

PhD Thesis

*Development of Immunosuppressive Indoleamine
2,3-dioxygenase 1 Inhibitors for Chemo-
Immunotherapeutic Advancement*

*A dissertation submitted to the
Indian Institute of Technology Guwahati
as partial fulfilment for the Degree of
Doctor of Philosophy in Chemistry*

By

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To
My Beloved Family



Declaration

28th May, 2021

I hereby declare that the thesis entitled “**Development of Immunosuppressive Indoleamine 2,3-dioxygenase 1 Inhibitors for Chemo-Immunotherapeutic Advancement**” is compilation of research work which was carried out by me under the supervision of Prof. Debasis Manna, Department of Chemistry, Indian Institute of Technology Guwahati. This thesis has been submitted by me to the Department of Chemistry, Indian Institute of Technology Guwahati, for the award of the degree of Doctor of Philosophy. In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

I further declare that this work has not been submitted anywhere else for any degree, diploma, associateship or membership etc. of any Institute or University to the best of my knowledge.



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To whom it may concern

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(Prof. Debasis Manna)



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Abstract

This thesis “***Development of Immunosuppressive Indoleamine 2,3-dioxygenase 1 Inhibitors for Chemo-Immunotherapeutic Advancement***” deals with small molecules for the immunotherapeutic treatment in life threatening disease. This thesis has been arranged into five chapters based on the experimental results which were obtained during the research period.

Chapter 1 elaborates the functions of indoleamine 2,3-dioxygenase 1 (IDO1) enzyme in cancer. This chapter also discuss the importance of developing small molecule-based inhibitors for the domain, IDO1 and gives a small overview of reported inhibitors up to date.

Chapter 2 describes 1*H*-indazole derivatives as a suitable inhibitor of IDO1 enzyme. This chapter has explored that C3-substitution in indole ring generates the substrate, L-tryptophan alike motif which binds with active pocket to behave as antagonist of the enzyme.

Chapter 3 elucidates histidine mimic triazole derivatives which binds with prosthetic heme group in the IDO1 active pocket to show potent inhibitory activity. In addition, rejuvenation of IDO1 mediated immune-suppression with potent triazoles has been discussed in this chapter. *In vivo* antitumor efficacy of the triazole derivatives also has been conversed.

Chapter 4 articulates quinine-based apo-IDO1 targeting inhibitors which displayed robust and selective inhibition in lower nanomolar concentration range. In this chapter, perturbation of IDO1 activity by aqueous heme-inhibitor complex has been exemplified for the first time.

Chapter 5 conveys a comprehensible outcome of a delivery vehicle which has selectively delivered chemo and immunotherapeutic, IDO1 inhibitor into the cancer cells. The induction of immunogenic cell death (ICD) by both chemo and immunotherapeutic drugs in *in vivo* model has been reflected in this chapter.







List of Abbreviation

ADME	Absorption, distribution, metabolism, excretion
S _a	Active site
APC	Antigen-presenting cells
K _a	Association rate
BHK	Baby hamster kidney
K _d	Binding affinity
<i>Bin1</i>	Bridging Integrator-1
BSO	Buthionine sulfoximine
CRT	Calreticulin
CLANs	Cationic lipid-assisted nanoparticle
CAR	Chimeric antigen receptor
CD	Circular dichroism
CD80	Cluster of differentiation 80
CPCSEA	Committee for the Purpose of Control and Supervision of Experimental Animals
CLSM	Confocal laser scanning microscopy
CAC	Critical aggregation constant
Cys	Cysteine
CYP	Cytochromes P450
CTL	Cytotoxic T lymphocytes
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DAMPs	Damage-associated molecular patterns
DCs	Dendritic cells
DFT	Density functional theory
CDCl ₃	Deuterated chloroform
DCM	Dichloromethane
DIAD	Diisopropyl azodicarboxylate
DMF	Dimethylformamide
DMAB	Dimethylaminobenzaldehyde
DMSO	Dimethyl sulfoxide

K _D	Dissociation constant
DTT	Dithiothreitol
Dox	Doxorubicin
DMEM	Dulbecco's Modified Eagle Medium
DLS	Dynamic light scattering
EAC	Ehrlich ascites carcinoma
ESI-MS	Electrospray ionization-mass spectrometry
λ_{em}	Emission wavelength
ECL	Enhanced chemiluminescence
EtOAc	Ethyl acetate
EDTA	Ethylenediaminetetraacetic acid
eIF2 α	Eukaryotic initiation factor 2 α kinase
λ_{ex}	Excitation wavelength
FBS	Fetal Bovine Serum
FESEM	Field emission scanning electron microscopy
FETEM	Field emission transmission electron microscopy
FOXp	Forkhead box protein
GCN2	General control nonderepressible 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gly	Glycine
GSH	Glutathione
EC ₅₀	Half maximal effective concentration
IC ₅₀	Half maximal inhibitory concentration
HPLC	High performance liquid chromatography
HRMS	High-resolution mass spectrometry
His	Histidine
HSA	Human serum albumin
HCl	Hydrochloric acid
d _H	Hydrodynamic diameters
H ₂ O ₂	Hydrogen peroxide
HOBt	Hydroxybenzotriazole
ICD	Immunogenic cell death
IDO1	Indoleamine 2,3-dioxygenase 1

K _i	<i>Inhibitor</i> constant
S _i	Inhibitory substrate binding site
IFN- γ	Interferon gamma
IL6	Interleukin 6
IP	Isoelectric point
Ile	Isoleucine
IPTG	Isopropyl β -D-1-thiogalactopyranoside
JAK	Janus kinases
Leu	Leucine
LiOH	Lithium hydroxide
LB	Luria-Bertani media
MgCl ₂	Magnesium chloride
mTORC	Mammalian target of rapamycin
MALDI-TOF	Matrix assisted laser desorption ionization-time of flight
T _m	Melting temperature
mRNA	Messenger Ribonucleic acid
MeOH	Methanol
K _m	Michaelis constant
miR	Micro RNA
MD	Molecular dynamic
NAs	Nano-aggregates
NDI	Naphthalene-diimide
NK	Natural killer cells
CHES	N-Cyclohexyl-2-aminoethanesulfonic acid
EDC.HCl	N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride
NFK	N-Formylkynurenin
DIPEA	N,N-Diisopropylethylamine
NSCLC	Non-small cell lung cancer
NF- κ B	Nuclear factor kappa B
N ₂	Nitrogen
NMR	Nuclear magnetic resonance
OD	Optical density

O ₂	Oxygen
PD-L1	Programmed death-ligand 1
PDAC	Pancreatic ductal adenocarcinoma model
PME	Particle mesh Ewald
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cell
Phe	Phenylalanine
PMSF	Phenylmethanesulfonyl fluoride
PMA	Phorbol myristate acetate
PBS	Phosphate-buffered saline
DPPC	Phospholipid dipalmitoylphosphatidylcholine
H ₃ PO ₄	Phosphoric acid
PDT	Photodynamic therapy
PEG	Polyethylene Glycol
KCl	Potassium chloride
KOH	Potassium hydroxide
KPB	Potassium phosphate buffer
PD-1	Programmed cell death protein 1
PI	Propidium iodide
PGE ₂	Prostaglandin E ₂
PDB	Protein Data Bank
PpIX	Protoporphyrin IX
QM	Quantum mechanics
ROS	<i>Reactive oxygen species</i>
RBC	Red blood cells
Treg	Regulatory T cells
RU	Response units
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute media
Ser	Serine
STAT	Signal transducer and activator of transcription
siIDO1	Small interfering RNA targeting IDO1
NaCl	Sodium chloride

Na ₂ S ₂ O ₄	Sodium dithionite
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
NaH	Sodium hydride
Na ₂ SO ₄	Sodium sulfate
SARs	Structure-activity relationship
SPR	Surface plasmon resonance
THF	Tetrahydrofuran
TMS	Tetramethylsilane
TLC	Thin layer chromatography
tRNA	Transfer ribonucleic acid
TGF-β	Transforming growth factor beta
TCA	Trichloroacetic acid
TES	Triethylsilane
Tris	Tris(hydroxymethyl)aminomethane
TFA	Trifluoroacetic acid
TCEP	Tris(2-carboxyethyl)phosphine
Tyr	Tyrosine
Trp	Tryptophan
TDO	Tryptophan 2,3-dioxygenase
TNF-α	Tumor necrosis factor alpha
TDLNs	Tumor-draining lymph nodes
Th	T helper cell
Thr	Threonine
K _{cat}	Turnover number
UV-Vis	Ultraviolet–visible
UGT	Uridine 5'-diphospho-glucuronosyltransferase
H ₂ O	Water
DPH	1,6-diphenyl-1,3,5-hexatriene
HBTU	(2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MOPS	3-(N-morpholino)propanesulfonic acid

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

For symbols/units

α	Alpha
Å	Angstrom
atm	Atmosphere
β	Beta
C	Celsius
J	Coupling constant
°	Degree
δ	Delta
Hz	Hertz
K	Kelvin
MHz	Megahertz
m	Meta
μg	Micro gram
μM	Micro mole
mL	Millilitre
mV	Mili Volt
min	Minute
nM	Nano mole
o	Ortho
p	Para
ppm	Parts per million
%	Percent
π	Pi
ps	Pico second
s	Second

Chapter 1

Introduction of IDO1 and Its Inhibitor Towards Immunotherapy

Arresting of host immune-signal is a breakthrough for high mortality in several diseases including cancer. Immunotherapy evokes a powerful therapeutic strategy through inducing ICD.¹ However, tumors stimulate development of several immunosuppressive mechanisms which counteracts the effect of ICD. Few immuno-checkpoint inhibitors like PD-1, CTLA-4 antibody etc. have exhibited a promising approach for cancer treatment by rehabilitating ICD. But, the clinical outcomes for immuno-checkpoint antibody affected a low response in tumor microenvironment.² Additionally, IDO1 and TDO in tumor tissues and TDLNs also interferes in immunosuppression mechanism.³ Overexpression of IDO1 and TDO depletes local tryptophan concentration which activates several kinase pathways to arrest the proliferation of tumor CTL in cancerous microenvironment.^{4, 5} Furthermore, IDO1 has been recognised as an immunomodulator in cancer and IDO1-mediated immunosuppression has 'hogged the lime light' of cancer immunotherapy in recent years.

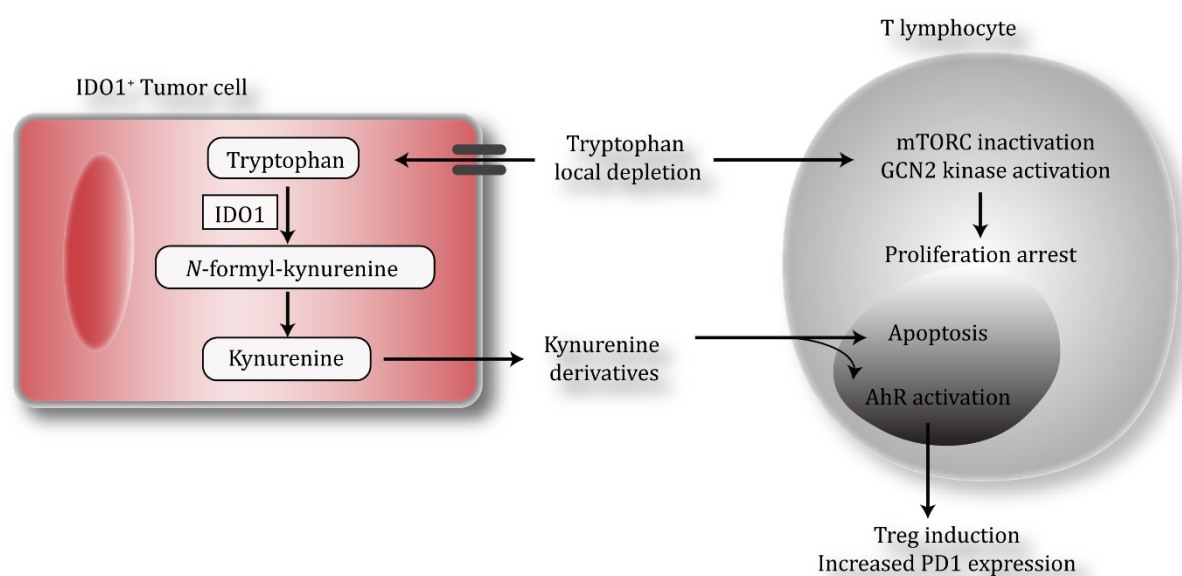
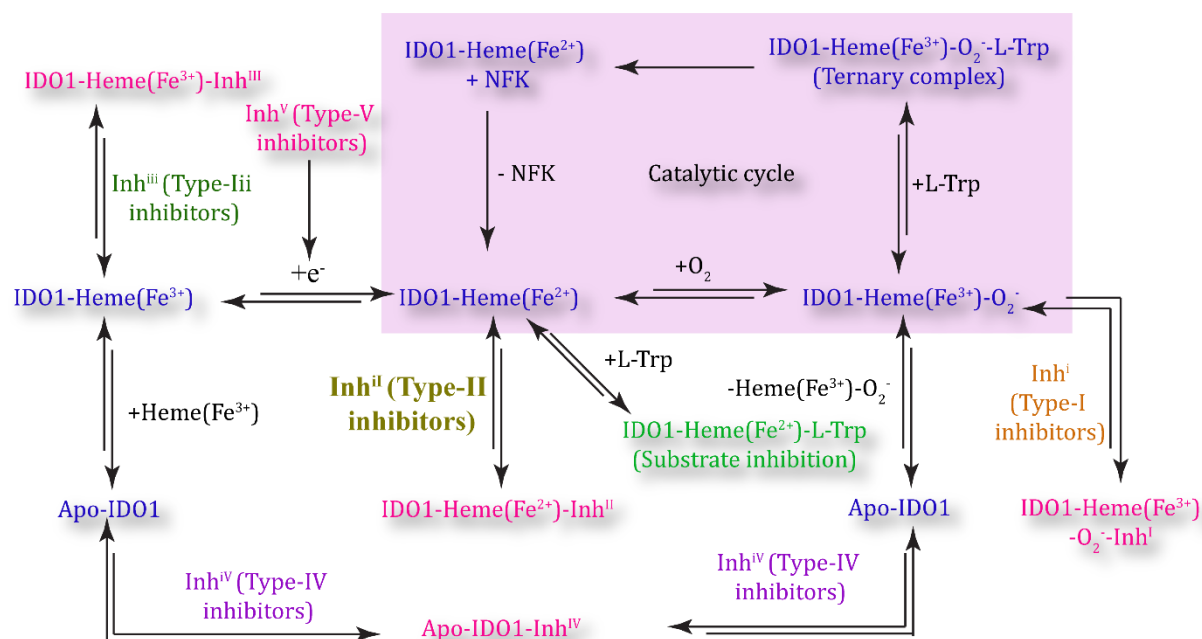


Figure 1.1. Proposed mechanisms by which IDO1-mediated tryptophan degradation inhibits local T cell responses in tumors.

The enormous number of inhibitors with diverse structural entities enriched IDO1 as a potential therapeutic target. Diverse structural classes, including *N*-



Scheme 1.1 IDO1 ligand binding, redox equilibria, and catalytic cycle (colour box). Five different inhibitor types are defined based on their preferential binding to oxygen-bound holo-IDO1 (type I, Inh^I), ferrous holo-IDO1 (type II, Inh^{II}), ferric holo-IDO1 (type III, Inh^{III}), apo-IDO1 (type IV, Inh^{IV}) and catalytic pathway inhibitor (type V, Inh^V). Apo-IDO1 is formed primarily by dissociation of free ferric or oxygen-bound ferric heme. L-Trp recruitment before oxygen binding leads to an unproductive complex (substrate inhibition).

hydroxyamidine, imidathiazole, tryptophan, triazole, imidazole and others were reported as potent IDO1 inhibitor. There are several modes of binding the inhibitors in the IDO1 active pocket. By considering the binding mode, inhibitors are also classified in few categories.⁶ But the outcome of phase 3 trial (ECHO-301), epacadostat, an IDO1 selective inhibitor with pembrolizumab has generated the ambiguity of IDO1 inhibition in cancer-immunotherapeutic treatment. Although clinical trials with the combination of IDO1 inhibitors/immune checkpoint inhibitors have been reinitiated, there is a need to develop new inhibitors or a convenient strategy to reinvestigate the IDO1 inhibition mechanism to execute clinical benefits.⁷ Numerous evidences support the additional benefits of dual chemo and immunotherapeutic drugs to the antitumor signals. Few antineoplastic drugs like doxorubicin, oxaliplatin and others induce ICD through the expression of CRT on

dying tumor cell surface. ICD facilitates the action of DCs against cancerous cells and boosts antitumor immune response.⁸ Several side effects and poor tumor targeting effects of chemotherapeutic drugs have impeded its application in cancer treatment. But lately, the cancer-immunotherapy research has incorporated chemo drugs with IDO1 inhibitors in several clinical trials. Amphiphile-based advanced nano-delivery systems have exhibited a promising tool to transport therapeutic agents into the targeted tissues selectively. The growing number of nano-carriers have been utilized to overcome the limitation of chemotherapeutic agents in triggering anti-immunosuppression response.^{8,9}

Aim of the Thesis

In the present thesis, we have focused on developing small molecule for robust and selective inhibition of IDO1. In addition, we have explored the therapeutic potential of our synthesised inhibitors for the immunotherapeutic treatment of cancer. The entire work is arranged into four chapters as given below. In **chapter 2** we have focused how the substrate, L-Trp mimic indazole derivatives execute its selective IDO1 inhibitory potency. **Chapter 3** mainly deals how triazole derivatives mitigates IDO1 mediated tumor growth. **Chapter 4** elaborates how quinine-based IDO1 inhibitors targets both apo-IDO1 and free heme for the inhibition process. **Chapter 5** corroborates how a delivery vehicle improves chemo and immunotherapeutic, IDO1 inhibitors efficacy for the treatment of cancer.

Chapter 2

Identification of 1*H*-Indazoles Derivatives as Potent IDO1 Inhibitors

The detailed mechanistic investigation of tryptophan catabolism by IDO1 enzyme has revealed that the involvement of C2-C3 double bond of the indole ring.¹⁰ 1*H*-indazoles was structurally similar with the replacement of C2-C3 double bond of indole to C3-N2 double bond.¹¹ Hence, 1*H*-indazoles was a suitable replacer of indole ring of the substrate, *L*-tryptophan. The extensive structural investigation of indazole moiety hypothesised that the 3-substituted 1*H*-indazole might be suitable for IDO1 inhibition. In an attempt, we synthesized a small series of 3-substituted 1*H*-indazole derivatives and examined their IDO1 inhibitory potency. Additionally, several other experiments were conducted with potent 3-substituted 1*H*-indazole to evaluate the

therapeutic potential regarding IDO1 inhibition. Couple of 3-aryl 1*H*-indazole derivatives exhibited low-micromolar level inhibitory activity against purified human IDO1 enzyme. Spectroscopic-based binding studies indicated that the 1*H*-indazoles preferably interacted with the ferric-IDO1 enzyme. Overall, C3-substitution in the 1*H*-indazole ring might interrupt further radical reaction with Fe³⁺-superoxide intermediate leading to a better approach in designing mechanism-based IDO1 inhibitors.

