Development and Molecular Characterization of Novel HIV-1 Associated Topoisomerase II β Kinase Inhibitors during HIV-1 Replication

DOCTOR OF PHILOSOPHY

by

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DECLARATION

The research work presented in the thesis entitled "**Development and Molecular Characterization of Novel HIV-1 Associated Topoisomerase II β Kinase Inhibitors during HIV-1 Replication"** has been carried out by me in the Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, Hyderabad, Telangana under the guidance of Prof. Anand K. Kondapi. I hereby declare that this work is original and has not been submitted in part or full for any other degree or diploma of any other University or Institution.

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CERTIFICATE

This is to certify that thesis entitled **"Development and Molecular Characterization of Novel HIV-1 Associated Topoisomerase II β Kinase Inhibitors during HIV-1 Replication"** is a record of bonafide work done by **Mr. D. A. Kiran Kumar**, a research scholar for the Ph.D. programme in the Department of Biotechnology and Bioinformatics, University of Hyderabad under my guidance and supervision. The thesis has not been submitted previously in part or full to this or any other University or Institution for the award of any degree or diploma. I recommend his thesis for submission towards the partial fulfillment of 'Doctor of Philosophy' degree in Biotechnology.

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This thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for award of any degree or diploma.

Further, the student has the following publication (s) before submission of the thesis/monograph for adjudication and has produced evidence for the same in the form of the acceptance letter or the reprint in the relevant area of his research: (Note: at least one publication in referred journal is required the research)

Kammari K, **Devaraya K**, Bommakanti A, Kondapi AK. 2017. Development of pyridine dicoumarols as potent anti HIV-1 leads, targeting HIV-1 associated topoisomeraseIIβ kinase. Future Medicinal Chemmistry 9:1597-1609.Chapter of the dissertation where this publication appears: Volume 82, issue 3. **Future Medicinal Chemmistry:** (ISSN Number: 1756-8927)

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3	**EMBO Conference (Poster Presentation)** **Inhibition of Topo IIβKHIV affects HIV-1 reverse transcription** Kiran Devaraya, Kurumurthy Kammari, S L Balakrishna and Anand K Kondapi.	17-21 September 2017
4	**Indian Association for the cultivation of Science (IABS 2018) (Poster Presentation)** **Pyridine analogues as potential inhibitors of HIV-1 associated Topoisomerase II β kinase and Reverse Transcription during the HIV-1 Infection** Kiran Devaraya, Kurumurthy Kammari, B Akhila and Anand K Kondapi.	1-3 February 2018
5	**International Conference on Combat HIV 2019 (Poster Presentation)** **Potential inhibitors of HIV-1 associated Topoisomerase II β kinase affects the Reverse Transcription events of HIV-1** Kiran Devaraya, Kurumurthy Kammari, B Akhila and Anand K Kondapi.	19-21 January 2019

Further, the student has passed the following courses towards fulfillment of coursework (recommended by doctoral committee) on the basis of the following courses passed during his Ph.D. degree was awarded.

S.No.	Course code	Name	Credits	Pass/Fail
1	BT 801	Seminar	1	Pass
2	BT 802	Research ethics and management	2	Pass
3	BT 803	Biostatistics	2	Pass
4	BT 804	Analytical techniques	3	Pass
5	BT 805	Lab work	4	Pass

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D.A. Kiran Kumar.

INDEX

ABBREVIATIONS

AIDS	: Acquired Immuno-Deficiency Syndrome
AZT	: Azidothymidine (Zidovudine)
pM	: Picomolar Concentration
nM	: Nanomolar Concentration
µM	: Micro molar Concentration
mM	: Mill molar Concentration
WHO	: World Health Organisation
FDA	: Food and Drug Administration
Topo II	Topoisomerases II
TOPO IIβK_{HIV-1}	: HIV-1 associated Topoisomerase II β kinase
HAART	: Highly Active Anti-Retroviral Therapy
HIV	: Human Immunodeficiency Virus
IC_{50}	: 50% Inhibitory Concentration
CC_{50}	: 50% Cytotoxic Concentration
QSAR	: Quantitative Structure Activity Relationship
3D-QSAR	: 3- Dimentional Quantitative Structure Activity Relationship
CoMFA	:Comparative Molecular Field Analysis
MAPK	Mitogen Activated Protein Kinase
$TCID_{50}$	50% Tissue Culture Infectious Dose

CHAPTER-I

Introduction

Introduction:

HIV-1(**Human Immunodeficiency Virus-1)** belongs to genus lenti virus, family retroviridaeand subfamily orthoretrovirinae(1).HIV frequently associated with Acquired Immune Deficiency Syndrome (**AIDS**), a paradigmin humans where the immune organization fails and finally takes to the high-risk opportunistic infectionsin the life(35). According to the **WHO/UNAIDS,** globally37.9 million peoplewere infected with **HIV-1**/**AIDS,**in which adults were 36.2 million and children were1.7 million. Worldwide an estimation of newly infected people with **HIV-1** were1.7 millionand 32.0 million people have died from AIDS-related illnesses(23.6 million–43.8 million) since from the beginning of this epidemic disease(59).

India is the third largest country in having the HIV infected cases when compared with the world's epidemiology. HIV/AIDS prevalence rate in India is lower than in many other countries, the epidemic status in India is having an estimation of 0.30%in HIV-1prevalence, in which adults are aged between 15-49 years and children are below 15 years (59).

India HIV/AIDS Epidemic Status:

Indicator	Global	India
People living with HIV (All ages)	36.9 M	2.14 M
New HIV Infections (All ages)	1.8 M	88,000
PLHIV on ART	21.7 M	1.23 M
AIDS-Related Deaths	0.940 M	69,000

Table 1: The above table is showing the epidemiological status of HIV-1 in India in comparison with the world **(59).**

HIV-1viral genome structure and their function:

HIV-1 belongs to the genus lenti virus within the family of retroviridae and subfamily Orthoretrovirinae(1). It is a sphere shapedvirusparticle and around 100 nm in size. HIV-1contains two copies of single-stranded positive-sense RNA which are non-covalently linked and enclosed in a cone-shaped capsid composed of viral protein **p24(1).**The HIV-1 is having 9.8kb genome containing set of structural genes (***Gag, Pol and Env***), enzymes (**RT, IN** and **PR**), accessory genes(***nef, vif, vpr and vpu***) and a pair of regulatory genes (***tat and rev***) that help to regulate viral replication(1). The whole genome of HIV-1 is driven by Long Terminal Repeat (LTR) sequences which are present on both ends of the genome(1), all these components of virus will help in the viral replication and propagation.

Figure-1: HIV-1 genome

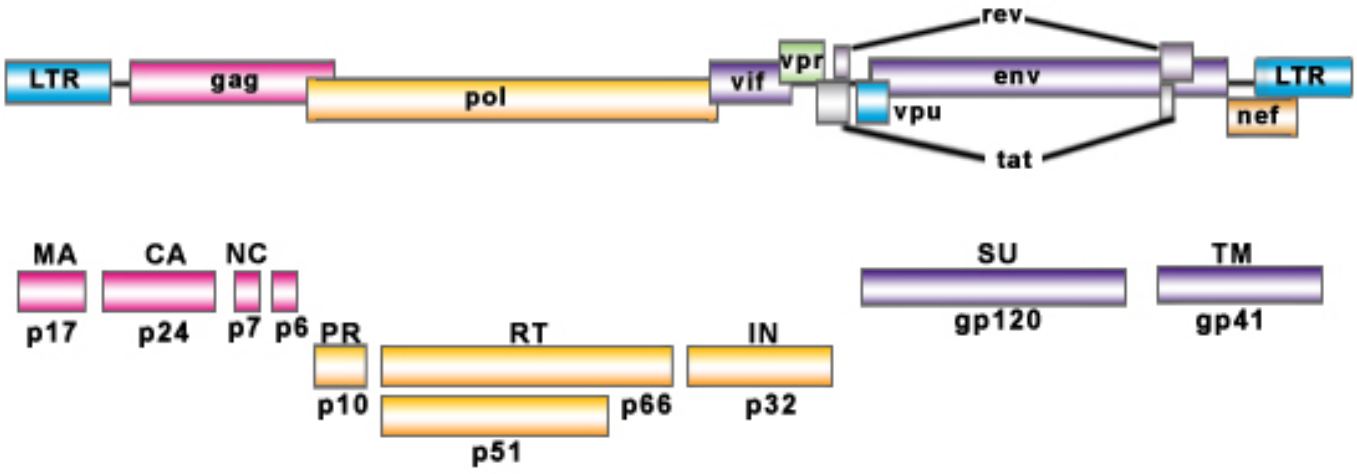


Fig.1: Above figure is showing the genome size of the HIV-1which is about 9.8kb, 3 structural genes (gag, pol and env) and 3 enzymes (RT, IN, PR) and six accessory genes (tat, vif, nef, rev, vpr and vpu) that help to regulate viral replication.

Figure 2: HIV-1 virion

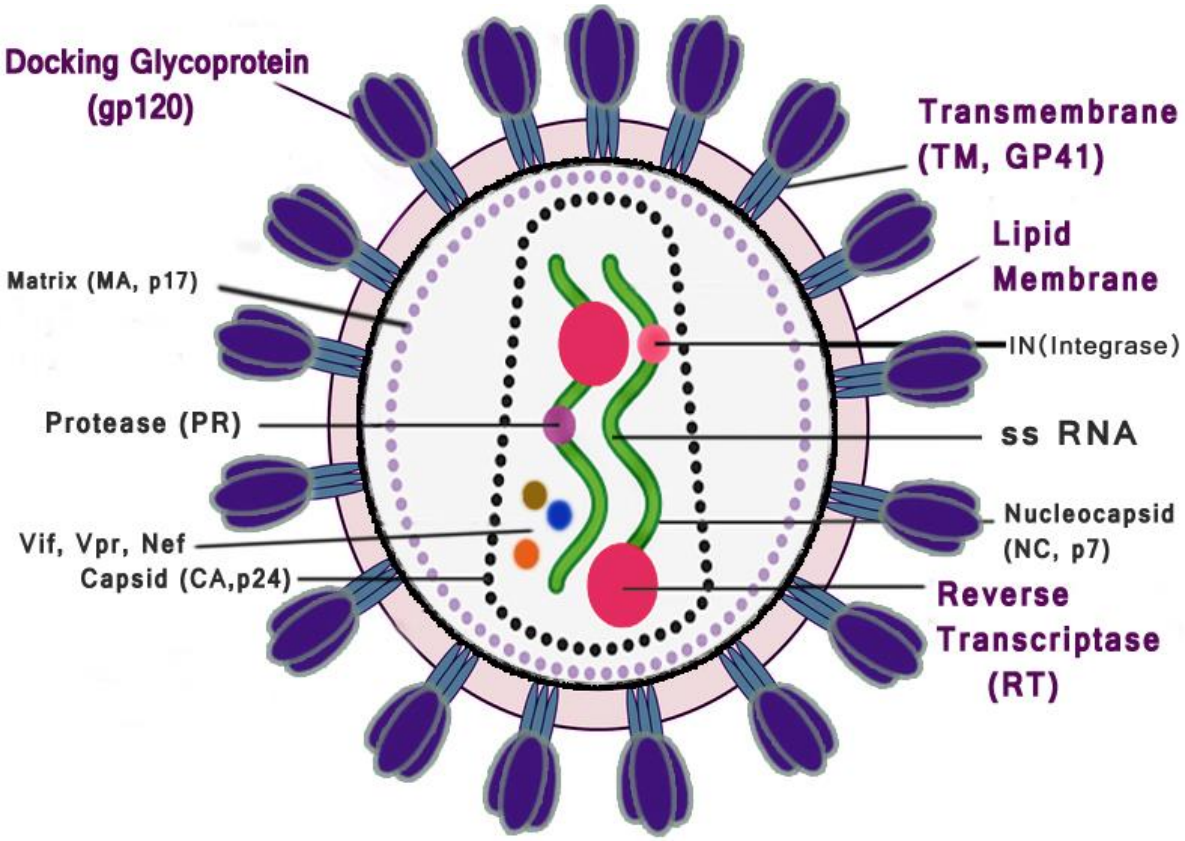


Fig.2: Above figure is showing virus structure of HIV-1, virus particle is composed of two copies of positive-sense single stranded RNA enclosed in a conical capsid composed of viral protein **p24** and three major structural genes **gag, pol** and **env.**

Structural Genes:

Structural genes ***Gag*, *Pol*,** and***Env***are involved in the formation of virus particle (1) initially these proteins are synthesized as precursors of poly-proteinsand later these areconvertedinto mature proteins by viral or cellular proteases.

Gag*:** The ***gag gene produces a 55-kDa Gag precursor polyprotein which is processed to p24 Capsid(CA)protein,p17 Matrix(MA)protein,p7 Nucleocapsid (NC) protein, p6 protein, Spacer Peptide 1 (SP1) and Spacer Peptide 2(SP2) by viral protease during maturation (37), (133).

Pol:Initially the Pol polypepide is a Gag-Pol precursor fusion polyprotein (p160) later it is processed to viral enzymes p55-Reverse Transcriptase (RT), p15-RNase H, p31-Integrase (IN) and p10-HIV Protease (PR)by viral proteases(71)(145).

Env: envgene codes a polyprotein gp160, which undergoes glycosylation and cleaved by host proteases in the Golgi complex of the host cell, the gp160 polyprotein undergoes a post-translational process to producegp41,transmembrane (TM) protein and gp120, surface glycoprotein(SU)to form the envelope of virus which helps in the attachment and fusion with host cell membrane (71).

Enzymatic proteins:

Reverse Transcriptase (RT): RT is encoded by ***pol*** gene and has three activities:

1. RNA-dependent DNA polymerase activity, 2. Ribonuclease H activity and 3. DNA-dependent -DNA polymerase activity. All the activities collectively permit the viral enzyme to convert the single-stranded genomic RNA to double-stranded cDNA of virus(105).

Integrase (IN): HIV-1 Integrase (IN) is a 32kDa protein produced from *pol* gene essential for the process ofviral cDNA integration into the host genome(145).

Protease (PR): Mature HIV-1 protease (PR) exists as a 22kDa homodimer, it is a retroviral aspertyl protease which is required for the cleaving of Gagand Gag-Polprecursor polyproteinsto structural proteins(53), (107).

Regulatory proteins:

The two regulatory proteins of HIV-1 are coded by the genes ***rev*** and ***tat***which are very crucial for the viral propagation.

Trans-activator of transcription (Tat): Tat is transcriptional trans activator, crucialfor the replication of HIV-1.Tat is viral subtype specific protein expressed as 86 to 101 amino acid.

It is a regulatory protein that binds to Trans Activation Response (TAR) element which is located at the 5'terminus of HIV-1 RNAs. Tat binds to the TAR that will activates the transcription and up-regulationof HIV-1 RNA alongwith RNA polymerase II (33)(50)(60).

Regulator of virion (Rev): Rev is a regulatory protein that binds to RNA, with a molecular weight of 13 kDa. It will bind to the Rev Response Element (RRE), a complex RNA secondary structurewith 240-base region present inHIV-1 genome, Rev-RRE complex will helps in the export of in-completeand un-spliced viral RNAs from the nucleus to the cytoplasmof the cell which is indeed required for RNA splicing mechanisms and HIV-1 replication (42)(13).

Accessory Proteins:

The Four accessory proteins are coded by the genes ***nef , vif, vpr*** and ***vpu***that are crucial for the viral replication ofHIV-1.

Negative regulatory factor (Nef): Nefis a myristoylated protein with a M.W. of 27 kDa. Nef is the foremost viral protein that can be detected during the infection of HIV-1. Nef is having multiple activities such as perturb the T-cell activation, down regulation of cell surface expression of CD4 and class I MHC (Major Histocompatability Complex) molecules present on

the immune cells of host, such as infected APCs (Antigen Presenting Cells) and T-Lymphocytes(38), (79), (110).

Viral infectivity factor (Vif): Vif is a 23kDa accessory protein which is necessary for the replication of virus in the peripheral macrophages, lymphocytes and other immune cells (82). Vif inhibits the host protein family like APOBEC by triggering the degradation mechanism of ubiquitination pathway(89).

Viral protein R (Vpr): Vpr is a regulatory protein associated with shuttling of nucleo-cytoplasmic functions in the virion. It is specifically requiredfor the nuclear import of pre-integration complex (PIC) (31)(114). Vpr also involves in arresting the G2 cell cycle in proliferating cells (115)(113) , thereby activating the DNA repair mechanism which may leads to the viral DNA integration into the host genome (51), (54).

Viral protein U (Vpu): Vpu is a 16 kDa polypeptide that is present in the internal membrane of the cell membrane (39), Vpu helps in down modulation of CD4 and enhances the releasing of viral particle (66), (93), (120).

Life cycle of HIV-1:

HIV-1 life cycle consists of several steps, whichincludes entry, reverse transcription, integration, transcription,assembly and budding

Entry (Binding and Fusion):

HIV-1 begins its life cycle when it invades the host cells which express the $CD4^+$ (Cluster of Differentiation 4) molecules on their surface such as T lymphocyte, macrophages, monocytes and dendritic cells. Viral entry initiates with the fusion of viral envelope gp120 protein to the $CD4^+$receptor along with co-receptors CXCR-4 (Chemokine Receptor-4) and CCR5 (Chemokine Receptor-5) on the host cell membrane, the interaction between gp120 and $CD4^+$ induces a conformational change in gp41transmembrane protein that folds itself into a six helical bundle structure which helps in fusion of virus to the host cells, subsequently release of viral capsid into the cytosol of host cell.

Reverse Transcription: The process of conversion of ss RNA to ds cDNA mediated by a viral coded enzyme Reverse Transcriptase is known as Reverse Transcription.

Integration: Pro viral DNA integration into the host genome mediated by viral enzyme integrase is known as Integration. The Pre Integration Complex (PIC) formation with the help of host and viral proteins that is translocated to the nucleus and the integration of viral DNA into the host genome. Integratedviral DNA into the host genome is called provirus. Provirus remains in the inactive latency stage for a prolonged period or it may produce new viral particles of HIV-1.

Transcription: The active state of provirus utilizes the host RNA polymerase to synthesize the short strand of viral RNA (mRNA), thus synthesized mRNA will translate new viral proteins by utilizing the host cell.

Assembly: Assembly of new virions proceeds when the RNA molecules and HIV-1 proteins come together to form icosahedral virus particles in the cytoplasm of host cell. As the Gag (p55) and Gag-Pol (p160) polyproteins are processedto matured viral proteinsby viral protease. Once all the essential proteins are synthesized together with the viral RNA genome a new viral particle is assembled and ready for further maturation and release.

Maturation and Budding: The newly assembled virus particles undergo maturation in the host cell cytoplasm and released out by a process of budding mechanism from an infected T cell.

Figure 3: Life cycle of HIV-1

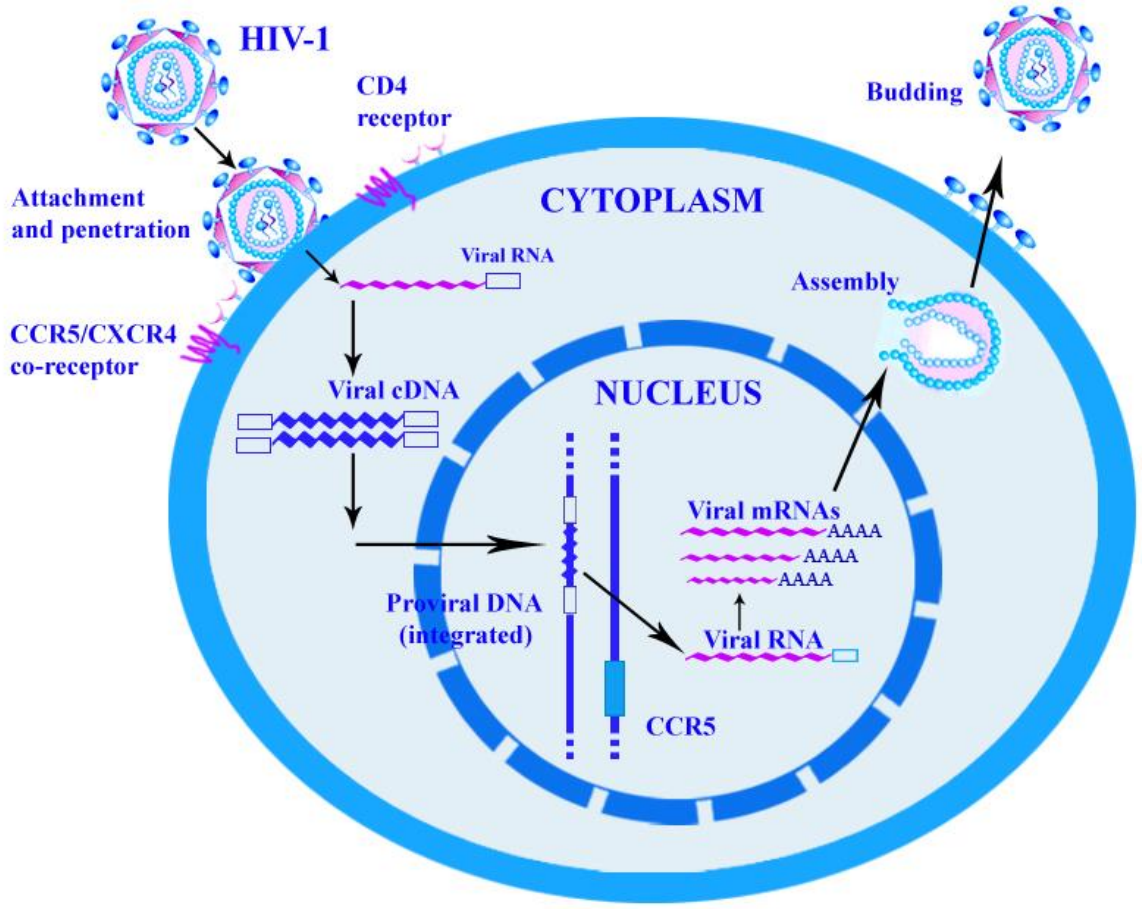


Figure 3: Schematic diagram showing the various stages of HIV-1 life cycle:

1) Entry, 2) Reverse Transcription, 3) Integration, 4) Transcription 5) Assembly

6) Maturation and Budding

HIV-1 and Host protein interactions:

During HIV-1 infection, the virus will infect specifically to certain type of cells known as viral tropism. HIV-1 infects different types of host immune cells particularly $CD4^{+}$T cells, dendritic cells, microglia, monocytes and macrophages (57). Macrophage-tropic (M-tropic) and Non-syncytia inducing (NSI or R5 viruses) strains will use β-chemokine receptor (CCR5) for their infection (23), (109), CCR5 co-receptor is used by almost all the primary HIV-1 isolates. T cell-tropic (T-tropic) and syncytia inducing (SI or X4 viruses) strains will use α-chemokine receptor (CXCR4) for their infection (10), (109). Dual-tropic strains of HIV-1 are transitional strains which uses both the co-receptors, CCR5 and CXCR4 for the viral fusion and these viruses are termed as X4R5 (3).

HIV-1 virus will interact with many host proteins for their replication and propagation.APOBEC3Gis an enzyme belongs to cytidine deaminases family serves as host defence mechanism by interacting with reverse transcription process; it deaminates cytidine to uridinein viral DNA transcripts that leads to either degradation or formation of hypermutated defective proviruses (129).LEDGF is other host protein that interacts with the HIV-1 integrase and helps in cDNA integration into host genome (95). Various cellular elements which bounds to viral Integrase (IN) were recognized (137) but their intact role is not known. Some of them are Integrase Interactor 1 (INI1, hSNF5) (61), (104), Histone Deactylase 1 (HDAC1) SIN3A-Associated Protein (SAP18),(117) and Survival Motor Neuron (SMN) interacting protein-1 (Gemin2)(45).INI1 and SWI/SNF complex required chromatin remodeling (128) and their direct interaction with the HIV-1 IN domain in Gag-Pol polyproteinis required for Tat mediated transcription (144).HostcomponentsSIN3A-HDAC1 complex binds to other members HDAC1, SIN3A, SAP18, andSAP30are specifically packed into virion (117). HDAC1 involves in

bothviral uncoating and reverse transcription process resulting in effectivecDNA synthesis of HIV-1. Gemin2 isessential foranegative strand (strong stop) synthesis during early reverse transcription processor integration of viralcDNA, indicating itsinteraction with either PICS orRT(45). It recruitsRNA helicase A (DHX9)(108), in association with SMN complex (100). A RNA binding nuclear protein, Human antigen R(HuR) involves in shuttlingnucleo-cytoplasmic functions (91)with high affinity andspecificity towards AU-rich elements (AREs) (91), (124).A-kinase anchor protein 1 (AKAP1)binds to both regulatory subunit of Protein Kinase A(PKA, a cAMP dependent kinase)and Reverse Transcriptase (RT) which anchors them to several membranes in the cell (30). Cyclin-dependent kinase (CDK) 2 regulatesthe RT by phosphorylating on threonine increasing its efficiency and stability leading to enhanced viral fitness. Similarly, another hostprotein HMGI(Y), involves in pre-integration complexes (PICs) and covalent strand transfer process during reverse transcription of HIV-1 (29).

Table 2: HIV-1 and Host protein interactions:

S.No	Viral Proteins	Host Proteins	Function
1	Gp41	Host Membrane	Promotes viral entry
2	Gp120	$CD4^+$ and CCR5/CXCR5	Promotes viral entry
3	RT	AKAP149, APOBEC3G, HUR	Promotes Reverse Transcription
4	Integrase	APOBEC3G, LEDGF, p300, INI1, hSNF5, SAP18, HDAC1 and Gemin2	Promotes Integration of HIV-1 Genome
5	Rev	CRM1, DEAD/H Box Proteins Staufen-2, Topoisomerase II	Exports the viral RNA from nucleus to cytoplasm
6	Tat	CREB, CGNS,SWI/SNF, p300	Promotes Integration and Transcription
7	PR	BAF, DNA Topoisomerase I LEDGF, TNOP3,	Promotes Viral maturation and Budding
8	Nef	AIP1, HCK, MAPK	Down regulation of $CD4^+$, Increases the Viral Infectivity and Viral Budding
9	Vif	APOBEC3G, CBF-β, HP68,	Counteracts the host anti-viral factors and stimulates Reverse Transcriptase
10	Vpr	DCAF-1, p300, Importin-α, TFIIB	Promotes the nuclear importation of pre-initiation complex
11	Vpu	CD4, WD boxes of β-TrCP	CD4 degradation and promotes virion release

Table 2: Table is showing the HIV-1 and Host protein interactions during the virus replication and progression at different stages of life cycle

Table 3: DNA Topoisomerases subfamilies:

Subfamily	Distribution of Topoisomerases in organisms
IA	Bacteria (Topoisomerases I and III), Yeast (DNA Topoisomerases III), Drosophilla melanogaster (DNA Topoisomerases III α and III β), Mammalian DNA Topoisomerases III α and III β
IB	Eukaryotic DNA Topoisomerase I, Mammalian Mitochondrial DNA Topoisomerase I and Pox virus Topoisomerase
IC	Archaebacteria, (Example: Methanopyrus kandleri).
IIA	Bacterial gyrase, DNA Topoisomerase IV, Phage T4 DNA Topoisomerase, Yeast DNA Topoisomerase II, Drosophila DNA Topoisomerase II and Mammalian DNA Topoisomerase II α and II β
IIB	Sulfolobus shibatae DNA Topoisomerase VI

Table 3: Above table is showing the distribution of DNA Topoisomerases subfamilies in various organisms

DNA Topoisomerases:

Topoisomerases are the enzymes which resolves the topological constrains in the DNA from oneform to another form in the chromosome (18)of both prokaryotes and eukaryotes(94), (125).Topoisomerases are involved in the maintenance of genomic stability by regulating the DNA replication, transcription and segregation of chromosomes (94). These enzymes are classified as Type I and Type II Topoisomerases, whereas subfamilies were named as IA, IB and IC, IIA and IIB. Type I Topoisomerases do not require ATP hydrolysis in releasing the DNA super coils whereas Type II Topoisomerases utilizes ATPhydrolysis in releasing the super coils of DNA. In prokaryotes DNA Gyrase is a Type II Topoisomerasewhich is similar to the eukaryotic enzyme in catalytic activity. Invertebrates and lower eukaryotes possess only one Type II Topoisomerases where as in eukaryotes two exclusive isoforms are present, TopoII α and βwhich help in cellular division and development(125).

In mammals, Topo II is distributed uniformly on the chromosomal regions of 17q21-22 and 3p24 and its requirement is unique during segregation of daughter chromosomes in mitotic and meiotic cell division (119). Basically Topo II exists in 170 kDa α and 180 kDa β isoforms, structurally both isoforms are similar but are different in biochemical, genetical and immunological aspects (26). TopoII α expression is seen in proliferating cells and highly regulated during cell division whereas Topo IIβ is expressed in all cell types associated with non-proliferating functions (17). Activity of Topo II α is high in the karyoplasm during G_2/M phase of the cell cycle whereas Topo II β is dispersed in the karyoplasms of interphase and it is present in the cytoplasm during mitosis of cell cycle, the activity is consistent over all phases of cell cycle (88). The major functions of Topo II are to control the topological constrains in DNA

strands and promotes the cell cycle by maintaining the integrity of the cell during the DNA replication process.

Regulation of Topoisomerases:

Activity of TopoII isoforms are regulated by means of phosphorylation and dephosphorylation (73), (118). In the previous studies, it is widely reported that Topo II phosphorylation is connected with activation and regulation of enzymes involves in the progression of cell cycle. Topo II is identified as a major phosphoprotein of mitosis during cell division (55). Topo II helps in chromosomal condensation preferably via phosphorylation and dephosphorylation (36). In Topo II, C-terminal region is the crucial target for the regulatoryphosphorylation and its phosphorylation increases the specific activity by 2-3 folds which enhances the ATP hydrolysis during decatenation of DNA (36). Along with C-terminal region phosphorylation sites are present on N-terminal region whose phophorylation helps in the progression of cell cycle. It was reported that phosphorylation of serine 1212 of TopoII α helps in resolving constraint in DNA topologyprogressively from the centromere to the chromosome arm duringchromosome condensation of metaphase stage (55). Phosphorylation of Topo II is important in cellular growth and stringently regulated throughout in all the stages of cell cycle. Phosphorylation of Topo II α is high in G1 phase and reaches maximal during G2/M stage of cell cycle whereas Topos II β is expressed uniformly in all the stages of cell cycle (88). Cellular Topo II is reported to be over expressed in rapidly proliferating cancer cells (9), (52), (123).

