized CM5 sensor chip, in-vitro IRAK4 kinase assay and IRAK4 immunocomplex kinase assay showing indicated IC_{50} value.	111
6.11	Molecular orientation of VNL within the active site binding cleft of IRAK4, 3D visualization displaying VNL occupation in the active site cavity of IRAK4, 2D docking image exhibiting VNL-IRAK4 non-covalent interactions and kinase activity measured in wild-type (IRAK-4-WT) and mutated IRAK4 (IRAK4-Y262A, IRAK4-D329A, and IRAK4-V263F) using myelin basic protein (MBP) as substrate.	112 115
6.12	C57BL/6 mice pre-treated twice with vehicle (saline) or VNL (1.5 mM/g bw, serum TNF-α and IL-1β levels measured in these mice by ELISA (n=4), RT-qPCR analysis of iNOS, IL-1β, MCP-1, and IL-6 mRNA expression in the peritoneal macrophages (n=4), Flow cytometric analysis of CD80 and CD206 levels in F4/80+ peritoneal macrophages (n=4), flow cytometic analysis of CD80 and CD206 levels in F4/80+ monocytes isolated from the peritoneum, expression analysis showing abundance of pIRAK4 and pNF-κB (S536) levels in the peritoneal macrophages of these mice (n=4) and proposed schematic model indicating the molecular target of vanillin in TLR4 signaling.	

ABBREVIATIONS

Abbreviation	Full form
T2D	Type 2 Diabetes
IDF	International Diabetes Federation
WHO	World Health Organization
SFA	Saturated Fatty Acids
FFA	Free Fatty Acid
AT	Adipose Tissue
TLR4	Toll-like Receptor
AR	Adenosine Receptor
NECA	5'-N-ethylcarboxamidoadenosine
I3M	Indirubin-3`-monoxime
VNL	Vanillin
CYT	Cytisine
IRAK4	Interleukin-1 receptor-associated kinase 4
T1D	Type 1 diabetes
IR	Insulin resistance
FPG	Fasting and postprandial plasma glucose
OGTT	Oral glucose tolerant test
ER	Endoplasmic reticulum
DAMP	Damage-associated molecular pattern
GPCR	G-protein coupled receptors
CDK	Cyclin-dependent kinases
GSK-3β	Glycogen synthase kinase-3β
NF-κB	Nuclear factor kappa B
BMI	Body Mass Index
CKD	Chronic kidney disease
NCD	Non-communicable disease
WOF	World Obesity Federation
T2D	Type 2 diabetes mellitus
IR	Insulin resistance
FFA	Free fatty acid
AT	Adipose tissue
WAT	White adipose tissue
vWAT	Visceral white adipose tissue
sWAT	Subcutaneous white adipose tissue
TNFα	Tumour necrosis factor alpha
IL6	Interleukin 6
IL1β	Interleukin 1beta
MCP-1	Monocyte chemoattractant protein-1
CRP	C-reactive protein
ATMs	Adipose tissue macrophages
CLS	Crown like Structure
PAMPs	Pathogen-associated molecular patterns
TLRs	Toll-like receptors
NLRs	NOD-like receptors

NF-κB	Nuclear factor kappa B
STAT3	Signal transducer and activator of transcription 3
nM	Nanomolar
μM	Micromolar
ATP	Adenosine triphosphate
AMP	Adenosine monophosphate
ENTs	Equilibrative nucleoside transporters
CNTs	Concentrative nucleoside transporters
ARs	Adenosine receptors
A_1	Adora1
A_{2A}	Adora2a
A_{2B}	Adora2b
A_3	Adora3
NECA	5'-N-ethylcarboxamidoadenosine
ALRs	AIM2-like receptors
NOD	Nucleotide-binding oligomerization domain
LRR	Leucin Rich Repeats
RIG-1	Retinoic acid-inducible gene 1
RLRs	retinoic acid-inducible gene 1- like receptors

Abbreviation	**Full form**
PRRs	Pattern recognition receptors
CLRs	C-type lectin receptors
TIR	Toll/IL-1R homology
DC	Dendritic cells
NK	Natural killer
NTD	Amino-terminal domain
DAMPs	Damage associated molecules patterns
ER	Endoplasmic reticulum
LPS	Lipopolysaccharide
TLR4	Toll-like receptor 4
LBP	LPS binding protein
CD14	Cluster of differentiation 14
GPI	Glycosylphosphatidylinositol
MyD88	Myeloid differentiation factor 88
TRIF	TIR-domain containing adaptor molecule
TIRAP/MAL	TIR domain containing adaptor molecule/ MyD88-adaptor-like
TRAM	TRIF-related adaptor molecule
SARM	Sterile α- and armadillo-motif-containing protein
PIP2	Phosphatidylinositol (4,5) bisphosphate
SMOC	Supramolecular organizing center
IRAK	IL-1R-associated kinase

DD	Death domain
KD	Kinase domain
Ub	Ubiquitin
TRAF6	TNF receptor-associated factor 6
TAK1	TGF-β activated kinase 1
TAB2	TGFβ-activated kinase 1 binding protein
IKK	IkB kinase
NEMO	NF-κB essential modulator
AP-1	Activator protein-1
CREB	cAMP response element-binding protein
ISGs	IFN stimulated genes
DAGs	Diacylglycerols
ROS	Reactive oxygen species
MIP-2α	Macrophage inflammatory protein 2 alpha
PBMNC	Peripheral blood mononuclear cells
SFA	Saturated fatty acid
HFD	High fat diet
FetA	Fetuin A
PKC	Protein kinase C
IRS-1	Insulin receptor substrate-1
IRS-2	Insulin receptor substrate-2

Abbreviation	**Full form**
A	Adsorption
D	Distribution
M	Metabolized
E	Excreted
FDA	Food and Drug Administration
B	Biological source
N	Natural product
NB	Natural product “Botanical”
ND	Derived natural product
S	Synthetic molecule
V	Vaccine
CML	Chronic myelogenous leukemia
CDKs	Cyclin-dependent kinases
GSK-3β	Glycogen synthase kinase-3β
AD	Alzheimer’s disease
6BIO	6-bromoindirubin-3’-oxime
7BIO	7-bromoindirubin-3’- oxime
FLT3	FMS-like tyrosine kinase-3
AML	Acute myeloid leukemia
STAT3	Signal transducer and activator of transcription 3
PDAC	Pancreatic ductal adenocarcinoma

HUVECs	Human umbilical vein endothelial cells
BAT	Brown adipose tissue
UCP-1	Uncoupling protein-1
I3M	Indirubin-3'-monoxime
VSMCs	Vascular smooth muscle cells
T_q	Thymoquinone
LC	Lung cancer
OS	Osteosarcoma
NO	Nitric oxide
PGE_2	Prostaglandin E_2
NHEJ	Non-homologous end joining
MIC	Minimum inhibitory concentration
NO	Nitric oxide
ALI	Acute lung injury
EHC	Enterohepatic circulation
NP	Nanoparticles

Abbreviation	**Full form**
CHO	Chinese hamster ovary
KRP	Kreb's Ringer Phosphate
LDH	Lactate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
ELISA	Enzyme-linked immunosorbent assay
ChIP	Chromatin immunoprecipitation
SPR	Surface plasmon resonance
KD	Equilibrium dissociation constant
FACS	Fluorescence-activated cell sorting
QSAR	Quantitative structure activity relationship
ADMET	Absorption, Distribution, Metabolism, Excretion and Toxicity