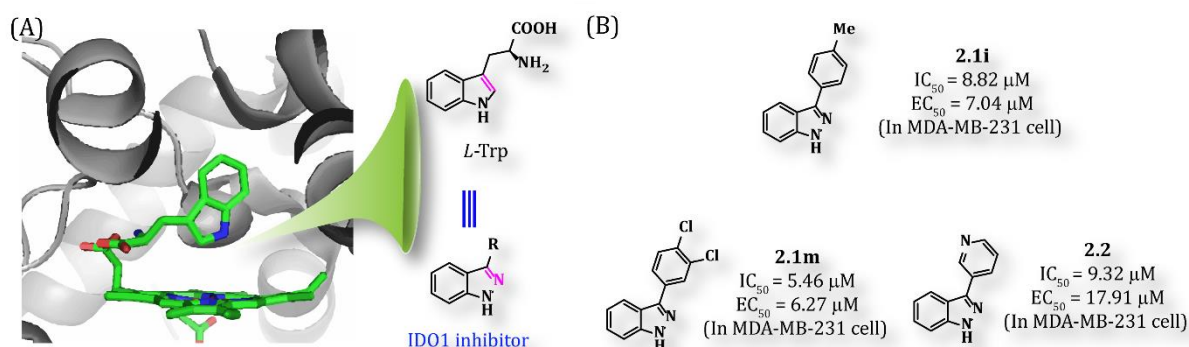


Figure 2.1 Background of designing 1*H*-indazole derivatives as IDO1 inhibitor (A). Image shows the substrate L-Trp binding into IDO1 active pocket. Potent indazole derivatives and their IDO1 inhibitory activity with purified and cellular enzyme (B).

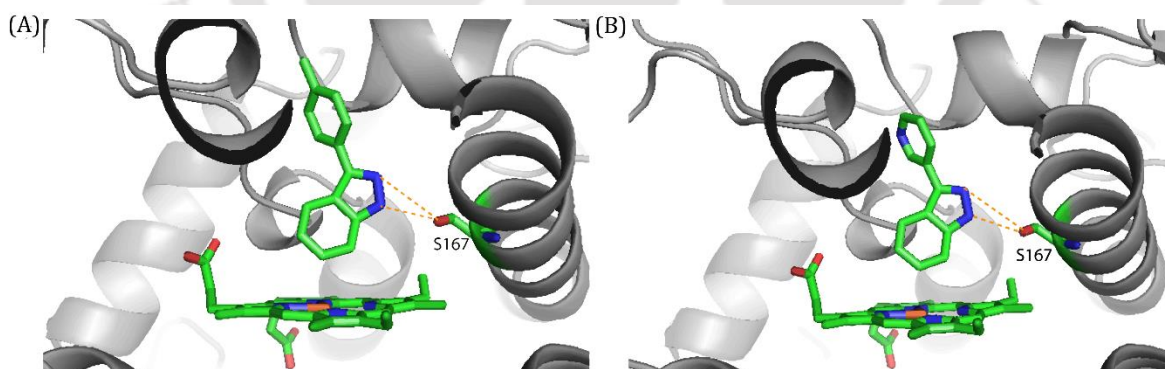


Figure 2.2 Probable mode of interaction of the compounds **2.1i** (A), and **2.2** (B) with the IDO1 enzyme (4PK5). The model structures were generated using AutoDock 4.2.0. Residues involved in interactions through hydrogen bond formation were shown using dashed lines (yellow). Images were generated using PyMol.

Chapter 3

4,5-Disubstituted 1,2,3-triazoles Regulates T Cell Activity and Mitigates Tumor Growth through Effective IDO1 Inhibition

Though, 1*H*-indazole-based inhibitors bound IDO1 active pocket to inhibit the enzyme, the heme binding properties of the developed compounds were not impressive. Hence, in an endeavour to identify effective IDO1 inhibitor, we focussed to develop a new class of IDO1 inhibitors. The 1,2,3-triazole scaffold had been used as an alternative to imidazole scaffold for its efficacy in providing better specificity for IDO1 enzyme over other heme-containing proteins TDO.^{12,13} We hypothesize that the 4,5-disubstituted 1,2,3-triazole could provide higher inhibitory potency and specificity for the IDO1 enzyme because of its probable interaction with both heme-group and Ser-167, a crucial residue in substrate/inhibitor binding to the active site of IDO1 enzyme. Besides, apposite occupation of the active site by the 4,5-disubstituted 1,2,3-triazole derivatives could be advantageous for their higher selectivity and better inhibitory activities for the IDO1 enzyme.

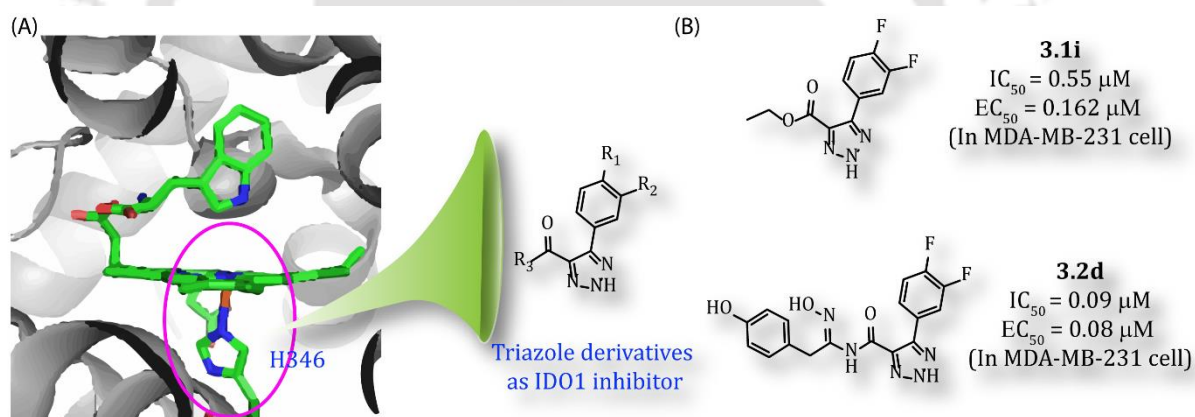


Figure 3.1 Background of designing 4,5-disubstituted 1,2,3-triazole derivatives as IDO1 inhibitor (A). Image shows the substrate L-Trp binding into IDO1 active pocket and H346 bind with heme iron. Potent triazole derivatives and their IDO1 inhibitory activity with purified and cellular enzyme (B).

This study described the synthesis of 4,5-disubstituted 1,2,3-triazole derivatives in IDO1 inhibition. The comprehensive biochemical and biophysical studies identified 4,5-disubstituted 1,2,3-triazole compounds as the potent and selective inhibitors IDO1 enzyme. Additionally, this studies also demonstrated controlled regulation of T cell -

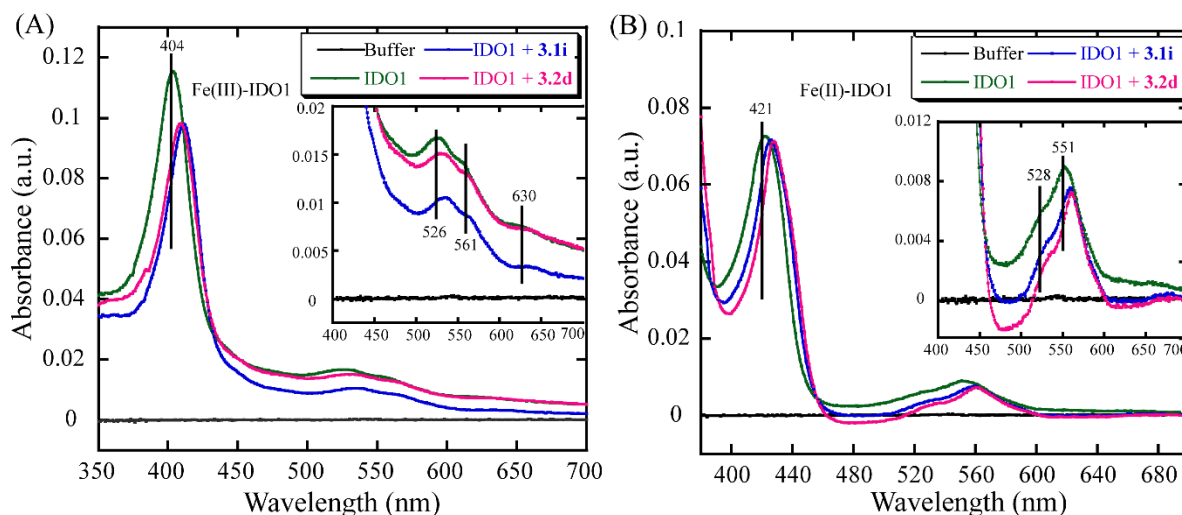


Figure 3.2 Absorption spectra of ferric-IDO1 (A) and deoxyferrous-IDO1 enzyme (B) in the absence and presence of the compounds (20 μ M) in 100 mM phosphate buffer at pH 6.5. [IDO1] = 0.65 μ M. The ferrous-deoxy reaction environment was generated by adding $\text{Na}_2\text{S}_2\text{O}_4$ to the solution under N_2 atmosphere.

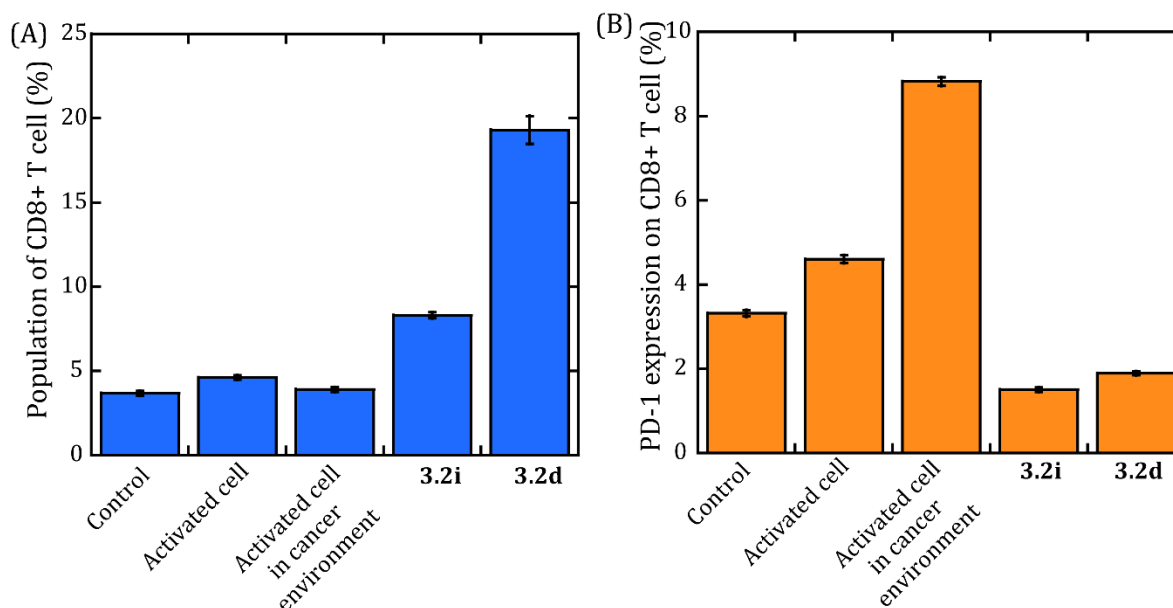


Figure 3.3 T cell activity studies in the presence of the compounds. Regulation of the CD8+ T cells in the tumor micro-environment ($P < 0.0001$) in the presence of the compounds (A). Regulation of the PD-1 expression on CD8+ T cells in tumor micro-environment ($P < 0.0001$) in the presence of the compounds (B). The results were found statistically significant post statistical analysis with one-way ANOVA.

activity to display excellent *in vivo* antitumor efficacy in female Swiss albino mice by the potent 4,5-disubstituted 1,2,3-triazole compounds. Overall, this study described the first example of 4,5-disubstituted 1,2,3-triazole-based IDO1 inhibitors with potential applications for immunotherapeutic studies.

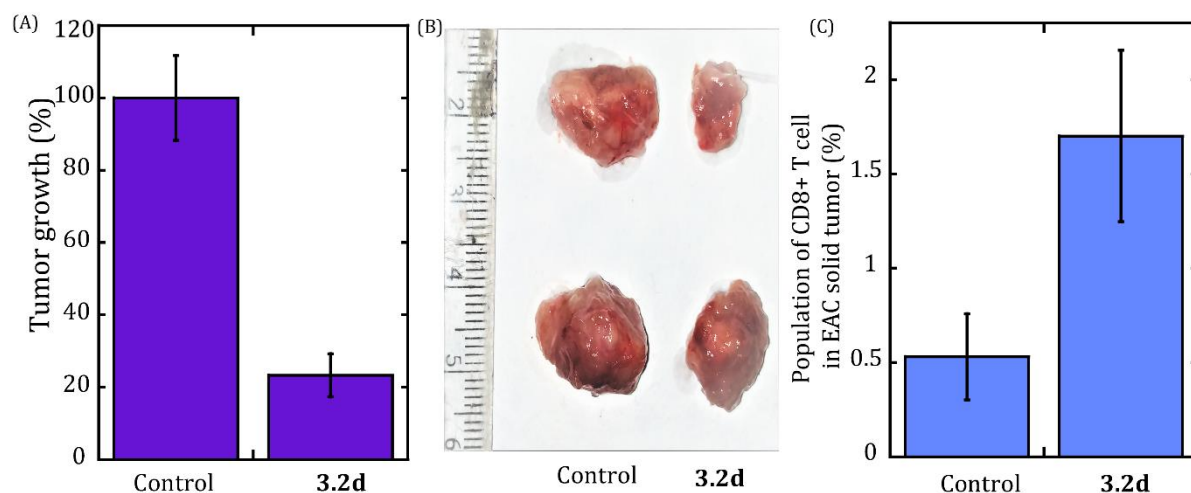


Figure 3.4 The effect of compounds (5 mg/kg body weight) on the growth of EAC solid tumor model in female Swiss albino mice (A, B). The compounds were injected intravenously at alternate days from the 5th day of the tumor implant. CD8+ T cell population *in vivo* solid tumor (C).

Chapter 4

Inhibition of Immunosuppressive IDO1 Enzyme by Targeting the Heme and Apo-form

Coordination with heme-iron and inherent binding affinity to the L-Trp binding pocket is the most common IDO1 inhibition mechanism of the reported inhibitors, including our previous indazoles and triazoles derivatives. Recently, it was demonstrated that heme-binding to the IDO1 enzyme is a dynamic and reversible process and around 85% of IDO1 exists as the heme-free conformation of the enzyme (apo-IDO1) under the cellular environment.¹⁴ Subsequently, the accumulation of free heme-cofactor due to the formation of apo-IDO1 in tumor cells over-activates the other heme-containing proteins, augments the expression of heme exporters and also affects the turnover rate of the IDO1 enzyme, suggesting that not only the heme and its precursor but also the heme biosynthetic pathway is stimulated in cancer cells. The elevated level of the free-heme group is also responsible for the increase in

the basal production of ROS, which facilitates the survival of cancer cells.¹⁵ Hence, the development of apo-IDO1 ligand and reduction of the level of free heme could be advantageous and a practical one in controlling the side effects due to the overexpression of IDO1 and generation of free heme in cancer cells. The quinine and its derivatives are known to bind the heme *via* its quinoline moiety and prevent hemozoin formation during malaria.¹⁶

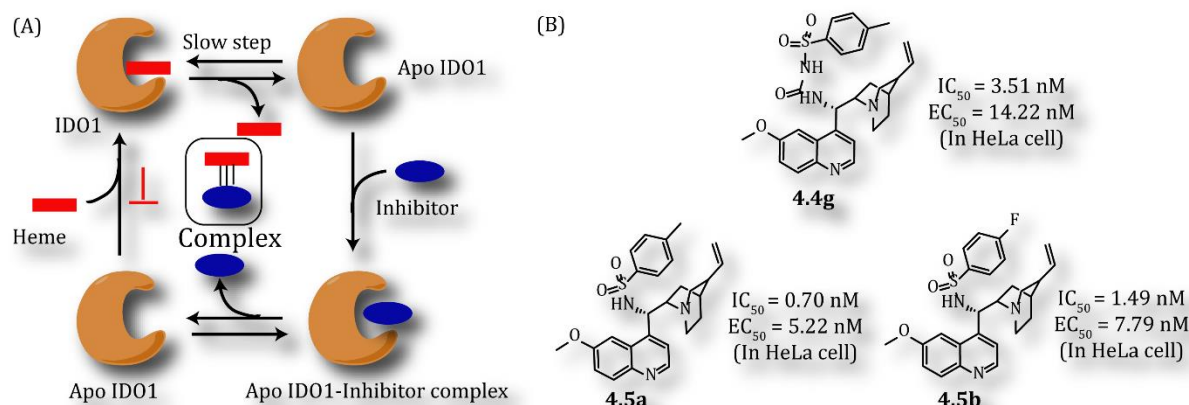


Figure 4.1 A schematic diagram depicts the mode of IDO1 inhibition in the presence of quinine derivatives (A). Potent quinine derivatives and their IDO1 inhibitory activity with purified and cellular enzyme (B).

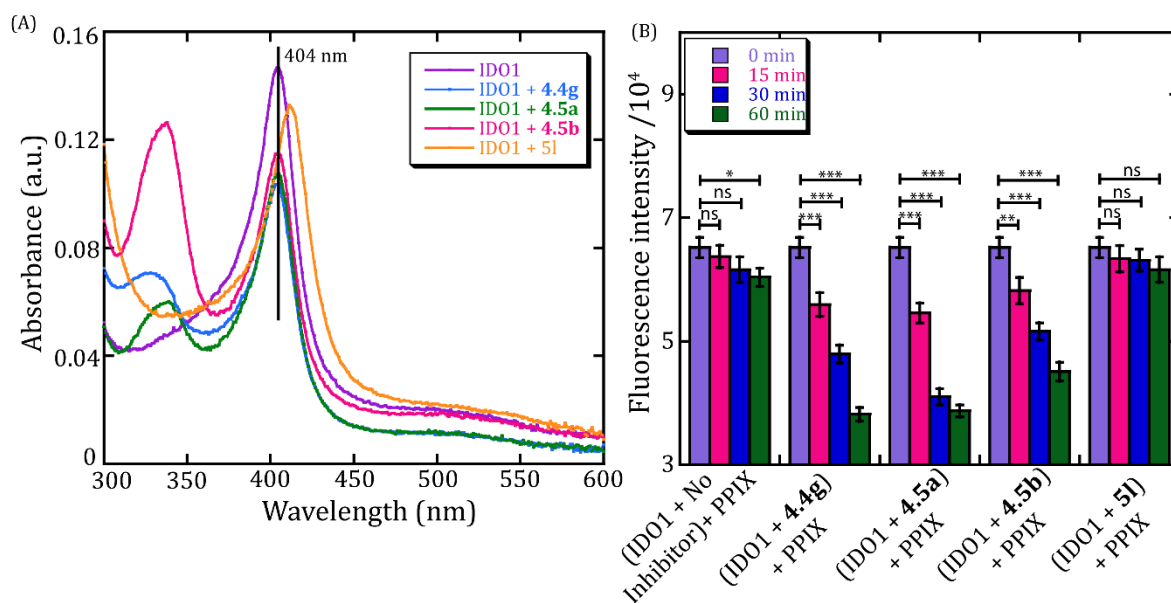


Figure 4.2 Absorption spectra of ferric-IDO1 enzyme (0.9 μ M) in the absence and presence of the compounds (10 μ M) in 100 mM phosphate buffer at pH 6.5 after 15 min incubation (A). The change in PpIX fluorescence upon the addition of incubated

IDO1 (0.75 μ M) in the presence and absence of compounds (25 μ M) at various times (B). The statistical analysis was performed with one-way ANOVA ($P < 0.05$).