Role of Topoisomerases in Viral Infections:

In the earlier studies it was reported that Topoisomerases are involved in the progression of viral infections. In Chlorella virus, PBCV-1 is known for having smallest type II Topos (75) which is having exceptionally high DNA cleavage activity (24), (32). In bacteriophage phiX174 DNA gyrase activity helps in DNA packing into the phage particle (4), (46) and in bacteriphage Mu, DNA gyrase is required for effective replicative transposition and its binding site present in the center of genome (98). *In-vitro* studies on SV40, a polyomavirus shown that Topo II acts as a swivelase in the late stages of chromosomal replication (56). An inhibitory study of Topo II by ICRF-193 has prevented the replication of herpes simplex virus 1 (HSV-1) (47). Host Topo II is necessary for the replication of herpes simplex virus 1 (HSV-1) in the late stages of infectious cycle (28). Topo II α is required for the transcription of late genes during untangling concatemeric DNA progeny in Herpes simplex virus-1 (2). Topo I is required during the replication of Herpes simplex virus 2 (HSV-2) and its inhibition with a specific inhibitor camptothecin (CPT) inhibited the viral replication (139). In Epstein-Barr virus, BGLF4 kinase phosphorylates the condensin in association with decatenation activity of Topo II during chromosomal condensation (76). Inhibition of Topo II by using antisense-mediated and inhibitor based studies showed that there was direct correlation between the depletion of Topo II and decrease in the replication ability of HIV-1, thus indicating the involvement of Topo II in the progression of HIV-1 replication (12), (69). Phosphorylation of Topos II α and βis required during initial stages of HIV-1 infection (68). Further shown that Topo II α phosphorylation activity is high at 8 hour and 32 hours of post infection, where as phosphorylation of β isoform is high at 4 hours and 64 hours onwards of post infection (68). Topo II helps in progression of

HIV–1 replication, in which TopoII β promotesHIV-1 replication and strongly associated with reverse transcriptionand other intermediates of HIV-1 replication (80).

Role of Protein Kinases in Viral Infection:

Protein kinases play an important role in phosphorylation of various proteins that arerequired to inducedistinct changes in several pathways of cell. In humans around 518 genes are coding for kinases but the diverse evolution in the family of kinase enables the participation of only 300 conserved residues of catalytic domain(85), (122). The ATP-binding catalytic domain is responsible for the transfer of ATP γ-phosphate to the substrate protein. Kinase phosphorylation is one of the important mechanisms by which many viral as well as cellular protein factors are regulated. Such regulation can have impact on basic functions of cells in mammals, including the changes in cell signaling, macromolecular complexes assembly, nucleo-cytoplasmic shuttling, proteins binding to DNA and regulation of many enzymatic activities (64). Kinases are encoded not only by host genome but also by viral genome themselves. Phosphorylation is the major implication during the post translational modification of host as well as viral proteins that will help in the progression of viral replication (64).

Many reports and earlier studies have proved that kinase phosphorylation is very crucial during the life cycle of viruses. HSV-1 and HSV-2 (Herpes Simplex Viruses) codes a ribonucleotide reductase which exhibits a Serine/Threonine kinase (STK) activity that autophosphorylate itself and also phosphorylates α-casein and histone 11-s that helps in the viral replication (20), (96). Similar kinases are present in cytomegalo virus and varicella zoster virus which help in the replication of viruses (49), (103). In Hepatitis B virus, HBx is a viral regulatory protein activates the Src kinase which specifically phosphorylates tyrosineresidues in stimulation of viral polymerase activityessential for viral infection (11), (65).In HIV-1 infection, viral proteins are

phosphorylated which plays an essential role to regulate the life cycle (15). Along with structural and accessory proteins of HIV-1, several other proteins are phosphorylated in course of its replication and propagation (15). Mitogen Activated Protein Kinase (MAPK) phosphorylates specific serine and threonine residues of Vif protein which plays an important role in HIV-1 infectivity and replication (140), (142). Previous studies reported that p17Gag, Nef, and Revof HIV-1 are phosphorylated by Protein Kinase C (PKC)(8), (16), (48). Vpuis phosphorylated bycasein kinase II during the release of virion from the plasma membrane of infected cell (111). Gag MAis phosphorylated by HIV-1associated serine/threonine kinase(15). Other HIV-1 protein such as Rev, Tat, $p17^{Gag}$ and Nef proteins are directly phosphorylated by MAPK to regulate the HIV-1 infection and progression (141).

Phosphorylation of Topoisomerases II:

In the previous studies, it was widely reported that Topo II phosphorylation is connected with activation and regulation of enzymes during cell cycle division and progression.It has been reported that several cellular kinases phosphorylate Topo II isoforms and tightly regulate the catalytic activity of these enzymes (73)**.** *In-vivo* studies on Topos II shown that phosphorylation of heterodimers are significantly at low levels when compared to homodimeric α enzymes, but both the enzymes are phosphorylated by casein kinase IIthat is added exogenously, the phosphorylation results suggested that there was an alternative regulation of these Topo II isoforms (43). An etoposide (VP-16) studies in human leukemia (K562) cells has shown that Beta II PKC plays a role in modulating the DNA binding activity of Topo II by means of phosphorylation (106). Casein kinase II (CK-II) and Protein Kinase C (PKC) uses a common mechanism to phosphorylate the C-terminal regulatory domain of Topo II which modifies the catalytic activity by increasing the rate of ATP hydrolysis (22). Proline-directed kinases

phosphorylate the serine-proline motifs on the C-terminal domain of Topo II α and its activity is regulated based on the cell-cycle progression (132).

In several aspects, Topo II α is a potential target for ERK kinase (ERK-1 and ERK-2) and which is highly phosphorylated during the metabolism of nucleic acids at mitosis of cell cycle. An *in-vitro and in-vivo* studies revealed that ERK-2 regulates Topo II *α* via MKK/ERK pathways suggesting that phosphorylated form of Topo II *α*is required for the chromatin modulation and during re-organization events of mitosis and other stages of the cell cycle progression (112).

Phosphorylation of Topoisomerase II during HIV-1 infection:

During the infection of HIV-1 not only the CD_4 receptor but also the co-receptor plays a vital role in pathogenesis by triggering many kinase pathways. Binding of different chemokine receptors (CCR5/CXCR4) to specific (R5/X4) tropic viral gp120 activates an array of signal transduction pathways contributing to HIV-1 infection (34), (135). CCR5 receptor present on the macrophages will activate the Src kinase Lyn pathway which concomitantly activates the MAPK (Mitogen-Activated Protein Kinase) pathway further a cascade of kinase phosphorylation takes place that leads to the production of TNF-α by macrophages which contributes to the HIV-1 pathogenesis (121).A characteristic feature of HIV-1 infection is host immune activation and inflammation with elevated levels of TNF-α in the plasma and tissues (97). Previously *Env* protein gp120 mediated CCR5/Lyn/Erk1/2 was known though activation of Topo II isoforms post infection is still not clear. Acknowledging the undisputed role of Topo II family in viral replication, both isoforms α and β carryout different set of functions. Over expression of both TopoII isoforms are reported post infection. Despite of concurrent activation of both isoforms through phosphorylation post infection, different pattern of activation and longevity was observed depicting differential roles in viral infection progression (68). In similar studies in

active and resting T cells, Topo II β levels show significant difference in post-infection, while Topo II α does not differ. This anomaly has further been studied extensively and time interval studies were able to provide a cellular snapshot in terms of Topo II β interaction; an elusive activity was observed and identified as Topo II β kinase activity (101).

Topo II found to be highly phosphorylated form in the infected cells of HIV-1 (87) and mainlyTopo II α and β phosphorylationis observed during initial stages of HIV-1infection (68). Purified Viral lysate reported to be associated with Topo II isoform-specific phosphorylation. Kinase fraction preferentially phosphorylating Topo II alpha isoform was sensitive to MAP kinase inhibitors, while Kinase fraction resistant to known panel of kinase inhibitors was preferentially phosphorylated Topo II beta (68). A thorough characterization of purified Topo II beta kinase activity revealed the presence of a 72 kDa protein ($TopoII\beta_{HIV\text{-}1}$). $TopoII\beta_{HIV\text{-}1}$is a serine and threonine kinase. $TopoII\beta_{HIV\text{-}1}$seems to be resistant to notable wide-range panel of kinase inhibitors (101). The aim of the thesis is to understand active site properties of $TopoII\beta HIV_{\text{-}1}$by development of various inhibitors.

Topoisomerase II beta kinase:

Purified viral lysate reported to be associated with Topo II isoform-specific phosphorylation. Earlier studies have shown that Topo IIβ is found to undergo phosphorylation during HIV-1 infection. A 72 kDa protein was purified from virus concentrates that exhibited a prominent Topo II β phosphorylating activity, indicating the kinase existence in the purified virus concentrates (101). Thus we termed this kinase as HIV-1 associated TopoII β kinase (Topo IIβK_{HIV}) and analysis of various enzymatic properties have shown that the β kinase is a serine/ threonine kinase, thus identified as a Ser/Thr kinase (STK). HIV-1 Associated Topo II β Kinase exhibited resistant to common wide-range inhibitors of kinase such as PD98059 and staurosporine(68). In the previous studies we synthesized and tested several pyridine derivatives towards Topos II β kinase $_{HIV}$ where they inhibited the viral replication (101),further development of various organic frame works using pyridine coumarin derivates as pharmacophore lead to identification of novel potent Topo II beta kinase inhibitors with anti-HIV-1 activity(62).

Antiretroviral Therapy:

Currently the Antiretroviral Therapy (ART) is a most potent treatment to control the spreading of HIV-1 and is recommended as a key treatment for HIV/AIDS. ART is related to the usage of two or more drug combinations in the treatment of HIV-1. AZT (Azidothymidine) is the first ever drug used in the Antiretroviral Therapy (ART) which is a NRTI (Nucleoside Reverse Transcriptase Inhibitors) approved by USFDA (130). Initially single drug treatment (monotherapy) was used to treat the AIDS, which was not sufficient and effective treatment to decrease the viral load. Later in 1990s, Highly Active Anti Retroviral Therapy (HAART) was employedwhich requires a combination of three or more different classes of inhibitors (drugs)that will lead to cessation of the HIV-1 replication at various stages in the host cell. HAART was efficiently reduced the progression of HIV/AIDS in many HIV patients (81). Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NRTIs), Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs), Protease Inhibitors (PIs) and Fusion Inhibitors (FIs) are employed in HAART (143). Due to several limitations and side effects, still scientific groups are working on new drug developments to eradicate the HIV/AIDS from the world.

According to WHO/UNAIDS programme 62% of people living with HIV were receiving antiretroviral treatment till 2018. Monitoring, prevention and timely response to population levels of HIV-1 drug resistance (HIVDR) is critical. WHO/UNAIDS as aimed a 90–90–90 target for 2030 i.e. 90% of people infected with HIV-1should notice their status, 90% of HIV-1 positive people should access the treatment and 90%of the people getting treatment should suppress the HIV-1 viral loads(41).

Figure 4: HIV-1 life cycle and Drug Targets

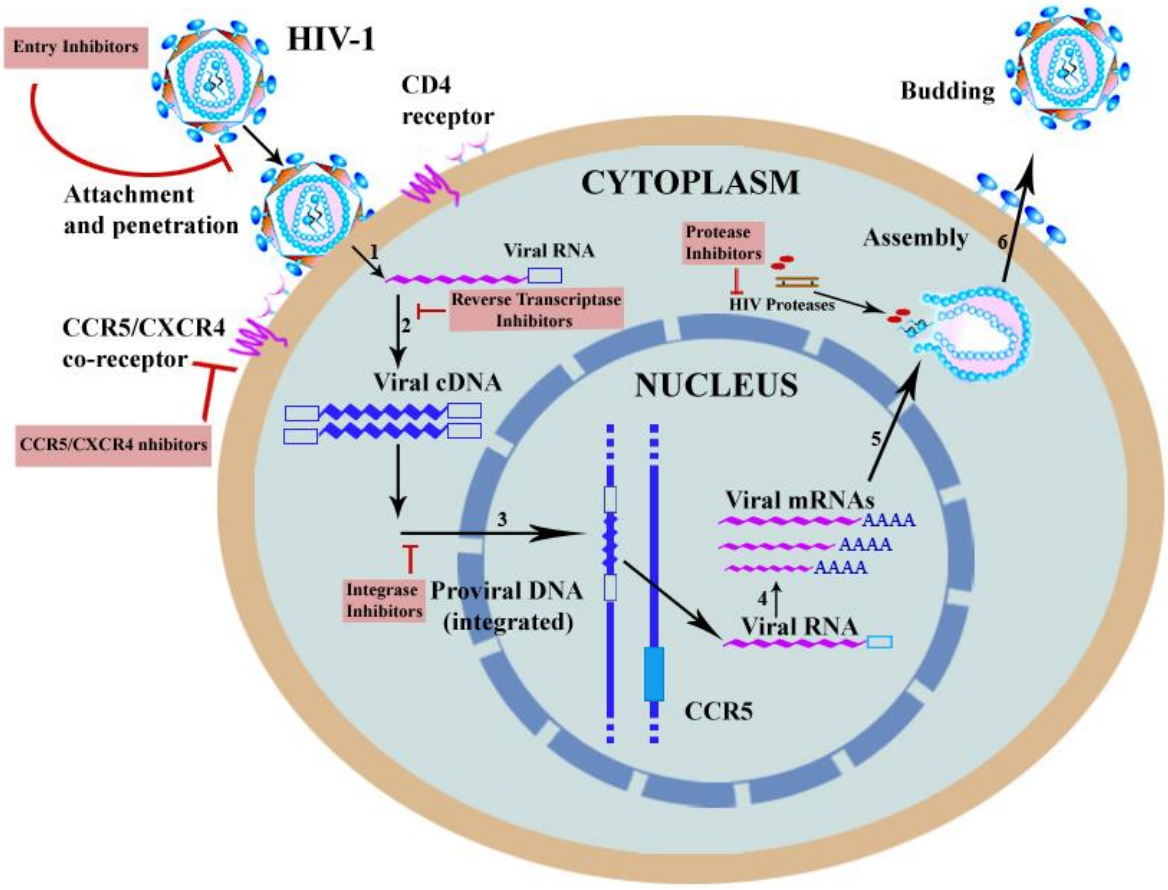


Fig. 4: Figure showing the various drug targets at different stages of HIV-1 life cycle 1) Entry inhibitors, 2) Co-receptor inhibitors, 3) Reverse Transcriptase inhibitors, 4) Integrase inhibitors and 4) Protease Inhibitors

Table 4: Drug Classes of HIV-1

4A: HIV-1 drug classes and their mode of action:

S.No.	Drug Class	Mode of Action
1	**Nucleoside reverse transcriptase inhibitors (NRTIs)**	Inhibits extension of polymerase activity
2.	**Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)**	Inhibits Reverse transcriptase
3.	**Protease Inhibitors (PIs)**	Targets HIV Proteases
4.	**Integrase Inhibitors (INSTIs)**	Targets Integrase and inhibits integration into the host
5.	**Fusion Inhibitors (FIs)**	Targets the gp120 receptors and inhibits the fusion
6.	**Chemokine receptor antagonists (CCR5 antagonists)**	Inhibits the entry into the host

4B: Pharmacologic drug classes, current in clinical preclinical and R & D include:

Drug Class (Type of Inhibitors)	NRTI	NNRTI	PI	Fusion Inhibitor	CCR5 Antagonist	Integrase Inhibitor
1	Abacavir	Delavirdine	Atazanavir	Enfuvirtide	Maraviroc	Raltegravir
2	Didanosine	Efavirenz	Darunavir	**Fixed-dose Combinations:**		
3	Emtricitabine	Etravirine	Fosamprenavir	Zidovudine/ lamivudine		
4	Lamivudine	Nevirapine	Indinavir	Zidovudine/lamivudine/abacavir		
5	Stavudine		Lopinavir	Abacavir/lamivudine		
6	Tenofovir		Nelfinavir	Emtricitabine/tenofovir		
7	Zidovudine		Ritonavir	Efavirenz/emtricitabine/tenofovir		
8			Saquinavir			
9			Tipranavir			

Table 4: Table 4A showing the different classes of FDA approved pharmacological drugs and their mode of action which inhibit the various stages of HIV-1 life cycle. 4B showing the current different classes of HIV-1 drugs in use.

Rationale of the Study:

Several reports have shown that Topo II α and β phosphorylation was recognized during HIV-1 infection. Our earlier studies have shown that purified viral concentrates of HIV-1 was having significant phosphorylating activity of Topo II β, indicating the existence of a 72 kDa kinase protein in the purified concentrates of virus. We termed this kinase as HIV-1 associated Topos II β kinase (TopoIIβK_{HIV-1}) and have been identified as a specific Ser/Thr kinase (STK), which was observed that the kinase is resistant to common broad-range inhibitors of kinases. In the previous studies we synthesized and tested several pyridine derivatives inhibiting TopoIIβ$K_{HIV-1,}$ exhibited inhibition of HIV-1 replication. Kinase activity of Topo II α and β was inhibited by small molecules that can decrease the progression of viral infection and when the kinase protein is expressed only during infection of HIV-1. A series of synthetic molecules from distinct backbone showed significant inhibitory action on HIV-1 Associated Topo II β Kinase (TopoIIβK_{HIV-1}).

Present study deals with the development and molecular characterization of novel HIV-1 associated TopoII β kinase inhibitors during infectivity of HIV-1 that will lead to block the important pathway in the life-cycle of HIV-1. Understanding how these inhibitors prevent virus replication by targeting to HIV-1 associated Topo IIβ Kinase and events of Reverse Transcription. Further broad spectrum activity of inhibitors against viral strains provides important information for lead development.

The following objectives were framed based on the above rationale:

1. **Bioactivity of Pyridine derivatives against Topo II β kinase and HIV-1 replication.**
2. **Analysis of activity of Bis-chalcone derivatives against Topo II β kinase and HIV-1 replication.**
3. **Characterization and molecular action of Topo II β kinase during early replication of HIV-1.**
4. **Wide-Range (Broad-Spectrum) activity of HIV-1 inhibitors against different isolates of HIV-1.**

CHAPTER-II

Materials and Methods

Materials:

Antibodies:

- Mouse anti-human Topo IIβ monoclonal antibodies were from BD Biosciences, USA
- Anti-mouse IgG secondary Ab (Millipore, MA, USA)
- Biotin linked Phosphoserine primary Ab (Sigma)
- Streptavadin HRP secondary Ab (B. Genei)

Enzymes:

- Calf-Intestinal Alkaline Phosphatase (CIAP) Promega, USA
- Casein Kinase II(CKII), Promega, USA

Molecular Biology Materials:

- Mono Q matrix, Amersham Biosciences
- Protease inhibitors were purchased from Sigma-Aldrich Co., USA
- 0.2 and 0.45 μm syringe filter were from PALL Life Sciences, USA.
- PVDF (Polyvinylidene Flouride) membrane from PALL Life Sciences, USA.
- Protein A/G agarose beads (B. Genei).
- PCR Master mix from
- HIV-1 p24 Antigen Capture Assay was performed with ABL(Advanced Bioscience Laboratories)ELISA kit purchased from, Kensington, MD, USA.

Radioactive Materials:

- Research grade γ-32P ATP and 32P was purchased from JONAKI/BRIT, CCMB Campus, Hyderabad.

Biochemicals & Cell Culture Materials:

- DMEM and RPMI-1640 was purchased from Invitrogen, USA; phosphate-free RPMI-1640 was purchased from MP Bio, Ohio, USA; was
- MTT reagent was procured from Hi-Media, India
- Cell culture dishes, flasks and tubes were procured fromCorning Inc. USA
- Cell culture grade bottles and 96-well plates were procured from Corning Inc.USA.

Cells & Viruses:

Cell types:

- Sup T1 (CD4 +ve T cells)
- U937 (macrophage cell line with CD4 +)
- HL2/3 (Recombinant expressing gp120 on surface)
- SKNSH (Neuroblastoma with Chemokine receptors)

Viruses:

- 91/UG/273 – Subtype A
- pNL4-3 – Subtype B
- BAL – Subtype B
- 93IN101 – Subtype C

Methods:

Cell:

SupT1 and U937cells were used and propagated in RPMI-1640 complete media(10% FBS) maintained in incubator with 5% CO_2 and 37ºC.

HL2/3 and SKNSH cells were used and propagated in DMEM complete media (10% FBS) maintained in incubator with 5% CO_2 and 37ºC.

Virus Culture:

HIV-1 strains, 93IN101, 91/UG/273and NL4-3 were cultured with SupT1 cells for viral infection where as BAL virus was infected in U937 with cry preserved viral stock, infection was maintained for 5-6 hours, infected cells were pelleted and washed twice with 0.1 M PBS, resuspended the cells back to complete media (RPMI-1640 with 10% FBS). On 4^{th} day the virus titer was estimatedby p24 ELISA kit (Advanced Bioscience Laboratories (ABL) USA). Viralsupernatants of infected cell culture were stored in cryopreservation container and were used for infection experiments.

TCID-50:

HIV-1 viral titers are typically monitored by *in-vitro* culture method by quantitative measure of p24 antigen production in Sup T1 cells acutely infected with an increasing dilution of virus supernatant, typicallyTCID 50 equivalent p24in nanogram of virus stock was used for each acute infection.

Preparation of viral lysate:

Virus culture supernatants from 4th day of infected cells were pooled and overlaid on 30% sucrose cushion 1:1 (v:v) at 4 °C in OAK Ridge tube and centrifuged for 2 hours at 18,000 rpm under 4 °C. The supernatant was disposed and the thin pellet was rinsed once with TBS (0.1M). Then the pellet was suspended in 1/10 - 1/100th the original volume in lysis solution [20 mM Tris (pH 7.5), 0.1 mM EDTA, 1 mM MgCl2, 500 mM KCl, 1% Triton X-100, 5% glycerol, 0.1% SDS, 0.1 mM β-mercaptoethanol, 0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin]. The viral lysate was dialyzed against TBS (0.1M) for 24 h in a 12-kD cut-off membrane and with periodical change of the TBS. The dialysate was further concentrated, collected and stored at -80 °C.

Kinase purification:

Purification of HIV associated kinase was done by infecting the supT1 cells with 2ng equivalent of HIV-1 93IN101 virus isolate, after sufficient count 4-6 million of infected cells were generated, cells were pelleted down at 4^0C, pellet was discarded and supernatant was passed through 0.2um filter syringe. Carefully supernatant was layered on the surface of 1.5mL Eppendorf which contains 20% cold sucrose solution half of its volume and centrifugation was done for 2 hours. Pelleted virus was lysed in lysis buffer and centrifuged at 800 rpm, after lysis the virus pellet was suspended in PBS buffer and stored at -70^0C.

Virus lysate suspended in PBS was then thawed in ice and purified with MONO Q anion exchange column. MONO Q beads were packed at a height of 1-1.5cm and column is equilibrated with 20 bed volume of 1X TBS, flow rate was maintained as 6-7mL/min, then 100uL of viral lysate was loaded on the top of column carefully. Washing was done by 1X TBS (3.0mL). Final elution was done by gradually increasing the concentration of NaCl i.e. 20mM,

100mM, 400mM, 800mM NaCl (each 2mL only) respectively. All these steps were performed at 4^0C, fractions were collected and stored in ice, 30uL from each fraction of flow through from all these washes and eight elutes was resolved on the 10% SDS-PAGE followed by silver staining to visualize the bands.

Cell Cytotoxicity of compounds:

All the synthesized compounds were checked for Cytotoxicity with MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) in SupT1 cells. Briefly, SupT1 cell were propagated in complete media (RPMI 1640 with 10% FBS), approximately 0.02 X 10^6cells were seeded in 100µL of complete media. After overnight incubation cell viability was determined by MTT reagent with 5mg/mL concentration. 10uL MTT was dispensed to all the 96 wellsof the plate and incubated for 4 hours in cell culture incubator with 5% $CO_2$and 37^{o}C. After incubation 100uL of DMSO was dispensed to all the 96 wells of the plate and gently agitated and kept in darkness for 30 minutes at RT. At 570 nm absorbance was measured in a micro titer plate reader, each assay was repeated thrice and in triplicates.

Cell lysate preparation:

The Sup T1 cell lysates were prepared by incubating the cells in RIPA buffer [50mM Tris–Cl (pH 7.4), 1% Triton X-100, 1%sodium deoxycholate, 0.1% SDS, 1 mM EDTA, pH 7.0, 150 mMNaCl and inhibitor cocktail (1% aprotonin 1% pepstatin,1mM PMSF,1mM Na_3VO_4, and 50 mMNaF)] for 30min on ice and sonicated at 40% amplitude, pulse of 15 seconds for 2 times. The sonicated cell lysate was centrifuged at 12,000 rpm for 15min at 4^0C. The supernatants were collected and protein estimation was done by using Bradford's method. Lysates containing Topo II was dephosphorylated by incubating the total protein at 100 ug concentration with 1U of Calf-

Intestinal Alkaline Phosphatase enzyme in CIAP buffer for 30 min at 37 °C. NaF with final concentration of 10mM was added to the above mixture to stop the reaction. The lysate containing dephosphorylated Topo II was used further in*in-vitro*kinase phosphorylation assays.

Protein Dephosphorylation:

The Protein Dephosphorylation of Topo II β has been done to identify the degree of phosphorylation by the HIV associated Kinase in the In vitro phosphorylation assay. The Topo II β present in the crude extract is treated with Calf-Intestinal Alkaline Phosphatase (CIAP) in the conditions of CIAP buffer at 37^{o} C for 30 minutes. The reaction will be stopped by using 50 mM sodium fluoride (NaF).

In vitro phosphorylation assay:

In vitro phosphorylation assay was validated by taking the dephosphorylated Topo II β in a kinase reaction mixture containing kinase, 10 µl (100 µM ATP) of cold ATP and 5 µCi of hot ATP [γ-^{32}P]in the presence of drug incubated at 37°C for 30 minutes. Two micro liters (2ng) of Topo II β antibody was mixed with the lysate and kept for overnight incubation at 4°C. Next day 6% protein A/G–agarose beads wereadded to the lysate. The lysate was incubated for 1 hour at 4° C under slow agitation, and thenspun at 2000 rpm for 5min. The beads sediment was washed with TBS for twice and precipitated with 40 µl of 5% trichloroacetic acid (TCA).10 µl of precipitate was spotted on discs of Whatman paper and dried for overnight at 37°C. On the next day liquid scintillation count was done to measured γ-^{32}P. In all the experiments specific Casein Kinase II (CK-II) which phosphorylates both Topo II isoforms was used as a positive control. In each experiment samples were run in triplicates and performed thrice.

Anti-Viral Assay:

Anti-viral activities of the compounds were tested using the p24 antigen capture sandwich ELISA method (p24 assay). SupT1 cells with 99% confluence were seeded in 24 well plates and infected with viral strain (HIV-$1_{93IN101}$), at a final concentration corresponding to 1ng/mL and then drug with a various concentration was added to the wells. The cells infected with virus were incubated in the 5% CO_2 and 37^0C incubator for 5 hours. After incubation, cells were sedimented at 350xg for 10 minutes and resuspended the pellet in fresh complete media (RPMI1640 containing 10% FBS). Resuspended infected SupT1 cells further incubated for 96 hours in 5% CO_2cell culture incubator.After 96 hours the supernatant was collected and viral titer was estimated by using p24 ELISA kit (Advanced Bioscience Laboratories (ABL), USA).

Analysis of proviral DNA synthesis in the presence of drug:

Provirus inhibition was analysed with various compounds by conventional PCR method. SupT1 cells with 99% confluency were seeded in 24 well plates and infected with viral strain (HIV-$1_{93IN101}$)at a final concentration corresponding to 1ng/mL and then drug with a varying concentration was added to the wells. After 5 hours of time point the infected cells were harvested and genomic DNA was isolated by standard phenol/chloroform method and analyzed for the amplification of proviral DNA by specific primers SK 38/39 codes for HIV-1 gag region.

Analysis of RT DNA synthesis in the presence of drug:

Viral strain (HIV-$1_{93IN101}$)was infected to SupT1 cells for 1 hour in the presence of drugs at various concentration and the samples were collected after 1hour. Cytosolic DNA was isolated by cytoplasmic extraction buffer and DNA was extracted by phenol/chloroform method. PCR analysis was done using specific primers amplifying the intermediates of Reverse Tanscription

(RT) namely Strong Stop (SS) DNA, First Strand Transfer (FST), Second Strand Transfer (SST) and Full Length Minus Strand (FLMS).

Silver Stain:

Acrylamide gels were stained with silver staining method (19), after electrophoresis gels were fixed with fixative solution (30% Methanol and 10% Acetic Acid) for overnight and the next day gels were rinsed with 20% ethanol solution. The gels were washed with deionised water for 5 minutes at least thriceand then rinsed with sensitizer $Na_2S_2O_3$ (0.02% Hypo) for 1 minute, after that gels were rinsed briefly with deionised water water and transferred to 0.2% $AgNO_3$ for 30 minutes, again the gels were rinsed with double distilled water for 10-20 seconds and developed with a solution of 0.3% Na2CO_3, and 0.4% HCOH for 5 to 10 min. Development was then stopped with a 5% Acetic Acid solution for 5 minutes.