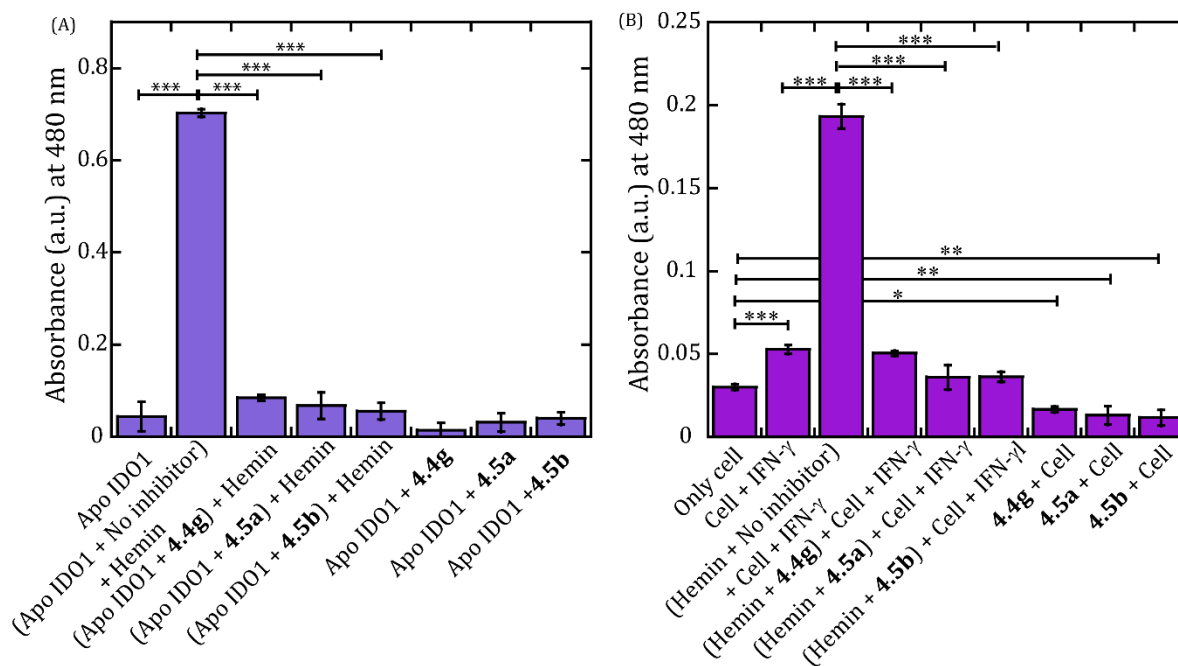


Figure 4.3 The activity of apo-IDO1 in the absence and presence of compound and/or hemin (A). Activities of the compounds in the absence and presence of extracellular hemin in HeLa cells (B). Activities were calculated by HPLC-based kynurenin assay. The statistical analysis was performed with one-way ANOVA ($P < 0.05$).

Inspired by the heme-binding ability of the quinine, we developed a series of quinine derivatives apposite for binding to apo-IDO1. Comprehensive biophysical and cellular studies showed that quinine derivatives effectively inhibit the activity of IDO1. Mechanistic studies revealed that the potent quinine derivatives competed with heme for binding to apo-IDO1 and perturbed its reversible binding propensity to apo-IDO1 *via* the formation of a heme-inhibitor complex. This IDO1 inhibitory pathway could provide new avenues to immunotherapy-based drug discovery strategies.

Chapter 5

Selective Co-delivery of IDO1 Inhibitor and Chemotherapeutic Drug Improves Immunogenic Cell Death of Cancer Cell

The outcome of phase 3 trial (ECHO-301), epacadostat, an IDO1 selective inhibitor with pembrolizumab has generated the ambiguity of singly IDO1 inhibition in

cancer-immunotherapeutic treatment. Numerous evidences support the additional benefits of dual chemo and immunotherapeutic drugs to the antitumor signals. Few antineoplastic drugs like doxorubicin, oxaliplatin and others induce ICD through the expression of CRT on dying tumor cell surface.³ Several side effects and poor tumor targeting effects of chemotherapeutic drugs have impeded its application in cancer treatment. Hence, there is a need to develop a convenient strategy to improve chemo and immune drug's efficacy in cancerous cells selectively. Of late, stimuli-responsive NAs revealed a promising strategy for the delivery of antineoplastic drug specifically for cancer treatment.^{17, 18, 7}

In this study, we designed and synthesized a novel GSH responsive amphiphile, which generated a stable nanoaggregate in aqueous environment. A wide range of biophysical experiments supported the dual loading and delivery of doxorubicin and immunotherapeutic drug, IDO1 inhibitor. It also exhibited selective drug release profile in GSH environment. Additionally, the potent NAs exhibited selective cellular cytotoxicity and improved dual chemo and immune-drug uptake process particularly in cancer cells. Moreover, the immunogenic antitumor efficacy was also augmented with the dual drug loaded NAs in the female Swiss albino mice model.

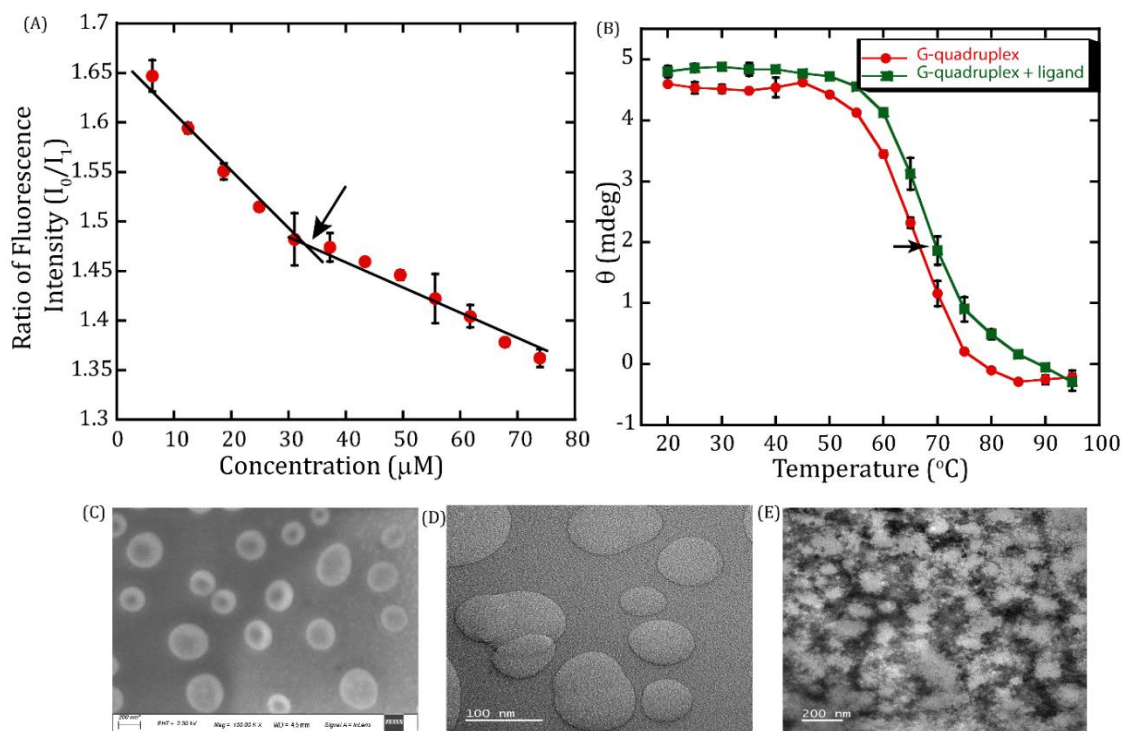


Figure 5.1 CAC of the peptide-amphiphile in aqueous solution (A). The fluorescence intensity of NDI core is monitored with increasing concentration of amphiphile. G-

quadruplex stabilisation in presence of **C6-NDI-SH** ligand (B). CD-based G-quadruplex (10 μ M) melting assay in absence and presence of ligand (15 μ M). FESEM (C) and FETEM (D) image of NAs. FETEM image of NAs in presence of GSH (E).

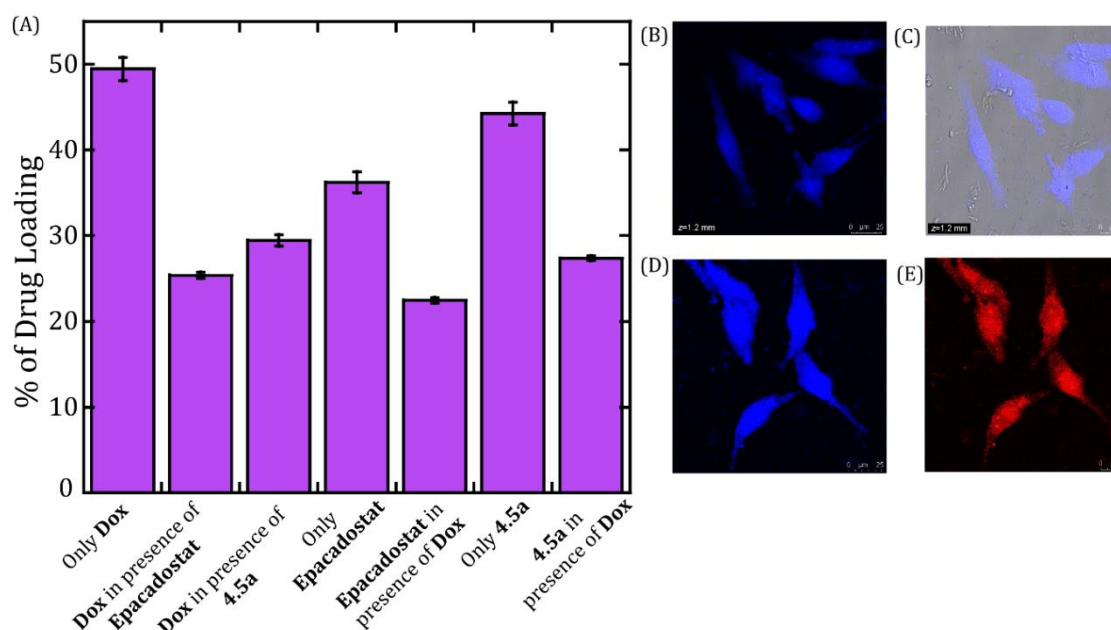


Figure 5.2 Different drug loading efficacy of the NAs (A). Cellular uptake of NAs and drug loaded NAs in MDA-MB-231 cells. Confocal image of MDA-MB-231 cells after 8 h incubation of NAs (B, C). Confocal image of MDA-MB-231 cells after 8 h incubation of **Dox@NAs** (D, E).

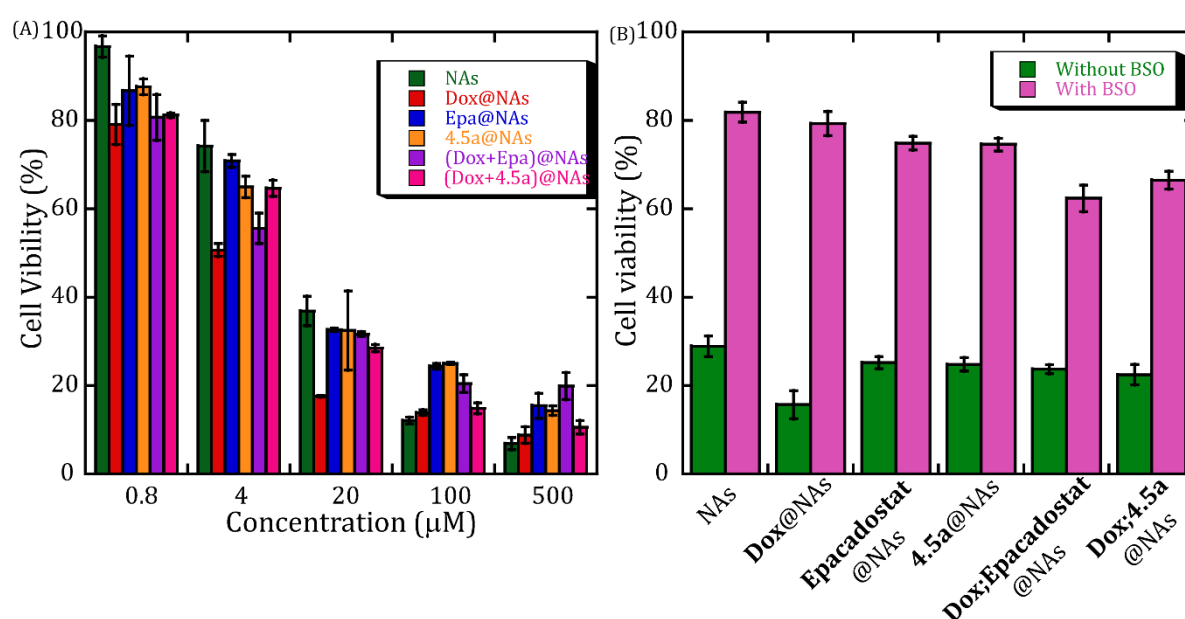


Figure 5.3 Cellular toxicity of drug@NAs in MDA-MB-231 cells after 72 h incubation (A) and in presence of BSO (B).

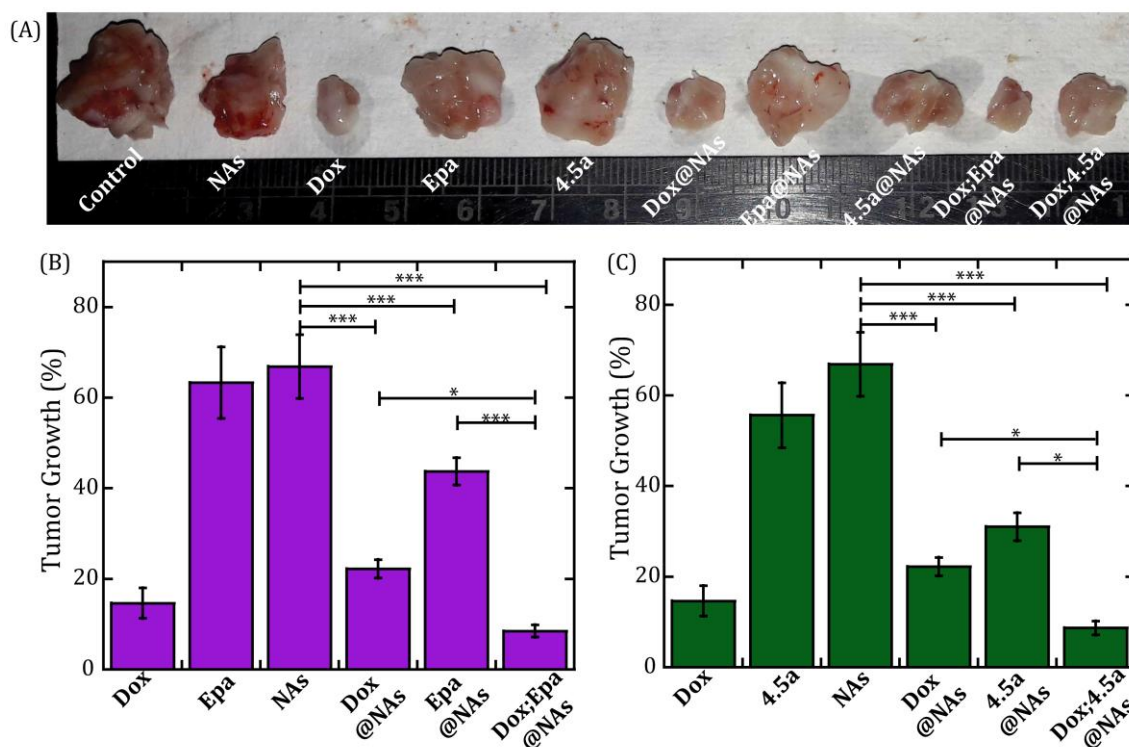


Figure 5.4 Size of solid tumor in the female Swiss albino mice after 15th day of the treatments (A). The Ehrlich ascites carcinoma (EAC) cells were injected into the thigh muscle of the Swiss albino mice to grow the tumor. Level (%) of tumor growth after the 15th day of the treatments (B and C).

Conclusion

In this thesis, we have successfully described the role of IDO1 in immunosuppression process in different life-threatening diseases especially cancer. Additionally, the importance of IDO1 inhibition has been described in **Chapter 1**. The L-Trp mimic *1H*-indazole derivatives (in **Chapter 2**) shows moderate IDO1 inhibitory activity. Though, *1H*-indazole derivatives occupies active pocket, it is unable to bind heme-iron which might be a possible reason for its moderate inhibition activity. The 4,5-disubstituted 1,2,3-triazoles (In **Chapter 3**) binds with both ferric and ferrous-heme to perturb IDO1 activity. Triazoles also have shown T cell activity which controls tumor growth. Apart from the heme binding inhibitors, dual apo and heme targeting quinone derivatives have shown a robust and selective IDO1 inhibitory activity (In **Chapter 4**). To improve the efficacy of IDO1 inhibitor along with chemotherapeutic drug in cancer cell, a novel peptide-based NAs have been designed (In **Chapter 5**). The NAs selectively deliver chemo and immuno drugs and improve ICD of the

cancer cells. Overall, this thesis deals with different IDO1 inhibitors for the improvement of cancer immunotherapy.

Future Prospects

This thesis elucidates different potent IDO1 inhibitors and novel delivery techniques for improvement of drug efficacy into the targeted cells. Initial biophysical and cellular data implies their therapeutic potential against immunotherapeutic treatments. Best inhibitory potency has been obtained with apo-IDO1 targeting quinine derivatives (a natural product derivative, having drug likeness properties). Hence, these quinine derivatives can have profound application in therapeutic use in the near future. But several other experimental evidences remain to conclude their potential against several life-threatening diseases. Recently, delivery vehicles have been tremendously used for targeted delivery of drugs in cancer cells. We have also developed a stimuli-responsive vehicle which can deliver dual chemo and immune drug to the cancer cells. The vehicle has also shown target specific toxicity specifically in cancer cells which gives additional benefit for inducing apoptosis in cancer cells. This arena of vehicle toxicity can be explored in the future. Hope, these inhibitors and technique could be the 'spot-light' of cancer immunotherapy in due time.

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Chapter 1

Introduction of IDO1 and Its Inhibitor Towards Immunotherapy





1.1. Introduction

Arrest of host immune-signal is a breakthrough for high mortality in several diseases including cancer. Boosting our immune system to fight against cancer added a new dimension to the battle against cancer and its immunotherapy can be considered as 'fifth pillar' in this regard.¹ The drawbacks of the traditional approach like surgery, chemo, radiation and targeted therapy in cancer research can be upgraded by using alternative immunotherapy. Immunotherapy leads the cancer research through specific and adaptive action of the immune cell towards the targeted milieu. Immunotherapy evokes a powerful therapeutic strategy through inducing ICD.² However, tumors stimulate development of several immunosuppressive mechanisms which counteracts the effect of ICD. The binding of cellular surface protein, CD80 or PD-L1 on cancerous tissues with CTLA-4 or PD-1 on immune cells involves immune-hijacking mechanism. Few immuno-checkpoint inhibitors like PD-1, CTLA-4 antibody etc. have exhibited a promising approach for cancer treatment by rehabilitating ICD. But, the clinical outcomes for immuno-checkpoint antibody affected a low response in tumor microenvironment.³ On that account, an auxiliary strategy has been demanded to restore the immune system to oppose cancer like life threatening disease. In addition, IDO1 and TDO in tumor tissues and TDLNs also interferes in immunosuppression mechanism.⁴ Overexpression of IDO1 and TDO depletes local L-Trp concentration which activates several kinase pathways to arrest the proliferation of tumor CTL in cancerous microenvironment.^{5, 6} Furthermore, IDO1 has been recognised as an immune-modulator in cancer and IDO1-mediated immunosuppression has 'hogged the lime light' of cancer immunotherapy in recent years.

The enormous numbers of inhibitors with diverse structural entities enriched IDO1 as a potential therapeutic target. Diverse structural classes, including *N*-hydroxyamidine, imidathiazole, tryptophan, triazole, imidazole and others had been reported as potent IDO1 inhibitor.⁷ These inhibitors bind the IDO1 active pocket in diverse modes to perturb IDO1 activity.⁸ But recently the outcome of phase 3 trial (ECHO-301), epacadostat, an IDO1 selective inhibitor with pembrolizumab has generated the ambiguity of IDO1 inhibition in cancer-immunotherapeutic treatment. Although clinical trials with the combination of IDO1 inhibitors/immune checkpoint

inhibitors have been reinitiated, there is a need to develop new inhibitors or a convenient strategy to reinvestigate the IDO1 inhibition mechanism to execute clinical benefits.⁹

This entire chapter describes a brief journey of the recent involvement of IDO1 enzyme in cancer immunotherapeutic treatment. Herein, it also describes the details mechanism of IDO1 involvement in immune suppression process into the tumor microenvironment and the development strategy for several IDO1 inhibitors. This chapter also survey the reported potent IDO1 inhibitors with their clinical importance. Finally, we have proposed our hypothesis to inhibit IDO1 for reliving the deadly diseases, cancer.

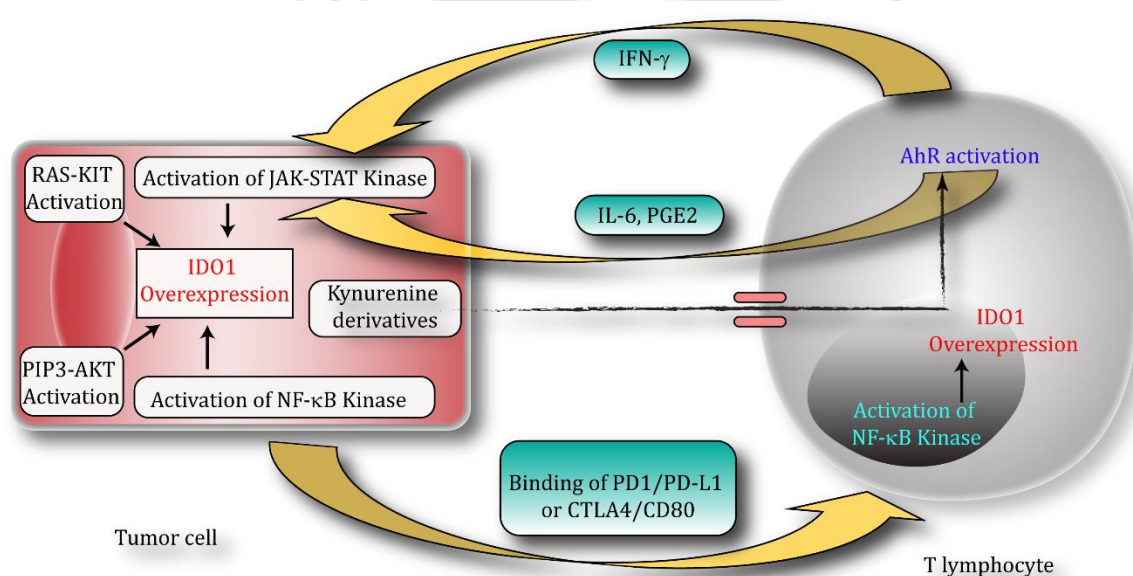


Figure 1.1 Upstream regulation of IDO1 enzyme in cancer microenvironment by several signalling pathways.

1.2. Upstream regulation of IDO1

The current research reveals that tumor associated immunogenic microenvironment is manipulated by excessive production of IDO1 enzyme (2,3).¹⁰ In adult humans, IDO1 is expressed in most of the tissues under physiological conditions, but its expression crosses borderline in several cancer cells, immune cells and stromal cells. Overexpression of IDO1 is laid out by few uncontrolled signalling pathways which includes majorly IFN- γ loop. During inflammation, immune-cells releases several inflammatory molecules, such as IFN- γ , TGF- β , TNF- α , PGE2 and DAMPs. IFN- γ compromises its anticancer function and activates JAK-STAT

pathways to overexpress IDO1.¹²⁻¹⁴ The kynurenine metabolites activate AhR in immune-cells to produce IL6 and PGE2 which further activates JAK-STAT pathway. In addition, few immune checkpoints PD-1/PD-L1 and CTLA-4/CD80 interactions also upregulates IDO1 through upstreaming of NF- κ B signaling pathways in both immune-cell and tumor cell. TGF- β stimulates IDO1 production in CD80 dendritic cells (48).¹⁵ COX-2 and PGE2 also triggers PKC and PI3K pathways which actuates IDO1 in cancer milieu. RAS-Kit signalling pathways like multiple upstream pathways regulates IDO1 expression in cancerous tissues (40-42).¹⁶⁻¹⁸ Diverse upstream pathways regulate cancer-suppression gene, *Bin1* production which further participates in IDO1 overexpression.¹⁶ Overall, cancer milieu enriches with IDO1 enzyme through several cellular signaling pathways.

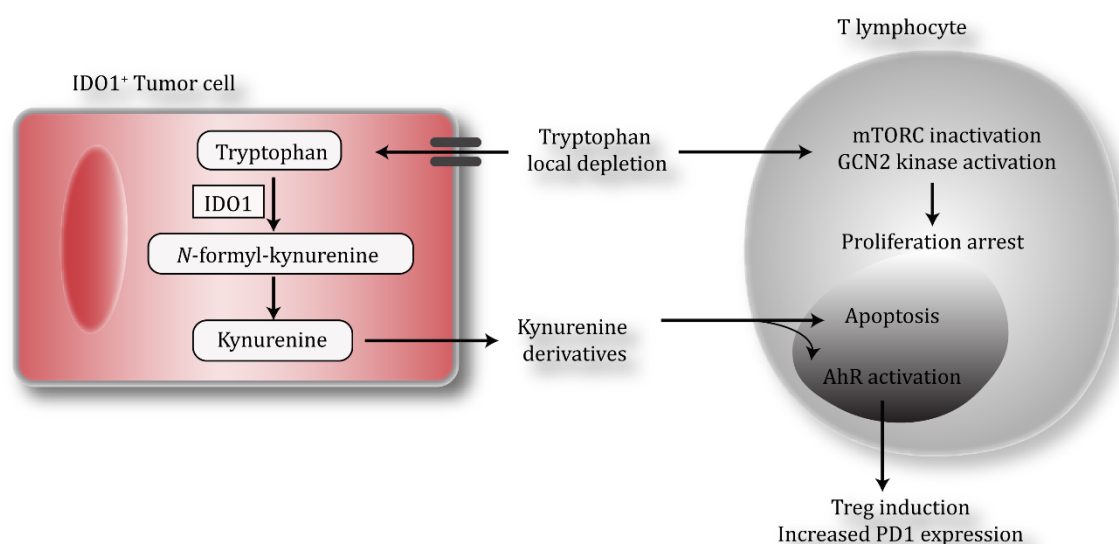


Figure 1.2 Mechanisms of IDO1-mediated L-Trp degradation which inhibits local T cell responses in tumor microenvironment.

1.3. IDO1 mediated immunosuppression and inflammation towards cancer

The participation of IDO1 in cancer occurs mainly through two major aspects. Firstly, cancer cells express IDO1 unboundedly and secondly, overexpressed IDO1 activates several immunosuppressive and inflammatory pathways in cancer microenvironment to secure the cancer cells.^{19,20} The immunosuppressive action of IDO1 enzyme mainly lies into the catabolism of an essential amino acid, L-Trp. Several metabolites like kynurenine, kynurenic acid, 3-hydroxy kynurenine, excitotoxin quinolinic acid and others mainly regulates local immune responses.

IDO1 activates three major effectors GCN2, mTORC and AhR to immunosuppression in cancer milieu. IDO1 mediated depletion of Trp level and accumulation of uncharged Trp-tRNA activates GCN2 kinase, which activates eIF2 α via phosphorylation. GCN2 activation attenuates RNA transcription and protein synthesis specifically in T effector cells, resulting in the cell cycle arrest and/or apoptosis.^{13, 21} Inhibition of fatty acid synthesis in human primary T cells via IDO1-activated GCN2 also suppresses T cell proliferation and effector function.²² Dysregulation of body Trp level by IDO1 also regulates the function of mTORC which triggers autophagy and anergy in T cells in tumor microenvironment.^{12, 23} Interference of IDO1-mediated mTORC suppression enhances Treg cell function and reduces the Th1, Th2, and Th17 lineages differentiation.^{24, 25} Kynurenine and its downstream metabolites, kynurenic acid have been recognized by intracellular receptor, AhR.²⁶ AhR regulates the functions of a plethora of cells of both the innate and adaptive immune system like DCs, macrophages, NK cells, ILCs, Th17 cells, Th22 cells, and Treg cells. AhR also promotes the transcription of IL-10 and IL-6.^{27, 28} IL-6 further amplifies IDO1-mediated kynurenine production and AhR activation. IL-10 promotes the proliferation, maintenance and differentiation of FOXP+ Treg cells and enhances the immune-escaping of cancer cells.²⁹⁻³¹ IDO1 not only sticks into the immunosuppressive mechanism for cancer activity, but also behaves as a crucial operator of 'tumor-promoting' inflammation. In addition, IFN- γ also participates in anticancer functions via decreasing of the tumor neovascularization.^{32, 33} The IFN- γ mediated IDO1 expression promotes the cancer progression via the suppression of IFN- γ level in cancer milieu that reflects on anti-neovascular effect of IFN- γ . The IL-6 has been also implicated as a pro-neovascular factor of cancer.³⁴ As a consequence, involvement of IDO1 in pro-tumorigenic inflammatory neovascularization is another key modulating path of cancer activities. Thus, overexpression of IDO1 enzyme is a witness for cancer survival against immune-cells.

1.4. Subduing of IDO1 functions in preclinical development

Involvement of IDO1 in immune suppression during cancer development urge to an attractive therapeutic remedy in cancer treatment. Trimming the functions of IDO1 with IDO1 expression inhibitors, IDO1 vaccination and IDO1 inhibitors have been recognised the fruitful approach against cancer. IDO1 expression was

downregulated by miR-153 in colon cancer cell. Only miR-153 failed the apoptosis of cancer cell, but tumor growth rate was significantly suppressed in combination with CAR presenting T cells in mice model.³⁵ Some IDO1 peptides evoked specific CD8⁺ T cells which recognised and killed IDO1-expressing cancerous cell.³⁶ Another IDO1-derived human leukocyte antigen A2-restricted epitope able to reduce Treg cell population in advanced metastatic NSCLC patients after sixth dose of the vaccine. The promising IDO1 vaccination had promoted several clinical trials in combination with ipilimumab, CTLA-4 inhibitors or nivolumab, PD-1 monoclonal antibody.^{37, 38} Currently, few IDO1 inhibitors in combination with immune checkpoint inhibitors have strongly manifested their therapeutic potential and come in the spotlight of immune-oncology field. Several potent IDO1 inhibitors with versatile structural scaffolds, including L-Trp, quinone, imidazole, imidathiazole, *N*-hydroxyamidine, triazole and others have been reported.⁷ But only few of them displayed their therapeutic potential. The appalling failure of epacadostat in the phase III trials by Incyte and Merck with KEYTRUDA® (pembrolizumab) simply directed the IDO1 research towards the combination therapy.⁹ In consequences, there is a demand of discussion on inhibition mechanism for better understanding of designing IDO1 inhibitors.

1.5. IDO1 mediated L-Trp catabolism

Understanding of L-Trp catabolism is important for clinical aspects as it is the key step of the IDO1-mediated immune suppression mechanism. The initial and rate-determining step of L-Trp catabolism i.e. the kynurenine pathway is mainly controlled by hIDO1, hTDO and hIDO2 enzymes. Continuous-flow resonance Raman spectroscopy and several QM/MM simulation studies support that both hIDO1 and hTDO follows the two-step ferryl-based mechanism where the reaction starts with the natural substrate, O₂ and Trp binding to the deoxy ferrous enzyme to form the active ternary complex.³⁹⁻⁴² The ternary complex rapidly converts to ferric superoxide electronic configuration which further generates Trp-epoxide and ferryl-type intermediate (via an alkylperoxo transition state). The recombination of ferryl moiety with Trp-epoxide generates the product NFK and transfers the enzyme into inactive ferric-IDO1.⁴³ The relatively inert ferryl intermediate requires favourable regio-orientation with respect to the Trp-epoxide to perform the reaction.

Altogether, the natural substrate, O₂ and the redox state of heme are responsible for IDO1/TDO-mediated Trp catabolism.

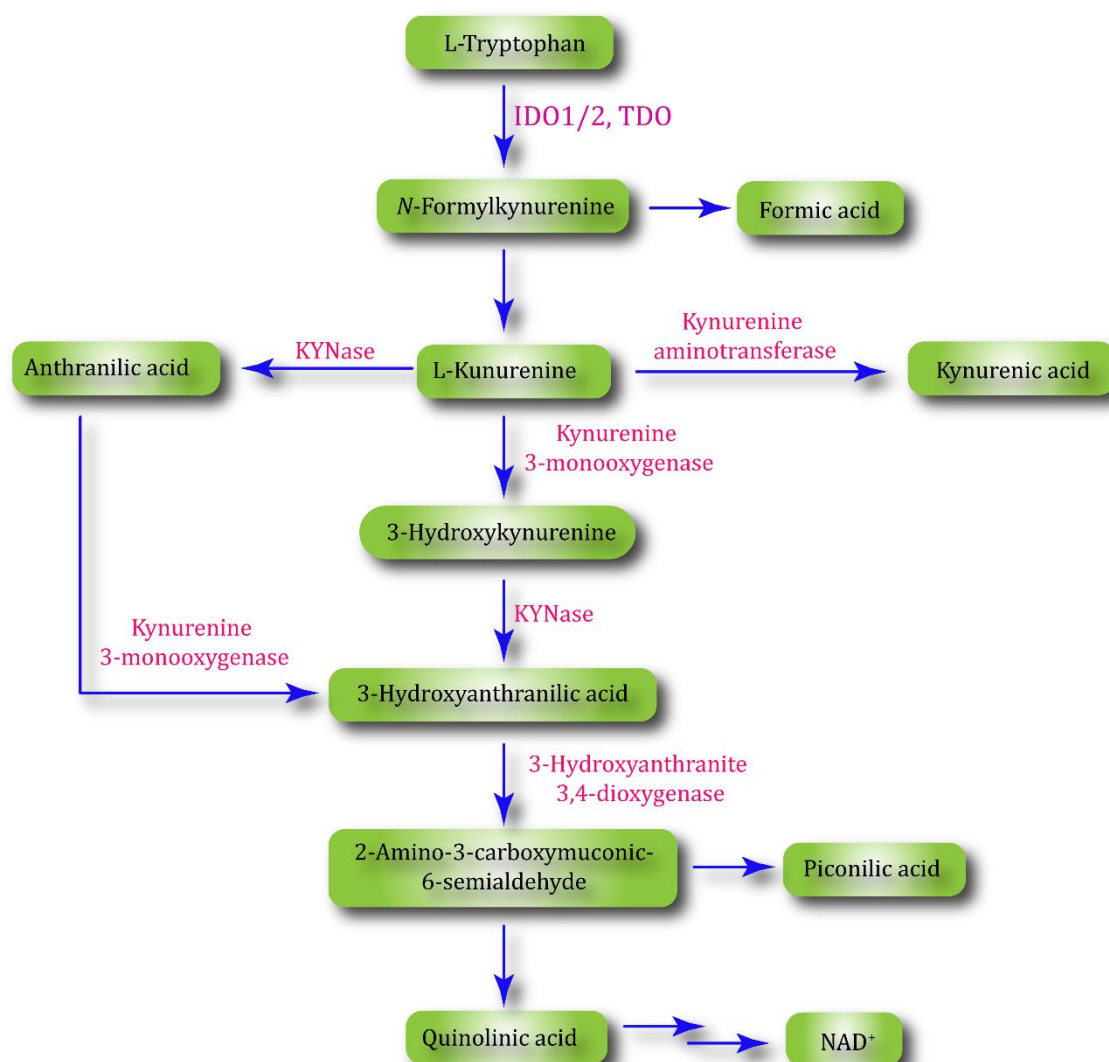


Figure 1.3 Schematic representation of L-Trp catabolism by IDO1, IDO2 and TDO in kynurenine pathways.

The active site of IDO1 enzyme has an influential role in Trp catabolism process. The catalytic site of IDO1 is composed of a prosthetic heme group (Heme-Porphyrin IX), S_a (containing partially hydrophobic pocket A and partially hydrophilic pocket B) to the distal site of heme group and a unique S_i site to the proximal site of heme group. Trp binds the S_a site of IDO1 where the ammonium group makes a H-bonding with 7-propionate group of heme. The carboxylate group participates in H-bonding with Arg231 in pocket B. The indoleamine moiety of the Trp is H-bonded to Ser167 through a bridging water and also participates in hydrophobic interaction with

residues in pocket A. The occupancy of S_i site with Trp induces structural change of S_a site which perturbs the recombination process of the ferryl-intermediate with Trp-epoxide. Additionally, Trp binding in S_i site facilitates the movement of Phe270 residue to block water tunnel which perturbs O_2 delivery into the S_a site during enzyme turnover.⁴⁴ In addition, heme cofactor in IDO1 is dynamic and reversible and it depends on oxidation state of heme-iron.⁴⁵ Overall, the oxidation state of heme group and catalytic site of IDO1, mainly Ser167, Arg231 and Phe270 residues are important in Trp binding and IDO1-mediated specific catabolism process.

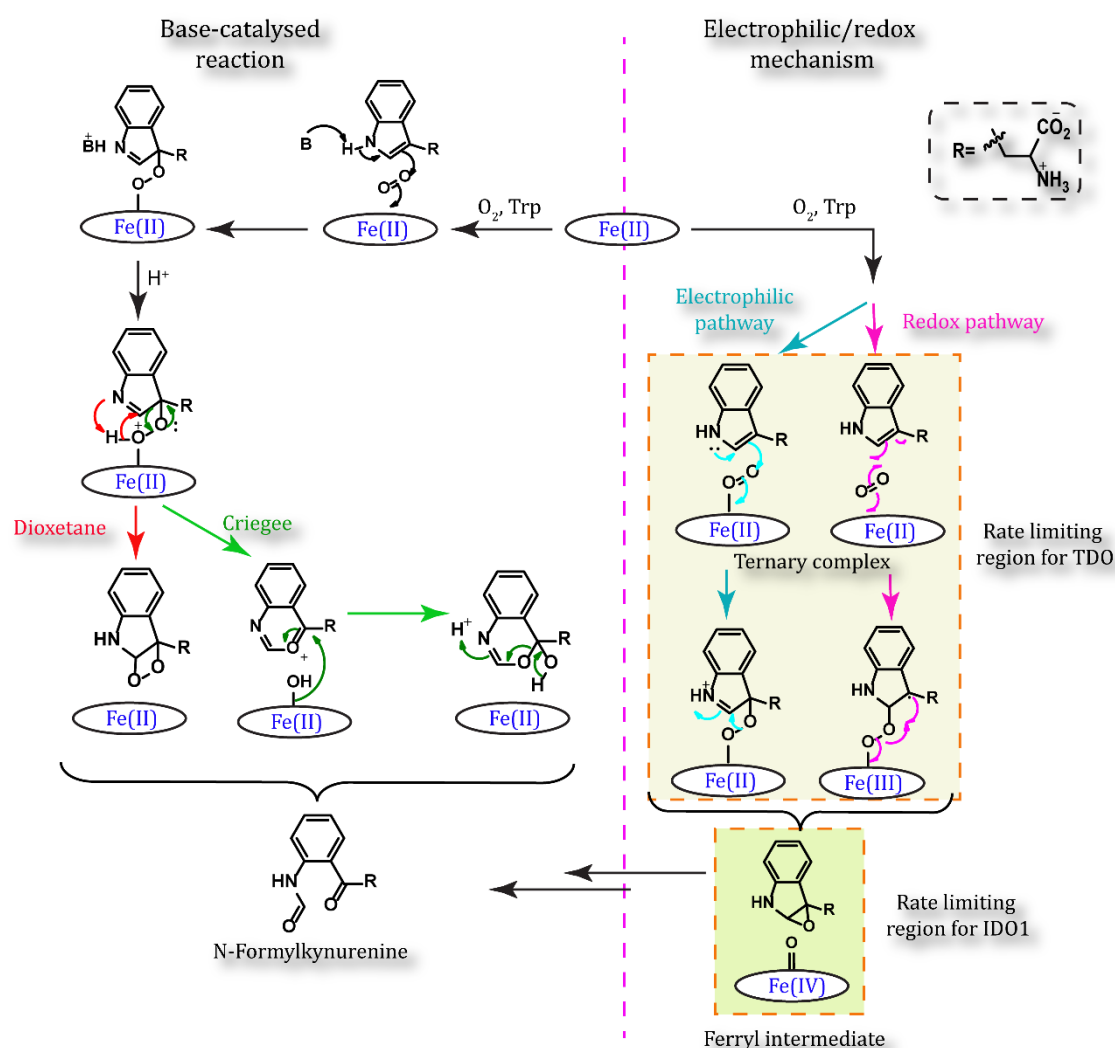


Figure 1.4 Mechanism of L-Trp catabolism by dioxygenase enzyme.