Western Blotting:

For Immune precipitation 100 µg of total protein samples were used and resolved on 8% SDS-PAGE. Sample preparation was done by using 6X loading dye containing [0.125 M Tris-Cl (pH 6.8), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% bromophenol blue]and samples were boiled for 5 min at 100 ºC. Electrophoresis was carried with 1X [0.025 M Tris HCl, 0.192 M Glycine and 0.1% SDS (pH 8.3)] electrode running at 100 V. After electrophoresis gel was transferred onto nitrocellulose membrane (Pall) using the Transfer unit apparatus (Bio-Rad, USA) by Towbin et al. method (125). Initially, the gel and membrane was equilibrated in 1X Towbin buffer (0.025 M Tris HCl, 0.192 M Glycine and 20% methanol) for 30 min andgel transferredon to nitrocellulose membrane for overnight at 35 V at 250 mA under 4ºC. Next day the blocking of the blot was done to block non-specific binding by treating it with blocking solution (5% BSA w/v) in 1X Tris-buffered saline (TBS) [0.01 M Tris-Cl (pH 7.4), 0.15 mM NaCl) for 2 h at room temperature. After blocking,

the blot was rinsedwith 1X TBST (TBS containing 0.1% Tween-20). The blot was then treatedwith1:4000 diluted primary antibody of mouse anti-human Topo II β (BD Biosciences, USA) or Biotinylated phosphoserine primary antibody (Sigma, USA) and incubated for overnight at 4 ºC. Next day the blot was washed twice in TBST for 10 minutes and a single wash in TBS, the blot was incubated again with diluted (1:20,000) goat anti-mouse IgG secondary antibody conjugated to HRP (horseradish peroxidase) (Millipore, USA) for 2 h at room temperature.After incubation blot was washed again with TBST (twice) and TBS (single). ECL substrate (Thermo Scientific, USA) was used to develop the blots and chemiluminescence image was developed in chemidoc instrument (Bio-Rad).

Immune precipitation:

For immune precipitation100ug protein extract was taken and 250 ng ab/1 mg Topo II β primary Ab (BD Biosciences , USA) was added to total proteinand incubated for overnight in 4ºC under slow agitation, next day 6% protein A/G agarose beads were mixed and incubated for 2 hours. The lysate was spun at 2000 rpm for 5 minutes and supernatant was discarded, the settled beads were washed twice with 0.1% TBST and checked on 8% SDS-PAGE.

CC_{50} and IC_{50} Calculation:

All the experiments were conducted in triplicates at least twice independently. The data taken for calculation is the average of the replicates. The CC_{50} and IC_{50} values of all the inhibitors were calculated and compiled the data by taking the broad-range (1 pM to 1 mM) of inhibitor concentrations. All the CC_{50} and IC_{50} values were calculated by using the Graph Pad Prism 8 XML Project.

Densitometric analysis

Densitometric analysis was done by taking the variations in relative expression of various DNA and proteins. By using Image J software,DNA and proteinexpression was quantified, which is a public domain image analysis and processing software downloaded from the website National Institute of Health (NIH). All the experimental image values are normalized with loading controls and normalization of RT intermediates was done with GAPDH.

Table 5: Primer Sequences

S.No	Primer	Forward Primer	Reverse Primer	Purpose
1	Sk38/39	TAATCCACCTATCCCAGTAGGAGAAT	TTTGGTCCTTGTCTTATGTCCAGAATGC	Proviral DNA synthesis
2	SS	CTGGGAGCTCTCTGGCTAACTAG	GGCGCCACTGCTAGAGATTTTCC	Reverse Transcription Events
3	FST	CTTCAGCTACCACCGCTTGAG	CTTTGACCACTTGCCACCCATC	Reverse Transcription Events
4	SST	TACCAATGCTGCTTGTGCCTGG	GAAAGTCCCCAGCGGAAAGTCC	Reverse Transcription Events
5	FLMS	GCGCCCGAACAGGGACTTGAAAG	ACGCTCTCGCACCCATCTCTCTCC	Reverse Transcription Events
6	GAPDH	TACCACCGCTTAGGAGAATTTGAG	TCCAGAATGCCTTTGACCACTTGC	Standard Control

Table 5: Above table is showing the different primers used for PCR experiments

CHAPTER-III

Bioactivity of Pyridine derivatives against Topo II β kinase and HIV-1 replication

Introduction:

During the lentiviral infection many specific kinases are activated for their replication and propagation. HIV-1 can activate the multiple kinase signaling pathways within the host cells to facilitate the viral entry and for successful replication. Right from the viral entry to the budding it employs several host cellular proteins for the successful virion formation. As earlier studies have shown that Topos II are phosphorylated during the HIV-1 infection and specifically Topos IIβ is phosphorylated in the primary phase of HIV-1 life cycle. It was reported that phospho form of Topos IIβ supports in theexport of viral genomic RNA and associated with reverse transcription events.

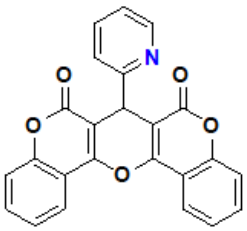
Kinase inhibitors are one of the targets to eradicate HIV-1infection; these inhibitors will interrupt the phosphorylation steps in various stages of viral replication. A diverse class of organic molecules have shown inhibitory activity on HIV-1, among the high prevalence kinase inhibitors pyridine sub-structure has shown a broad-spectrum activity (72)**.**In the earlier studiesTopo II α and β phosphorylation was characterized (68). Sensitivity of these two-kinase activities were analysed in the presence of various inhibitors, the results showed that Topo II α kinase is sensitive to Map kinase inhibitorswhile Topo II β exhibits very low sensitivity with the known kinase inhibitors (68).Recentstudieshave shown that Pyridine derivatives have inhibitory activity towards the HIV-associated TopoIIβ Kinase (TopoIIβK_{HIV})activity. Further pyridine derivatives have shown significant inhibitory activity on TopoIIβK_{HIV} along with inhibition of HIV-1replication, thus suggesting that TopoIIβK_{HIV} as a possible target for developing the anti-HIV-1 molecules.Thus, pyridine derivatives have showed an inhibition of TopoIIβK_{HIV} *in-vitro* and anti-HIV-1 activity(101), further development of various organic frame works using coumarin

derivatives as pharmacophore lead to identification of novel potentTopoIIβK_{HIV}inhibitors with anti-HIV-1 activity(62).

In the present study, we have further analysed the molecular activity of pyridine coumarin derivativesin inhibition of TopoIIβK_{HIV}activity and HIV-1 replication *in- vitro*.

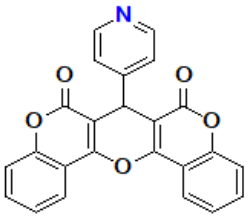
Figure 3.1:

UHAKKM-1



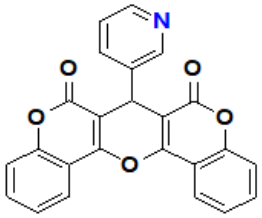
2-Pyridylidene-4,4'- epoxy dicoumarin

UHAKKM-2



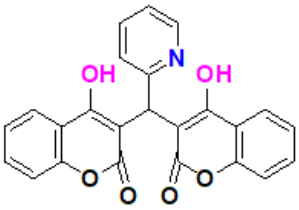
4-Pyridylidene-4,4'- epoxy dicoumarin

UHAKKM-3



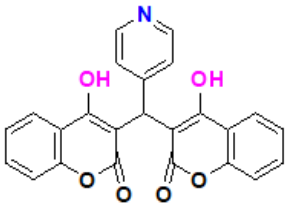
3-Pyridylidene-4,4'- epoxy dicoumarin

UHAKKM-4



3,3'-(pyridin-2-ylmethylene)bis(4-hydroxy-2*H*-chromen-2-one)

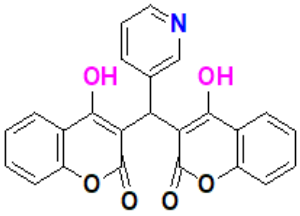
UHAKKM-5



3,3'-(pyridin-4-ylmethylene)bis(4-hydroxy-2*H*-chromen-2-one)

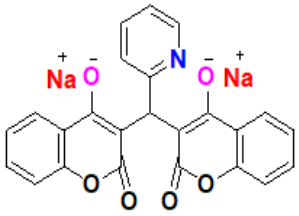
Molecules were synthesized as per Kurumurthy *et.al.* (2017), further biological characterization for antiviral and molecular action was carried out

UHAKKM-6



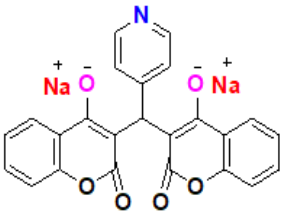
3,3'-(pyridin-3-ylmethylene)bis(4-hydroxy-2*H*-chromen-2-one)

UHAKKM-7



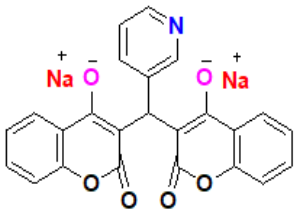
sodium 3,3'-(pyridin-2-ylmethylene)bis(2-oxo-2*H*-chromen-4-olate)

UHAKKM-8



sodium 3,3'-(pyridin-4-ylmethylene)bis(2-oxo-2*H*-chromen-4-olate)

UHAKKM-9



sodium 3,3'-(pyridin-3-ylmethylene)bis(2-oxo-2*H*-chromen-4-olate)

Molecules were synthesized as per Kurumurthy *et.al.* (2017), further biological characterization for antiviral and molecular action was carried out

3D QSAR Model:

Based on structure and activity data from previous study(63), contours were developed using 3D-QSAR. The desirable and undesirable substitutions in terms of steric or electrically charged groups to the core molecule can be understood. In this model the 4^{th} and 5^{th} positions of the pyridine ring favor addition of steric groups, while the addition such that the conformation aligns the hydrophobic group between 3^{rd} and 4^{th} positions results in decreased binding affinity. Equivalently an electropositive group addition at 5^{th} position corresponding to the alignment conformation between 4^{th} and 5^{th}positions elevates the affinity. The structures were designed to match the contours and the coumarin moiety was placed each at 4, 5, 6 position of the ring for compounds UHAKKM-4, UHAKKM-5 and UHAKKM-6 respectively. From the hypothetical pocket model proposed (63) it could be understood that the interaction of 2, 3 positions with the kinase hinge region, restricted the substitutions to only the above mentioned positions. The coumarin moiety acts as a hydrophobic bulky group which on substitution in the favorable region increased the activity of the compound. Similarly the presence of oxygen atoms on the coumarin group resulted in satisfying the electrostatic constraints. Owing to this feature, the three synthesized compounds showed high kinase antagonism.

Pyridine epoxy dicoumarin derivatives (UHAKKM-1 to 3) and pyridine dicoumarol derivatives (UHAKKM-4 to 6) both the series of molecules have shown high inhibition of Topo IIβK_{HIV} activity at low concentration but surprisingly have not shown anti-HIV-1 activity surprisingly at immense concentration (μM). This may be due to lack of flexibility and permeability through membranes. Hence to overcome these problems intra molecular hydrogen bonding was removed in the molecules UHAKKM-4 to UHAKKM-6 which made the formation of disodium pyridine

dicoumarate salt compounds UHAKKM-7 to UHAKKM-9, acquired the properties to permeate through plasma membrane and reach the intracellular targets.

Figure 3.2: QSAR Model

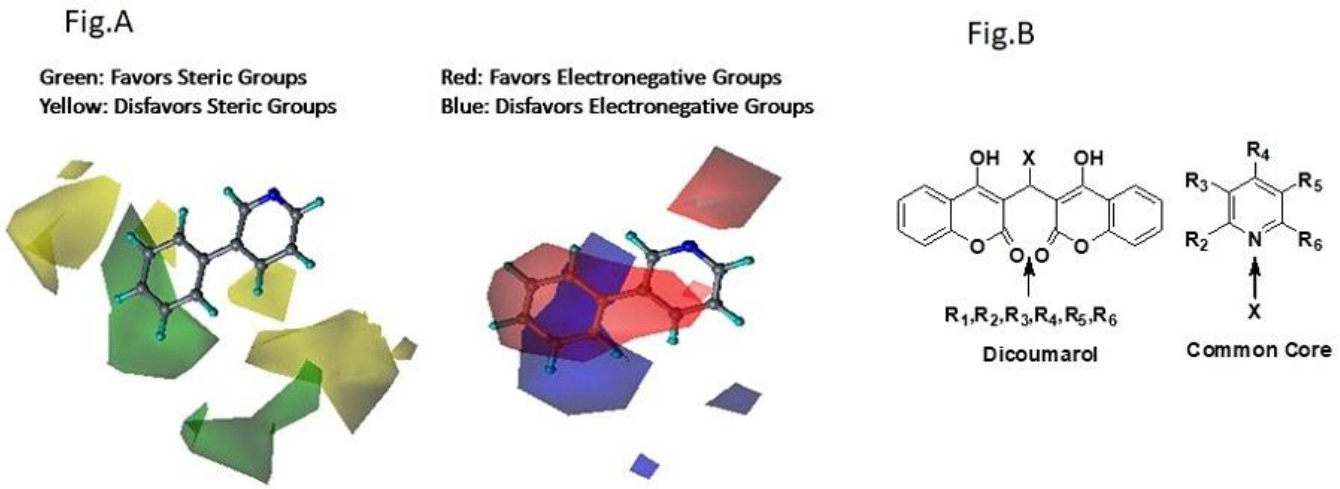


Fig 4: 3D QSAR model A) CoMFA model depicting the Steric Electrostatic contour of active molecules. The 3D contour maps around the highest activity molecule (3-Phenyl Pyridine) generated by CoMFA analysis of the derivatives. (i) Regions where hydrophobic substitution enhances (green) or reduces (yellow) the binding affinity. (ii) The color coding's indicating regions where electronegative substituent enhances (red) or reduce (blue) the binding affinity. **B)** The structures of derivatives with a coumarin moiety at different positions of the core pyridine ring (X) (R1, R2, R3, R4, R5, R6 = Dicoumarol) designed based on the contours.

QSAR Model was prepared by Dr. Akhila Bommakanti as per Kurumurthy *et.al.* (2017), further biological characterization for antiviral and molecular activity was carried out.

Results:

Kinase purification:

Kinase a 72kDa protein was purified from viral lysate, which was collected from infected SupT1 cell culture. The lysate was loaded onto MONO Q beads an anion exchange column, washing was done by 1X TBS (2.0mL) and final elution was done by gradually increasing the concentration of NaCl i.e. 20mM, 100mM, 400mM, 800mM NaCl (each 2mL only) respectively and the protein fractions along with flowthrough were collected and checked for purity on 10% SDS-PAGE by silver stained with 0.02% of silver nitrate ($AgNo_3$).Results in Fig 1 shows that a 72 protein, $TopoII\beta K_{HIV}$ is purified at 800 mM NaCl to the homogeneity.

Figure 3.3: Kinase Purification

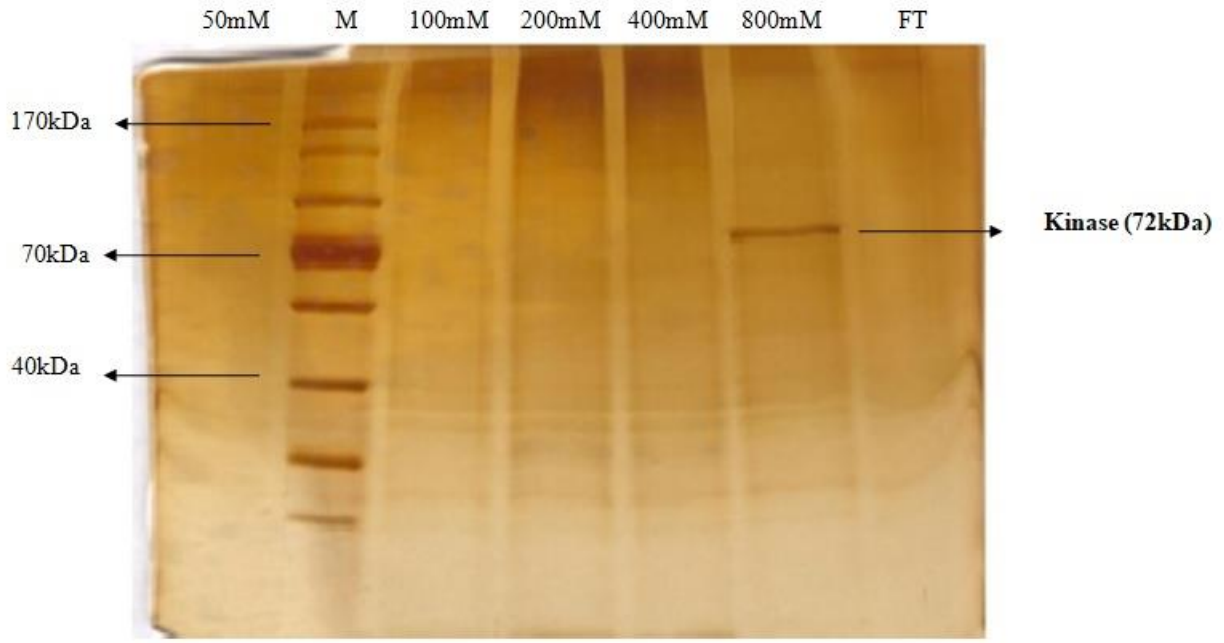


Fig.3.3: Above Fig.3.3 shows the 72kDa protein as a purified kinase, gel was stained with 0.02% silver nitrate, kinase protein (72kDa) was seen in the eluted fraction at 800mM concentration of NaCl (lane6), when compared with the other fractions 50mM (lane1), 100mM (lane3), 200mM (lane4), 400mM (lane 5) and flowthrough (lane 7). Protein Marker was used as standard control (Lane2).

Immuneprecipitation of Topo II Beta:

Immuneprecipitation (IP) and western blot studies were carried out for TopoII Beta. Sup T1 cells were lysed and supernatent was collected, protein estimation was done by Bradford method. Around 100µg total proteincontaining cell extract was taken for each experiment, 1.0µl of mouse anti-human TopoII β monoclonal antibody and 6% protein A/G–agarose beads were used for immuneprecipitation.Protein in IP samples was eluted and resolved on 8% SDS-PAGE and protein was visualized by staining with 0.02% of silver nitrate ($AgNO_3$) and confirmed with specific TopoII β Antibody by Western Blot, the results in Fig 3.4 show the presece of a single band of TopoII β.

Figure 3.4: Immuneprecipitation of Topo II Beta

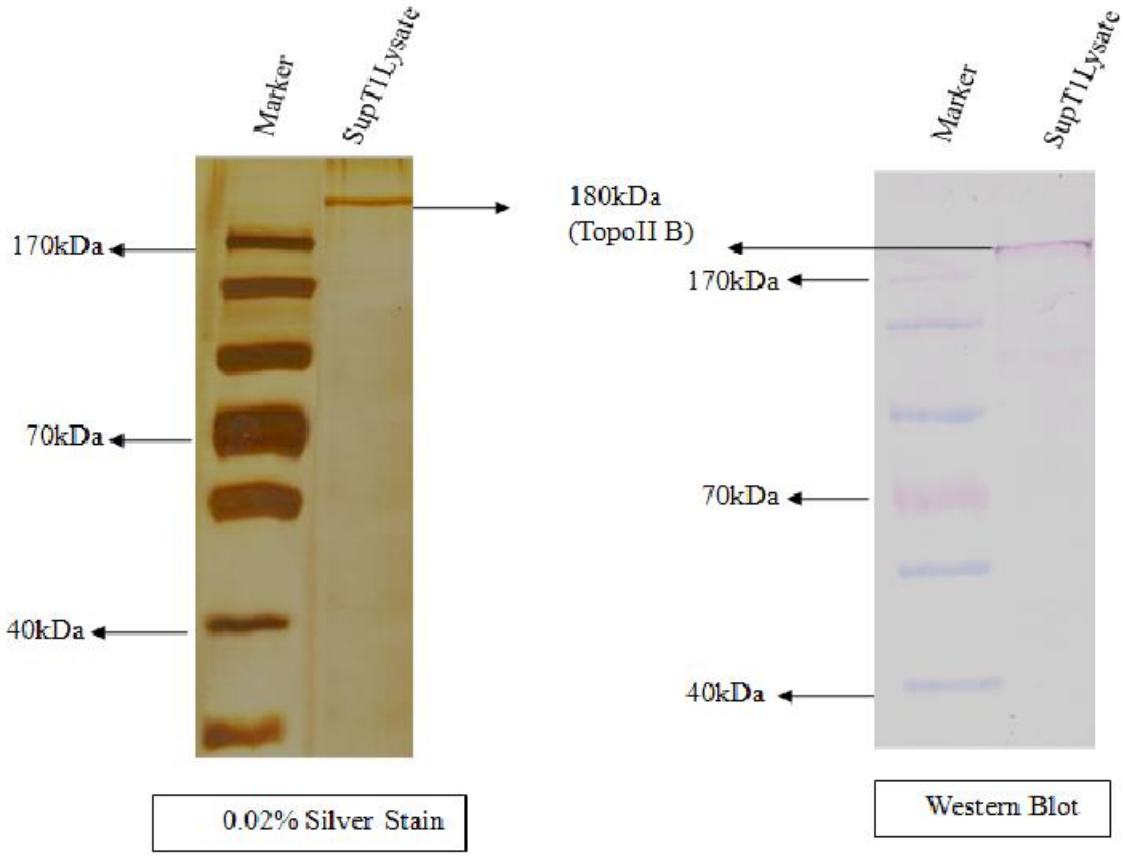


Fig.3.4: In the silver stain (figure.A), immumeprecipitation of supT1 lysate is clearly showed Topo II Beta protein band as 180kDa when resolved on 8% SDS-PAGE and developed with 0.02% of silver nitrate ($AgNo_3$), where as in the Western blot analysis (Fig.B) confirmed by mouse anti-human 180kDa TopoII Beta monoclonal antibody (BD).

***In-vitro* phosphorylation assay:**

Immunprecipitated Topo II β is treated with calf-intestinal alkaline phosphatase (CIP) in the presence of dephosphorylation buffer at 37^{o} C for 30 minutes to remove endogenous phosphate present in Topo II β, dephophorylated Topo IIβ (dpTopoIIβ). Activity of TopoIIβ$K_{HIV}$was analyzed by its incubation with dpTopoIIβ in a kinase buffer containing 5μCi of [γ -32p] ATP. Kinase activity was monitored at increasing concentrations of TopoIIβK_{HIV}i.e. 0.25μg, 0.5, 1.0 and 2.5μg. The results in Fig. 3.5 show a concentration dependent phosphorylation of TopoIIβ by TopoIIβK_{HIV}.

Figure 3.5: *In-vitro* phosphorylation assay

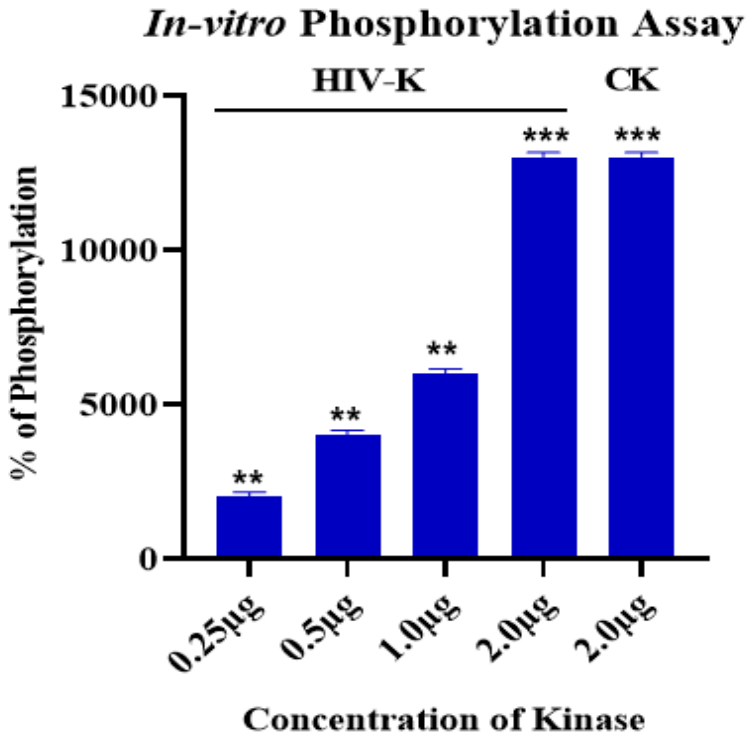


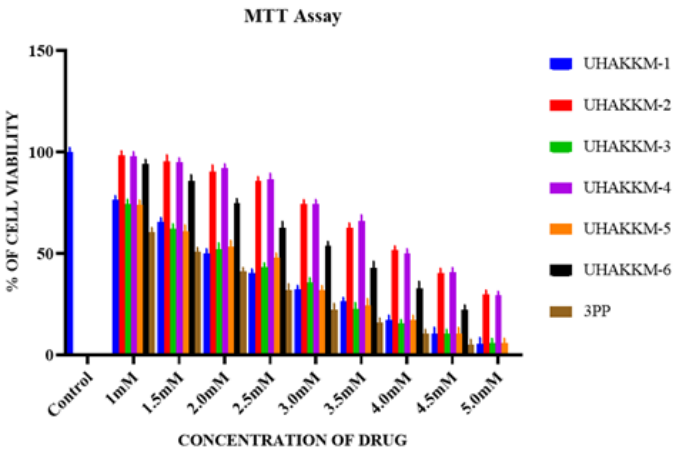
Fig 3.5: Above result shows that concentration dependent phosphorylation by HIV-1 Kinase (HIV-1K), Topo II β is preferentially phosphorylated by HIV-kinase in the presence of 5µCi of [γ -32p] ATP at different concentrations (0.25µg, 0.5, 1.0 and 2.0µg). 2.0µg kinase was showing high proportion of phosphorylation (***P<0.001) and 0.25µg was showing less phosphorylation (**P<0.001). Thus the result suggests that, as the concentration of kinase is increased phosphorylation of Topo II β is increased. Casein Kinase (CK) was used as Reference Standard (***P<0.001).

MTT Assay (Cytotoxicity Assay):

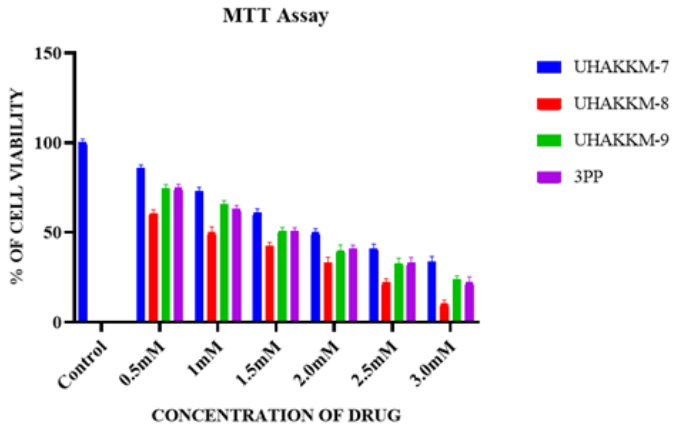
Approximately 0.02 X 10^6cells were seeded in 100µL of complete media per each well in 96 well plate and kept for overnight incubation, on the next day, drugs of indicated concentration was added and incubated in 5% CO_2 incubator for 24 hours, next day10µL of MTT (5mg/mL) reagent was mixed to whole 96 well plate and furtherincubated for 4 hours, then 100µL of DMSO was added to all the 96 wells, after gently agitation the platewas kept in darkness for half-an-hour at RT. At 570 nm absorbance was measured in a micro titerplate reader, samples were run in triplicate and each experiment was repeated thrice. The results in Fig. 3.6 show that all drugs tested exhibit no significant toxicity <10µM. Hence, molecules were analyzed below 10µM.

Figure 3.6: MTT Assay (Cytotoxicity Assay)

A



B



C

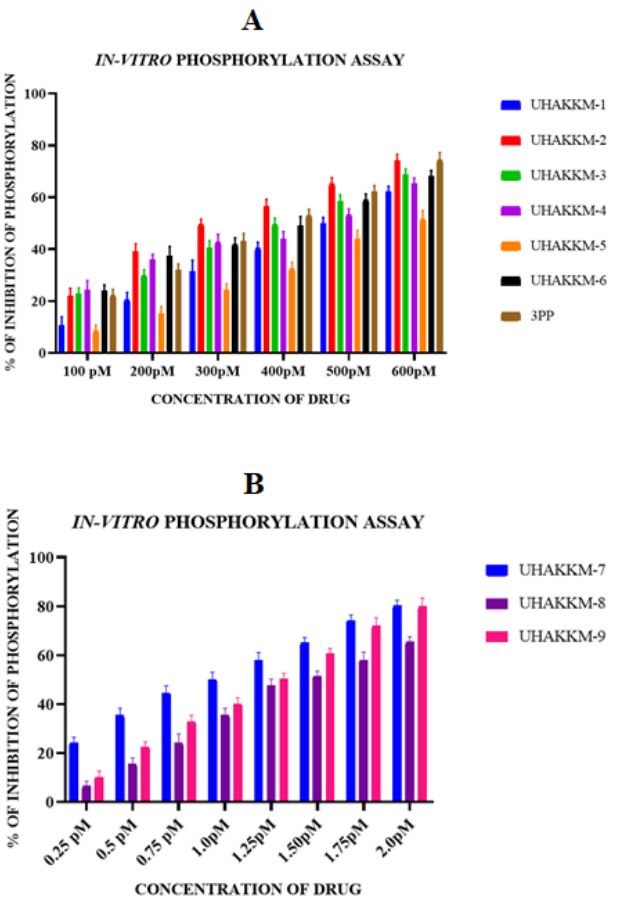
Drug	CC_{50} (mM)
UHAKKM-1	2.09± 0.62
UHAKKM-2	3.89± 0.74
UHAKKM-3	1.94± 0.55
UHAKKM-4	4.04± 0.44
UHAKKM-5	2.30± 0.58
UHAKKM-6	3.23± 0.56
UHAKKM-7	2.06± 0.33
UHAKKM-8	1.04± 0.32
UHAKKM-9	1.55± 0.65
3-Phenyl Pyridine(Control)	1.50 ± 0.029mM

Fig. 3.6: Cytotoxicity Assay was performed in supT1 cells for the pyridine drugs with different concentration as shown in the Fig.3 In above results it was shown that all the drugs are showing above 60% viability at 1mM concentration and at 1mM concentration no significant cytotoxicity observed, thus below this concentration we can use the drugs for anti-HIV activity.