1.6 Classification of mechanism-based IDO1 inhibitors

By observing the redox dependent Trp catabolism mechanism in presence of O_2 , the mode of binding of the inhibitors in the IDO1 active pocket might be an essential step for inhibition process. By considering the several modes of binding of

the inhibitors in the IDO1 active pocket, they are classified in a few categories.⁷ Firstly, the type I inhibitor which participates in IDO1 inhibition via competitive inhibition mode with respect to substrate L-Trp. Type I inhibitor does not participate in direct binding with heme iron in the active site. Secondly, the inhibitor, which competes with O₂ to bind the ferrous-heme group in the active site of IDO1. This type of inhibitor is classified as type II. Type III inhibitor binds inactive ferric-heme group to inhibit IDO1. Type IV inhibitor competes IDO1 active site with labile heme group. This type of inhibitor shows time and temperature dependent IDO1 inhibition. Lastly, type V inhibitors inhibit IDO1 via the reduction of active ferrous-heme to inactive ferric heme group. All the reported inhibitors can be categorised under one of these classes.⁷

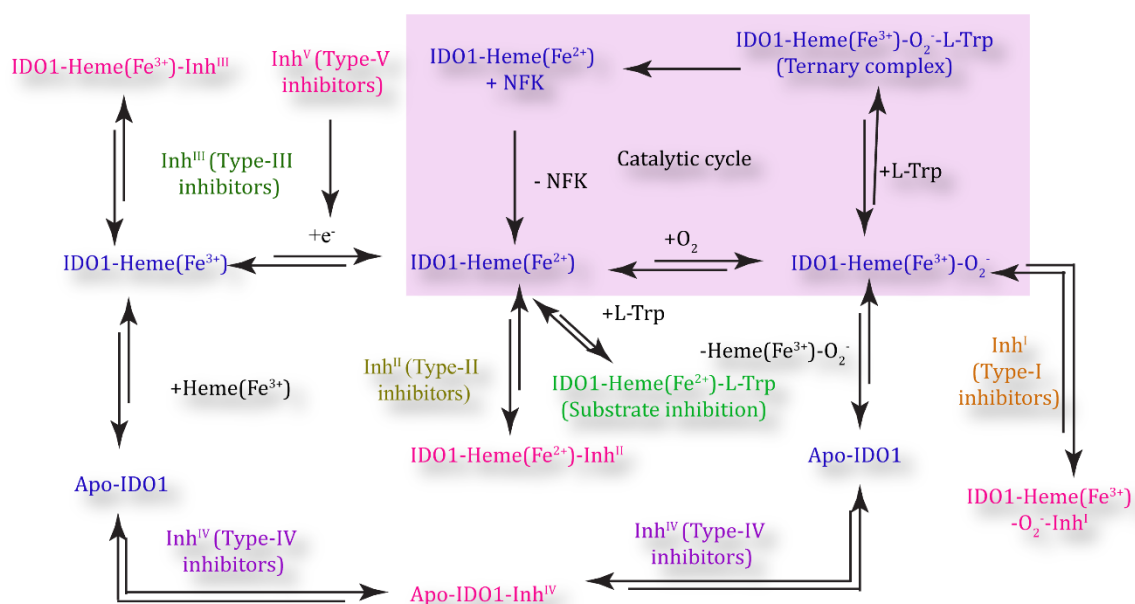


Figure1.5 Schematic representations of redox equilibrium, catalytic cycle and ligand binding with IDO1 enzyme

1.7. Mechanism-based IDO1 inhibitors

The enormous number of small molecules with diverse scaffold have been reported as potential IDO1 inhibitors. In order to probe into the class under which reported inhibitor fall, there is a need to carefully scrutinize their experimental data. The active site binding primarily confirms from NFK production kinetics in presence of IDO1 inhibitors. An inhibitor that binds to heme cofactor would appear competitive against O₂, but non-competitive for L-Trp.⁴⁶ The inhibition kinetics for

inactive ferric IDO1-bound inhibitors or substrate inhibition at higher concentration generates complication for assessment of true inhibition mechanism. Thus, NFK-mediated inhibition kinetics fail to display the convincing mechanism for the potent inhibitors. The optical absorbance spectroscopy of IDO1 is another convenient tool to characterise the binding mode of inhibitor in active site. The heme group of IDO1 is mainly responsible for showing optical properties and the spectrum is very sensitive to both the oxidation state and spin state of heme iron.⁴⁷ The specific Soret band (~ 400 nm) with the α and β band (between 500-600 nm) are the characteristic band for each oxidation and spin state of heme iron.⁴⁸ The ferrous-IDO1 mainly displays a high spin heme (~ 428 nm) and ferric-IDO1 shows both high and low-spin spectrum (~ 405 nm).^{44, 49} Binding of inhibitors to IDO1 active pocket changes the electronic environment of heme-iron and porphyrin which reflects on the displacement of Soret peak, as well as α and β band. There are several reports on binding study of the inhibitors with both ferrous and ferric-IDO1 in N_2 environment. Both the Soret peak also alters its position upon addition of inhibitors. But this binding study is not convening because of the absence of another heme binding substrate, O_2 which participates into the catabolic process through heme binding. Recently, the O_2 dependent absorbance kinetics for both ferric and ferrous-IDO1 in presence of inhibitors demonstrated a mimicked catabolic process and convey true their inhibition mechanism. Altogether, computation of Soret peak in the presence of inhibitors confirms the direct binding with heme iron in the IDO1 active pocket.

The detailed investigation of IDO1 inhibition action of L-Trp analogue, 1-methyl-L-tryptophan (L-1MT) showed a competitive mode of inhibition with respect to the substrate L-Trp in O_2 controlled system.⁴⁶ L-1MT was also non-competitive with respect to another substrate O_2 . In contrast, L-1MT showed a weak binding with purified IDO1 enzyme.⁵⁰ Besides, there was no direct evidence of L-1MT binding with heme iron i.e., no shift of 405 nm Soret peak of ferric-IDO1, 428 nm Soret peak of ferrous-IDO1 in N_2 system or 411 nm Soret peak of ferrous-oxy-IDO1. Hence, L-1MT is classified as type I IDO1 inhibitor. Another compound, D-1MT (indoximod), the stereoisomer of L-1MT also exhibited a comparable UV-Vis absorption pattern analogous to L-1MT.⁵¹ Similarly, the binding experiment of PF-06840003 with purified hIDO1 demonstrated no shifting of ferric-IDO1 peak at 405 nm and ferrous-IDO1 peak at 427 nm in absence of O_2 but displayed the depletion of Soret peaks. The

observed depleted Soret peaks might be rationalised by the change of polarity in the active pocket upon binding of PF-06840003.⁵² Altogether, PF-06840003 binds to IDO1 active pocket during inhibition but failed to directly bind the ferrous-heme in presence of O₂. Hence D-1MT and PF-06840003 are also classified as type I inhibitor likewise L-1MT.

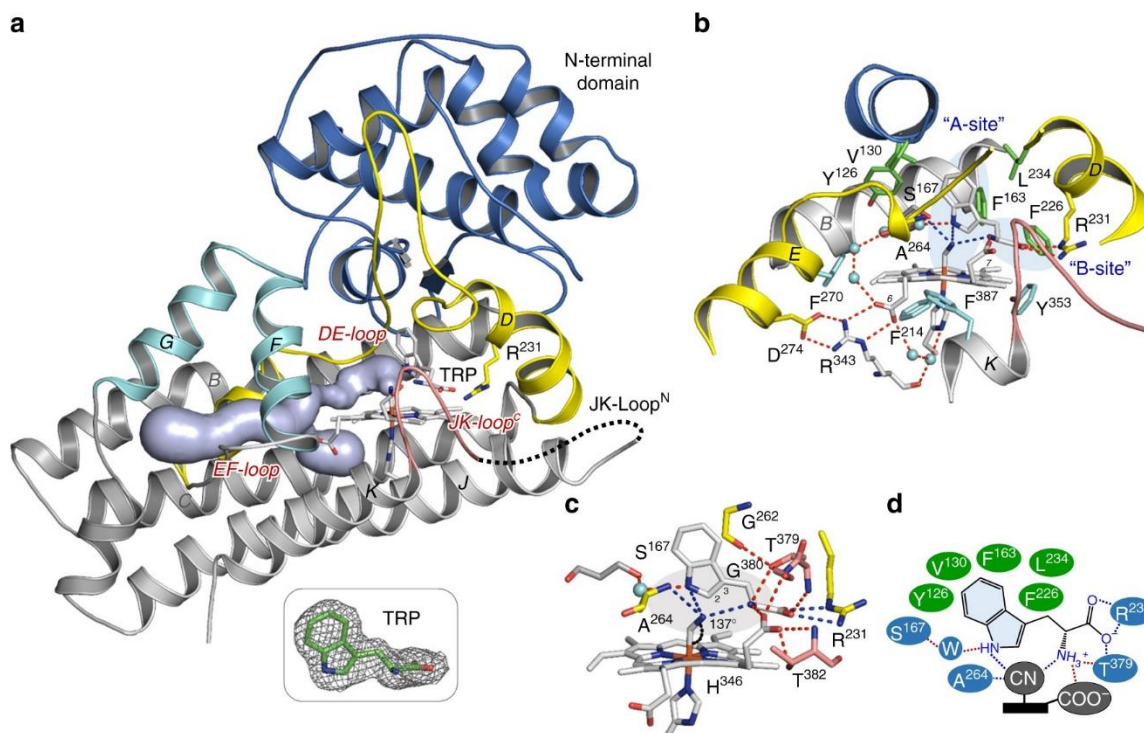


Figure 1.6 Binding mode of L-Trp. Crystal structure of the hIDO1-CN-Trp complex (a). Views of the active site, Sa. (b, c). The Trp interacts with the protein matrix, as well as the heme, via various hydrophobic and polar interactions (d).

The hydroxyamidine chemotype, Incyte-5l (INCB14943) exhibited competitive mode of IDO1 inhibition with both substrates, L-Trp and D-Trp.^{53, 54} The UV-Vis binding analysis of Incyte-5l with IDO1 displayed the shifting of ferric and ferrous-IDO1 soret peak to 412 nm and 419 nm respectively in N₂ environment.⁴⁹ Additionally, Incyte-5l demonstrated the shifting of Incyte-5l bounded ferric-IDO1 peak at 411 nm to 418 nm in presence of the reducing agent within 20 minutes.⁷ Hence, it can be proposed that Incyte-5l selectivity binds ferrous-IDO1 with higher affinity in inhibition process and thus it is included in type II IDO1 inhibitor. Another hydroxyamidine-based IDO1 inhibitor, epacadostat (INCB024360) was developed by Incyte from the lead, Incyte-5l. The electronic properties of IDO1-epacadostat

complex, displaying the Soret peak maxima for ferric and ferrous complexes at 412 and 419 nm, respectively, resembled Incyte-51. Hence, likewise Incyte-51, epacadostat is also included in type II inhibitor. Thus, type II inhibitors bind into IDO1 active pocket and complex with ferrous-heme to inhibit IDO1 into the catabolic process.

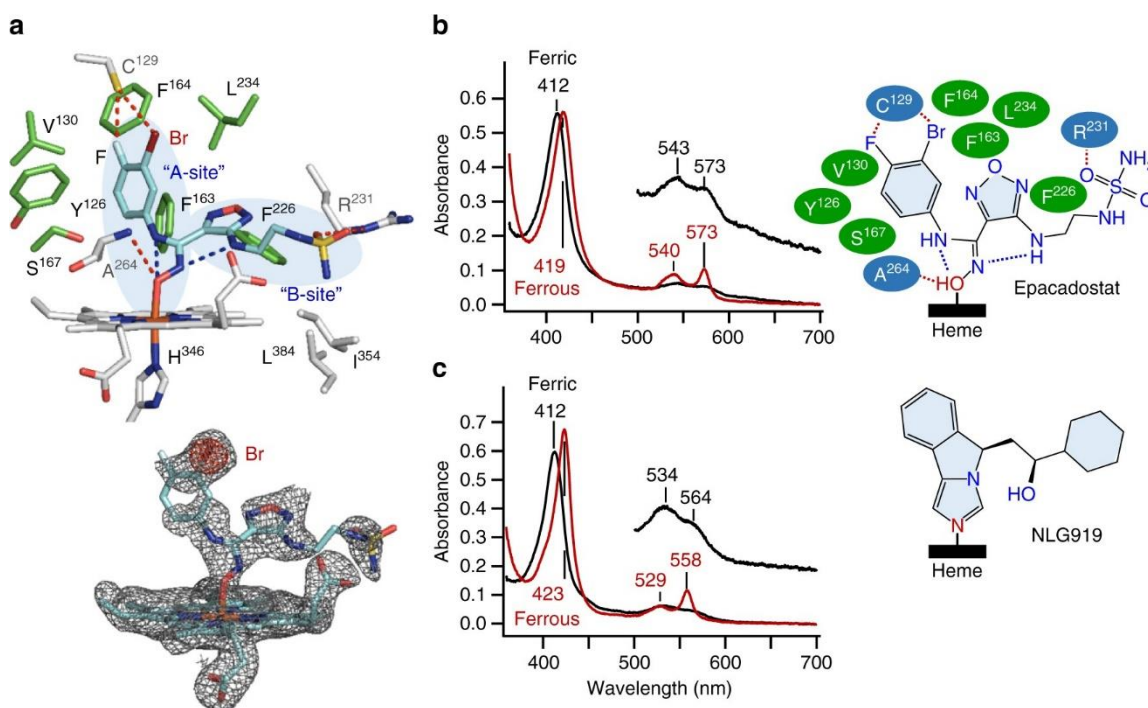


Figure 1.7 Binding mode of epacadostat and spectral markers for O and N-based inhibitors. Crystal structure of the hIDO1-epacadostat complex (a). Optical absorption spectra of hIDO1 in complex with epacadostat or NLG919 (b, C).

The absorption spectra of both ferric and ferrous IDO1 in presence of imidazole derivative, 4-phenylimidazole (4PI) shifted the Soret peak at 412 and 423 nm, respectively into a N₂ control system.⁵⁵ The ferric-IDO1-4PI complex also failed to shift Soret peak upon addition of reducing agent.⁷ Additionally, the intactness of ferric-IDO1-4PI peak at 411 nm absorption peak revealed the stability of ferric-IDO1-4PI complex over 4 hours. All of the above observation projects 4PI as both type III inhibitor as it selectively binds and stabilise ferric-IDO1. The imidazoleisoindole derivative, NLG0919, developed by NewLink Genetics manifested IDO1 binding in the active site and shifted both ferric and ferrous Soret peak at 412 and 423 nm, respectively in inert (N₂) environment.⁴⁹ But, there was no report of NLG0919-IDO1 absorption kinetics in absence and presence of reducing agents. As NLG0919 is an

analogue of 4PI, it can be categorised as type III inhibitor. Navoximode (GDC0919) is also in the similar class of NLG0919 and hence GDC0919 has also been sorted into type III IDO1 inhibitor family. The triazole scaffold-based IDO1 inhibitor, MMG0358 showcased the Soret peak of IDO1 at 415 nm in absence and presence of the reductant in O₂ control system. The ferric-IDO1-MMG0359 complex also failed to shift Soret peak upon addition of reducing agent. Thus, MMG0358 is classified as type III inhibitor.⁸ Overall, type III inhibitors bind into IDO1 active pocket and selectively complex with ferric-heme to inhibit IDO1 into the catabolic process.

The aminotriazole core, Vertex-AT displayed non-competitive mode of inhibition with the varying concentration of D-Trp. Vertex-AT displayed the shifting of ferric IDO1 Soret peak at 414 nm via the formation of ferric-ligand complex in presence of O₂. But the reducing environment generated a tight ferrous-ligand complex at a much slower rate (>2 h) and Soret peak shifted to 426 nm which was like Incyte-5l. The UV-based IDO1 binding kinetics of Vertex-AT after 4-hour equilibration period supported the higher affinity of Vertex-AT towards ferrous IDO1.⁴⁷ But recently the depletion of the Soret peak at 425 nm was observed after reaching a maximum at 90 minutes.⁸ Additionally, Incyte-5l shifted the ferrous-IDO1 peak within 20 minutes, whereas Vertex-AT had taken >2 h. Hence, considering the above experimental evidences, Vertex-AT is placed into both type II and type III inhibitors.

The UV-Vis binding kinetics of BMS-986205 supported that the effective and selective inhibition occurred through the generation of apo-IDO1. The redox state dependent depletion of both the Soret peak in presence and absence of reducing agent confirmed the heme dissociation in a time dependent manner during the incubation of BMS-986205 with IDO1.⁴⁵ The binding of BMS-986205 with IDO1 does not equate with previous inhibitors and it creates a novel inhibition mechanism for the robust and selective inhibition of IDO1. The novel BMS-986205 mediated inhibition has convened it into type IV apo-IDO1 targeting inhibitors.

The quinone derivatives, menadione and β -lapachone also displayed IDO1 inhibitory potency in enzymatic assay. But, the less potency in cellular IDO1 inhibition assays of menadione and β -lapachone generated a quart city of true inhibition mechanism. The UV-Vis binding analysis of both the compounds with IDO1 exhibited no shifting of Soret peak regardless of the absence or presence of reducing

agents. The UV-Vis binding kinetics of both the compounds with IDO1 is analogous with the no inhibitor case.⁸ The result argued against active site binding during IDO1 inhibition process for both the compounds. There was no ferrous-IDO1 peak in presence of reducing agent with both the compounds supporting that no significant amount of active IDO1 was present at any point of time. In summary, both the compounds disabled the reducing potency of the system i.e. ascorbate and methylene blue system. The discussed evidences of menadione and β -lapachone mediated IDO1 inhibition has generated a new inhibitory mechanism and both the compounds are categorised as type V redox inhibitors.

In general, type I inhibitors fails to computing both ferric and ferrous Soret peak after their binding in IDO1 active pocket. Due to changing polarity of the environment, the depletion of Soret peak might be observed for type I inhibitors. Type II and III inhibitors exhibits the changing of Soret peak position after their binding with heme iron in the active pocket. The apo-IDO1 targeting type IV inhibitors have been displayed the time dependent depletion of both the Soret peak during their binding with IDO1. Type V inhibitors conveys unfaltering of Soret peak like no inhibitors during their inhibition actions.

1.8. Structural analysis of inhibitors for selective inhibition mechanism

The potent IDO1 inhibitors contains important scaffolds to accommodate themselves into the active pockets. The analysis of fundamental moiety in the potent inhibitor might convey their inhibition mechanism for the binding in the active pocket and classify themselves into a specific type inhibitor. Indoximod (D-1MT) is a Trp analogue with methyl substitution on indole's nitrogen. Several evidences support indoximod as a IDO1 enzymatic pathway inhibitor and it is not a direct IDO1 binding inhibitor. The bio-isoster of indoximod, L-1MT bind to the oxygen-bound active state of ferrous IDO1 during the inhibition process. The different observation between two stereoisomers directed the analysis on the structural importance of both D-and L-1MT. The docking analysis with ferric IDO1 with indoximod has indicated its bounding mode into IDO1 active pocket where *N*-methyl indole ring place into hydrophobic 'pocket A' with 'sandwich' composed of Tyr126 and Phe163.⁵⁶ *N*-methyl group places itself orthogonally to heme plane, alike L-Trp binding pose (PDB: 6E46). Hence D-1MT lacks the H-bonding interaction with

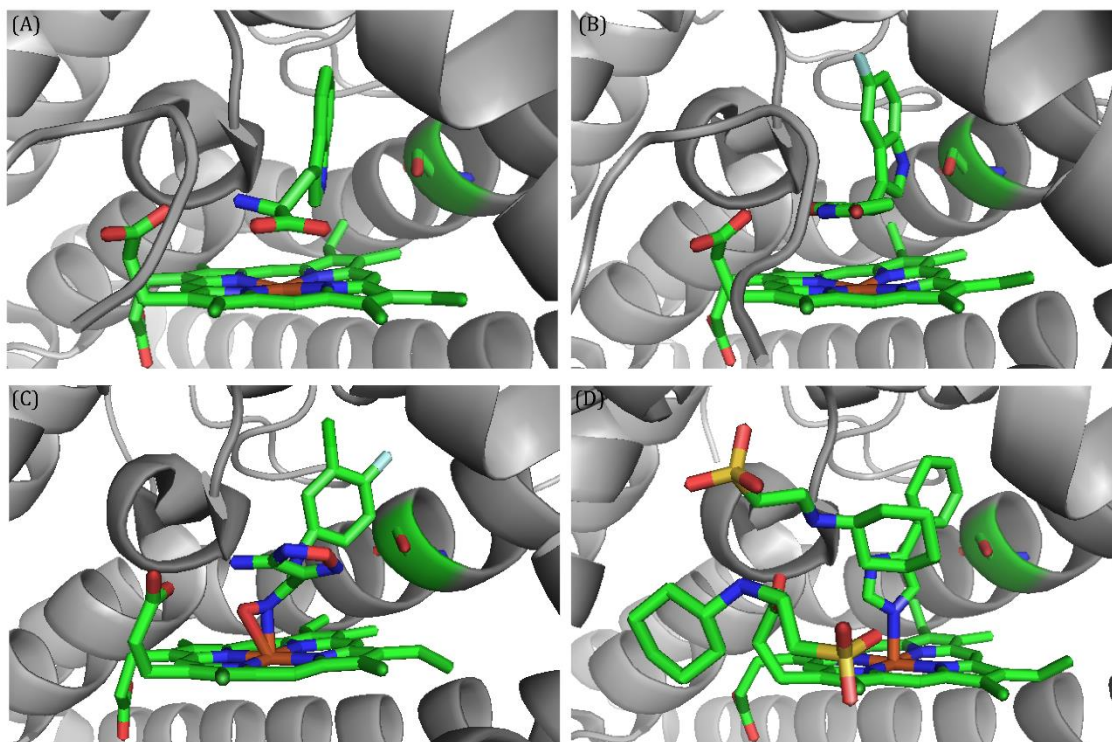


Figure 1.8 Binding mode of substrate and potent inhibitors into the IDO1 active pockets. Complex of Trp and ferrous IDO1 (PDB code: 6E46) (A). Complex of PF-06840003 (PDB code: 5WHR) (B), Incyte-51 (PDB code: 5XE1) (C) and 4PI (PDB code: 2D0T) (D) with IDO1 enzyme.

Ser167. The α -amino groups participate in H-bonding with 7-propionate group of the heme co-factor. A similar binding pose is also observing for L-1MT where it shows H-bonding with Gly262 residues with no significant difference in scoring function compared to indoximod. Overall, the binding studies supports indoximod and L-1MT are non-heme binder inhibitor. The indole derivative, PF-06840003 also binds IDO1 active pocket in similar fashion of the substrate L-Trp or indoximod where indole ring occupies lipophilic 'pocket A' (PDB: 5WHR) and participates in strong π - π interactions with several aromatic groups.⁵² The indole NH indulges in H-bonding directly with Ser167 residues. The installation of the succinimide group in PF-06840003 gives the additional H-bonding interaction with 7-propionate group as well as Ala264 and Thr379 residues. Though the succinimide moiety is parallel to the heme plane within a short distance, there is no direct interaction between PF-06840003 and iron atom. Interestingly, another crystal structure (PDB: 6PZ1)

successfully demonstrated the binding ability of PF-06840003 with the S_i site of IDO1.⁵⁷ Overall, the indole derivatives, the type I inhibitors, lack the heme-iron binding atom in a suitable direction amounting to no direct iron binding within the active IDO1 pockets.

The Incyte-51 (INCB14943) composes of hydrophobic phenyl group, hydroxylamine moiety along with amine substituted furazan moiety. The initial modelling of INCB14943 bound IDO1 displays oxygen atom of hydroxyamine group coordinates with ferrous atom into active site.⁵⁴ The intramolecular H-bonding of INCB14943 generates the cis-conformation of hydroxyamine group which facilitates proper fitting of the compound into the active pocket. The phenyl ring is placed into 'pocket A' and the amine substituents on the furazan moiety interacts with the propionate group. Recently, the INCB14943 bound IDO1 co-crystal (PDB: 5XE1) has supported dock pose of the compound into the active pocket but, nitrogen atom in the oxime moiety interacts with heme iron atom.⁵⁸ In addition, chloro-substitution on phenyl ring participated in halogen bonding with Cys129 in 'pocket A'. INCB14943 modification of furazan-amine with the sulfamide group generates epacadostat (INCB24360). The crystal structure of epacadostat also supports the intramolecular H-bonding in cis-conformation.⁵⁹ The co-crystal structure of epacadostat with IDO1 (PDB: 5WN8) has displayed the proper binding mode into the active pocket.⁴⁹ The hydroxyamine-oxygen atom coordinates with heme iron. The intramolecular H-bonding interaction and Ala264 residue helps the coordination of the oxygen atom with heme iron. Similar to INCB14943, the halogen substitution on phenyl group participates in halogen bonding and interacts with Cys129 residue within 'pocket A'. The extended sulfamide group occupies 'pocket B' and stabilises through additional H-bonding with Arg231 residue. Overall, the intramolecular H-bonding of *N*-hydroxyamine moiety creates a suitable scaffold to bind the active pocket. The borderline ferrous atom in heme might favour the binding of soft hydroxylamine ligand into the active pocket and displays the type II inhibition mechanism.⁶⁰

The first co-crystal of IDO1 and small molecule-based inhibitor was reported with imidazole-based small molecule, 4PI (PDB: 2D0T).⁶¹ 4PI contains a phenyl ring with an imidazole moiety. The co-crystal structure supports the binding of 4PI with heme group through imidazole scaffold. The phenyl group occupies the lipophilic

'pocket A' where it is stabilised through π - π interaction with the residues. The buffer molecule, CHES also occupies active site and participates in salt-bridge interaction with propionate and Arg231 groups. Further development of imidazole scaffold yielded NLG919 inhibitor which composes of imidazoleisoindole core, a hydroxyl group and a cyclohexane moiety. The detailed analysis of NLG0919 co-crystal structure with IDO1 (PDB: 5EK3) supports NLG0919 binds heme group through imidazole nitrogen.⁶² The imidazoleisoindole moiety has placed into 'pocket A' and participates in extensive hydrophobic interaction with the residues. Additional hydrophobic interaction was also identified among Phe226, Ile354, Leu384 residues and 1-cyclohexylethanol moiety has extended in 'pocket B'. The intramolecular H-bonding between the hydroxyl group and isoindole nitrogen gives a distinct configuration which ensue additional H-bonding of hydroxyl group with 7-propionate and Ala264 residue. Another analogue of NLG0919, GDC0919 (navoximode), with additional 6-fluorine substitution on imidazoisoindole moiety and hydroxy substitution on cyclohexane scaffold is also a potent IDO1 inhibitor. The co-crystal structure of navoximode with IDO1 (PDB: 6O3I) corroborates similar pose of interaction within the active IDO1 pocket alike NLG0919 analogue.⁶³ Additionally, the terminal hydroxyl-substitution participates in H-bonding with Ser235 residue. The C6-fluorine substitution displays a strong interaction with Gly262 residue. The MMG-0358 compound carries a triazole ring and a substituted phenyl ring. The MMG-0358 bound IDO1 crystal structure (PDB: 6R63) reveals ferric-hemen binding into the active pocket.⁸ The substituted phenyl ring is placed into 'pocket A' where the chloride atom is surrounded by Cys129, Leu234, and Gly262. The hydroxy group participates into H-bonding with Ser167 residue. MMG-0358 also occupies the S_i site of IDO1.⁶⁴ The triazole is in equilibrium with its deprotonated form. Interestingly, the QM calculation for the binding of deprotonated triazole in MMG-0358 showed a stronger complex with IDO1. Hence, MMG-0358 binds the active pocket like 4PI. In conclusion, the imidazole or triazole scaffold-based inhibitor binds IDO1 active site with strong H-bonding milieu and proper orientation of imidazole or triazole nitrogen which facilitates heme binding. The hard ligand, imidazole or triazole might be responsible for the proclivity to the hard metal, ferric ion which helps the type III inhibition mechanism.⁶⁰

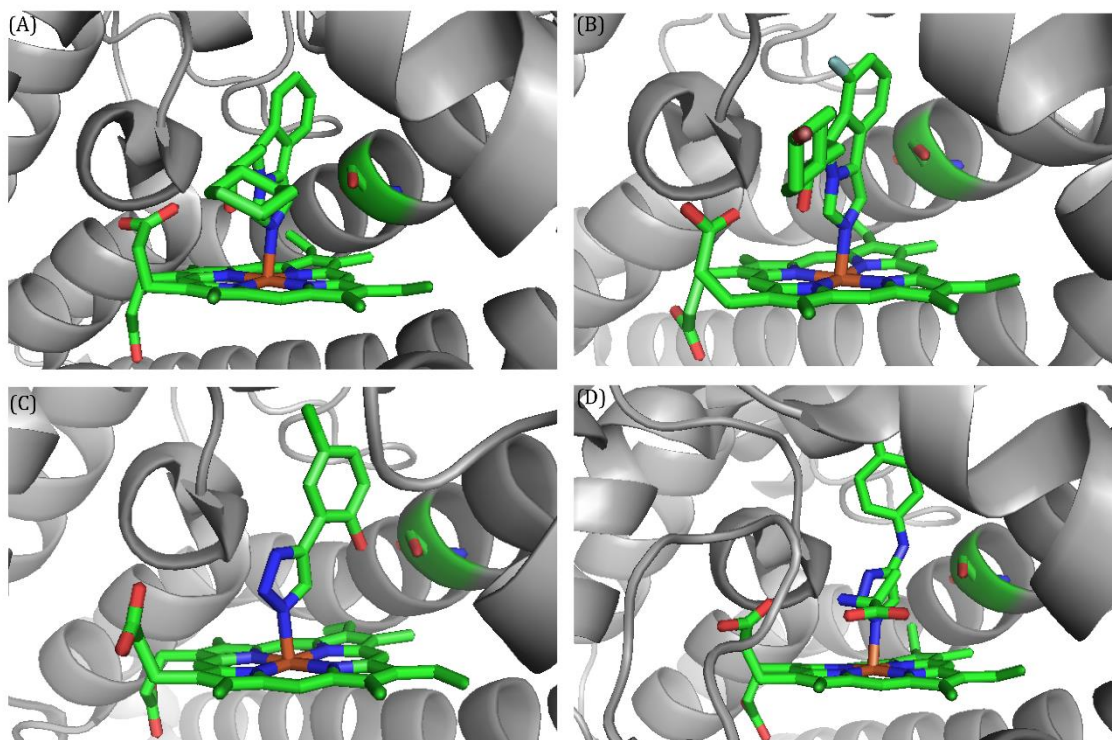


Figure 1.9 Binding mode of potent inhibitors into the IDO1 active pockets. Complex of NLG0919 (PDB code: 5EK3) (A), navoximode (PDB code: 6O3I) (B), MMG-0358 (PDB code: 6R63) and Vertex-AT (PDB code: 6F0A) (D) with IDO1 enzyme.

Vertex-AT, another IDO1 inhibitor, holds a 4-amino substituted triazole moiety and a chloro-substituted phenyl ring. Vertex-AT also binds the IDO1 active pocket (PDB: 6F0A) and the triazole moiety participates in heme interaction.⁴⁷ The chlorophenyl group has placed into hydrophobic 'pocket A'. 4-amino group is H-bonded with Ser167 residue. The additional buffer molecule also participates into salt-bridge interaction between triazole group and propionate group, Arg231 and Thr379 residue like 4PI compound. The detailed structural investigation deduces that the hard nitrogen atom in triazole moiety interacts with the-ferric atom like in MMG-0358, but the interacting buffer molecule may convert triazole moiety into a borderline ligand that interacts preferably with ferrous atom. Hence, Vertex-AT participates in the dual, type II/III inhibition mechanism.

The suicide inhibitor, BMS-986205 comprises of quinoline, cyclohexane and phenyl- propenamide scaffolds.⁶⁵ The heme deficient co-crystal structure of BMS-986205 and IDO1 (PDB: 6MQ6) supports apo-IDO1 binding mechanism. The co-crystal reveals that the quinoline group extends to S_i pocket of IDO1. The π -stacking

and H-bonding interactions also observed between BMS-986205 and Phe270, His343 residues, respectively. The phenyl and propenamide group have placed into S_a pocket and propenamide moiety establishes H-bonding interaction with Ser167 and His346 residues. The cyclohexane moiety occupies heme position. The detailed mechanism has concluded that different binding trajectory of BMS-986205 mainly responsible for apo-IDO1 targeting phenomena. Initially, solvent-exposed surface binding of BMS-986205 unfolds the active pocket and accelerates the release of heme group (PDB: 6DPQ). The hydrophobic quinoline and elongated cyclohexane substitution are responsible for this surface exposed interaction. Finally, the transition from kink (PDB: 6DPR) to bent conformation (PDB: 6MQ6) of cyclohexane moiety creates a heme-dissociated BMS-986205 bound pockets which perturbs heme rebinding process. Overall, an inhibitor containing the elongated hydrophobic quinoline like fused cyclic compound which has a propensity to bind both S_a and S_i pockets, is mainly responsible for showing type-IV apo-IDO1 targeting inhibition mechanism.

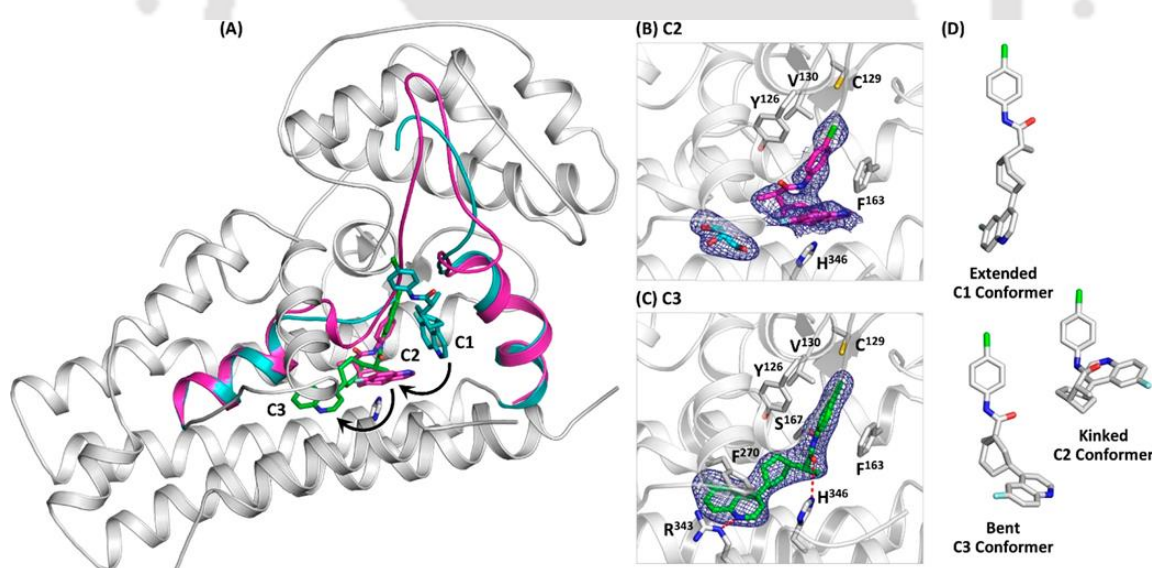


Figure 1.10 Sequential movement of BMS-986205 from the C1, C2, to C3 sites. The BMS-986205 bound in the C2 and C3 sites (magenta and green sticks, respectively) are superimposed in the C1 structure to illustrate the inhibitor binding trajectory (A). Expanded view of the C2 site and (magenta sticks) and glycerol (cyan sticks) (B) and the C3 site and the bound BMS (green sticks) (C) with bound BMS-986205. Three BMS-986205 conformers (D).

At a glance, binding mechanism of indole analogue i.e. substrate L-Trp like molecule place into type I inhibitors. Whereas the soft or borderline chelating ligand like hydroxylamine moiety identifies as type II inhibitor which preferably binds with borderline ferrous ion. Similarly, the hard ligand imidazole or triazole sticks with hard ferric ion and distinguishes themselves in type III categories. The elongated hydrophobic molecule binds to solvent-expose pocket of IDO1 and shows type IV inhibition mechanism. Additionally, the redox active quinone derivatives lies into type V inhibitors.