In-Vitro Phosphorylation Assay in the presence of Drug:

Dephosphorylated Topo II present in the reaction mixture was incubated with TopoIIβ$K_{HIV.}$in the presence of 10 µl of cold ATP (100 µM stock) and 5 µCi of hot ATP [γ -^{32}P] in 1X Kinase buffer for 30 min at 37 °C. 3 micro liters (3 ng) of mouse anti-human Topo II β monoclonal antibody was mixed to the lysate and incubated for 1 hour at 37 ° C, after incubation 6% protein A/G–agarose beads were mixed to the lysates and incubated at 4° C for 1hour with slow agitation, after incubation the reaction mixture was sedimented at 2000 rpm for 5min. The sedimented beads was washed with TBS for twice and precipitated with 40 µl of 5% Tri-Chloro Acetic acid (TCA). 10 µl of precipitate was spotted on discs of whatman paper and dried for overnight at 37° C. Next day ^{32}P was measured by liquid scintillation. All the compounds have shown the inhibition of phosphorylation activity of TopoIIβ$K_{HIV.}$ Among the compounds UHAKKM-7 being highly active followed by UHAKKM-8, 9 and 2. Thus results Fig. 3.7 confirm that the compounds possess significant activity against TopoIIβK_{HIV}.

Figure 3.7: *In-Vitro* Phosphorylation Assay in the presence of Drug:



C

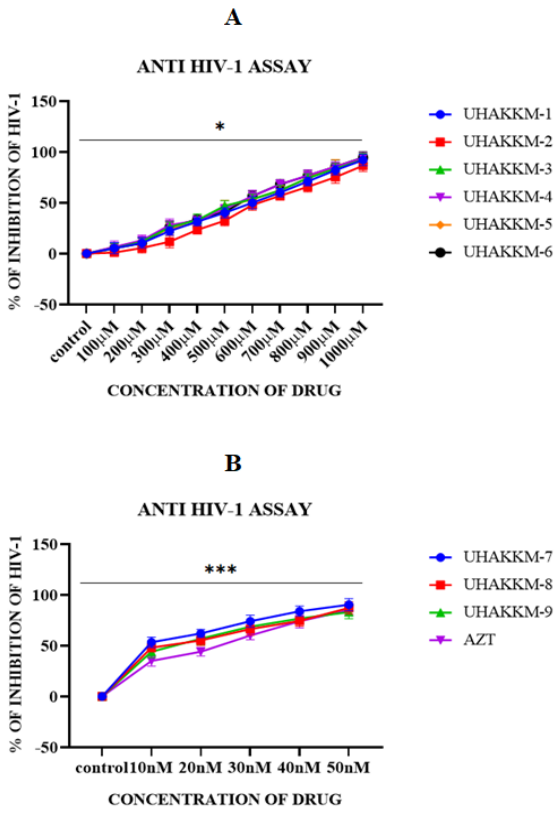
Molecules	IC-50 (pM Concentration)
UHAKKM-1	$5.0\pm0.21x10^2$
UHAKKM-2	$3.3\pm0.1x10^2$
UHAKKM-3	$4.2\pm0.34x10^2$
UHAKKM-4	$4.7\pm0.4x10^2$
UHAKKM-5	$5.8\pm0.14x10^2$
UHAKKM-6	$4.2\pm0.21x10^2$
UHAKKM-7	1.04±0.03
UHAKKM-8	1.32±0.06
UHAKKM-9	1.22±0.05
3-Phenyl Pyridine (Control)	$3.8\pm 0.22X10^2$

Fig.3.7: Above results shows the phosphorylation inhibition of kinase of different drugs in the presence of radiolabel γ -32[P] ATP. In Fig. 3.7 graphs in panel A and B are depicting the percentage of drug inhibition plotted against the concentration of drugs and panel C showing the IC_{50} concentration of all the pyridine coumarin derivatives. All the compounds have shown the phosphorylation inhibition activity, among the compounds UHAKKM-9 being highly active followed by UHAKKM-6, 8, 5 and 7. Thus results confirm that the compounds possess significant activity against kinase. Each experiment was performed in triplicates (***P<0.001) nd all data points represent an average of results from the triplicate experiments. In all the experiments 3-Phenyl Pyridine used as positive control (**P<0.001).

Anti-Viral Assay (p24 Assay):

SupT1 cells were challenged with HIV-$I_{93IN101}$ for 5 hours at various concentrations of drugs in triplicates. After 5 hours the samples were washed, pelleted and resuspended in RPMI complete media, further incubated for 96 hours. The virus replicated at 96 hours was estimated in supernatants in terms of p24 antigen capture assay using ELISA method. HIV-1 replicated in the absence of drug was considered zero percent inhibition, based on p24 in control infection percent inhibition was calculated at each drug concentrations (Fig. 3.8). Based on the viral inhibition in the presence of drug, IC50 was calculated and included in the Fig. 3.8. Among compounds, UHAKKM-7, 8, and 9 showed significant activities (Panel C and D), while other compounds though possess kinase inhibition, they did not exhibit anti-viral activity which was indicated in the Fig. 3.8 (Panel A and B).

Figure 3.8: Anti-Viral Assay (p24 Assay):



C

Molecules	IC_{50}
UHAKKM-1	0.60± 0.04mM
UHAKKM-2	0.74± 0.03mM
UHAKKM-3	0.62± 0.04mM
UHAKKM-4	0.55± 0.05mM
UHAKKM-5	0.58± 0.06 mM
UHAKKM-6	0.56± 0.05mM

D

Molecules	IC_{50}
UHAKKM-7	13.40 ± 0.02 nM
UHAKKM-8	16.80 ± 0.05 nM
UHAKKM-9	22.92± 0.04 nM
AZT	25.00 ± 0.038 nM

Fig 3.8: Above results shows the anti-viral activity all the active compounds. In Fig. 3.8 panel A and B graphs indicates the percentage of drug inhibition plotted against the various concentrations of drug, panel C and D shows the IC_{50} concentration of all the pyridine coumarin derivatives. Among compounds, UHAKKM-7, 8, and 9 showed significant activities (***P<0.001), while other compounds though possess kinase inhibition, they did not exhibit anti-viral activity at high concentration (*P<0.005). IC_{50} of all the active drug compounds 7, 8, 9 have shown anti-viral activity at nM concentration and AZT was employed as positive control. Each experiment was conducted in triplicates and repeated thrice. $IC_{50}$of the entire active drug compounds 7, 8, 9 have shown anti-viral activity at nM concentration and AZT was employed as positive control (***P<0.001).

Analysis of serine phosphorylation in vivo:

Viral strain of HIV-$1_{93IN101}$ was infected to SupT1 cells in the presence of high active drug compound (UHAKKM-7). After 5 hours of post infection cells were pelleted, lysed and lysates were collected. Lysates were prepared from uninfected and infected SupT1 cellsin the presence of Drug, further samples were analyzed by SDS-PAGE. Result in Fig 3.9 confirms that Topo II beta phosphorylation at serine is enhanced upon HIV-1 infection. Experiment was repeated twice and the results show that Drug UHAKKM-7 significantly inhibited Topo II beta phosphorylation at serine in infected cells, while no notable change was observed in uninfected cells suggesting the specific action of Drug UHAKKM-7 against TopoIIβK_{HIV}.

Figure 3.9: Analysis of serine phosphorylation in vivo:

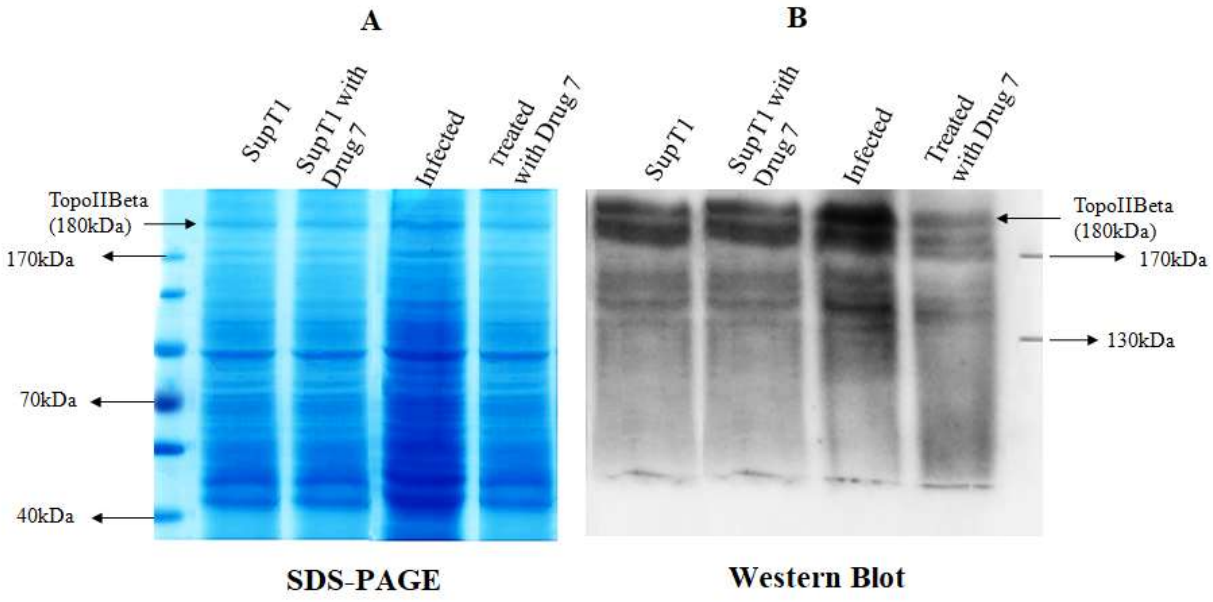


Fig 3.9: The SDS-PAGE analysis by Coomassie Brilliant Blue stain (Fig. 3.9A) and Western blot analysis (Fig. 3.9B) by Biotin linked phosphoserine Ab (Pri.Ab) and Streptavadin HRP Ab (Sec.Ab) shows the comparison of results in normal and infected SupT1 cells after treatment with active drug compound. Result confirms Topo II beta phosphorylation at serine is enhanced upon HIV-1 infection. Drug UHAKKM-7 significantly inhibited Topo II beta phosphorylation at serine in infected cells, while no significant change was observed in uninfected cells suggesting the specific action of Drug UHAKKM-7 against HIV-1 infection associated kinase activity.

Analysis of serine phosphorylation by Immune precipitation:

Viral strain of HIV-$1_{93IN101}$ was infected to SupT1 cells in the presence of high active drug compound (UHAKKM-7). After 5 hours of post infection cells were pelleted and lysed. Lysates were analysed by Western Blot. Phosphorylation of Topo II beta at serine is enhanced upon HIV-1 infection and when treated with active drug UHAKKM-7 significantly inhibited Topo II beta phosphorylation at serine in infected cells, where as no notable change was observed in uninfected cells suggesting the specificity in action of drug UHAKKM-7 against TopoIIβK_{HIV}.

Figure 3.10: Analysis of serine phosphorylation by Immune precipitation:

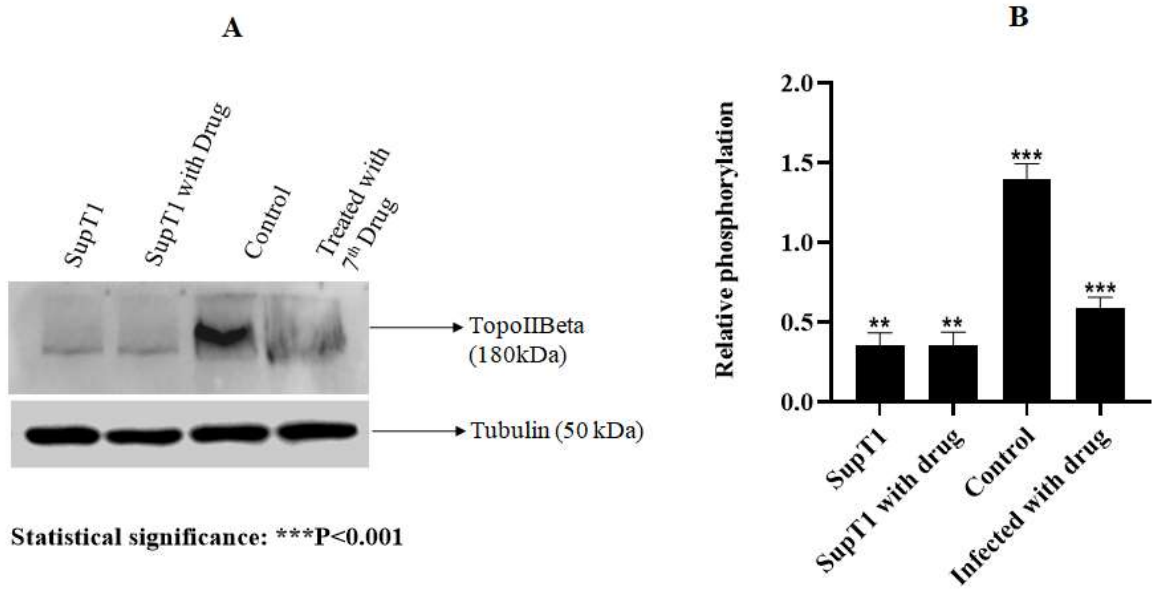


Fig. 3.10: Immunprecipitation of Topo II β was done with normal and infected SupT1 cell lysates which were treated with drug; further samples were analyzed by western blot with Biotin linked Phosphoserine Ab (Pri.Ab) and Streptavadin HRP Ab (Sec.Ab). Western blot analysis (Fig. 3.10 A) shows the Phosphorylation of Topo II beta at serine is enhanced upon HIV-1 infection in Control sample (***P<0.001) (Lane-3) and significantly inhibited Topo II beta phosphorylation at serine in test sample (***P<0.001) (Lane-4) which is treated with active drug UHAKKM-7 and Densitometry analysis was done by Image Software (Fig. 3.10 B). Thus results confirm specific action of drug UHAKKM-7 against Topo II β associated kinase activity.

Dose-dependent Inhibition of Serine Phosphorylation:

Infected SupT1 cells were pelleted and lysed after 5 hours of post infection with viral strain HIV-$1_{93IN101}$ at various concentrations of active drugs (UHAKKM-7, 8 and 9), Infected sample (control) and drug treated (test) samples were immune precipitated with Topo II βand serine phosphorylation studies were carried out with specific antibodies. Western blot analysis (Fig. 3.11) confirms the significant inhibition of Topo II beta phosphorylation at serine in test samples when compared with the control sample. Fig. 3.11 A clearly indicates the dose dependent inhibition of serine phosphorylation by active drug compounds UHAKKM-7, 8 and 9. Densitometry analysis by Image J Software in Fig. 3.11 B clearly indicates the phosphorylation inhibition at various concentrations (5, 10, 20 and 50nM) of active drug compounds. The results confirm residue specific action of active drug compounds against Topo II β associated kinase activity. Among the drugs, UHAKKM-8 showed highest inhibition followed by UHAKKM-7 and UHAKKM-9.

Figure 3.11: Dose-dependent Inhibition of Serine Phosphorylation

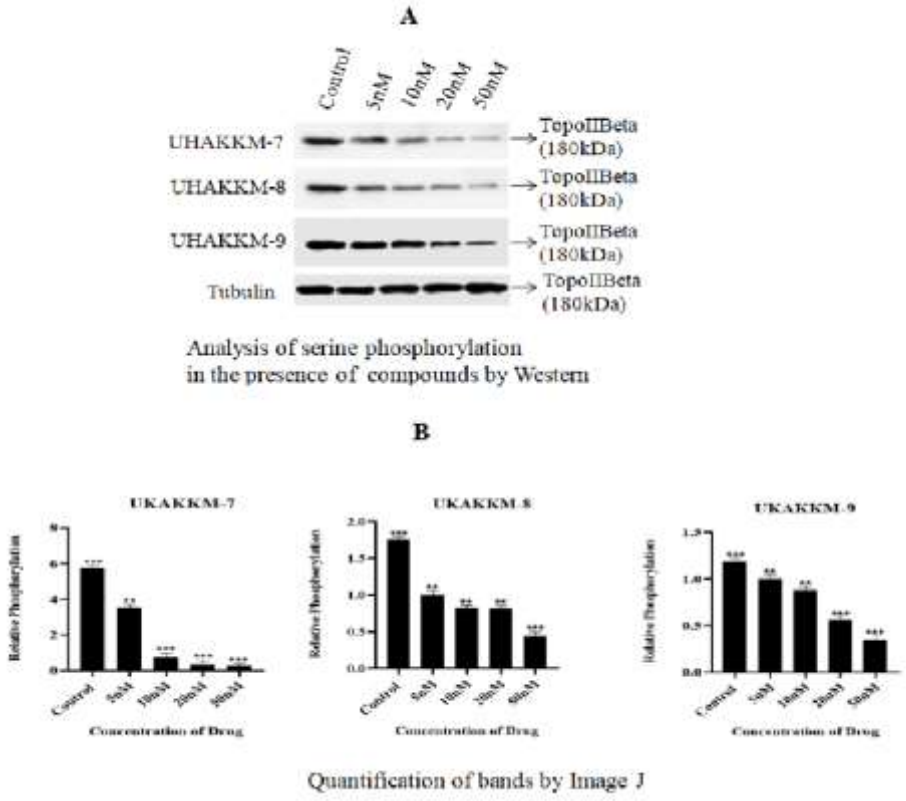


Fig 3.11: Control and test samples were Immunprecipitated with Topo II β and further samples were analyzed by western blot with Biotin linked Phosphoserine Ab (Pri.Ab) and Streptavadin HRP Ab (Sec.Ab). Western blot analysis (Fig. 3.11) confirms the significant inhibition of Topo II beta phosphorylation at serine in test samples when compared with the control sample (***P<0.001). Fig. 3.11 A clearly indicates the dose dependent inhibition of serine phosphorylation by active drug compounds UHAKKM-7, 8 and 9. Densitometry analysis by Image J Software in Fig. 3.11 B clearly indicates the phosphorylation inhibition at various concentrations (5, 10, 20 and 50nM) of active drug compounds (***P<0.001). Thus results confirm specific action of active drug compounds against Topo II β associated kinase activity. Among the drugs, UHAKKM-8 showed highest inhibition followed by UHAKKM-7 and UHAKKM-9.

Analysis of Proviral DNA Synthesis:

To study the extent of proviral DNA analysis promoted in the presence of compounds studied, the results in Fig 3.12 show that low kinase inhibitory active compounds UHAKKM-1, UHAKKM-2, and UHAKKM-3 exhibited no notable inhibition of proviral DNA synthesis at gag region. For further analysis, infection of viral strain ($HIV\text{-}1_{93IN101}$) to SupT1 cells in the presence of high kinase inhibitory active compounds (UHAKKM-7, UHAKKM-8 and UHAKKM-9) at two different concentrations 20nM and 50nM, after 5 hours of infection gag synthesis was analyzed using PCR. The result in Fig. 3.12 shows significant dose-dependent inhibition of proviral DNA synthesis by compounds UHAKKM-7, UHAKKM-8 and UHAKKM-9. Among the compounds, UHAKKM-7 and UHAKKM-8 exhibits higher activity than compound UHAKKM-9, which compares their activity against Topo II beta phosphorylating kinase.

Figure 3.12: Analysis of Proviral DNA Synthesis:

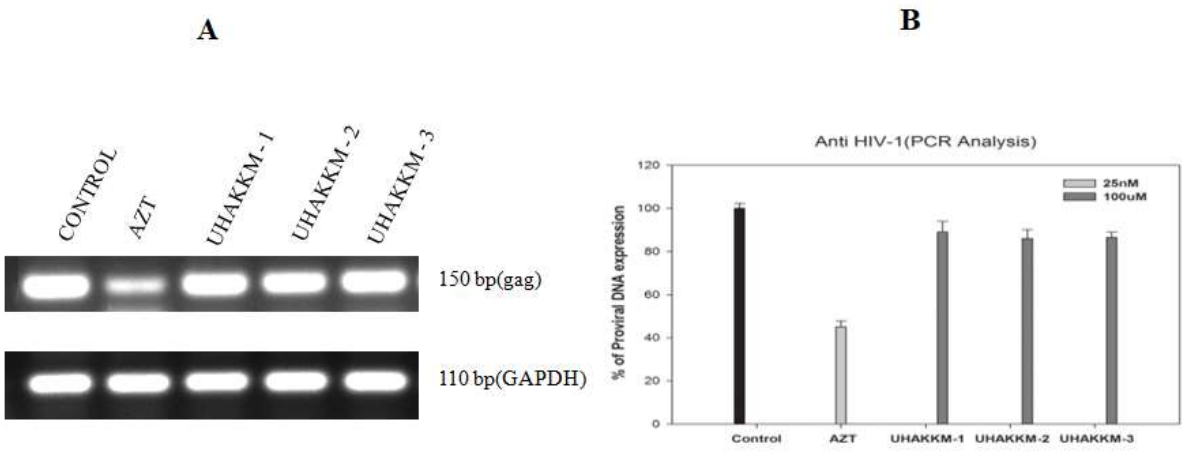


Fig 3.12: Analysis of Proviral DNA Synthesis: Above proviral DNA analysis shows the effect of drugs on the gag gene expression of HIV-1. Infected sample is used as control and infected sample in the presence of drug is test sample. AZT was used as positive control and GAPDH was used as PCR loading control. A) Anti HIV-1 activity of drug compounds UHAKKM-1, UHAKKM-2, and UHAKKM-3 (less active) are not showing affect on the amplification of gag region using sk38/39 primers even though the compounds are showing kinase activity. B) Densitometric analysis of gag gene expression deduced by image J software.

Figure 3.13: Proviral DNA Analysis of Active Compounds

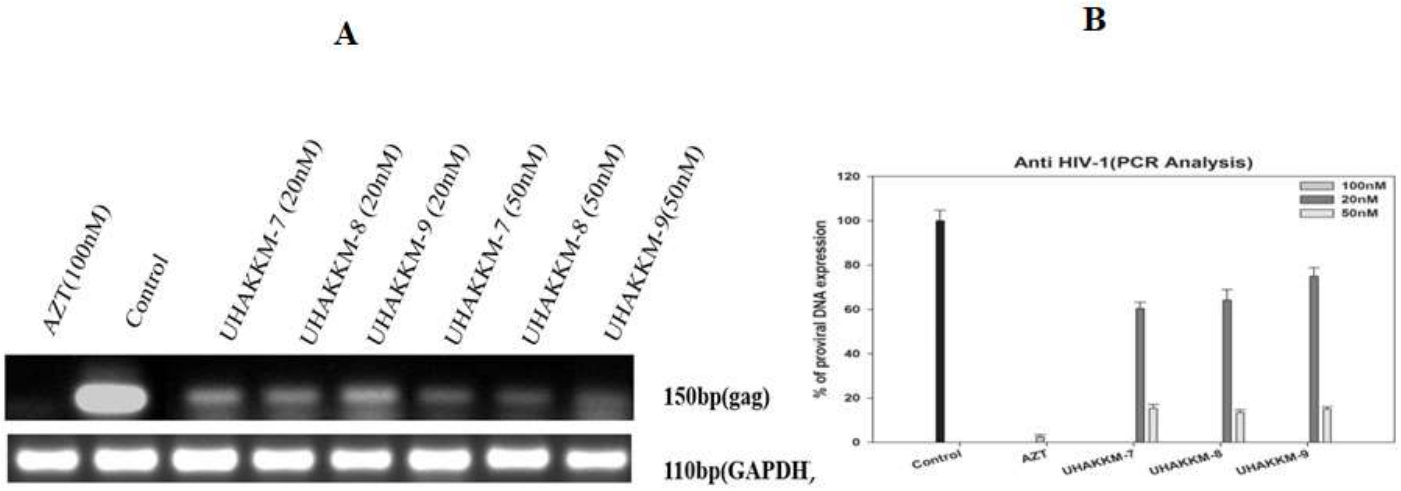


Fig. 3.13 Anti-HIV-1 Activity (Proviral DNA Analysis): HIV-1 acute infection conducted in the presence of 20 and 50 nM concentration and checked the proviral DNA amplification with specific primers sk38/39. Infected sample is used as control and infected sample in the presence of drug is test sample, AZT was used as positive control (25nM) and GAPDH was used as PCR loading control. A) Anti HIV-1 activity results shows significant dose-dependent inhibition of proviral DNA synthesis by compounds UHAKKM-7, UHAKKM-8 and UHAKKM-9. Among the compounds, UHAKKM-7 and UHAKKM-8 exhibits higher activity than compound UHAKKM-9, which compares their activity against Topo II beta phosphorylating kinase. B) Densitometric analysis of gag gene expression deduced by image J software.

Analysis of Proviral DNA Synthesis Active Compounds at IC_{50} Concentration:

Acute infection of HIV-1 was carried out in the presence of active compounds (UHAKKM-7, 8 and 9) at IC_{50} concentration and after 5 hours of post infection, amplification of proviral DNA was analyzed with sk38/39 primers which are specific to HIV-1 gag region by using PCR. Each sample was run in duplicates and experiment was repeated twice The results in Fig 3.14 confirms that these compounds are active and UHAKKM-7 exhibits highest activity.

Figure 3.14: Proviral DNA Analysis of Active Compounds (IC_{50} Concentration):

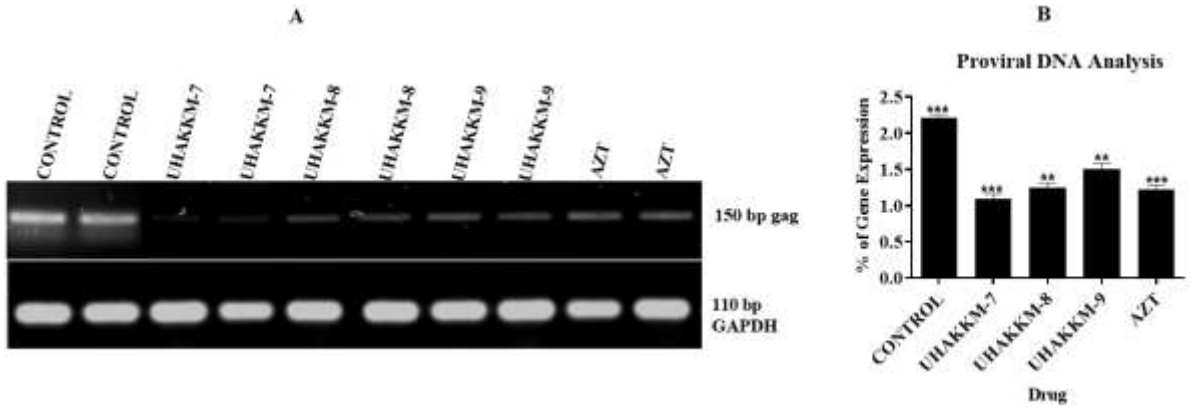


Fig 3.14: HIV-1 acute infection conducted in the presence of IC_{50} concentration and checked the proviral DNA amplification with specific primers sk38/39. Infected sample is used as control and infected sample in the presence of drug is test sample, AZT was used as positive control (25nM) and GAPDH (***P<0.001) was used as PCR loading control. A) Anti HIV-1 activity results shows significant inhibition of proviral DNA synthesis by compounds UHAKKM-7, UHAKKM-8 and UHAKKM-9 at IC_{50} concentration. Among the compounds, UHAKKM-7 (***P<0.001) exhibits higher activity than compounds UHAKKM-8 and UHAKKM-9 (**P<0.001), which compares their activity against Topo II beta phosphorylating kinase. B) Densitometric analysis of gag gene expression deduced by image J software.

Discussion:

During HIV-1 replication and propagation,Virus activates many host proteins by regulating by various post translational modification. Phosphorylation of virus-associated kinases are one the crucial steps in protein activation and regulation. In the earlier studies, it was shown that a series of pyridine dicoumarol derivatives were having potential anti-HIV-1 activity (62). These compounds specifically target the novel Topo IIβK_{HIV} that was shown to be responsible for phosphorylation of Topo II β (68) necessary for replication of HIV-1.

In this study, we have purified a 72 kDa protein from the viral lysates and showed a concentration dependent phosphorylation of Topo II β at serine residue by Topo IIβK_{HIV}. Coumarin derivatives were analysed for cytotoxicity, inhibition of Topo II$\beta K_{HIV,}$ and anti-HIV-1 activity. These compounds exhibited no significant toxicity below 10 micro molar concentration. Active compounds have shown the kinase inhibitory activity at pico molar concentration. Analysis of Anti-HIV-1 activity reveled that UHAKKM-1 to 6 exhibited low activity of anti-HIV-1 even at higher concentrations than 100µM. However, water soluble disodium pyridine dicoumarate molecules UHAKKM-7 to 9 have shown high inhibition at low concentration (IC_{50}<30nM). Results showed that among all the pyridine dicoumarol derivatives, UHAKKM-7 showed anti-viral activity with an IC_{50} of 15 nM, which was reported to be in the range of widely and clinically used, Azidothymidine (AZT) that has an IC_{50} of 25nM. It was already reported that Topo IIβ phosphorylation is enhanced during the HIV-1 infection which can be a new target for inhibitors to block HIV-1 replication. Based on the earlier reports and above results, we have screened these compounds for inhibitory activity of serine phosphorylation. Initially we have carried out the phosphorylation studies in crud extracts in the presence high active durg compound (UHAKKM-7) and later confirmed by immune precipitation studies. These results

confirmed that UHAKKM-7 significantly inhibits TopoIIβK_{HIV}, thus affecting Topo II beta phosphorylation at serine in infected cells, while no distinct change was detected in uninfected cells suggesting the specificity of action of the compound against Topo IIβK_{HIV}. Further we have performed dose-dependent studies with active anti HIV-1 molecules (UHAKKM-7, 8 and 9) for the inhibition of Topo II beta phosphorylation at serine residue, results confirmed that the Topo II β phosphorylation is inhibited in a dose-dependent mode at serine residue. In other set of PCR experiments, compounds UHAKKM-1, 2, 3, 4, 5and 6 have not shown significant proviral DNA synthesis, while the compounds UHAKKM-7, 8 and 9 have shown significance inhibition of proviral DNA synthesis in a dose dependent studies and confirmed at IC_{50} concentration. These results unequivocally confirm that the inhibition of Topo IIβK_{HIV} by UHAKKM-7, 8 and 9 is specific in infected cells and affects HIV-1 replication and proviral DNA synthesis. These results further indicated that Topo II β phosphorylation is crucial for proviral DNA synthesis. Hence, disodium pyridine dicoumarate salt compounds UHAKKM-7 to UHAKKM-9, acquired the properties to reach the intracellular targets and the placement of negative charges on enolic oxygen resulting from formation of salts enhances the activity. Thus pyridine ring negatively charged enolic oxygen and flexibility plays a crucial role in bioactivity of dicoumarol derivatives.

Conclusion:

In conclusion, a structure activity based evaluation of substituted pyridine derivatives lead to drug development against Topo IIβK_{HIV} catalyzed Topo II beta phosphorylation along with anti-HIV-1 activity. Among the dicoumarol studied, pyridine dicoumarol derivatives (UHAKKM-7, 8, and 9) showed significant inhibition of proviral DNA formation suggesting that the involvement of Topo IIβK_{HIV} catalysed Topo IIβ phosphorylation in proviral DNA synthesis.

CHAPTER-IV

Analysis of activity of Bis-chalcone derivatives against Topo II β kinase and HIV-1 replication.

Introduction:

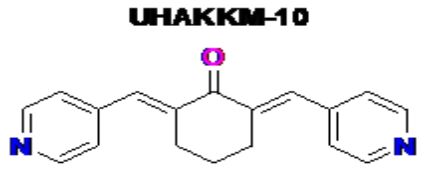
Bis-chalcones are Heterocyclic compounds present abundantly in nature and are very significant to life because their structural subunits are present in many natural products such as vegetables, fruits, vitamins, hormones, antibiotics etc. bis-chalcones are having great attention in the design of biologically active compounds (147).The natural and synthetic compounds have shown many interesting biological activities and clinically potential therapeutic agents to various diseases. Bis-chalcones show a wide-range of pharmaceutical activities viz. anti-bacterial, anti-inflammatory, anti-tuberculosis, anti-viral and anti-cancer properties (77). The pharmacological effects on cancerand suppression of NF-κB-mediated inflammation have been proved that chalcone derivatives are efficient bio-active compounds in recent reports (7), (138).

AChalcone derivative Trans-1,3-Di-Phenyl-2,3-Epoxy-Propane-1-one (DPEP) exerts the anti-inflammatory action by lowering the cytokine production, likeIL-1β, IL-6 and TNF-α. Thus the medicinal activity of DPEP was attributed by blocking NFκB activity and MAPK phosphorylation which play crucial role in the initiation and development of inflammatory responses (67). In another study, it was reported that concentration-dependent 2′,5′-Di-Hydroxy-2-Furfuryl-Chalcone (DHFC) have shown respiratory burst in neutrophils stimulated by formyl-L-methionyl-L-leucyl-phenylalanine (fMLP) also exhibit inhibition of PI3K activation and moderately suppress the phosphorylation of serine/threonine protein kinase i.e. Protein Kinase B (Akt), which is an effector of PI3K during the inhibition of inflammatory response (126). A novel flavonoid chalcone showed anti-HIV activity against H9 lymphocytic cell infection (134). Xanthohumol is a natural chalcone possesses properties of apoptotic activity which is a potential target in improving inflammation in the dendritic cells (136) and Xanthohumol have shown anti-HIV-1 activity in C8166 lymphocytes by inhibiting cytopathic effects (127).

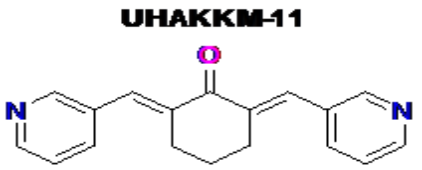
During HIV-1 infection large amount of TNF-α is released into the serum as a response to inflammation and disease progression (97).It is well known that TNF-α can stimulate the transcription componentsAP-1and NF-κB, which in turn mediate the downstream expression of TNF-α-responsive genes. AP-1 and NF-κB binds to specific sequence of DNA in Long Terminal Repeats (LTR) region which enhances the HIV-1 replication (102). Bis-chalcones have been proved in having the anti-HIV-1 activity (147).

In thisstudy we have analysed the molecular activities of Bis-chalcone derivatives on the early replication of virus namely Topo II β phosphorylation and inhibition of viral replication.

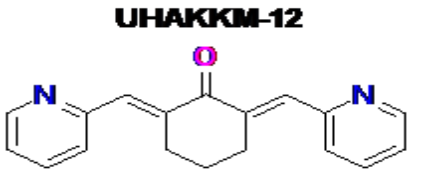
Figure 4.1: Structures of Molecules (Bis-Chalcone Derivatives)



(2*E*,6*E*)-2,6-bis(pyridin-4-ylmethylene)cyclohexanone



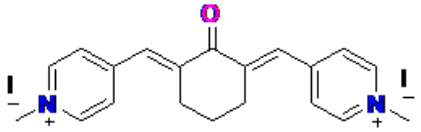
(2*E*,6*E*)-2,6-bis(pyridin-3-ylmethylene)cyclohexanone



(2*E*,6*E*)-2,6-bis(pyridin-2-ylmethylene)cyclohexanone

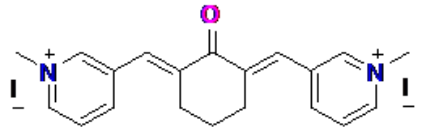
Molecules are synthesized as per Kurumurthy et al (2017), further biological characterization for antiviral and molecular action was carried out

UHAKKM-13



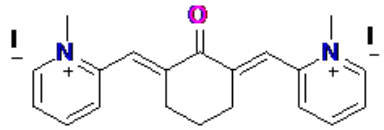
4,4'-(1*E*,1'*E*)-(2-oxocyclohexane-1,3-diylidene)bis(methan-1-yl-1-ylidene)bis(1-methylpyridinium) iodide

UHAKKM-14



3,3'-(1*E*,1'*E*)-(2-oxocyclohexane-1,3-diylidene)bis(methan-1-yl-1-ylidene)bis(1-methylpyridinium) iodide

UHAKKM-15



2,2'-(1*E*,1'*E*)-(2-oxocyclohexane-1,3-diylidene)bis(methan-1-yl-1-ylidene)bis(1-methylpyridinium) iodide

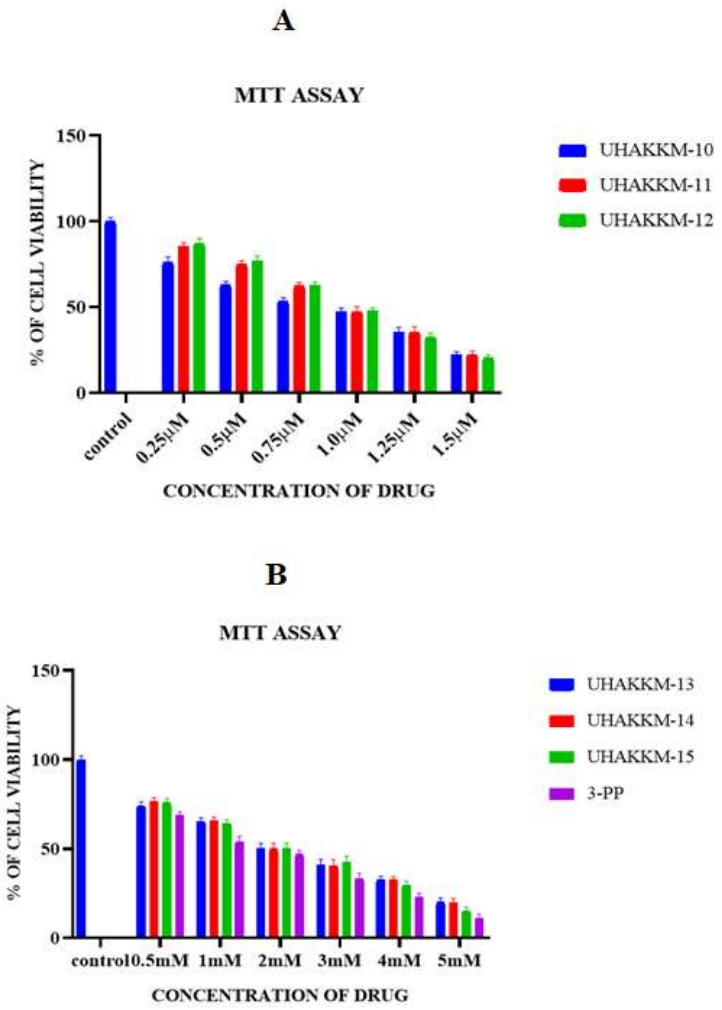
Molecules are synthesized as per Kurumurthy et al (2017), further biological characterization for antiviral and molecular action was carried out

Results:

MTT Assay (Cell Viability Assay):

Cytotoxicity assay was performed in supT1 cells for the Bis-chalcone derivatives with different concentration. Approximately 0.02 X 10^6cells were seeded in 100µL of complete media per each well in 96 well plate and kept for overnight incubation, on the next day drugs of different concentration was added and kept at incubation in 5% CO_2 incubator for 24 hours, next day after incubation 10uL MTT was dispensed to 96 well plate and again incubated for 4 hours, later 100uL of Dimethyl sulphoxide (DMSO) was added to all the 96 well plate, gently agitated and kept in darkness for half-an-hour at RT. At 570 nm, absorbance was measured in a micro titer plate reader, all the samples were run in triplicate and each experiment was repeated thrice. The result shows that all the molecules exhibited no significant cytotoxicity <10µM concentration, thus anit-HIV activity of molecules was analysed below 10µM concentration.

Figure 4.2: Cell Viability Assay (MTT Assay):



C

Drug	CC_{50}
UHAKKM-10	0.82± 0.044 uM
UHAKKM-11	0.96± 0.032 uM
UHAKKM-12	0.92± 0.056 uM
UHAKKM-13	2.02± 0.068 mM
UHAKKM-14	2.12± 0.086 mM
UHAKKM-15	2.04± 0.042 mM
3-Phenyl Pyridine(Control)	1.50 ± 0.029mM

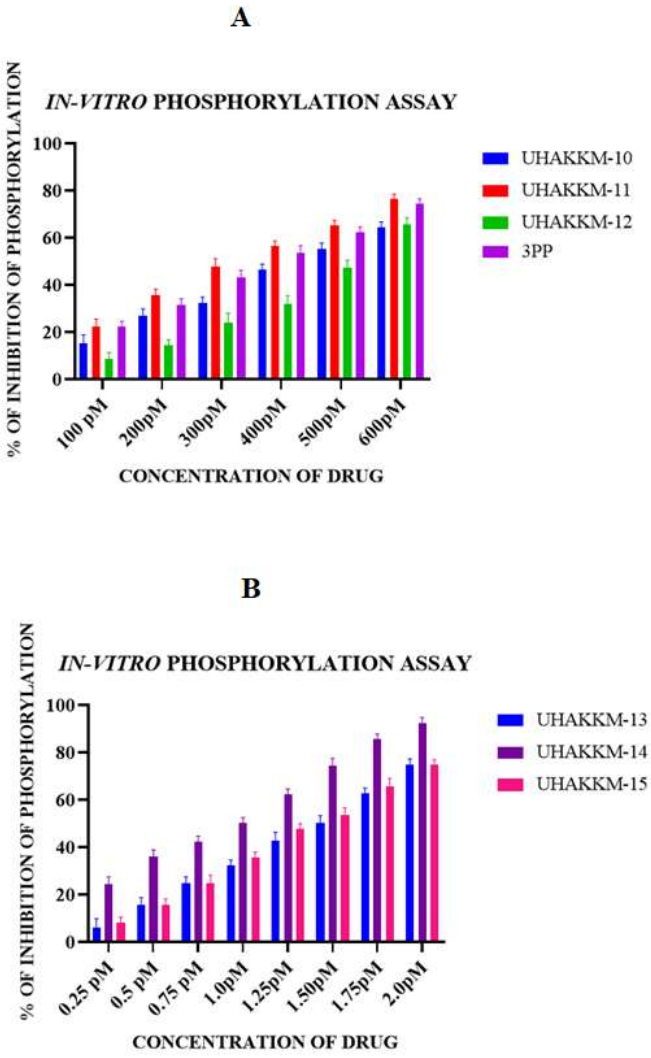
Fig.4.2: Cytotoxicity Assay was performed in supT1 cells for the bis-chalcone derivatives with different concentration as shown in the Fig.4.2 panel A and B graphs and the CC_{50} was indicated in the panel C table. In above results it was shown that all the drugs are showing above 60% viability at 0.75 µM (UHAKKM-10, 11 and 12) and 2.0 µM (UHAKKM-13, 14 and 15) concentrations, at 0.5 µM (UHAKKM-10, 11 and 12) and 1 Mm (UHAKKM-13, 14 and 15) concentrations no significant cytotoxicity was observed.

In-Vitro Phosphorylation Assay (Bis-chalcone derivatives):

Assay was performed by taking dephosphorylation Topo II β as substrate molecule in 1x kinase buffer containing reaction mixture of 5 µCi radiolabelled ATP [γ -^{32}P], 10 µl (100 µM ATP) of cold ATP and purified kinase in the presence of (Bis-chalcone derivatives) drugs. The reaction mixture was incubated at 37 °C for 30 min. Three micro liters (3 ng) of mouse anti-human Topo II β monoclonal antibody was mixed with reaction mixture and incubated for 1 hour at 37° C, after incubation 6% protein A/G agarose beads were mixed with lysates and incubated for 30 min with slow agitation at 37°C, centrifugation of the reaction tubes was done at 2000 rpm for 5min. The sedimented beads were washed with 1X TBS for twice and precipitated with 40 µl of 5% Tri-Chloro Acetic acid (TCA). 10 µl of precipitate was spotted on discs of Whatman paper and dried for overnight at 37° C. Next day ^{32}P was measured by liquid scintillation.

All the compounds have shown the inhibition of phosphorylation activity of TopoIIβK_{HIV}. Among the compounds UHAKKM-14 being highly active followed by UHAKKM-15, 13, 11, 10 and 12. Thus results in Fig 4.3 confirm that the compounds possess significant activity against TopoIIβK_{HIV}.

Figure 4.3: *In-Vitro* Phosphorylation Assay (Bis-chalcone derivatives)



C

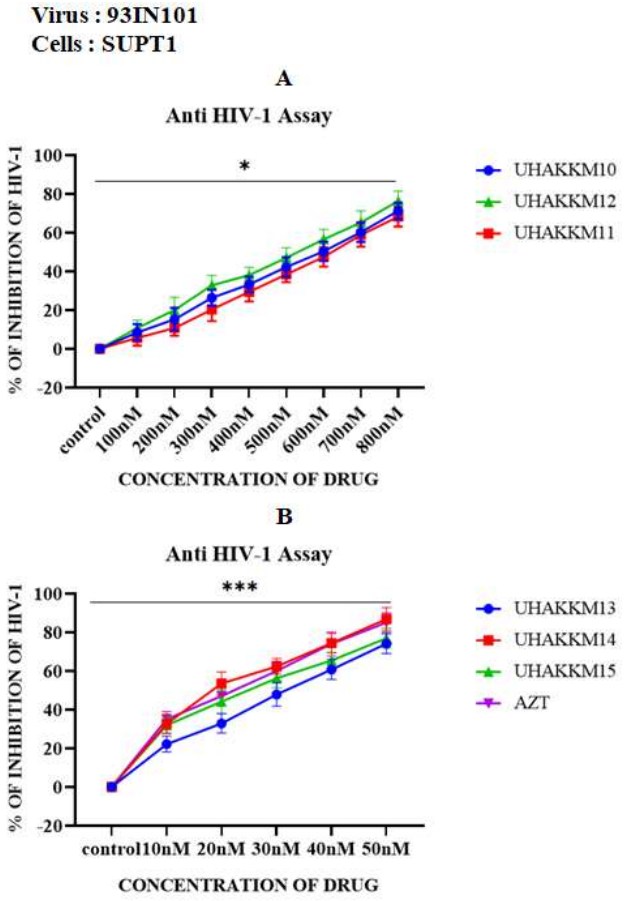
Molecules	IC-50 (pM Concentration)
UHAKKM-10	$4.4\pm0.33x10^2$
UHAKKM-11	$3.2\pm0.22x10^2$
UHAKKM-12	$5.5\pm0.26x10^2$
UHAKKM-13	1.5±0.29
UHAKKM-14	1.0±0.24
UHAKKM-15	1.4±0.56
3-Phenyl Pyridine (Control)	$3.80\pm 0.01X10^2$

Fig. 4.3: The results show the phosphorylation inhibition of kinase by different drugs in the presence of radiolabel γ -^{32}[P] ATP. Panal A and B graphs depict the percentage of drug inhibition plotted against the concentration of drugs and panal C table indicating the IC_{50} concentration of Bis-chalcones derivatives. Thus results confirm that the compounds possess significant inhibitory activity against TopoIIβKHIV. Among the compounds analysed, UHAKKM-14 being highly active followed by UHAKKM-15, 13, 12, 11 and 10 (***P<0.001).

Anti-Viral Assay (p24 Assay):

SupT1 cells were infected with HIV-I93IN101 for 5 hours in the presence of various concentrations of Bis-chalcones derivatives. After 5 hours the samples were pelleted, cleaned, and resuspended in culture plate containing complete media (RPMI with 10% FBS), further incubation was done for 96 hours, after which the viral titer was estimated by p24 ELISA method. HIV-1 replicated in the absence of drug was considered zero percent inhibition, based on p24 in control infection percent inhibition was calculated at each drug concentrations (Fig 4.4 Panel A and B). Based on the virus inhibition in the presence of drug, IC_{50} was calculated and included in the Fig 4.4 Panel C. All the drug compounds were used in nM concentration and AZT was employed as positive control. Among compounds UHAKKM-13, 14, and 15 showed significant activities.

Figure 4.4: Anti-Viral Assay (p24 Assay):



C

Drug	IC50
UHAKKM-10	0.58 ± 0.02 uM
UHAKKM-11	0.62 ± 0.04 uM
UHAKKM-12	0.56 ± 0.06 uM
UHAKKM-13	35.56± 0.56 nM
UHAKKM-14	23.42± 0.86 nM
UHAKKM-15	32.65± 0.42 nM
AZT	25.00± 0.58 nM

Fig.4.4: The result in Fig 4.4 shows that the bis-chalcones significantly inhibits HIV-1 replication in a dose-dependent manner. In panel A and B graphs representing the inhibition of virus in a dose-dependent manner at various concentration of drug compounds and in panel C table representing the IC_{50} of all bis-chalcones derivatives. Among compounds, UHAKKM-13, 14, and 15 showed significant activities, while compounds UHAKKM-10, 11 and 12 exhibited anti-viral activities at µM concentration (**P<0.001). IC_{50} of all the active drug compounds 13, 14, and 15 have shown anti-viral activity at nM concentration (***P<0.001).

Effect of Bis-chalocones on Proviral DNA synthesis:

SupT1 cells were given infection with viral strain (HIV-$1_{93IN101}$) under the presence of active Bis-chalcone derivatives (UHAKKM-13, UHAKKM-14, and UHAKKM-15) at IC_{50} concentration. After 5 hours of incubation at 37°C in 5% CO_2 incubator, samples were collected and Genomic DNA was separated by standard phenol/chloroform method.1 µg of DNA was used for PCR amplification with specific primers sk38/39 to the gag region of HIV-1. Anti HIV-1 activity results in Fig 4.5 shows a significant inhibition of proviral DNA synthesis by compounds UHAKKM-13, UHAKKM-14 and UHAKKM-15. Among the compounds, UHAKKM-14 exhibits higher activity than compounds UHAKKM-13 and UHAKKM-15, which correlates well with their inhibitory activity against the kinase, Topo IIβK_{HIV}.

Figure 4.5: Proviral DNA synthesis:

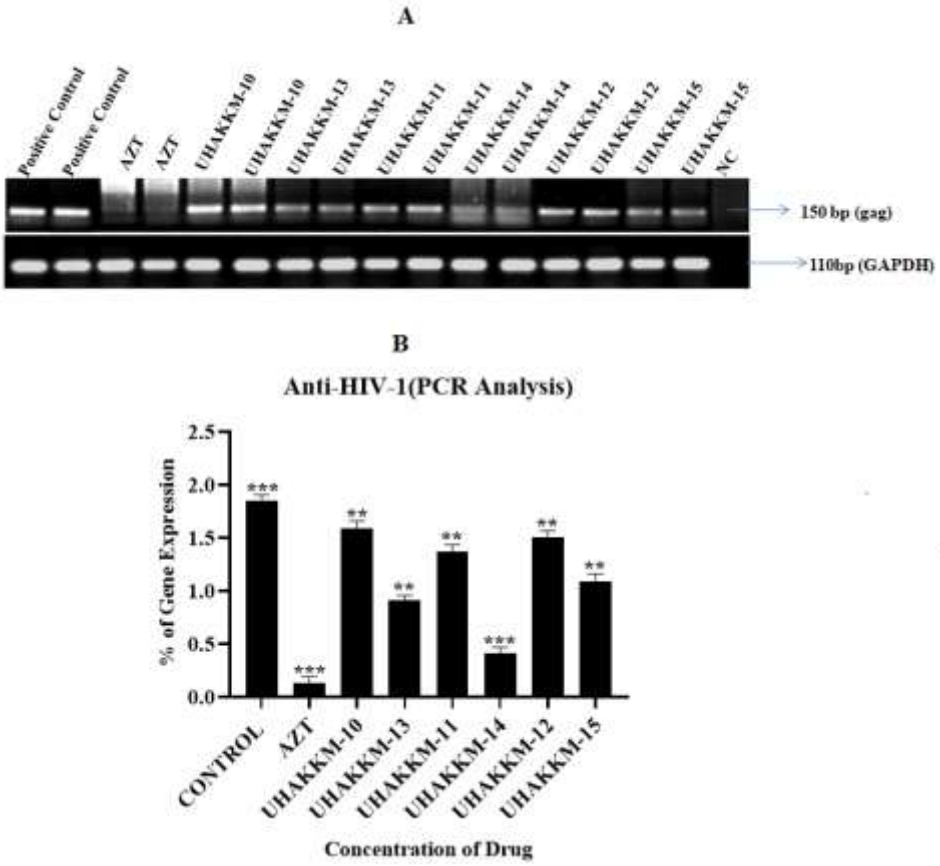


Fig.4.5: HIV-1 acute infection conducted in the presence of Bis-chalcone derivatives at IC_{50}concentration and checked the proviral DNA amplification with specific primers sk38/39. Samples were done in duplicates, infected sample is used as control and infected sample in the presence of drug is test sample, AZT was used as positive control (25nM) and GAPDH was used as PCR loading control (***P<0.001). Panel A shows the decrease in the amplification of gag gene at IC50 concentration of drugs when compared with Positive control (Infected Sup T1 without drug) and AZT (25nM). Among the compounds UHAKKM-14 have shown the highest activity (***P<0.001). Panel B indicating the densitometric analysis of gag gene expression deduced by image J software.

Dose Dependent Inhibition of Serine Phosphorylation:

Infected SupT1 cells were pelleted and lysed after5 hours of post infection with viral strain (HIV-$_{93IN101}$) at of various concentrations of active Bis-chalcone derivatives (UHAKKM-13, 14 and 15), Infected sample (control) and drug treated (test) samples were immunoprecipitated with Topo II β and serine phosphorylation studies were carried out with specific antibodies; Biotin linked Phosphoserine Ab (Pri.Ab) and Streptavadin HRP Ab (Sec.Ab) by western blot analysis. Thus results confirm specific action of active drug compounds against Topo II β associated kinase activity. Among the drugs, UHAKKM-15 showed highest inhibition followed by UHAKKM-14 and UHAKKM-13.

Figure 4.6: Dose Dependent Inhibition of Serine Phosphorylation

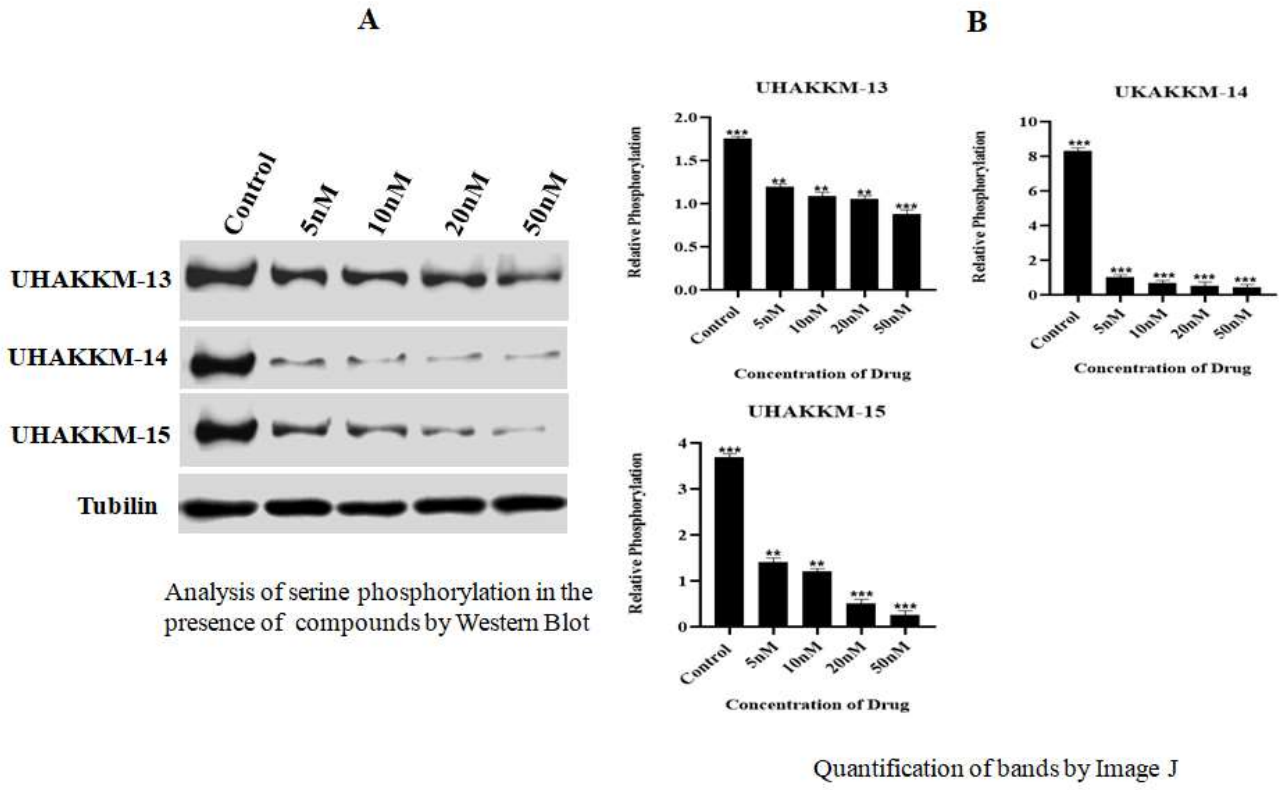


Fig.4.6: Control and test samples were Immunprecipitated with Topo II β and further samples were analyzed by western blot with Biotin linked Phosphoserine Ab (Pri.Ab) and Streptavadin HRP Ab (Sec.Ab). Results in Fig.4.6 clearly indicate the dose-dependent inhibition of serine phosphorylation by active drug compounds UHAKKM-13, 14 and 15, among the compounds UHAKKM-14 showed the highest activity (***P<0.001). In panel A western blot analysis confirms the significant dose dependent inhibition of Topo II beta phosphorylation at serine in test samples when compared with the control sample (***P<0.001) and panel B indicating the densitometric quantification of bands by image J software as presented in bar diagram. Indicates the phosphorylation inhibition at various concentrations (5, 10, 20 and 50nM) of active drug compounds.