1.9. Potency of IDO1 inhibitors

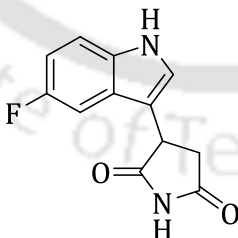
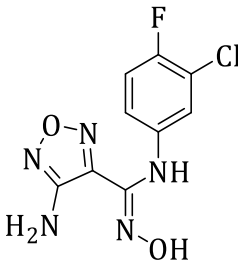
L-Trp or indole-based inhibitors are first known IDO1 inhibitors and the most widely used 1-methyl-D/L-tryptophan (1MT) inhibitors in preclinical studies. 1MT was reported by NewLink Genetics and shows moderate inhibitory efficacy against IDO1 with K_i of 34 μM .^{51,66} Additional research supports only L-1MT binds with IDO1 during inhibition, whereas D-1MT exhibits no inhibitory effects. But, the preclinical data of D-1MT presents exciting anticancer activity via reactivation of T-cells functions.⁶⁷ The recent research supported that the participation of D-1MT in reverse mTORC1 inhibition under L-Trp-depletes milieu.¹² High-throughput mass spectrometry assay able to find PF-06840003 as selective IDO1 inhibitor with an IC_{50} value of 0.41 μM .⁵² The stereochemistry analysis supports R-enantiomer is the potent inhibitor with IC_{50} of 0.2 μM , whereas S-enantiomer shows IC_{50} of 38 μM . But the rapid enantiomeric exchange of PF-06840003 in plasma indicates the racemic participation in preclinical and clinical advancement. PF-06840003 also exhibits excellent IDO1 inhibitory activity in both HeLa cells and THP1 cells with IC_{50} of 1.8 and 1.7 μM , respectively. In 2016, PF-06840003 went to clinical trial by Pfizer Pharmaceuticals to evaluate the antitumor potency in patients with malignant gliomas. Incyte-51 (INCB14943) is the potent compound among the novel hydroxylamides series of inhibitors with IC_{50} of 67 nM and 19 nM in enzymatic and cellular IDO1 inhibition assay, respectively.⁵⁴ But details pharmacokinetics studies concluded the poor oral bioavailability of Incyte-51 and hence it fails clinical trials. The modified compound, epacadostat (INCB024360) displays the clinical lead compound. The epacadostat shows selectively high IDO1 inhibitory potency in both HeLa cell and enzymatic assay with IC_{50} of 7.3 and 74 nM, respectively.⁵⁹ The *in vitro*

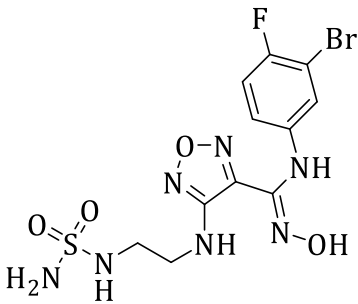
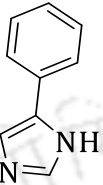
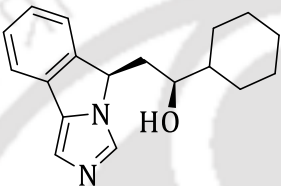
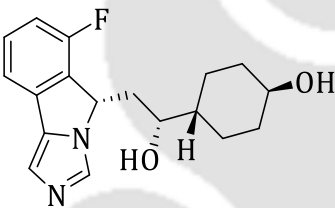
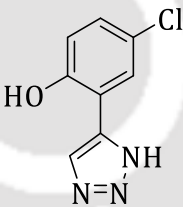
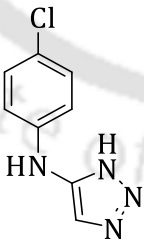
'ADME' data also concludes a good oral bioavailability and cell permeability in several model. Additionally, epacadostat exhibits the antitumor effect in the B16 melanoma model. However, the unfortunate failure of epacadostat/keytruda combination (in ECHO-301 phase III clinical trial) against metastatic melanoma halted the immunotherapy-related research on cancer. The 4PI compound discloses the IDO1 inhibitory potency with IC_{50} of 48 μM and the cellular IC_{50} of 140 μM .⁶¹ The imidazoleisoindole derivative, NLG0919 exhibits the IC_{50} of 38 nM in IDO1 enzymatic assay and EC_{50} of 61 nM against HeLa cell.⁶² The navoximod (GDC0919) displays the inhibitory potency with IC_{50} of 28 nM and 75 nM in enzymatic and cellular milieu respectively.⁶³ Navoximod also displays 10 to 20-folds selective IDO1 inhibition over TDO enzyme. Compound MMG0358 conducts IC_{50} of 330 nM against IDO1 enzyme without TDO activity.⁶⁴ MMG0358 also shows IC_{50} values of 80 nM and 2 nM on hIDO1 and mIDO1, respectively in a cellular assay without cellular toxicity. The aminotriazole derivatives, Vertex-AT was discovered as non-competitive IDO1 inhibitor by high-throughput screening and it shows inhibition potential with IC_{50} of 11 μM and 23 nM against biochemical assay and HeLa cell, respectively.⁴⁷ The BMS-986205 compound displays robust and highly selective IDO1 inhibitory potency with IC_{50} of 0.5 nM in HeLa cell. The naphthoquinone derivatives, menadione exhibits IDO1 inhibitory activity in sub-micromolar range with IC_{50} of 1.1 μM .⁶⁸ The non-immunogenic melanoma tumor graft model also demonstrates the robust anti-tumor activity of menadione via the activation of IDO1 mediated T cell immunity. The 1,2-naphthoquinone derivative, β -lapachone shows the IC_{50} of 0.44 μM .⁶⁸ β -lapachone represents a 10-fold greater IDO1 inhibitory potency over the basic 1,2-naphthoquinone pharmacophore. But it shows a reverse IDO1 activity in IFN- γ -induced HeLa cells.⁶⁹ Few quinone derivatives displays low micromolar/nanomolar IDO1 inhibitory potency in the enzymatic assay but fails in cellular assays. The measured IC_{50} value of quinone-based inhibitor greatly varies depending on the assay protocol.⁸ These quinone derivatives also an efficient substrate for the human quinone reductase NQO1 which reflected on NQO1-dependent redox cycling, leading to antitumor cytotoxicity.⁷⁰

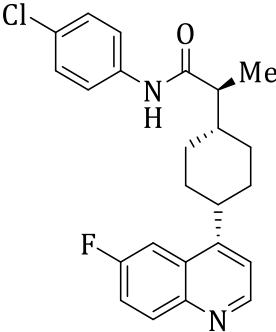
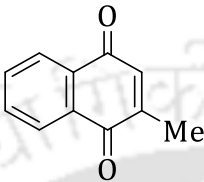
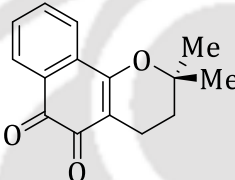
The experimental evidences for the inhibitory potency of reported potent IDO1 inhibitors follow a trend. The type IV apo-IDO1 targeting inhibitors shows robust inhibition even in pico molar concentration range. The inhibitory potency of type II

hydroxyamidine-based inhibitors falls into lowest nanomolar range. The type III imidazole or triazole-based inhibitors show IC_{50} below 100 nM. Whereas type I substrate like inhibitor makes a doubt though the additional binding instead of heme prefers the lower micromolar inhibitory efficacy. The type V redox mediated inhibitors are not suitable for true IDO1 binding and it also toxic to cell via activating several signalling pathways. The proper binding of inhibitor prefers accommodation within the active pocket, which is the driving force for showing higher potency. Additionally, the abundance of different state of IDO1 inhibitor might be another controlling factor for displaying robust cellular inhibition potency. 85% of cellular IDO1 is in apo-form and remaining contains the prosthetic heme group.⁴⁵ The catalytic cycle starts with ferrous heme and cytochrome b5, cytochrome P450 and NADPH mainly responsible for active ferrous-IDO1 production in cell (283-285).⁷¹⁻⁷³ The inactive ferric-IDO1 gets generated after the completion of a catalytic cycle. Hence, presence of ferric-IDO1 in cells is least and apo-IDO1 is maximum. Thus, targeting of apo-IDO1 inhibitors shows an exciting result in cellular inhibition process and ferrous and ferric-IDO1 targeting inhibitors follows the inhibition potency accordingly.

Table 1.1 Structure of potent IDO1 inhibitors with their inhibitory potency

Compound	Structure	Purified hIDO1 inhibition, IC_{50}	Cellular IDO1 inhibition, EC_{50}	Type
PF-06840003		0.2 μ M	1.8 μ M (In HeLa Cell)	Type I
Incyte-51		0.067 μ M	0.019 μ M (In HeLa Cell)	Type II

Epacadostat		0.074 μM	0.007 μM (In HeLa Type II Cell)	
4PI		48 μM	140 μM	Type III
NLG0919		0.038 μM	0.061 μM (In HeLa Type III Cell)	
Navoximod		0.028 μM	0.075 μM (In HeLa Cell)	Type III
MMG0358		0.330 μM	0.080 μM (In HEK-293 Cell)	Type III
Vertex-AT		11.000 μM	0.023 μM (In HeLa Cell)	Both Type II & Type III

BMS-986205		0.0005 μ M (In HeLa Type IV Cell)
Menadione		1.100 μ M Type V
β-Lapachone		0.440 μ M Type V

1.10. Off-target effect of reported IDO1 inhibitors

The helix-loop-helix transcription factor, AhR binds with several xenobiotic and translocate to nucleus to regulate several genes. The IDO1 mediated L-Trp metabolites generates in kynurenine pathway, are agonist of AhR. Likely, AhR binds with IDO1 promoter region and directly regulates IDO1 transcription process and positively regulated AhR-IDO1 loop. Interestingly, L-Trp memetic 6-formylindolo[3,2-b]carbazole, **FICZ** and 2-(1'*H*-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester, **ITE** are the high affinity natural agonist of AhR. Indole-3-carbinol, **I3C** also binds with AhR and amplify the IDO1 expression through upregulations of IL-6, PGE2. Additionally, AhR activation suppress adaptive immunity in tumor milieu via IL-10 mediated differentiation of Treg cells and regulatory dendritic cells. Overall, L-Trp memetic xenobiotics possessing polycyclic, planar molecular scaffold are suitable for AhR affinity and exhibits AHR mediated immune-tolerance effects.⁷⁴

Recent reports demonstrated that few of IDO1 inhibitors, currently in clinical trials also behaves as AhR agonist. Indoximod showed AhR affinity behaviour and stimulated AhR signalling process to induce the IDO1 gene in luciferase assays. Indoximod was able to enhance 19.6 fold AhR-directed luciferase activity in human



Several small molecules with diverse scaffolds have been reported as potential IDO1 inhibitors to control immune-modulating functions in the cancer milieu. But the outcome of the phase 3 trial (ECHO-301), epacadostat with pembrolizumab, generates the ambiguity of singly IDO1 inhibition.³ However, the clinical trials with the combination of IDO1 inhibitors/immune checkpoint inhibitors have been commenced, the reinvestigation of the IDO1 inhibition mechanism has become requisite to achieve pertinent clinical benefits. The IDO2 and TDO enzymes were, in the beginning, assumed to be the only hepatic enzyme responsible for metabolizing the Trp in the liver. However, recent studies revealed that TDO is also expressed in various cancer cells and associated with immune tolerance. Detailed cellular and in vivo studies demonstrated that TDO could be an additional target for inhibiting the L-Trp metabolism via the kynurenine pathway. Thus, both IDO1 and TDO dual inhibition are obligatory for tumor immunotherapy.

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IDO1 contains a unique substrate-binding site (S_i site) to the proximal site of the heme group, which is absent in TDO. The L-Trp binds to the S_a site of both the enzyme. The indoleamine moiety of the L-Trp is hydrogen-bonded to Ser167 through a bridging water molecule in the IDO1 pocket, whereas in TDO, it is directly H-bonded with His76 residue. The occupancy of L-Trp in the S_i site of IDO1 induces the structural change of S_a . Additionally, L-Trp binding in S_i site facilitates the movement of Phe270 residue to block water tunnel, which perturbs O_2 delivery into the S_a site during active IDO1 turnover.^{44, 49} However, the active pocket of TDO is highly substrate-specific, which reflect the smaller number of reported TDO inhibitors.

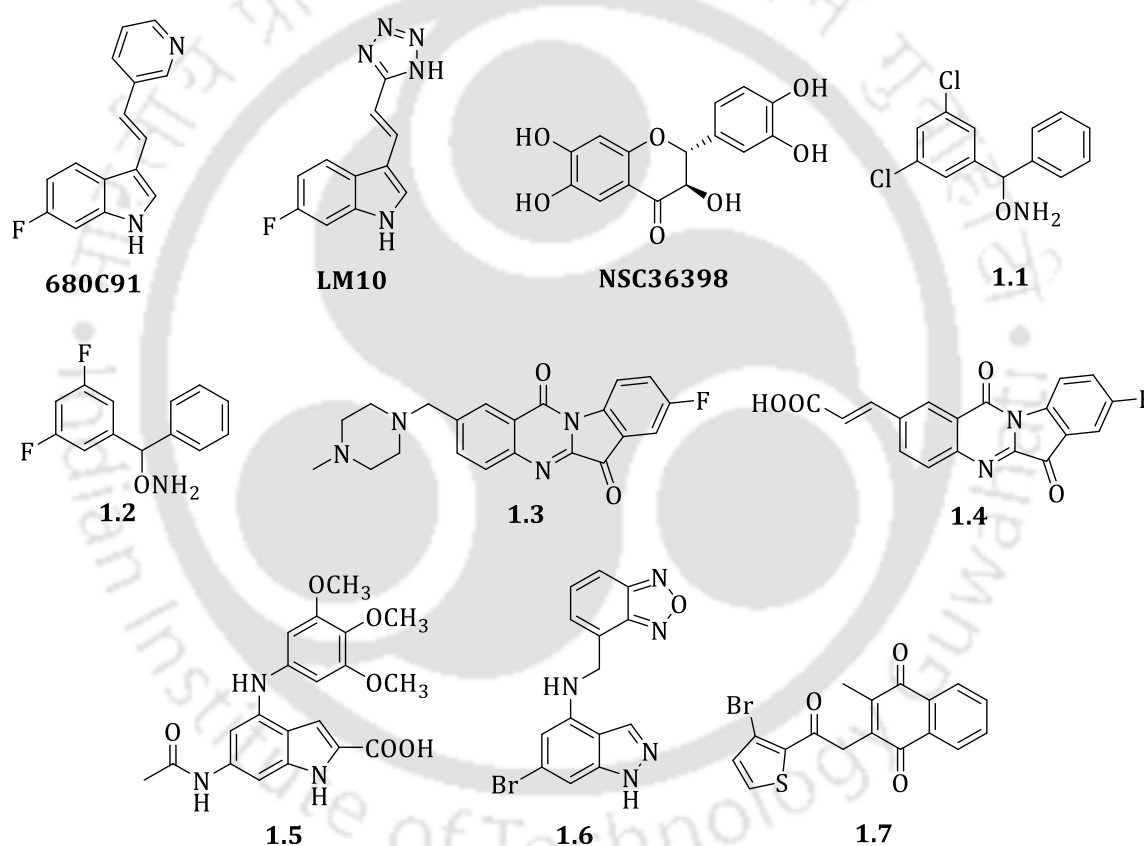


Figure 1.12 Reported dual IDO1 and TDO inhibitors.

Few indole derivatives (**680C91**, **LM10**, and **NSC36398**) showed selective TDO inhibitory potency. But, due to limited bio isostere of substrate L-Trp, TDO inhibitors were not explored. Recently, L-Trp induced-immunosuppression research prone to develop both the IDO1 and TDO dual inhibitors for a logical clinical aspect. A small number of dual inhibitors are enlisted in the literature. But the cell-based inhibition data of diaryl hydroxylamines-compounds (**1.1**, **1.2**) suggest most of the compounds

were pan inhibitors.⁷⁸ The *N*-benzyl/aryl-substituted tryptanthrin derivatives (**1.3**) directly interact with the IDO1, IDO2, and TDO enzymes and significantly induce the T cells proliferation and inhibit the catabolism of L-Trp via the kynurenine pathway.⁷⁹ Meanwhile, the cinnamic acid derivative of tryptanthrin **1.4** potentially inhibits the activities of IDO1 and TDO enzymes.⁸⁰ Recently, a series of indole-2-carboxylic acid derivatives and evaluated their inhibitory activities against both IDO1 and TDO enzymes.⁸¹ The SARs studies identified 6-acetamido-indole-2-carboxylic acid derivative **1.5** as a potent dual inhibitor of IDO1 and TDO enzymes. Compound **1.5** also showed the induction of T cell proliferation and suppression of growth of the tumors in the mouse B16F10 melanoma model. Besides the indole derivatives, the 1*H*-indazole derivatives also displayed dual IDO1 and TDO inhibitory activities. A series of 4,6-substituted-1*H*-indazole derivatives.⁸² The benzo-oxadiazole substituted 1*H*-indazole derivative **1.6** displayed potential IDO1 and TDO inhibitory activities against both purified enzymes and under cellular conditions. Interestingly, compound **1.6** suppressed the cellular IDO1 expression levels in IFN- γ -induced HeLa cells. However, compound **1.6** significantly suppressed the growth of tumors in the CT26 xenograft model. Recently, it was found that quinone as a potential scaffold for dual IDO1/TDO inhibitors through 3D shape similarity and pharmacophore in silico screening. The cellular assay revealed that quinone derivative **1.7** inhibits the activities of IDO1 and TDO enzymes.⁸³ However, the ambiguity related to the true enzyme inhibition mechanism of the redox responsive quinone moiety was not appropriately addressed. Compound **CMG017** effectively suppresses the production of kynurenine in various human and murine cancer cell lines than targeting either IDO or TDO enzymes. They demonstrated that compound **CMG017** reduced the kynurenine level in both tumors and plasma of tumor-bearing mice and intimidated the growth of tumors without displaying substantial toxicity. The compound **CMG017** also showed an induction of intratumoral infiltration of CD8⁺ effector T cells and caused substantial alternation in immune-related genes in TME. The combinatorial studies of compound **CMG017** and anti-PD-1 or anti-CTLA-4 showed resilient tumor reduction and long-term survival. However, the detailed structural information of compound **CMG017** was not disclosed. Recently, few dual inhibitors of IDO1/TDO are under clinical trials. Navoximod, in combination with a PD-L1 inhibitor (azolizumab), is under phase I clinical trial for the treatment of solid

tumors. The initial studies of the combination of navoximod and azolizumab against patients with advanced cancer revealed satisfactory tolerability, safety, and pharmacokinetics. Although navoximod is considered as a dual inhibitor of IDO1/TDO, it has 20-fold higher selectivity for the IDO1 over the TDO enzyme.⁸⁴ In 2019, clinical studies of compound **SHR-9146** (structure undisclosed), a dual inhibitor of IDO1/TDO was initiated. Further studies with compound **SHR-9146** in combination with a PD-1 inhibitor (SHR-1210) and/or VEGFR inhibitor (Apatinib) are currently under investigation for the treatment of advanced/metastatic solid tumors. The compound **DN1406131** (structure undisclosed), a dual inhibitor of IDO1/TDO, is also currently under clinical trials. Clinical studies of M4112 (structure undisclosed), a dual inhibitor of IDO1/TDO was recently terminated due to uninspiring pharmacodynamic results.

Altogether, the dual IDO1 and TDO inhibitors cover few natural product scaffolds like tryptanthrin, quinone, and the L-Trp's bio isostere indole, indazole derivatives. Few heterocyclic fused aromatic compounds also displayed their dual inhibition properties in both enzymatic and cellular assays. The dual IDO1/TDO inhibition are expected to be advantageous in entirely inhibiting the kynurenine pathway, which is indisputably reflected from the clinical trial initiation in kynurenine mediated immunosuppression process. Hence, the dual IDO1 and TDO inhibition demand new potent and selective inhibitors to rejuvenate the kynurenine-mediated immunosuppression process related to cancer and other diseases.

1.12. Selective delivery of IDO1 inhibitors

The shocking failure of phase III trials by Incyte and Merck for epacadostat with KEYTRUDA® (pembrolizumab) bent the IDO1 research towards improvement of drug efficacy, though the combination therapy with chemo or PDT. Recently, scientists were more focused to deliver IDO1 drug via modern nano-carrier which encapsulates suitable chemotherapeutic agent or bearing some sensitive scaffolds for co-treatment. A bio-responsive injectable gel which showed the sustain release of PD-1 antibody with D-1MT. The ROS responsive methionine group in the peptide hydrogel also participated in reducing the intra-tumoral ROS level. All the combination facts of thermos-gelling polypeptide hydrogel reflected on enhancement of antitumor efficacy compared with free drugs.⁸⁵ The tumor homing

peptide, arginyl-glycyl-aspartic acid conjugated IDO1 nano-inhibitors which exhibited pH-responsive disassembly of polypeptide along with esterase-catalysed drug release.⁸⁶ The improvement of *in-vivo* antitumor efficacy was distinguished by inhibition of intra-tumoral IDO1 activity with this nano-inhibitors. The chlorin-based nanoscale metal-organic framework was also designed which selectively destroyed tumors by releasing the loaded IDO1 inhibitors and PDT application without altering normal tissue damaged.⁸⁷ PDT application also used in a glutathione responsive porphyrin conjugated phospholipid. The IDO1 drug encapsulated porphyrin-phospholipid-based liposome was cleaved by glutathione reached tumors and released of loaded drugs. The prolonged of drug circulation and enhancement of drug accumulation in tumors exhibited the enhancement of antitumoral activity of loaded drug.⁸⁸ Some chemotherapeutic agents evoked the antitumor immune-response with the induction of immunogenic cell death. A cationic nano-delivery system which successfully delivered IDO1 siRNA with oxaliplatin, chemotherapeutic agent to both tumor tissue and tumor-draining lymph nodes.⁸⁹ This approach achieved the synergetic antitumor effects by controlling dendritic cell maturation, regulatory T cell's function etc. The co-delivery of docetaxel and NLG919 by the PVA-based nanogels were also reported.⁹⁰ Folic acid receptor on the nano-carrier selected the tumors and acidic degradation of the carrier enhanced the drug action on tumor tissue. In a chemo-immunotherapy approach, selective delivery of doxorubicin, immunogenic cell death-inducing chemo drug with indoximod was also observed.⁹¹ The self-assembly of phospholipid-conjugated dual-prodrug constructed the liposome. In summary, all the innovative nanocarrier-based approach gave a new dimension of IDO1 mediated cancer treatment.