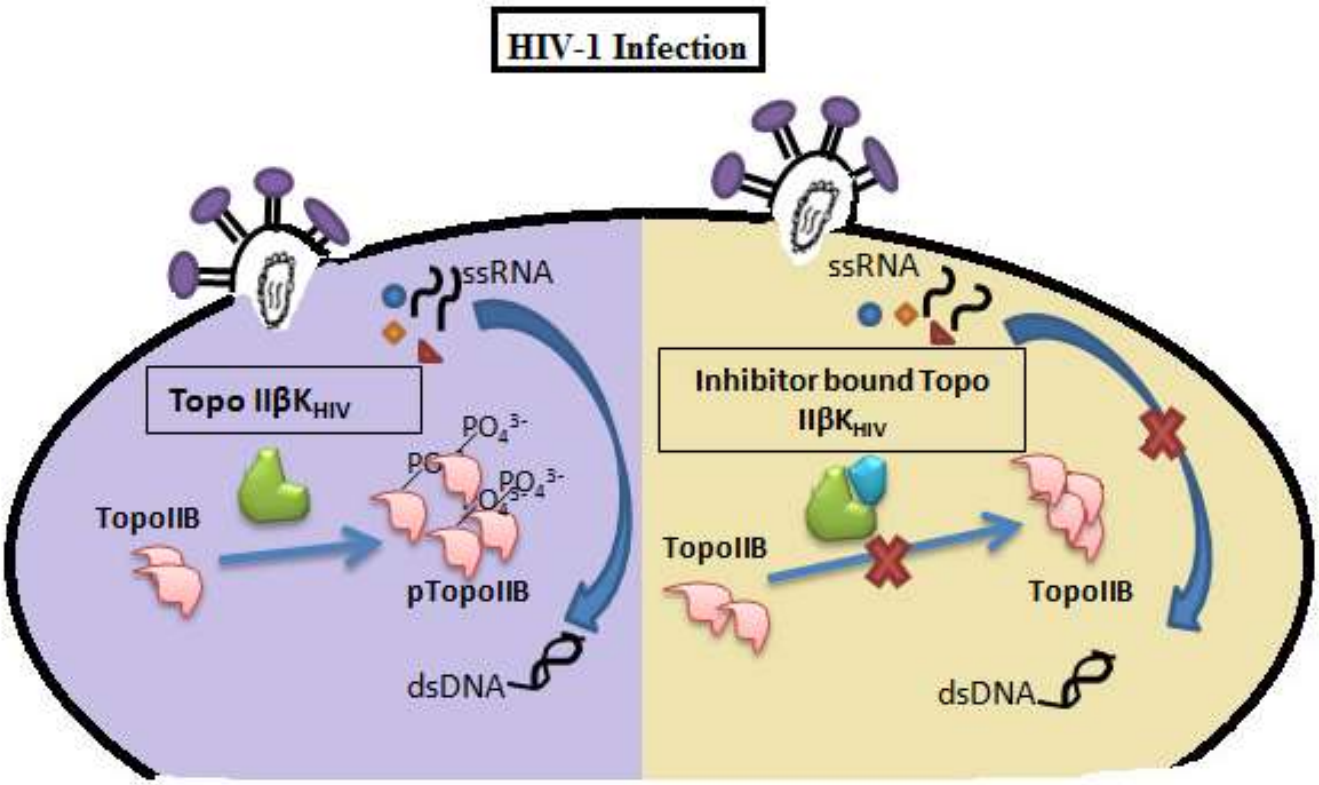
Discussion:

In the present study, we have tried to understand how these inhibitors affect early replication of virus by specifically targeting the novel Topo IIβK_{HIV}. We have analyzed the cytotoxicity of the compounds by cell viability assay; the results confirmed that compounds exhibited no significant toxicity below 10 micro molar concentrations of compounds. Action of compounds on Topo IIβK_{HIV-1} catalyzed phosphorylation of Topo II beta; the results showed that all compounds inhibit the activity assay to the extent of picomolar concentrations. Analysis of Anti-HIV-1 activity of the molecules revealed that UHAKKM-10 to 12 exhibits low anti-HIV-1 activity even at concentrations higher than 100µM, while water soluble active pyridine bis-chalcones molecules UHAKKM-13 to 15 have shown high inhibition. Amongst compounds, UHAKKM-15 exhibits highest anti-HIV-1 activity withIC_{50} below 20.65nM, which in close comparison with the activity of on par with the widely clinically used Azidothymidine (AZT) (IC_{50} of 25nM). Further characterization of compounds on specificity of the Topo IIβ phosphorylation at Serine amino acid by anti-phopho serine antibody showed that anti HIV-1 molecules UHAKKM-13, 14 and 15 significantly inhibited the Topo II beta phosphorylation at serine residue in a dose-dependent manner, wherein UHAKKM-15 showed highest inhibition followed by UHAKKM-14 and UHAKKM-13. Molecular analysis of action of Bis-chalcone derivatives UHAKKM-10 to 15 on HIV-1 early replication intermediate, proviral DNA synthesis showed those low Topo IIβKHIV inhibitory active molecules UHAKKM-10, 11 and 12 have shown lower inhibition of proviral DNA synthesis, while higher inhibitory UHAKKM-13, 14 and 15 showed highest activity against proviral DNA synthesis. In, summary, UHAKKM-13, 14 and 15 showed highest inhibition of TopoIIβK_{HIV} with specific action on phosphorylation of serine residue in Topo IIβ with anti-HIV-1 activity affecting proviral DNA synthesis.

Conclusion:

Molecular Analysis of active compounds, Bis-chalcone derivatives (UHAKKM-13, 14, and 15) has inhibited proviral DNA formation suggesting that TopoIIβK_{HIV} catalysed phosphorylation of TopoIIβ is required to promote the HIV-1 replication.

Model of Topo II β Kinase Inhibitors targeting the HIV-1 associated Topo II β Kinase:



(63)

CHAPTER-V

Characterization and molecular action of Topo II β kinase during early replication of HIV-1

Introduction

Due to the setbacks of current antiretroviral therapy, development of new antiretroviral drugs to eradicate off target effects, drug resistance and viral reservoirs is much needed (44, 86). A major hurdle in curing HIV-1 infection is due to its incorporation into the host genome and becomes latent. So, we focus on approach of targeting cellular rather than viral proteins. Even though many host proteins assist at various stages of HIV-1 life cycle; Maraviroc, as a single targetingdrugis in use. It binds to the co-receptor CCR5 during entry of HIV-1,thereby preventing its docking to viral gp120 leading to the failure of membrane fusion for viral entry (25). Broad screening analyses of genome has revealed a large number of host components which might have a functional role in the viral replication and thus provides a possible therapeutic targets (14), (70), (146). There are successful anti-viral defense mechanisms in the host because it's past interactions with retroviruses and 8% of the human genome is from retroviral LTR sequence (6). But HIV-1 has developed the propensity to impede with various "accessory" proteins such as Viral infectivity factor (Vif) and Viral protein U (Vpu)) or by avoiding the host mechanism by its high mutation rate and became efficient in replicating in the antagonistic milieu of the host cell (83), (92). Development of molecules which can inhibit the interaction of host and viral proteins during the initial stages of replication is one of the sensing strategies that may help to control HIV-1 infection.

Actually, HIV-1 entry is ideal step for intervention due to its distinct interactions withthe host cell membrane that can be prevented by specific drugs without entering into the cells (90). This will not only block HIV-1 incorporation into the host genome but also prevents the development of viral latency reservoirs. Theoretically viral replication of HIV-1 can be impeded at every stage of its cycle viz., HIV-1 viral uncoating and process of cDNA synthesis in reverse transcription,

viral integration, DNA transcription, assembly of viral particles and budding process to release virus from the cell membrane where all these constitute rational stages to arrest the HIV-1 infection whereas Reverse transcription is currently being investigated here.

It occurs in nucleoprotein organization called reverse transcription complex (RTC), contains viral RNA, primer as tRNA and synthesized nascent cDNA, viral factors and host factors. Host constituents histone deacetylasecomplex HDAC1-SIN3A and members HDAC1, SAP18, SAP30, and SIN3Awere also involved and packed in the virion (117). HDAC1 involves in viral uncoating and cDNA synthesis during reverse transcription process (74). Gemin2 is essential for an initial process of negative strand synthesis of reverse transcription (strong stop) or viral cDNA integration in to the host genome, indicating its interaction with either RT or PICS (45, 131). It recruits RNA helicase A (DHX9)(108), (131), that also associates with the Survival Motor Neuron (SMN) complex (45), (131).A RNA binding nuclear protein, Human antigen R (HuR) has nucleo-cytoplasmic regulating properties (40), (131) which shows high affinity and selectivity for Adenylate-Uridylate-rich elements (AREs) (19), (91), (124). A-kinase anchor protein 1 (AKAP1) which interacts with both regulatory subunit of protein kinase A (PKA) (cAMP-dependent kinase) and Reverse Transcriptase (RT) in anchoring these proteins to different membranes in the cell (30), (131). Cyclin-dependent kinase (CDK) 2 regulates the RT by phosphorylating on threonine increasing its efficiency and stability leading to enhanced viral fitness (78). Similarly, another host protein HMGI(Y) involves in covalent strand transfer process during Reverse Transcription (RT) of HIV-1 (29), (116).

Previous studies indicated the Topo II role in the infection of HIV-1 (84, 87). During infection, there was an increase in the phosphorylation levels of Topo IIα and β (68), (84). The phosphorylation is done by a specific serine kinase available in the purified virion of HIV-1 and

both forms are connected with pre-integration complexes (PICs) (68, 69). Inhibition of Topo II using inhibitors and their down regualtion by siRNA impaired the replication of HIV-1 by impedingthe formation of PICs (12), (69) and Reverse Transcription (RT) (99).

HIV-1 Reverse Transcription:

HIV-1 reverse transcriptase is one of the key players in the mechanism of infection and pathogenicity. In a retrovirus, conversion of single-stranded viral genomic RNA into cDNA by an enzyme Reverse Transcriptase is known as Reverse Transcription. It takes place in the host cell cytoplasm by coordinating with many host proteins which helps in the viral infection and progression of HIV-1

Figure 5.1: HIV-1 Reverse Transcription

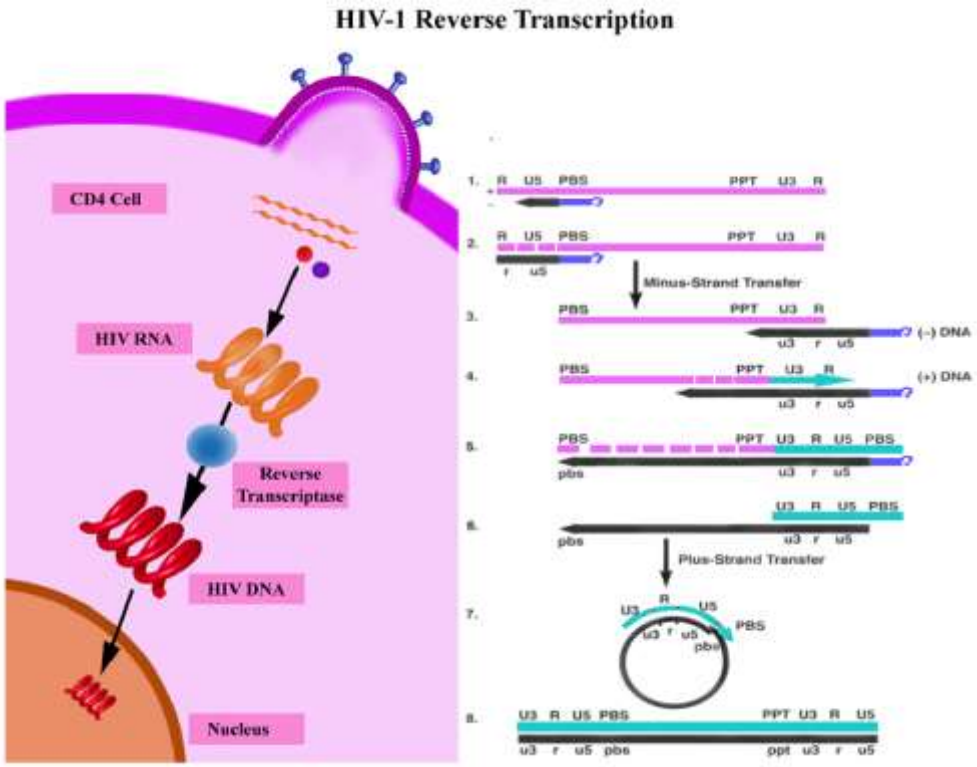


Fig.5.1: Above showing the Reverse Transcription process to convert viral RNA to cDNA in the presence of enzyme Reverse Transcriptase consists of Strong Stop (SS), First Strand Transfer (FST), Full Length Minus Strand (FLMS) synthesis and Second Strand Transfer (SST) to generate full length Plus Strand.

Results:

Analysis of Reverse Transcription (RT) intermediates:

Reverse Transcription (RT) involves a process of four intermediate steps i.e., SS, FST, SST and FLMS to convert viral RNA to cDNA by forming a Reverse Transcription Complex (RTC) in the cytoplasm. We have analyzed these RT events with specific primers, SupT1 cells were infected with HIV-I93IN101 for 1 hour, samples were collected after 1hour, cytosolic DNA was isolated and PCR analysis was done in the presence of SS, FST, SST and FLMS primers which are specific for RT intermediates.

Figure 5.2: Analysis of Reverse Transcription intermediates:

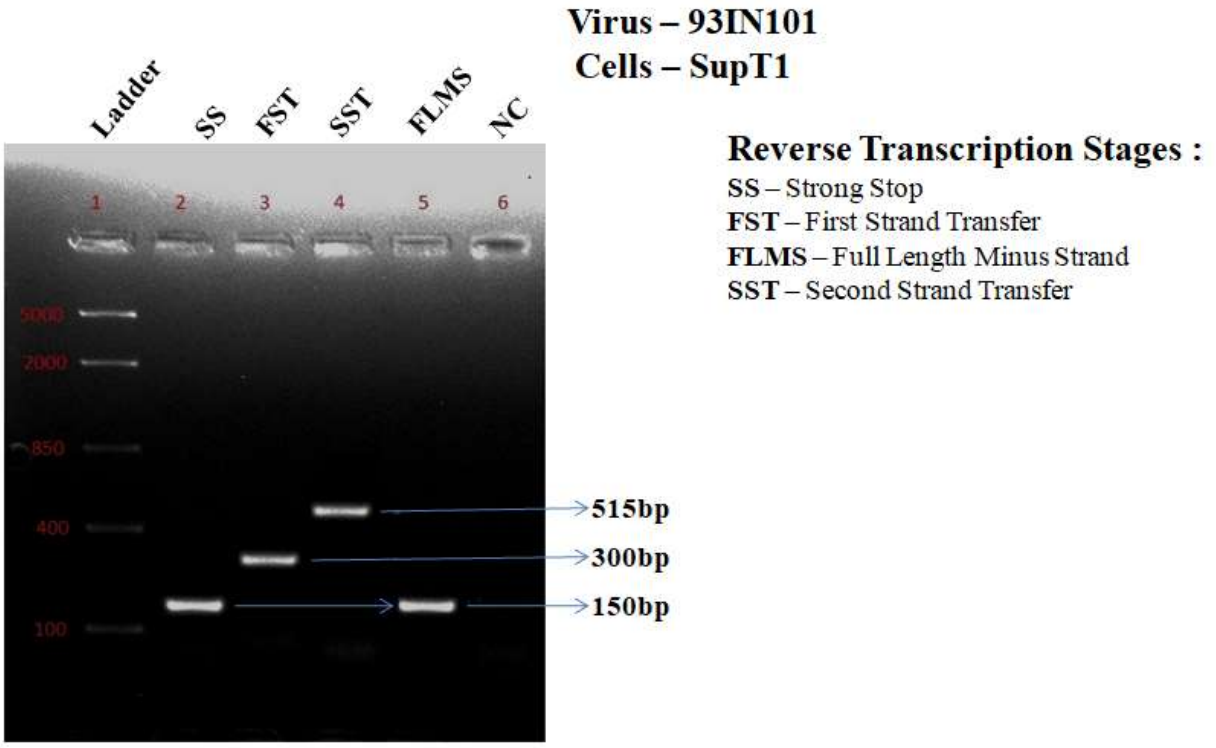


Fig. 5.2: PCR Experiment was done in control infection by infecting the SupT1 cells with HIV-1 93IN101 for 1 hour, cytosolic DNA was isolated and checked for the amplification of RT intermediates in the presence of specific Primers SS, FST, SST and FLMS. Above results confirmed that all the RT intermediates were amplified with control DNA samples in the presence of specific primers.

Inhibition of Reverse Transcription Events by synthesized HIV-1 TopoIIBeta Kinase Inhibitors:

SupT1 cells were infected with HIV-I93IN101 for 1 hour in the presence of active pyridine coumarin derivatives (UHAKKM-7, 8 and 9), at IC_{50} concentration. After 1hour samples were collected, Cytosolic DNA was isolated and PCR analysis was done in the presence of SS, FST, SST and FLMS primers which are specific for RT intermediates. Experiment was run thrice with a Statistical significance: ***P<0.001.

Figure 5.3: Analysis of Reverse Transcription (RT) intermediates (Pyridine Coumarin Derivatives):

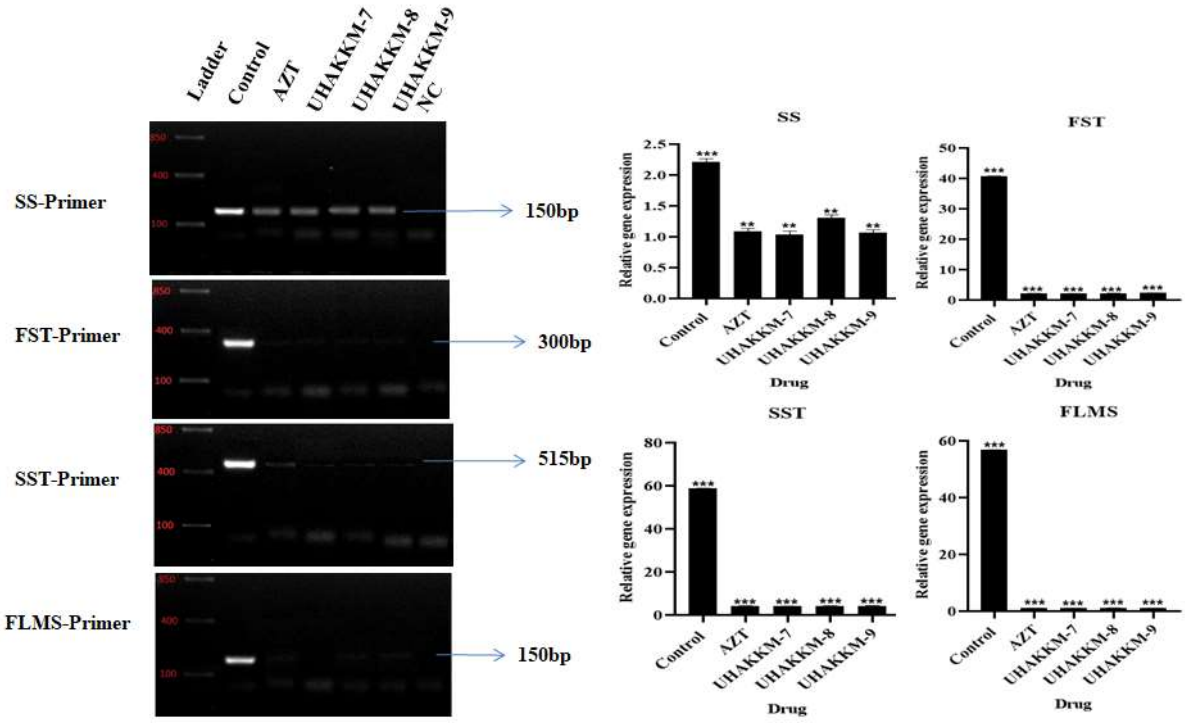


Fig.5.3: PCR Experiment was done by specific Primers SS, FST, SST and FLMS with cytosolic DNA isolated from infected SupT1 with HIV-1 93IN101as control where as infected SupT1in the presence of active pyridine coumarin derivatives (UHAKKM-7, 8 and 9) as test samples. AZT was used as Positive Control, without template as Negative Control (NC) and GAPDH was used as internal control. Above result shows the inhibition of FST, SST and FLMS intermediates completely at IC50 concentration of drug (***P<0.001) when compared with AZT (***P<0.001).

Inhibition of Reverse Transcription Events by synthesized HIV-1 TopoIIBeta Kinase Inhibitors:

SupT1 cells were infected with HIV-I93IN101 for 1 hour in the presence of active pyridine Bis-chalcone derivatives (UHAKKM-13, 14 and 15), at IC_{50} concentration. After 1hour samples were collected, Cytosolic DNA was isolated and PCR analysis was done in the presence of SS, FST, SST and FLMS primers which are specific for RT intermediates. Experiment was run thrice with a Statistical significance: ***P<0.001.

Figure 5.3: Analysis of Reverse Transcription (RT) intermediates (Pyridine Bis-Chalcone Derivatives):

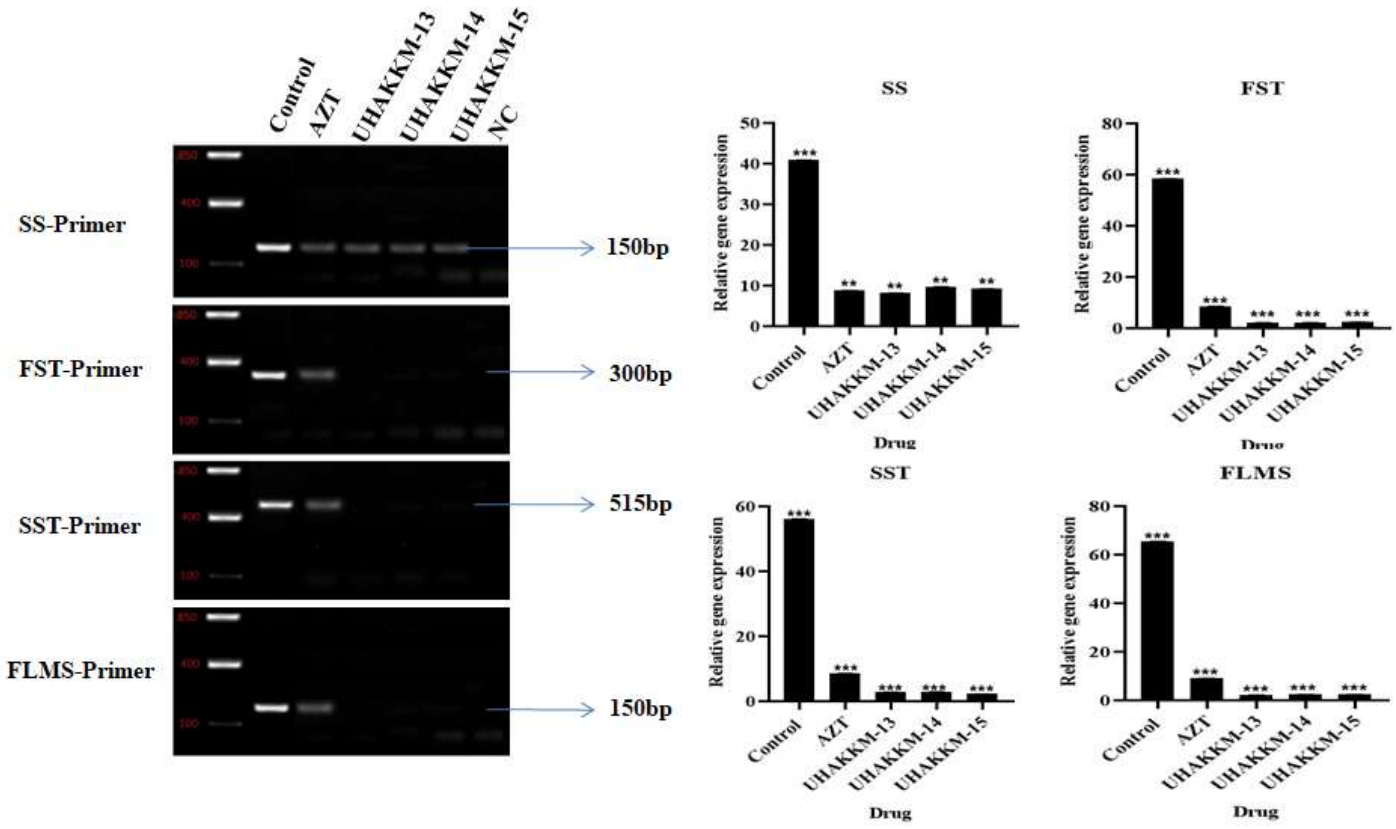


Fig. 5.4: PCR Experiment was done by specific Primers SS, FST, SST and FLMS with cytosolic DNA isolated from infected SupT1 with HIV-1 93IN101 as control where as infected SupT1 in the presence of active pyridine bis-chalcones derivatives (UHAKKM-13, 14 and 15) as test samples. AZT was used as Positive Control, without template as Negative Control (NC) and GAPDH was used as internal control. Above result shows the inhibition of FST, SST and FLMS intermediates completely at IC50 concentration of drug (***P<0.001) when compared with AZT and control samples (***P<0.001).

Discussion:

HIV-1 Reverse Transcription is a crucial process, conversion of ss RNA to cDNA by an enzyme Reverse Transcriptase takes place in cytoplasm. It occurs in nucleoprotein organization called reverse transcription complex (RTC) containing viral RNA, tRNA as primer and novel cDNA, viral factors and host factors. Previous studies shown that during the conversion of ss RNA to cDNA, a series of events takes place in Reverse Transcription process [41].

In the previous objectives both the series of molecules, pyridine dicoumarol (UHAKKM-7, 8 and 9) and bis-chalcone (UHAKKM-13, 14 and 15) derivatives have shown inhibitory affect against Topo IIβK_{HIV} and anti-viral activity with an IC_{50} at nano molar concentration, which is comparable with Azidothymidine (AZT) that has an IC_{50} of 25 nM. In this objective, we have analyzed the molecular activities of pyridine dicoumarol and bis-chalcone derivatives on different events of Reverse Transcription process. Both series of molecules have shown that formation of Strong Stop (SS) is not significantly affected, while progression of first strand transfer and subsequent events of reverse transcription was completely blocked. The result of these studies point out that the TopoII$\beta K_{HIV\text{-}1}$ catalyzed phosphorylated Topo II β is essential for progression of first transfer reaction. These results were correlated by our earlier studies using SiRNA mediated Topo IIβ-knockdown studies; where it was shown that first strand transfer was affected in cells devoid of Topo II β [27]. Thus, confirming that Topo II β phosphorylation is crucial requirement for progression of reverser transcription.

In the earlier studies we have designed and sequentially developed a series of pyridine derivatives with potential inhibitory action against Topo IIβK_{HIV} and anti-HIV-1 activity. Active pyridine compounds (UHAKKM-7, 8 and 9) showed anti-viral activity with an IC_{50} at Nano Molar concentration, which is comparable with Azidothymidine (AZT) that has an IC_{50} of 25

nM. In the present study pyridine coumarin derivatives shown inhibition of Strong Stop (SS) and downstream events of Reverse Transcriptase by TopoIIβK_{HIV} inhibitors point out importance of Topo IIβ phosphorylation in primer binding at strong stop and downstream events of Reverse Transcription formulations. Thus, there could be topological transitions during reverse transcription complex binding and progression of cDNA synthesis, which may be halted due the absence of active Topo IIβK_{HIV}.

In another set of experimental studies pyridine bis-chalcone derivatives (UHAKKM-13, 14 and 15) have shown inhibitory activity on reverse transcription events at nano molar concentration. All the compounds have shown inhibitory activity on SS (Strong Stop) stage and completely inhibited the downstream events of reverse transcription when compared with AZT and positive control.

Conclusion:

Results had shown that inhibition of first strand transfer reaction in the presence of Topo II beta kinase inhibitors from class of pyridine coumarin (UHAKKM-7, 8 and 9) and bischalcones (UHAKKM-13, 14 and 15) derivatives**.** Thus results suggest that Topo II beta phosphorylation is essential for stand transfer step of reverse transcription.

CHAPTER-VI

Broad spectrum activity of HIV-1 inhibitors against different HIV-1 isolates.

Introduction:

Among various infectious disease threats, we face from all microorganisms; viral infections are major global menace in the modern era because of their perpetuation rates and transmissibility. An essential factor that plays an important role is the absence of 'Wide-Range' antiviral agents. In some parts of the world, bacteria are still causing substantial epidemics but their pandemic threats such as *Yersinia pestis*, which causes plague has been significantly controlled in the era of antibiotics(21). But coming to viruses which pose epidemic risks, current therapeutic options are more limited. Even though the armamentarium of anti-viral drugs is promptly amplifying and targeting various viral families but only few have extent of activity which can be compared to the range of major anti-bacterial compounds, penicillin or sulfa (58).

Most anti-viral compounds are designed to block the activity of a unique viral protein which is specific to particular virus for example in case of HIV-1, medications are absolutely unique to HIV-1 proteins like the integrase, protease, reverse transciptase and gp41 except Nucleo(t)side Reverse Transcriptase Inhibitors (NRTIs) that overlap with viral families because of homology in proteins exist in viral families(5). Majority of anti-viral therapeutics are targeted in narrow ranges; but few molecules have properties of broad-spectrum in nature.

The lack of broad-spectrum anti-viral compounds is a major problem in medical emergencies caused by viral infections. The inherent challenges with viral class pathogens are making the development of broad-spectrum antiviral agent is difficult. At the same time, it should be highlighted that there is a necessity to stabilize wide-range activity versus toxicity in the host, like nucleos(t)ide analogs. The lack of wide-range and toxicity antiviral agents is due to the ability of viruses to employ host cell machinery for their activity. Advent of antiretroviral therapy (ART) has shown to control HIV-1 and adherence to it helps in postponing the

progression of AIDS disease, which results in longevity of near normal life. Despite of this, a substantial number of patients fail to adhere due to conflicting side effects, misuse of drug, mental ataxia, socioeconomic status, stigma and literacy etc. Availability of various possible treatments of HIV-1 at individual stage of the life cycle gives a chance to physicians for change in different regimens to reduce the adverse effects of drugs. To achieve this, progression of highly potent, wide-range anti-viral molecules should be the prime objective in the antiviral drug discovery and development. Almost 200 functional proteins of cell are directly or indirectly involved in the replication of HIV-1 (14), (27) and understanding their roles, associated pathways has aided in designing the advanced strategies for the development of wide-range anti-viral compounds.

Classification of HIV-1:

It is the most common that HIV-1 causes AIDS disease. There are Two Types of HIV, HIV-1 (Predominant and Infectious) and HIV-2 (Unusual and less Infectious). HIV-1 again divided into Group M (Major group) and Group N, O and P (Minor group). More than 90% of cases deriving from infection with HIV-1 group M. Group M is again classified into sub-types A-K. Among these, subtypes B and C are more prevalent in India and Western Countries.

Figure 6.1: Classification of HIV-1

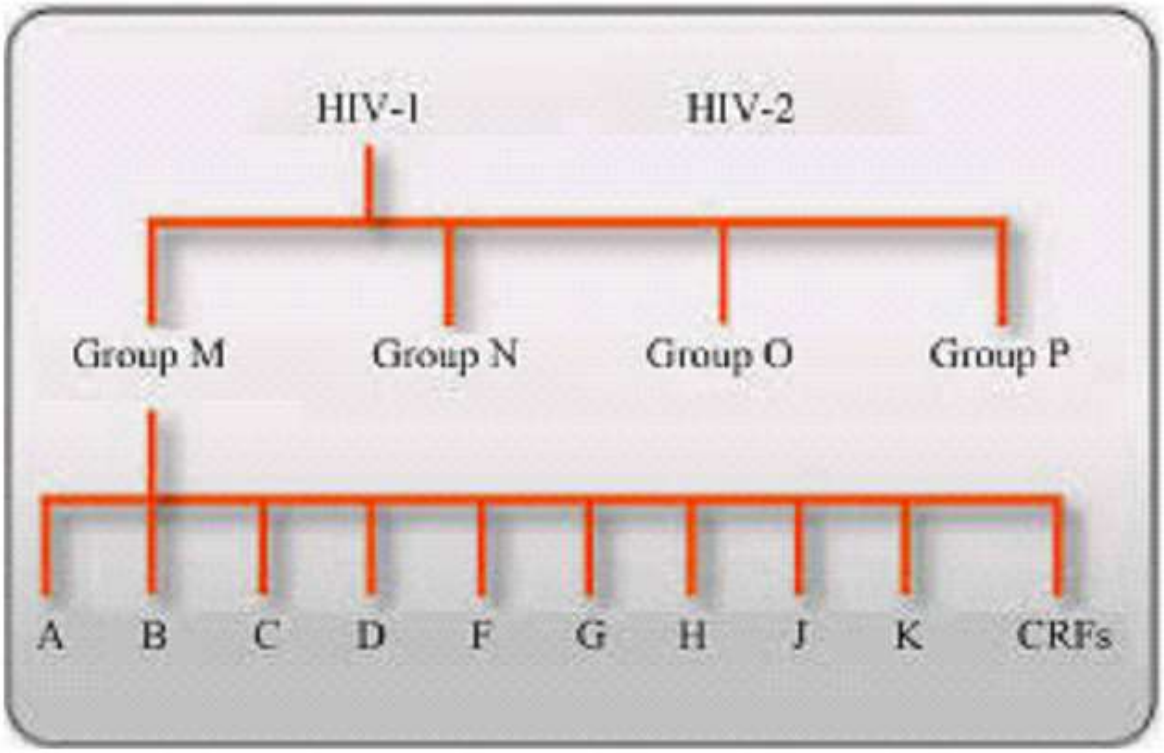


Fig. 6.1: Above figure is showing classification of HIV where two types are present HIV-1 and HIV-2. HIV-1 is the major type again divided into subtypes and subgroups.

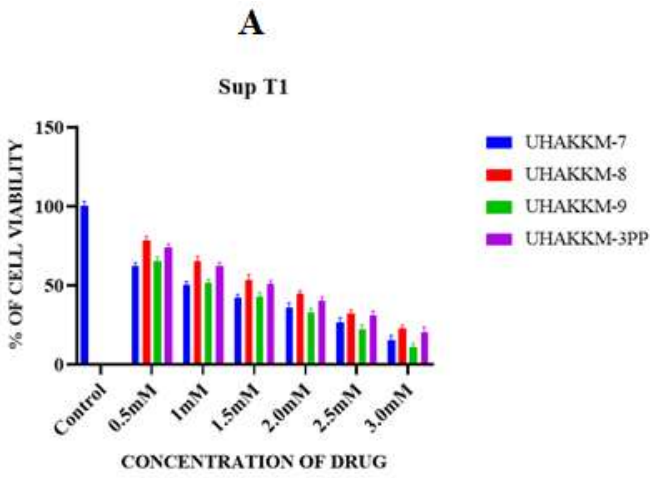
Results:

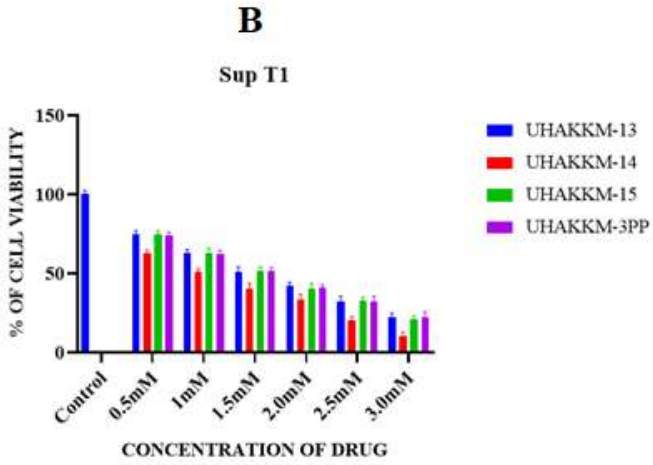
Cytotoxicity of selected active molecules in Sup T1 cells:

Approximately 0.02 X 10^6 SupT1 cells were seeded in 100µL of complete media per each well in 96 well plate and kept for overnight incubation, on the next day drugs of different concentrations were added to the plate and further incubated in 5% CO_2 incubator for 24 hours, next day 10uL MTT was added to all the of 96 well plates and further incubated for 4 hours, then 100uL of Dimethyl sulfoxide (DMSO) was dispensed to all the 96 wells of the plate, mixed gently and kept in darkness for half-an-hour at RT. At 570 nm absorbance was measured in a micro titer plate reader. All the samples were run in triplicate and each experiment was repeated thrice.

The result (Fig.6.2) shows that molecules are exhibited above 60% viability at 1mM concentration and at 0.5 mM concentration no significant cytotoxicity observed.

Figure 6.2: Cell Viability Assay (MTT Assay)





C

Drug	CC_{50} (mM)
UHAKKM-7	1.05± 0.62
UHAKKM-8	1.30± 0.74
UHAKKM-9	1.22± 0.26
UHAKKM-13	1.50± 0.38
UHAKKM-14	1.04± 0.28
UHAKKM-15	1.45± 0.22
3PP	1.52± 0.68

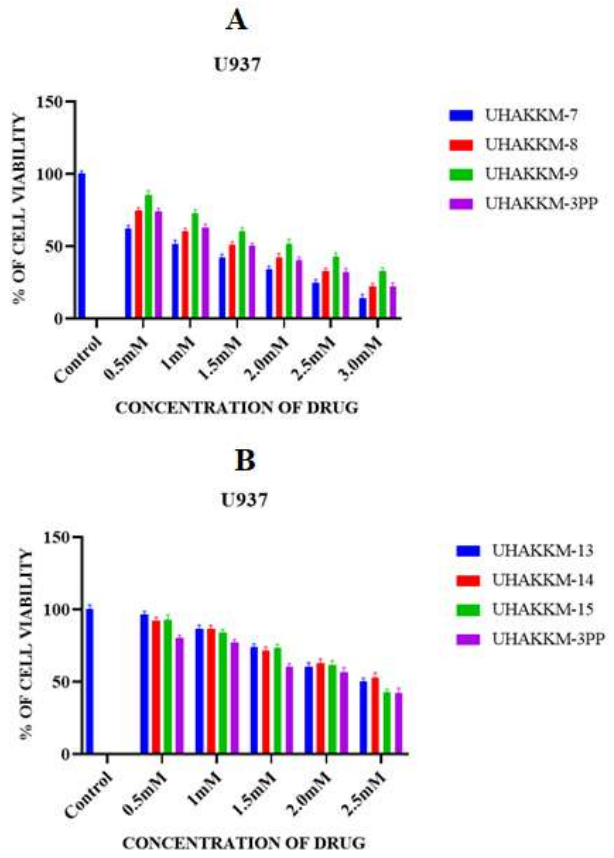
Fig. 6.2: Cytotoxicity Assay was performed in SupT1 cells for the pyridine dicoumarol (UHAKKM-7, 8 and 9) and bis-chalcone (UHAKKM-13, 14 and 15) derivatives at different concentrations to check the % of viability as shown in the Fig.6.2. The results in panel A and B graphs are representing the viability at different concentrations, panel C table indicating the CC_{50} of the molecules. All the molecules have shown above 60% viability at 1mM concentration and at 0.5 mM concentration no significant cytotoxicity observed.

Cytotoxicity of selected active molecules in U937 cells:

Approximately 0.02 X 10^6U937 cells were seeded in 100µL of complete media per each well in 96 well plate and kept for overnight incubation, on the next day drugs of different concentration were added to the plate and further incubated in 5% CO_2 incubator for 24 hours, next day 10uL MTT was added to all the of 96 well plates and further incubated for 4 hours, then 100uL of Dimethyl sulfoxide (DMSO) was dispensed to all the 96 wells of the plate, mixed gently and kept in darkness for half-an-hour at RT. At 570 nm absorbance was measured in a micro titer plate reader. All the samples were run in triplicate and each experiment was repeated thrice

The result (Fig.6.3) shows that molecules are exhibited above 60% viability at 1mM concentration and at 0.5 mM concentration no significant cytotoxicity observed.

Figure 6.3: Cytotoxicity Assay in U937 Cells (MTT Assay)



C

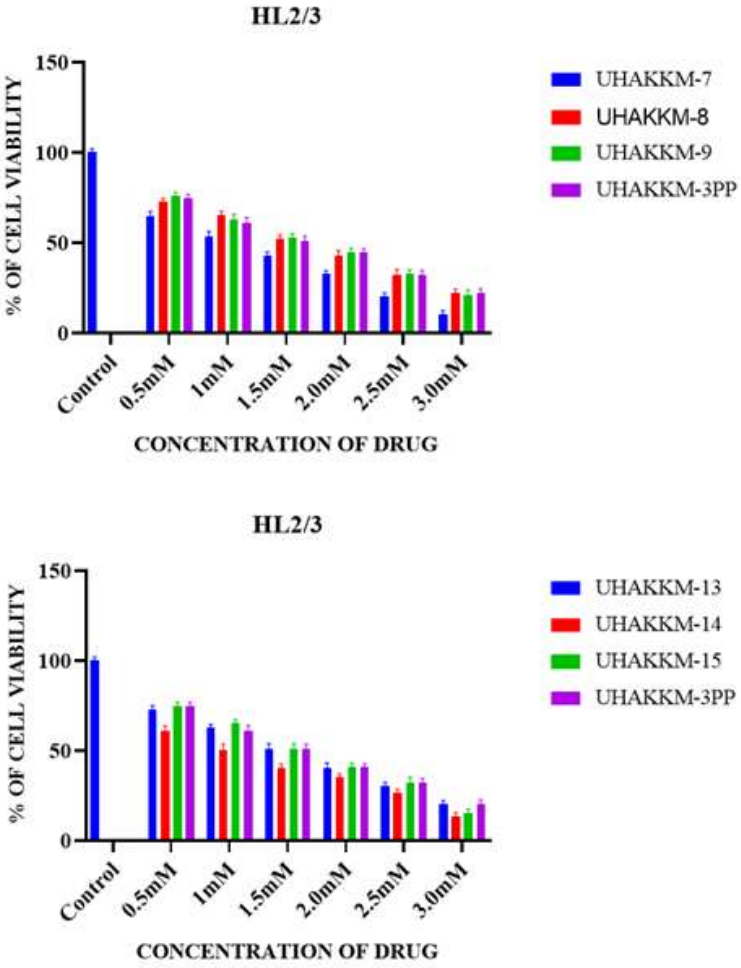
Drug	CC_{50} (mM)
UHAKKM-7	1.16± 0.15
UHAKKM-8	1.40± 0.24
UHAKKM-9	1.36± 0.33
UHAKKM-13	1.46± 0.56
UHAKKM-14	1.10± 0.29
UHAKKM-15	1.50± 0.62
3PP	1.50± 0.42

Fig. 6.3: Cytotoxicity Assay was performed in U937 cells for the pyridine dicoumarol (UHAKKM-7, 8 and 9) and bis-chalcone (UHAKKM-13, 14 and 15) derivatives at different concentrations to check the % of viability as shown in the Fig.6.3. The graphs in panel A and B are representing the viability at different concentrations, panel C table indicating the CC_{50} of the molecules. All the molecules have shown above 60% viability at 1mM concentration and at 0.5mM concentration no significant cytotoxicity observed.

Cytotoxicity of selected active molecules in HL2/3 cells:

Approximately 0.02 X 10^6HL2/3 cells were seeded in 100µL of complete media per each well in 96 well plate and kept for overnight incubation, on the next day drugs of different concentration were added to the plate and further incubated in 5% CO_2 incubator for 24 hours, next day 10uL MTT was added to all the of 96 well plates and further incubated for 4 hours, then 100uL of Dimethyl sulfoxide (DMSO) was dispensed to all the 96 wells of the plate, mixed gently and kept in darkness for half-an-hour at RT. At 570 nm absorbance was measured in a micro titer plate reader. All the samples were run in triplicate and each experiment was repeated thrice. The result (Fig.6.4) shows that molecules are exhibited above 60% viability at 1mM concentration and at 0.5 mM concentration no significant cytotoxicity observed.

Figure 6.4: Cytotoxicity Assay in HL2/3 Cells (MTT Assay)



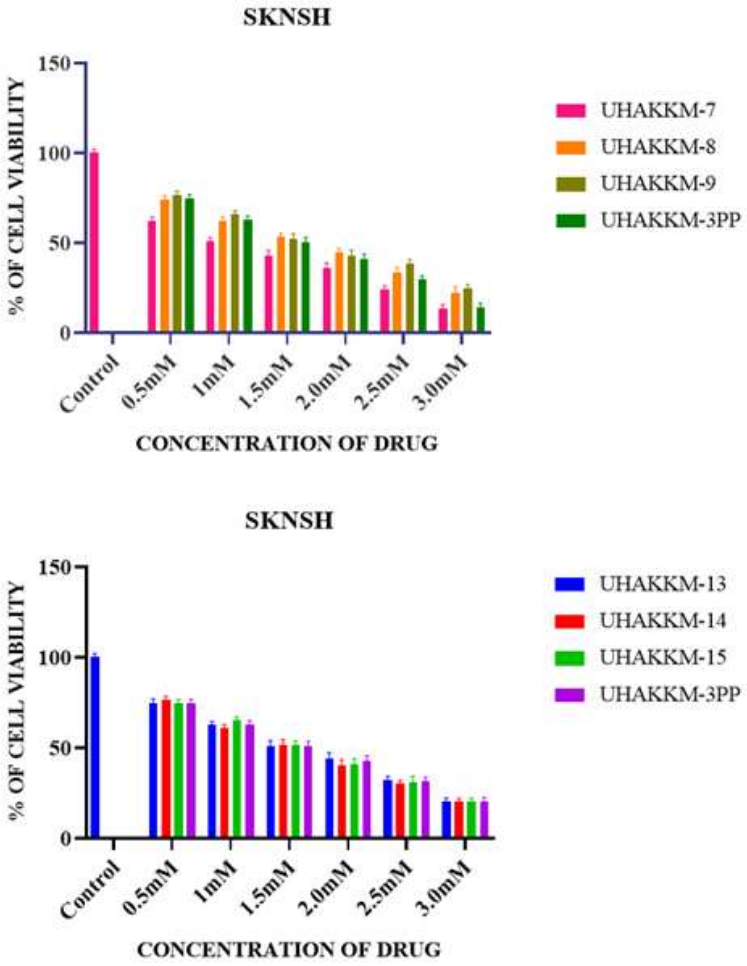
Drug	CC_{50} (mM)
UHAKKM-7	1.12± 0.32
UHAKKM-8	1.34± 0.44
UHAKKM-9	1.28± 0.22
UHAKKM-13	1.46± 0.74
UHAKKM-14	1.05± 0.33
UHAKKM-15	1.58± 0.48
3PP	1.46± 0.58

Fig 6.4: Cytotoxicity Assay was performed in HL2/3 cells for the pyridine dicoumarol (UHAKKM-7, 8 and 9) and bis-chalcone (UHAKKM-13, 14 and 15) derivatives at different concentrations to check the % of viability as shown in the Fig.6.3. The graphs in panel A and B are representing the viability at different concentrations, panel C table indicating the CC_{50} of the molecules. All the molecules have shown above 60% viability at 1mM concentration and at 0.5 mM concentration no significant cytotoxicity observed.

Cytotoxicity of selected active molecules in SK N-S-S cells

Approximately 0.02 X 10^6 SKNSH cells were seeded in 100µL of complete media per each well in 96 well plate and kept for overnight incubation, on the next day drugs of different concentration were added to the plate and further incubated in 5% CO_2 incubator for 24 hours, next day 10uL MTT was added to all the of 96 well plates and further incubated for 4 hours, then 100uL of Dimethyl sulfoxide (DMSO) was dispensed to all the 96 wells of the plate, mixed gently and kept in darkness for half-an-hour at RT. At 570 nm absorbance was measured in a micro titer plate reader. All the samples were run in triplicate and each experiment was repeated thrice. The results in Fig 6.5 (Panel A and B) and Table 6.4 (Panel C) show that all the drugs are showing above 60% viability at 1mM concentration and at 0.5 µM concentration no significant cytotoxicity observed.

Figure 6.5: Cell Viability Assay (MTT Assay)



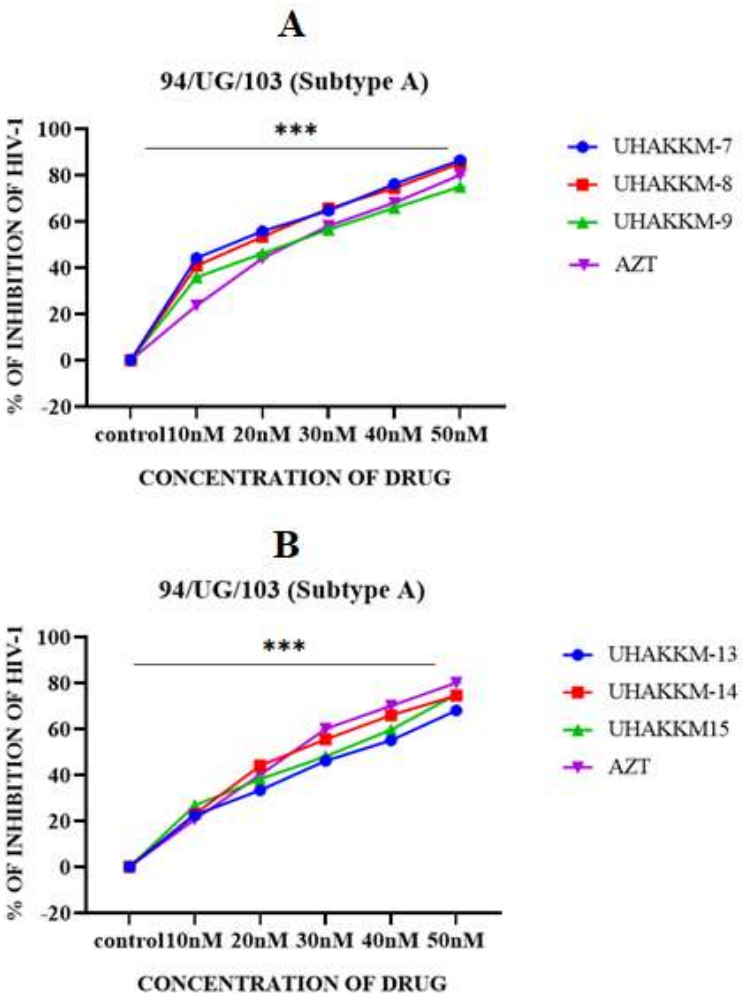
Drug	CC_{50} (mM)
UHAKKM-7	1.04± 0.24
UHAKKM-8	1.28± 0.66
UHAKKM-9	1.35± 0.56
UHAKKM-13	1.55± 0.42
UHAKKM-14	1.19± 0.38
UHAKKM-15	1.58± 0.68
3PP	1.50± 0.40

Fig 6.5: Cytotoxicity Assay was performed in SKNSH cells for the pyridine dicoumarol (UHAKKM-7, 8 and 9) and bis-chalcone (UHAKKM-13, 14 and 15) derivatives at different concentrations to check the % of viability as shown in the Fig.6.5 The graphs in panel A and B are representing the viability at different concentrations, panel C table indicating the CC_{50} of the molecules. All the molecules have shown above 60% viability at 1mM concentration and at 0.5 mM concentration no significant cytotoxicity observed.

Anti-Viral activity of active molecules against Subtype A, HIV-$I_{94/UG103}$:

Viral strain of HIV-$I_{94/UG103}$ (Subtype-A) was infected to SupT1 cells at various concentrations of pyridine coumarin (UHAKKM-7, 8 and 9) molecules and bis-chalcone (UHAKKM-13, 14 and 15) derivatives for 5 hours. After 5 hours the samples were washed, pelleted and resuspended in RPMI complete media, further incubated for 96 hours. On 4th day the viral titer was estimated by p24 ELISA method. Results in Fig.6.6 indicated that graphs (Panel A and B) represents the percentage of drug inhibition plotted against the various concentrations of drug and Table (Panel C) shows the IC_{50} concentration of all the pyridine coumarin and bis-chalcone derivatives. Among compounds, UHAKKM-7, 8, 9 and 14 showed highest activity at nM concentration and AZT was employed as positive control. All the samples were run in triplicates and each experiment was performed for thrice (Statistical significance: **P<0.001).

Figure 6.6: Anti-Viral activity of active molecules against Subtype A, HIV-$I_{94/UG103}$



C

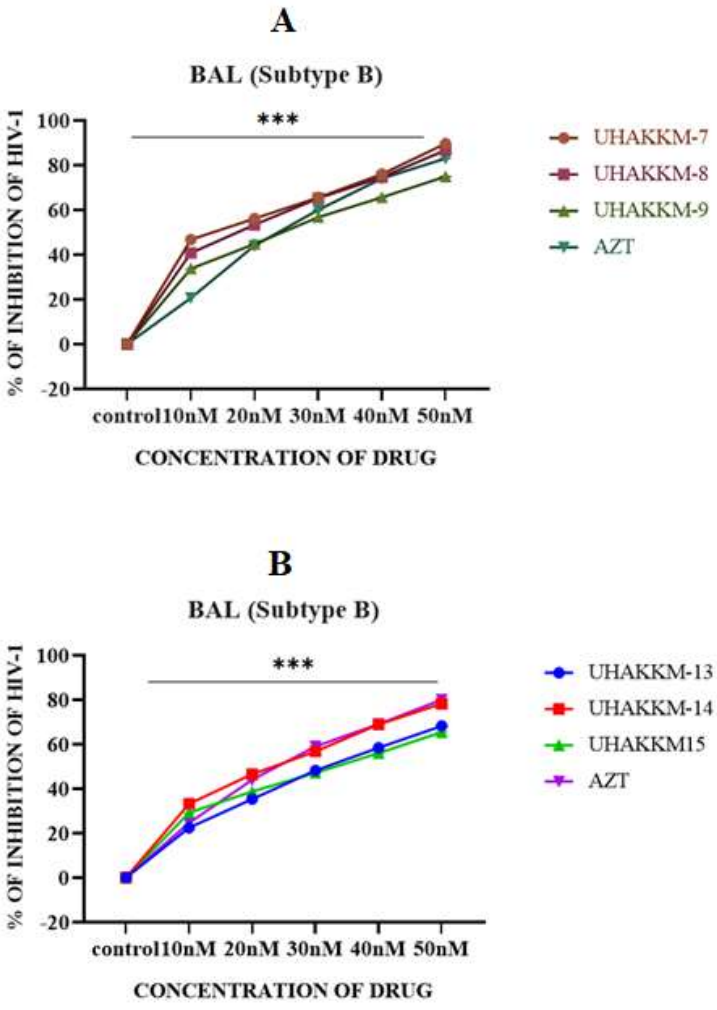
Drug	IC_{50} (nM)
Control (AZT)	25± 0.44
UHAKKM-7	15.40± 0.56
UHAKKM-8	17.00± 0.24
UHAKKM-9	23.50± 0.86
UHAKKM-13	33.20± 0.62
UHAKKM-14	24.50± 0.56
UHAKKM-15	32.00± 0.22

Fig. 6.6: Above results shows the anti-viral activity of all the active compounds. The results in the Fig.6.6 confirm the activity of drug compounds at nM concentration and AZT was employed as positive control. In panel A and B graphs indicating the viral inhibition at various concentrations and in panel C table indicates the IC50 of molecules against Subtype A virus HIV-$I_{94/UG103}$. All the active molecules have shown significant anti-HIV-1 activity (**P<0.001).

Anti-Viral activity against macrophage tropic HIV-1_{BaL} (Subtype-B) Virus:

U937 cells were infected with HIV-I BAL (Subtype-B) in the presence of various concentrations of pyridine coumarin (UHAKKM-7, 8 and 9) molecules and bis-chalcone (UHAKKM-13, 14 and 15) derivatives. After 5 hours the samples were washed, pelleted and resuspended in RPMI complete media, further incubated for 96 hours. On 4th day the viral titer was estimated by p24 ELISA method. Results in Fig.6.7 indicated that graphs (Panel A and B) represents the percentage of drug inhibition plotted against the various concentrations of drug and Table (Panel C) shows the IC_{50} concentration of all the pyridine coumarin and bis-chalcone derivatives. Among compounds, UHAKKM-7, 8, 9 and 14 showed highest activity at nM concentration and AZT was employed as positive control. All the samples were run in triplicates and each experiment was performed for three times. (Statistical significance: **P<0.001).

Figure 6.7: Anti-Viral activity against macrophage tropic HIV-1_{BaL} (Subtype-B) Virus



C

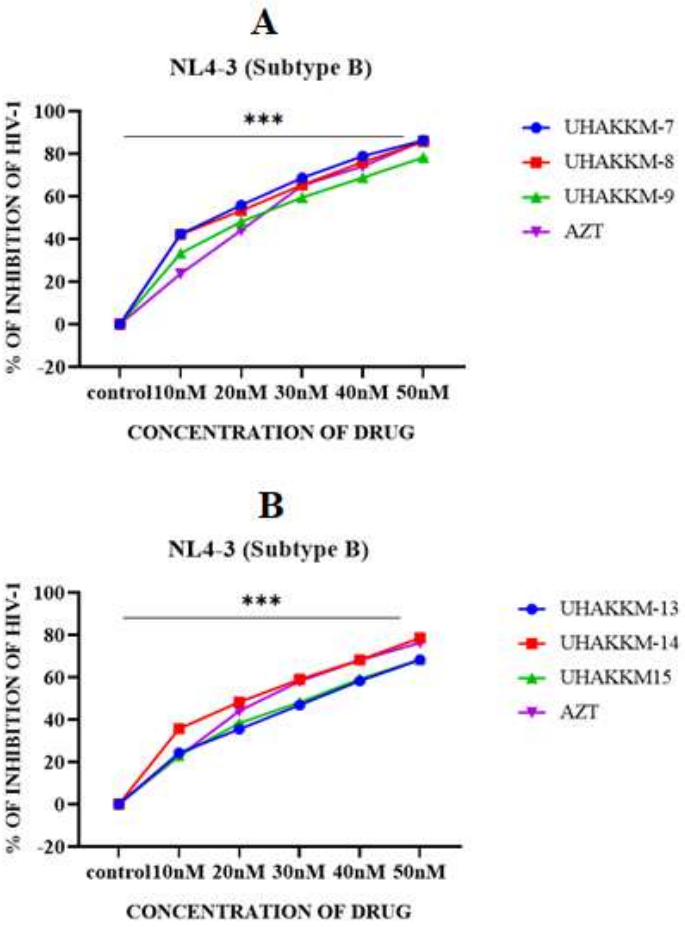
Drug	IC_{50} (nM)
Control (AZT)	25± 0.22
UHAKKM-7	14.50± 0.26
UHAKKM-8	18.50± 0.66
UHAKKM-9	24.00± 0.33
UHAKKM-13	32.00± 0.58
UHAKKM-14	24.50± 0.44
UHAKKM-15	33.50± 0.68

Fig. 6.7: Above results shows the anti-viral activity of all the active compounds. The results in the Fig.6.7 confirm the activity of drug compounds at nM concentration and AZT was employed as positive control. In panel A and B graphs indicating the viral inhibition at various concentrations and in panel C table indicate the IC50 of molecules against Subtype B virus HIV-1_{BaL}. All the active molecules have shown significant anti-HIV-1 activity (**P<0.001).

Anti-Viral activity against Subtype B virus HIV-1_{NL4-3}:

Viral strain of HIV-1_{NL4-3} (Subtype-B) was given infection to SupT1 cells at various concentrations of pyridine coumarin (UHAKKM-7, 8 and 9) molecules and bis-chalcone (UHAKKM-13, 14 and 15) derivatives for 5 hours. After 5 hours the samples were washed, pelleted and resuspended in RPMI complete media, further incubated for 96 hours. On 4th day the viral titer was estimated by p24 ELISA method. Results in Fig.6.8 indicated that graphs (Panel A and B) represents the percentage of drug inhibition plotted against the various concentrations of drug and Table (Panel C) shows the IC_{50} concentration of all the pyridine coumarin and bis-chalcone derivatives. Among compounds, UHAKKM-7, 8, 9 and 14 showed highest activity at nM concentration and AZT was employed as positive control. All the samples were run in triplicates and each experiment was performed for thrice (Statistical significance: **$P<0.001$).

Figure 6.8: Anti-Viral activity against HIV-1_{NL4-3} (Subtype B) virus



C

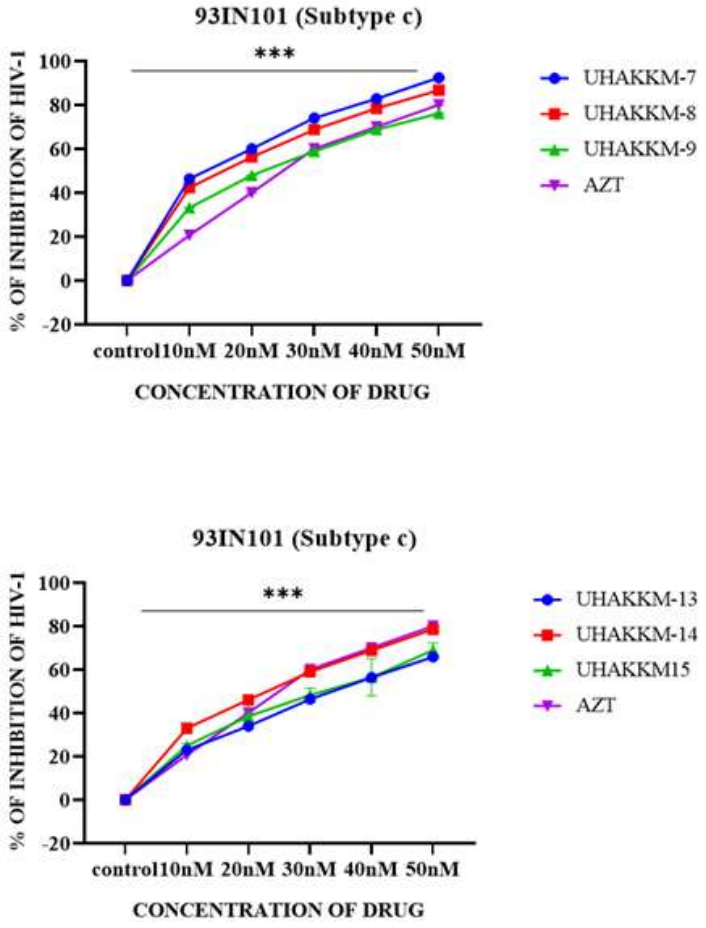
Drug	IC_{50} (nM)
Control (AZT)	25± 0.42
UHAKKM-7	14.76± 0.38
UHAKKM-8	17.50± 0.85
UHAKKM-9	22.00± 0.56
UHAKKM-13	33.50± 0.44
UHAKKM-14	23.94± 0.62
UHAKKM-15	32.08± 0.58

Fig. 6.8: Above results shows the anti-viral activity of all the active compounds. The results in the Fig.6.8 confirm the activity of drug compounds at nM concentration and AZT was employed as positive control. In panel A and B graphs indicating the viral inhibition at various concentrations and in panel C table indicating the IC50 of molecules against Subtype A virus HIV-1_{NL4-3} (***P<0.001).

Anti-Viral activity against HIV-$I_{93IN101}$ (Subtype C) Virus:

Viral strain of was HIV-$1_{93IN101}$ (Subtype-C) given infection to SupT1 cells various concentrations of pyridine coumarin (UHAKKM-7, 8 and 9) molecules and bis-chalcone (UHAKKM-13, 14 and 15) derivatives for 5 hours. After 5 hours the samples were washed, pelleted and resuspended in RPMI complete media, further incubated for 96 hours. On 4th day the viral titer was estimated by p24 ELISA method. Results in Fig.6.9 indicated that graphs (Panel A and B) represents the percentage of drug inhibition plotted against the various concentrations of drug and Table (Panel C) shows the IC_{50} concentration of all the pyridine coumarin and bis-chalcone derivatives. Among compounds, UHAKKM-7, 8, 9 and 14 showed highest activity at nM concentration and AZT was employed as positive control. All the samples were run in triplicates and each experiment was performed for thrice (Statistical significance: **P<0.001).

Figure 6.9: Anti-Viral activity against HIV-$I_{93IN101}$ (Subtype C) Virus



Drug	IC_{50} (nM)
Control (AZT)	25± 0.26
UHAKKM-7	13.50± 0.32
UHAKKM-8	16.40± 0.55
UHAKKM-9	23.40± 0.74
UHAKKM-13	35.20± 0.33
UHAKKM-14	22.40± 0.56
UHAKKM-15	33.50± 0.22

Fig. 6.9: Above results shows the anti-viral activity of all the active compounds. The results in the Fig.6.9 confirm the activity of drug compounds at nM concentration and AZT was employed as positive control. In panel A and B graphs indicating the viral inhibition at various concentrations and in panel C table indicating the IC50 of molecules against Subtype A virus HIV-$1_{93IN101}$ (***P<0.001).

Viral p24 Expression of various isolates of HIV-1:

Infection carried out for six hours using HIV-1 93IN101, NL4-3, 91/UG/273 and BAL in SupT1 and U937 cells in the presence of IC_{50} concentration of active compounds pyridine coumarin (UHAKKM-7, 8 and 9) and bis-chalcone derivatives (UHAKKM-13, 14 and 15). Cells were pelleted and lysed, p24 expression was analyzed by western blot analysis. The results show significant decrease in p24 expression in all three viruses in the presence of pyridine coumarin (UHAKKM-7, 8 and 9) and bis-chalcone derivatives (UHAKKM-13, 14 and 15).Thus confirming that virus replication is inhibited in the presence of these compounds. (Statistical significance: **P<0.001).

Figure 6.10: Viral p24 Expression of different HIV-1 isolates

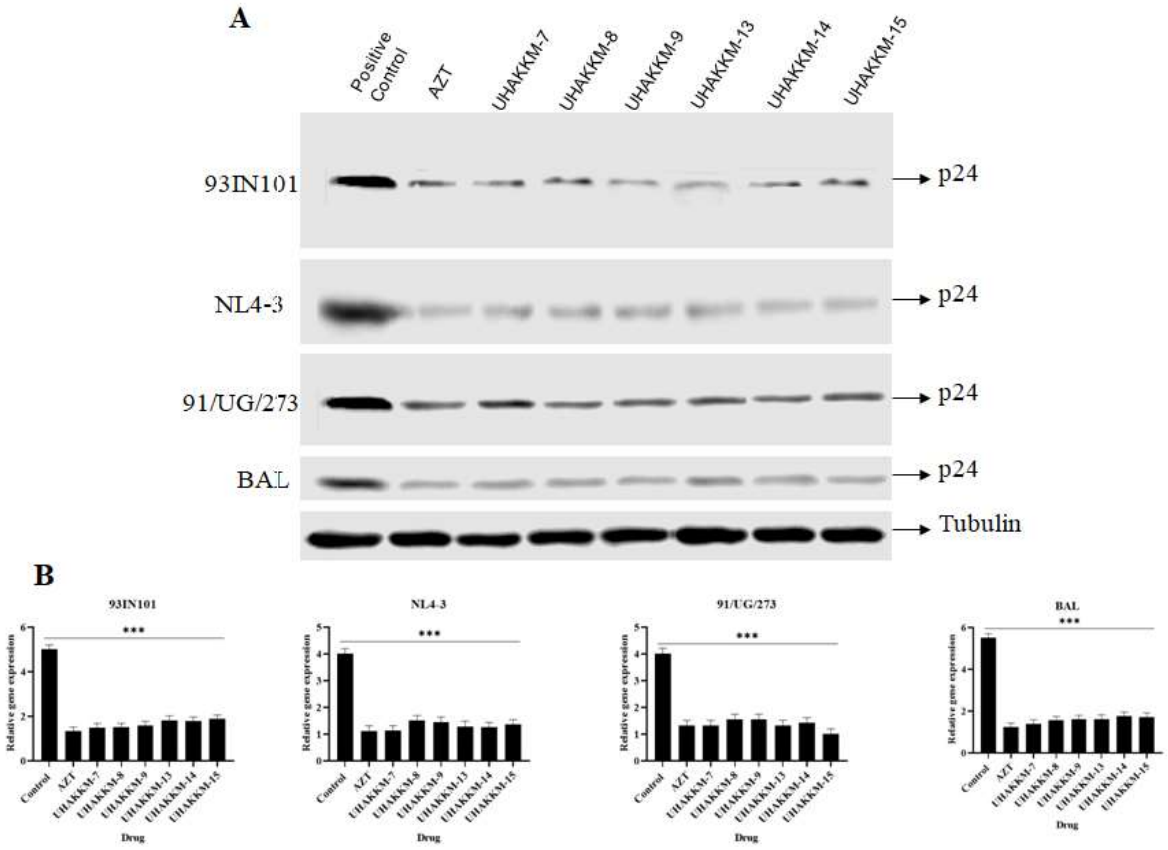


Fig. 6.10: Infected SupT1, U937 cells were pelleted and lysed after 6 hours of post infection with different strains; HIV-1 93IN101, NL4-3, 91/UG/273 and BAL in the presence of IC_{50} concentration of active compounds pyridine coumarin (UHAKKM-7, 8 and 9) and bis-chalcone derivatives (UHAKKM-13, 14 and 15). Infected sample (control), drug treated (test) samples were lysed and analyzed by SDS-PAGE. Panel A is showing western blot analysis was carried out with specific antibodies; HIV-1 viral p24 antibody (Pri.Ab) and anti-mouse IgG antibody (Sec.Ab). AZT was employed as positive control and Tubilin as internal control of all the samples. Panel B representing the densitometry analysis by Image J Software (***P<0.001).

Analysis of Proviral DNA by active compounds:

HIV-1 93IN101, NL4-3, 91/UG/273 and BAL acute infections were conducted at IC_{50} concentrations of active compounds pyridine coumarin(UHAKKM-7, 8 and 9) and bis-chalcone derivatives (UHAKKM-13, 14 and 15) and incubated in 5% CO_2 and 37°C incubator. Infected SupT1 and U937 cells were pelleted and lysed after 5 hours of post infection, samples were collected and Genomic DNA was extracted by standard phenol/chloroform method. DNA with 1 µg concentration was used for PCR amplification with specific primers sk38/39 to the gag region of HIV-1. All the samples were run at IC_{50} concentrations and each experiment was performed for thrice (Statistical significance: **P<0.001).

Figure 6.11: Analysis of Proviral DNA by active compounds

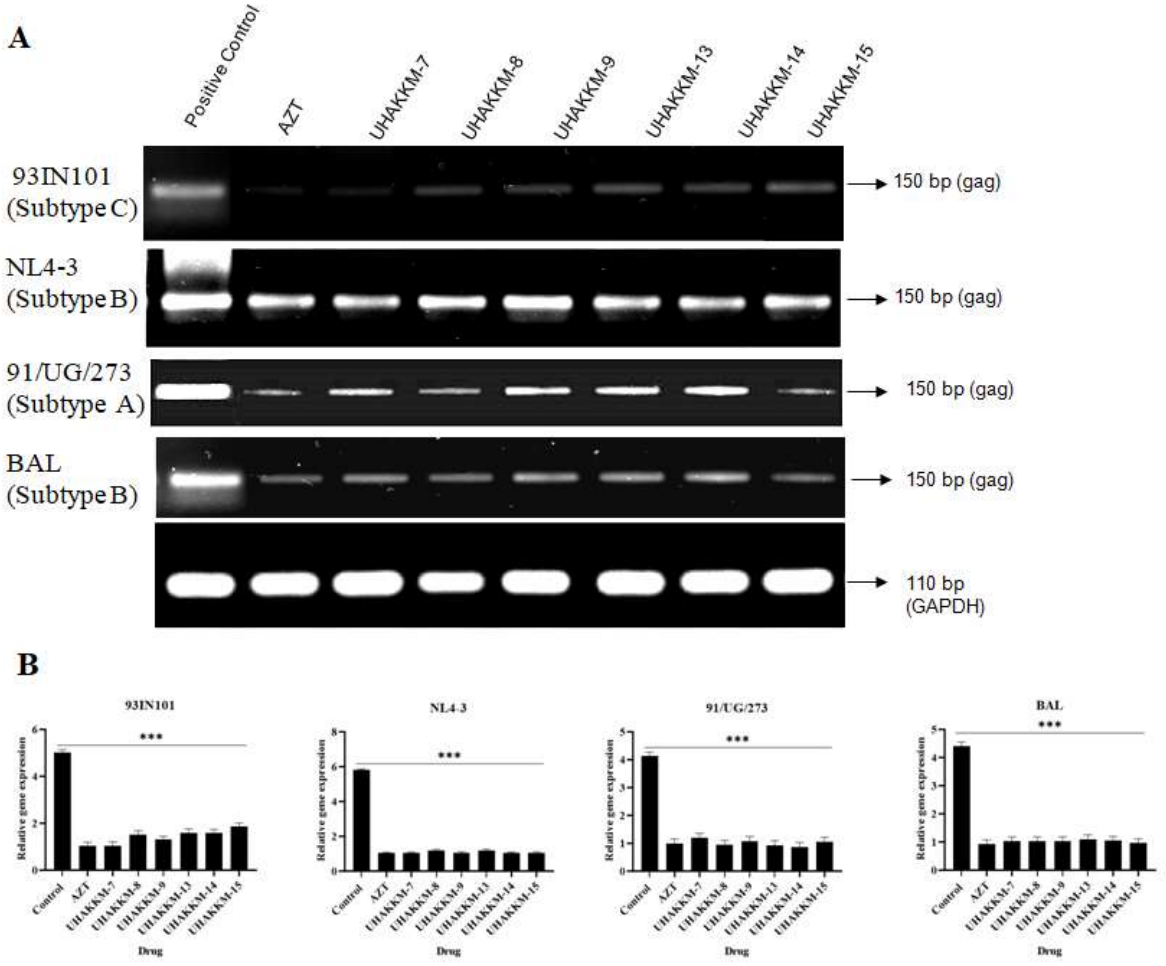


Fig.6.11: HIV-1 93IN101, NL4-3, 91/UG/273 and BAL acute infection was conducted in the presence of pyridine coumarin (UHAKKM-7, 8 and 9) and bis-chalcone derivatives (UHAKKM-13, 14 and 15) at IC_{50}concentration and checked the proviral DNA amplification with specific primers sk38/39. Infected sample is used as a control and infected sample in the presence of drug is test sample, AZT was used as positive control (25nM) and GAPDH was used as PCR loading control. A) Anti-HIV-1 activity results shows all the active compounds have shown high inhibition on 93IN101when compared with all the subtypes at IC_{50} concentration (***P<0.001) and Panel B representing the densitometry analysis by Image J Software.

Discussion:

In the previous objectives,the molecular action of the pyridine dicoumarol (UHAKKM-7, 8 and 9) and bis-chalcone (UHAKKM-13, 14 and 15) derivatives was analyzed. In the present study, we have checked the broad spectrum activity of active inhibitors against different HIV-1 isolates. We have checked the cytotoxicity of the compounds by cell viability assay in four different cell lines, SupT1, U937, HL2/3 and SKNSH. Cellswere incubated at various concentrations of drug compounds and results confirmed that compounds exhibited no significant toxicity below 0.5 mM concentrations [Fig.6.2 to Fig.6.5].Theanti-HIV-1activity of the molecules in inhibiting viral replication showed thatpyridine dicoumarol (UHAKKM-7, 8 and 9) derivatives have shown higher inhibition with IC_{50}<30nM against four viruses analyzed in this chapter. Whereas bis-chalcone (UHAKKM-13, 14 and 15) derivatives have shown higher inhibitionwith IC_{50}<30nM against HIV-$1_{93IN101}$, while other viruses (HIV-$I_{94/UG103,}$ HIV-$I_{B\ al,}$ HIV-$I_{NL4\text{-}}$3) they show an IC_{50}<50nM on all other viral isolates. Results showed that among all the pyridine dicoumarol and bis-chalcone derivatives UHAKKM-7showed anti-viral activity with an IC_{50} of 13.50nM,, which was seen tobe on par with the widely clinically used, Azidothymidine (AZT) that has an IC_{50} of 25nM. Anti-HIV-1 activity of all the active molecules reconfirmed with the inhibition of p24 expression by Western blot analysis. Results confirmed that all the active compounds have shown high inhibition on 93IN101 in when comparison with all other subtypes. Activity of active compounds by Anti-HIV-1 assay was further confirmed by the analysis of proviral DNA synthesis at gag region; results confirmed that the pyridine dicoumarol (UHAKKM-7, 8 and 9) and bis-chalcone (UHAKKM-13, 14 and 15) derivatives have inhibited proviral DNA synthesis confirming activity against all the HIV-1 viruses.All the active compounds have shown highly inhibition on HIV-1 $_{93IN101}$when compared with all the other viral isolates of HIV-1.

Among the viruses, HIV-$I_{94/UG103,}$ HIV-I_{NL4-3} are CxCR4-specifc cells and HIV-$I_{B\ al,}$is CCR5-specific cells, while HIV-$I_{93IN101}$ exhibits dual specific to both CCR5 and CxCR4 cells, we propose that these molecules are higher activity in this virus due to the phenotype of kinase expressed in HIV-$I_{93IN101}$infected cells. Also it is possible that these molecules are the leads from screening kinase isolated from HIV-$I_{93IN101}$ and anti-viral activity against HIV-$I_{93IN101,}$ hence there exist a bias towards HIV-$I_{93IN101}$ which could not be ruled out. Though molecules show higher activity in terms of IC_{50} against HIV-$I_{93IN101,}$ but overall performance of anti-viral efficacy against all four viruses evaluated in the thesis is significant and comparable to that of AZT.

Conclusion:

Analysis of broad-spectrum activity of kinase inhibitors towards Topo II beta from class pyridine UHAKKM-7, 8 and 9 and bis-chalcones UHAKKM-13, 14 and 15 derivatives showed significant inhibitory activity against on subtypes A, B and C of HIV-1 virus.Thus, suggesting wide-range anti-HIV-1 activity of these compounds.

Overall Summary:

Topo II is specifically phosphorylated throughoutthe infection of HIV-1 and its propagation, at the time of infection virus activates many host proteins by phosphorylating them. In the earlier studies it was shown that a series pyridine compounds specifically target the novel Topo IIβK_{HIV} that was shown to be responsible for phosphorylation of Topo II β (63) necessary for HIV-1 replication. Later a series of pyridine dicoumarol derivatives were having potential anti kinase and anti-HIV-1 activity (62). The consolidated summary of the thesis is to study the development and molecular characterization of pyridine dicoumarol and bis-chalcone derivatives against HIV-1-associated Topo II β kinase and HIV-1 early replication. In the objective-I we have analyzed the bio-activity of pyridine dicoumarol (UHAKKM-7, 8 and 9) which have shown high activity at low concentration (IC_{50}<30nM) against Topo IIβK_{HIV-1} which is known to play critical role in HIV-1 replication. In objective II tested the bis-chalcone derivatives (UHAKKM-10 to 15) against Topo IIβK_{HIV-1} all the compounds have shown anti HIV-1 activity at low concentration (IC_{50}<50nM). In the objective-I and II both the series of molecules have shown inhibitory affect against Topo IIβK_{HIV},anti-viral activity and affected proviral DNA synthesis. In objective-III, we have analyzed the molecular activities of pyridine dicoumarol and bis-chalcone derivatives on different events of Reverse Transcription process. Characterization of intermediates of reverse transcription by both the series of molecules have shown that formation of Strong Stop (SS) is not significantly affected, while progression of First Strand Transfer (FST) and furthersubsequent steps of reverse transcription were completely blocked. Results showed that various molecules having pyridine as pharmacophore exhibits significant inhibition of cDNA synthesis during reverse transcription events; Phosphorylated Topo II β is associated with reverse transcription complex and promote HIV-1 reverse transcription.

Molecular analysis of these inhibitors suggests the role of Topo IIβK_{HIV-1} in reverse transcription process and in the objective –VI broad spectrum activity of active pyridine dicoumarol and bis-chalcone derivatives on different HIV-1 subtypes was tested and results confirmed the activity of Topo II β kinase inhibitors from class pyridine dicoumarol derivatives UHAKKM-7, 8 and 9 and bis-chalcones UHAKKM-13, 14 and 15 with significant inhibitory activity against HIV-1 subtype A, B and C virus. Thus these compounds would provide a novel class of anti-HIV-1 therapeutics.

Objective-I:

In this objective, we have analyzed the molecular activities of these inhibitors, which affect the early replication of virus by specifically targeting the novel Topo IIβK_{HIV} which is a 72 kDa protein purified from the viral lysates. Compounds exhibited no significant cytotoxicity below 1 micro molar concentration which was checked in SupT1 cells. *Invitro*-Phosphorylation assay below the CC_{50} concentration have shown the kinase inhibitory activity at pico molar concentration. The anti-HIV activity of the molecules was tested and confirmed by using the p24-Assay (ELISA). Compounds, UHAKKM-1 to 6 showed low anti-viral activity of HIV-1 even at higher concentration of 100µM, whereas water soluble compounds (disodium pyridine dicoumarates) UHAKKM-7 to 9 have shown high inhibition at low concentration (IC_{50}<30nM). Results showed that among all the pyridine dicoumarol derivatives UHAKKM-7 showed anti-viral activity with an IC_{50} of 15 nM, which was seen to be on par with the widely used and clinically used, Azidothymidine (AZT) that has an IC_{50} of 25nM. Further we have screened these compounds (UHAKKM-7, 8 and 9) for inhibitory activity of Topo II beta phosphorylation at serine in a Dose Dependent studies by immune precipitation with Topo II β antibody and

confirmed with biotinylated anti-phophoserine antibody. Thus results confirmed that significantly inhibited Topo II beta phosphorylation at serine in infected cells, while no

important change was noticed in uninfected cells suggesting the specificity of action against HIV-1 infection associated kinase activity and also confirmed that the Topo II β phosphorylation is inhibited in a dose dependent method and among drugs analyzed, UHAKKM-8 showed highest inhibition followed by UHAKKM-7 and UHAKKM-9. In PCR experiments, compounds UHAKKM-1, 2, 3, 4, 5 and 6 have not shown the anti-HIV activity even though the compounds are having kinase activity where as the compounds UHAKKM-7, 8 and 9 have shown the high activity in a dose dependent studies and confirmed at IC_{50} concentration.

Objective-II:

In this objective we have studied and analyzed the activity of bis-chalcones, we have studied the affect of inhibitors on early replication of virus by specifically targeting the novel Topo IIβK_{HIV}. Cell viability of the compounds was checked by MTT assay in SupT1 cells. All the compounds exhibited no significant toxicity below 1 micro molar concentration. Compounds have shown the kinase inhibitory activity at pico molar concentration by *Invitro*-phosphorylation assay. The anti-HIV activity of the molecules in inhibiting viral replication was tested using the p24-Assay (ELISA). It was observed that UHAKKM-10 to 12 showed low anti HIV-1 activity even at concentrations higher than 100µM. However, water soluble disodium pyridine dicoumarate molecules UHAKKM-13 to 15 have shown high inhibition at low concentration (IC_{50}<30nM). Results showed that among all the pyridine dicoumarol derivatives UHAKKM-15 showed anti-viral activity with an IC_{50} of 20.65 nM, which was seen to be on par with the widely used and clinically used, Azidothymidine (AZT) that has an IC_{50} of 25nM. It was already reported that Topo IIβ phosphorylation is enhanced during the HIV-1 infection which can be a new target for inhibitors to stop HIV-1 replication. Based on the earlier reports and above results, we have screened these compounds for inhibitory activity of serine phosphorylation. We have carried out

the phosphorylation studies by immune precipitation with Topo IIβ antibody and confirmed with biotinylated anti-phophoserine antibody. The entire active anti HIV-1 molecules (UHAKKM-13, 14 and 15) were checked for the inhibition of Topo II beta phosphorylation at serine in aconcentration dependent phosphorylation studies.Thus results confirmed that the Topo II β phosphorylation is inhibited in a concentration dependent study and among drugs analyzed, UHAKKM-15 showed highest inhibition followed by UHAKKM-14 and UHAKKM-13. In another set of PCR experiments, Bis-chalconesderivativesUHAKKM-10-15 have checked for the anti-HIV activity, compounds UHAKKM-10, 11 and 12 have shown less activity even though the compounds are having kinase activity where as the compounds UHAKKM-13, 14 and 15 have shown the high activity at IC_{50} concentration.

Objective-III:

In this objective, we have analyzed the molecular activities of pyridine dicoumarol and bis-chalcone derivatives on different events of Reverse Transcription process. Both series of molecules have shown that formation of Strong Stop (SS) is not significantly affected, while progression of First Strand Transfer (FST) and furthersubsequent events of reverse transcription were entirely impeded. Thus, results of these studies point out that the TopoIIβK_{HIV} catalyzed phosphorylation of Topo II β is essential for progression of First Strand Transfer (FST) reaction. These results further confirmed by our earlier studies using SiRNA-mediated Topo II β knockdown studies, where it was shown that First Strand Transfer was affected in cells devoid of Topo II β. Thus, confirming theTopo II βphosphorylation is vital requirement for progression of reverse transcription.

Objective-IV:

In this objective, the broad-spectrum activity of pyridine dicoumarol and bis-chalcone derivatives was analyzed. Based on the prevalence of tropism in HIV-1 virus and its infectivity to a wide range of host immune cells, we have analyzed the toxicity of active compounds, pyridine coumarin (UHAKKM-7, 8, and 9) and bis-chalcone derivatives (UHAKKM-13, 14 and 15) in different cell lines (Sup T1, U937, HL2/3 and SKNSH) by cell viability assay (MTT assay) at increasing concentrations. These compounds exhibited no significant toxicity below 10 micro molar concentrations. Evaluation of anti-HIV-1 activity of compounds UHAKKM-7, 8 and 9 in cell lines indicated showed significant inhibition with IC50 below 30nM (IC_{50}<30nM) where as UHAKKM-13, 14 and 15 revealed anti-HIV-1 activity below a concentration of 50nM (IC_{50}<40nM). Pro-viral DNA and Western blot analysis showed significant inhibition by all the active compounds. All the active pyridine derivatives have shown inhibitory activity against the viral subtypes used in the study, but comparatively high inhibition was observed on HIV-$1_{93IN101}$ when compared with all the subtypes tested. Thus results confirmed the broad spectrum activity of Topo II β kinase inhibitors from class pyridine dicoumarol derivatives UHAKKM-7, 8 and 9 and bis-chalcones UHAKKM-13, 14 and 15 with significant inhibitory activity against HIV-1 subtype A, B and C virus.

Conclusion:

Evaluation of biological activity of pyridine derivatives and bis-chalcones showed that charged molecules of pyridine dicoumarol derivatives UHAKKM-7, 8 and 9 and bis-chalcones UHAKKM-13, 14 and 15 were active with following features:

- They significantly inhibited TopoIIβK_{HIV-1} catalyzed Topo II β phosphorylation at serine residues
- Affect proviral DNA synthesis
- Block First Strand Transfer reaction and downstream events during reverse transcription
- Inhibit HIV-1 subtype A, B and C viruses replication

All the above results demonstrate that Topo II β phosphorylation is essential at First Strand Transfer reaction during reverse transcription. Based on the observations, charge of molecule, pyridine-nitrogen orientation and solubility are important factors in conferring *in-vivo* biological activity.

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Publications

Research Article

Future Medicinal Chemistry

Development of pyridine dicoumarols as potent anti HIV-1 leads, targeting HIV-1 associated topoisomeraseIIβ kinase

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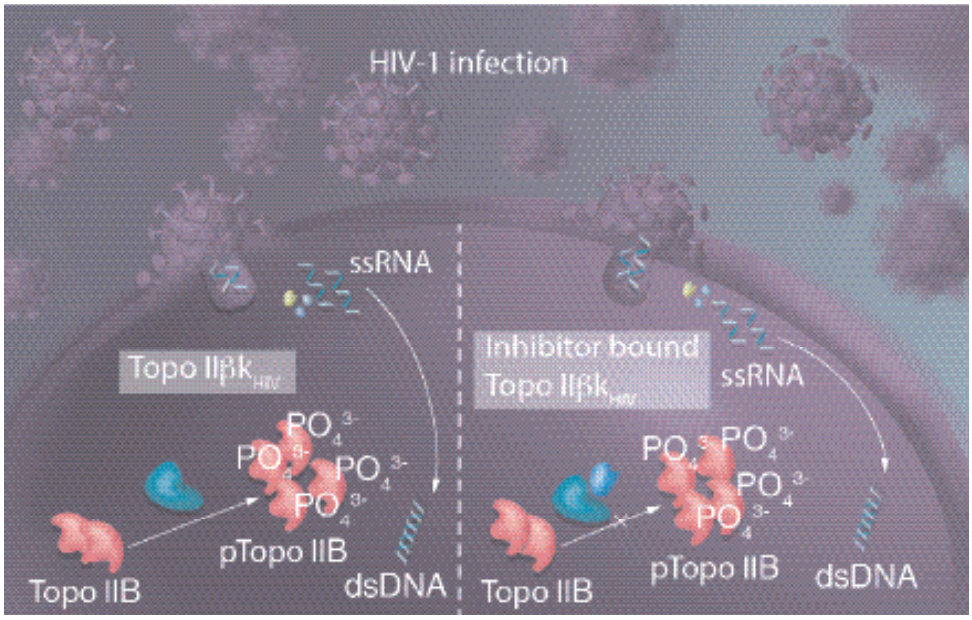
Aim: A structural study of a series of pyridine dicoumarol derivatives with potential activity against a novel Topoisomerase IIβ kinase which was identified in the HIV-1 viral lysate, compounds were designed and synthesized based on a 3D-QSAR study. **Materials & methods:** Based on QSAR model we have designed and synthesized a series of pyridine dicoumarol derivatives and characterized by spectral studies, all the molecules are biologically evaluated by kinase assay, cytotoxicity assay, ELISA and PCR method. **Result:** We demonstrated the achievement of water soluble disodium pyridine dicoumarate derivatives showing high anti-HIV-1 activity (IC_{50} <25 nM) which provides a crucial point for further development of pyridine dicoumarol series as HIV-1-associated topoisomerase IIβ kinase inhibitors for clinical application against AIDS. **Conclusion:** A new class of anti-HIV-1 lead compounds have been designed and tested. Further studies would result in development of novel and potential drugs.



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- **Development of Pyridine Dicoumarols as potent anti HIV-1 leads, targeting HIV-1associated TopoisomeraseIIβ Kinase. Future Med. Chem. (2017) 9(14), 1597–1609**

  Kurumurthy Kammari[a+], Kiran Devaraya[a+], Akhila Bommakanti[a] and Anand K Kondapi[a*]

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  Thechano Merry[a], Prabhakar Maddela[a,*], Kiran Devaraya[b], Anand K. Kondapi[b], Chullikkattil P. Pradeep[c]

Development and Molecular Characterization of Novel HIV-1 Associated Topoisomerase II β Kinase Inhibitors during HIV-1 Replication

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