1.13. Summary

IDO1 constructs a giant domain in cancer-immunotherapy because of its significant relation with immune system. The function of IDO1 directly correlates with immunosuppressive mechanism of CTLA4 or PD-1 or PD-L1 in cancer progression. Notably, IDO1 inhibition represents a therapeutic approach beyond the immune-checkpoint inhibitors in cancer immunotherapeutic treatment. Till now several classes of IDO1 inhibitors are reported by academic or pharmaceutical groups but few of them infiltrates into clinical trials. The branches of variable in

enzymatic screening system deviates selection of the true and potent IDO1 inhibitors. Recently, phase III ECHO-301/KEYNOTE-252 clinical trials of epacadostat with pembrolizumab generates the ambiguity, immune suppression role of IDO1. The disappointing efficacy of monotherapies for IDO1 inhibitors also suffers recent clinical trials. However, dual IDO1/TDO targeted inhibitors exhibits exciting preclinical data though the role of TDO in immunogenic cancer is not explored yet. The preclinical result of chemo or radiotherapy with IDO1 inhibitors might be effective in cancer battle as co-treatment. The involvement of nano-carrier with encapsulation IDO1 drugs improves the efficacy in tumor treatment was also impressive. Increasing the drug efficacy might be a novel approach to overcome the failure of IDO1-monotherapeutic drug. The specific drug action within the targeted position might be another concern as the IDO1 induction spread all through the non-hepatic cells. Apart from these, the multi component dependent IDO1 function remain a big concern and solving to a single solution might be a breakthrough of IDO1-mediated cancer-immunotherapy treatment. A long journey of IDO1 is still waiting to reach the milestone of advance cancer-immunotherapy treatment in the cancer battle.

1.14. Objective of research work

Based on literature, the details information of IDO1-mediated immune-suppression during cancer motivated us develop small molecule-based IDO1 inhibitors to control different immune-mediated diseases including cancer. The current thesis work has been arranged into four chapters. In **chapter 2** we have focused how the substrate, L-Trp mimic indazole derivatives execute its selective IDO1 inhibitory potency. **Chapter 3** mainly deals how triazole derivatives mitigates IDO1 mediated tumor growth. **Chapter 4** elaborates how quinine-based IDO1 inhibitors targets both apo-IDO1 and free heme for the inhibition process. **Chapter 5** corroborates how a delivery vehicle improves chemo and immunotherapeutic, IDO1 inhibitors efficacy for the treatment of cancer.

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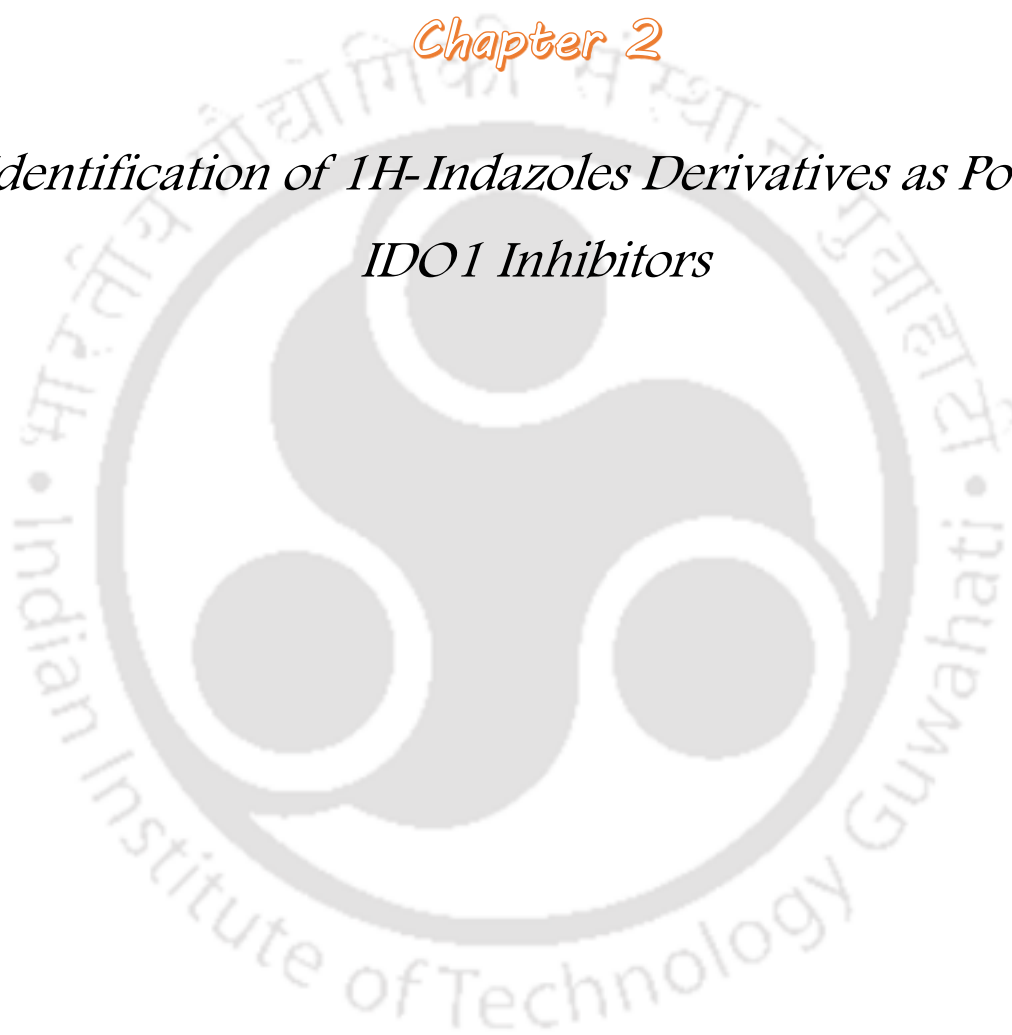
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Chapter 2

Identification of 1H-Indazoles Derivatives as Potent IDO1 Inhibitors





2.1. Background and objective of present work

The literature reviewed in first chapter discussed IDO1 as a validated target for the treatment of diseases that were associated with immune suppression, including cancer recently.¹⁻³ Overexpression of the immunosuppressive enzyme, IDO1 was associated with poor prognosis of patients for a wide range of malignancies.^{4,5} Recent studies in different animal models of cancer showed that inhibition of IDO1 activity could successfully pin down the anomalous growth of tumors.⁶⁻⁸ IDO1 inhibition also showed improved effect with radiotherapeutic and chemotherapeutic treatment. Hence, development of new classes IDO1 inhibitors is our interest.

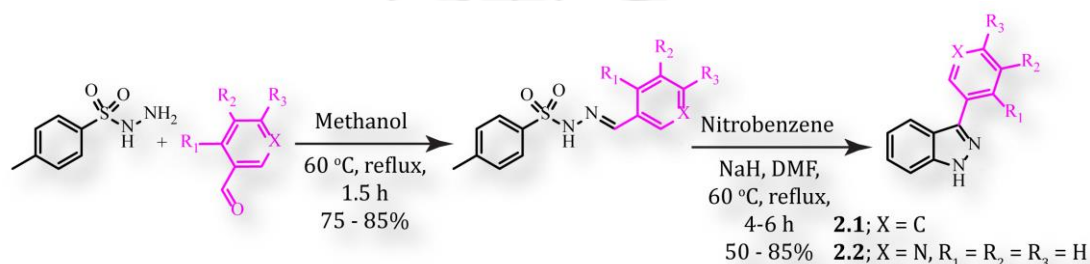
The different classes IDO1 inhibitors were known, containing the scaffolds of indole, quinone/iminoquinone, triazoles, hydroxyamidines, imidazole and others natural products.^{9,10} Most of the reported inhibitors failed or showed low potency in animal model. Several L-Trp-based inhibitors were also known as suitable IDO1 inhibitors, L1-MT among off them. The detailed mechanistic investigation of L-Trp catabolism by IDO1 enzyme was revealed that the involvement of C2-C3 double bond of the indole ring.^{11,12} 1*H*-indazoles was structurally similar with the replacement of C2-C3 double bond of indole to C3-N2 double bond. Hence, 1*H*-indazoles was a suitable replacer of indole ring of the substrate, L-Trp and recently 4- and 6-substituted 1*H*-indazole was reported as suitable IDO1 inhibitors.¹³ However, no indazole derivatives displayed higher potency against IDO1 enzyme. The details structural investigation, experimental evidences and molecular docking analyses arose the questions for proper binding of these 4- and 6-substituted 1*H*-indazole to the active site of IDO1 enzyme. In this regard, we hypothesised the 3-substituted 1*H*-indazole might be suitable alternative of 4- and 6-substituted 1*H*-indazole for IDO1 inhibition.

To find potent and selective IDO1 inhibitors, we synthesized a small series of 3-substituted 1*H*-indazole derivatives and examined their IDO1 inhibitory potency. Additionally, several others experiments were conducted with potent 3-substituted 1*H*-indazole to evaluate the therapeutic potential regarding IDO1 inhibition.

2.2. Results and discussions

2.2.1. Synthesis of 3-substituted 1*H*-indazole derivatives

The series of 3-aryl-1*H*-indazoles (**2.1a–2.1o** and **2.2**) were synthesized by reacting nitrobenzene with aryl-tosylhydrazone in DMF solvent with NaH as base. (Scheme 2.1).¹⁴ This metal free synthetic protocols allowed us to synthesize various halogen substituted 3-aryl groups containing 1*H*-indazoles derivatives. The corresponding aromatic aldehydes were used to synthesis aryl-tosylhydrazones. The similar reaction conditions were applied to synthesis compound **2.2** from 3-pyridinecarboxaldehyde. This favourable synthetic procedure was advantageous for large-scale production of 1*H*-indazole derivatives and their structural modification.



Scheme 2.1 Synthesis of 3-aryl-1*H*-indazoles from nitrobenzene and aryl-tosylhydrazone.

2.2.2. Inhibitory activity against purified human IDO1 enzyme

After synthesizing the 1*H*-indazole derivatives, the IDO1 inhibitory activities were initially evaluated by spectrophotometric method as described in literature.¹⁵⁻¹⁷ The UV-Vis spectra of the compounds with in the concentration range of 0.1 mM to 100 mM displayed no or negligible meddling with the enzymatic assay. The evaluated K_m and K_{cat} of the IDO1 enzyme were $24.86 \pm 3.9 \mu\text{M}$ and $5.23 \pm 0.32 \text{ s}^{-1}$, respectively with L-Trp. In order to optimize the potency of 1*H*-indazole scaffold, we mainly explored the 3-aryl structural modifications of the 1*H*-indazole core structures. We first inspected the importance of 3-aryl substitution of the 1*H*-indazoles on IDO1 enzyme activity (Table 2.1). The IC_{50} value of the reported compounds, L1-MT and 4-amino-*N*-(3-chloro-4-fluorophenyl)-*N'*-hydroxy-1,2,5-oxadiazole-3-carboximidamide were 203 and 95 nM, respectively which was consistent with the reported values under the experimental conditions.¹⁸ Among the tested 1*H*-indazoles derivatives, compound **2.1i** and **2.1m** showed modest IDO1 inhibition activity with IC_{50} value of 8.82 and 5.4 μM , respectively (Table 2.1). This observation suggested that the hydrophobicity and di-halogen substitution on 3-aryl ring of 1*H*-indazole scaffold might be vital to uphold the inhibition process through the interactions with

“pocket-A”. Overall, the intended inhibitory potency suggested that the 3-aryl modification of the 1*H*-indazole derivatives resulted in reasonable and/or trivial alteration in inhibitor potency. The installation of hetero aromatic moiety at the C3-position of the 1*H*-indazole scaffold in **2.2** was unproductive as well. Therefore, these data indicated that C3-substitution of the 1*H*-indazole might be a salient scaffold for potent IDO1 inhibitory activity.

Table 2.1 Inhibitory activity of the 3-aryl substituted 1*H*-indazoles against purified human IDO1 enzyme.

Compound		IDO1 inhibition, IC ₅₀ (μM) ^a
2.1a	R ₁ =R ₂ =R ₃ =H	19.62 ± 0.72
2.1b	R ₁ = F, R ₂ =R ₃ =H	21.73 ± 0.19
2.1c	R ₁ = Cl, R ₂ =R ₃ =H	10.73 ± 0.23
2.1d	R ₁ =R ₃ =H, R ₂ =F	12.16 ± 0.30
2.1e	R ₁ =R ₃ =H, R ₂ =Cl	13.91 ± 0.56
2.1f	R ₁ =R ₂ =H, R ₃ =F	13.83 ± 0.78
2.1g	R ₁ =R ₂ =H, R ₃ =Cl	17.58 ± 0.26
2.1h	R ₁ =R ₂ =H, R ₃ =Br	15.91 ± 0.26
2.1i	R ₁ =R ₂ =H, R ₃ =Me	8.82 ± 0.72
2.1j	R ₁ =R ₂ =H, R ₃ =OMe	19.64 ± 0.19
2.1k	R ₁ =R ₂ =H, R ₃ =NO ₂	21.55 ± 0.23
2.1l	R ₁ =H, R ₂ =R ₃ =F	12.93 ± 0.30
2.1m	R ₁ =H, R ₂ =R ₃ =Cl	5.46 ± 0.56
2.1n	R ₁ =H, R ₂ =Cl, R ₃ =F	15.36 ± 0.78
2.1o	R ₁ =H, R ₂ =Br, R ₃ =F	14.46 ± 0.26
2.2		9.32 ± 0.26

[a] IC₅₀ values are the mean of three independent assays.

Additionally, the IDO1 enzyme activities of selected 1*H*-indazoles were also reanalysed by HPLC-based assays for endorsement of their inhibition potency.¹⁹ The observed IC₅₀ values exposed that the inhibition activities follow a parallel pattern as

that were showed by the spectrophotometric method (Table 2.2). The differences in detected IC_{50} values between the HPLC-based and spectrophotometric assays could because of the precision reaction of *p*DMAB with NFK.²⁰ The HPLC-based assay also revealed **2.1i** exhibited moderate IC_{50} of 7.04 μ M and compound **2.2** presented IC_{50} of 17.91 μ M (Table 2.2).

In general, the C3-substituted 1*H*-indazoles was sensitive to IDO1 inhibition, possibly for the reason that of the restricted space in 'pocket-A' of the enzyme. Reasonable inhibiton potency of the 3-aryl-1*H*-indazole derivatives could be due to dealings of aryl moiety with the residues present in the interior of IDO1 binding 'pocket-A' and interaction of heme with 1*H*-indazole moiety. Any substitution in the aryl ring showed insignificant inhibitory effect on IDO1 activity. Presence of electron contributing methyl group and electron diminishing halogen substitution in the aryl moiety also displayed similar effects in their inhibitory potencies. All these results reinforced the hydrophobicity and projected binding mode for compound with IDO1 plays the foremost role in IDO1 inhibition.

Table 2.2 HPLC-based IDO1 inhibition assays of the selected compounds.

Compound	IDO1 inhibition, IC_{50} (μ M) ^a
1-L-MT	147.08 $\pm$ 13.67
2.1i	7.04 $\pm$ 1.19
2.1m	6.27 $\pm$ 0.52
2.2	17.91 $\pm$ 2.19

[a] IC_{50} values are the mean of three independent assays.

2.2.3. Binding analysis of 1*H*-indazole derivatives with IDO1 enzyme

The ligand-binding efficacy to the IDO1 enzyme were evaluated by UV-Vis spectral study.²¹ The optical properties of IDO1-heme-group depended to the local milieu of active site. The 1*H*-indazoles derivatives exposed their IDO1 inhibition capability, but unsuccessful to give any direct indication of ligand binding to the IDO1 active pocket. To find the ligand binding propensity, the UV-Vis spectra of ferric and deoxy-ferrous-IDO1 in the presence and absence of the compounds were investigated (Figure 2.1). The Soret peak at 404 nm for ferric-IDO1enzyme became

displaced by 2–5 nm in the presence of **2.1i**, **2.1m** and **2.2** compounds which was not that much significant. But, deoxy-ferrous-IDO1 enzyme aborted the substantial shift in Soret peak at 421 nm and Q-band 556 nm. Hence, the spectral properties suggested that lacking of direct binding of **2.1i**, **2.1m** and **2.2** to both ferric and ferrous-IDO1 enzyme. The slight depletion of both ferric and ferrous Soret peak was observed in presence of the compounds which signified the changing of active pocket's environment upon binding of compounds. The UV-Vis spectra of compounds in Tris-HCl buffer were not interfering in this region. The UV-Vis and HPLC-based inhibition assay, along with the UV-Vis spectral binding analysis of enzyme-compound solution specified that binding of these potent compounds was not off target to the IDO1 enzyme and compounds binds to the IDO1 active pockets without direct binding to heme group.

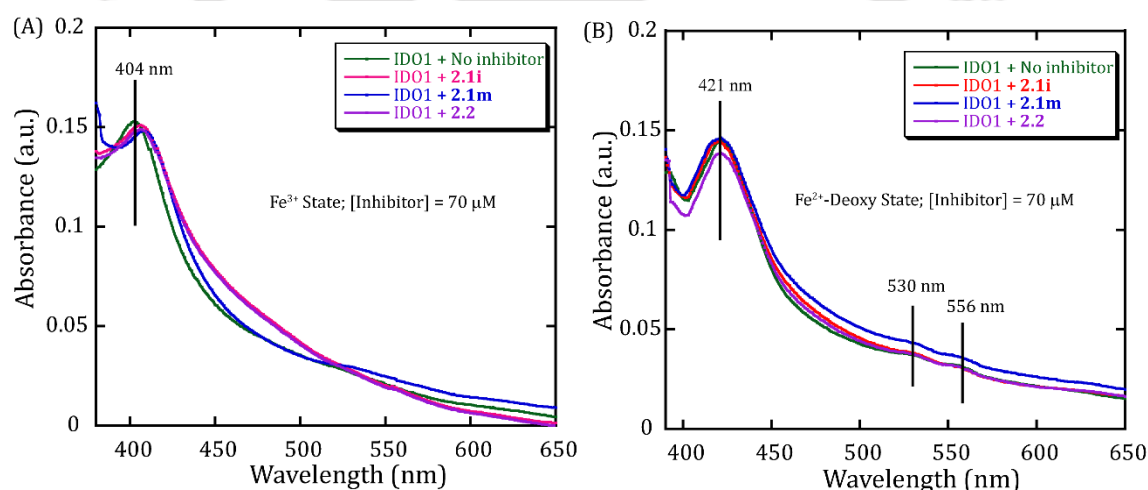


Figure 2.1 Absorption spectra of ferric-IDO1 (A) and deoxy-ferrous-IDO1 enzyme (B). in absence and presence of 70 μM compounds in Tris-HCl buffer (50 mM, at pH 8.0). IDO1 concentration was 5 μM. Na₂S₂O₄ was added to the solution under N₂ atmosphere to generate ferrous-deoxy reaction environment.

2.2.4. Cellular IDO1 inhibitory activities and toxicity of 1*H*-indazole derivatives

To explore the therapeutic potential of these selected 1*H*-indazole derivatives, cellular IDO1 inhibitory activities and toxicity were investigated in MDA-MB-231 cell. It was well documented that IFN-γ significantly expressed the native IDO1 enzyme from its mRNA in MDA-MB-231 cell.²² The cellular IDO1 inhibitory potencies of the compounds also followed analogous pattern alike against purified IDO1 enzyme

(Table 2.3). Under the similar experimental conditions, 4-amino-*N*-(3-chloro-4-fluorophenyl)-*N'*-hydroxy-1,2,5-oxadiazole-3-carboximidamide presented EC₅₀ values of 59 nM, which was in accordance with the former reported values.¹⁸ Though, smaller differences in inhibitory potency of these compounds could be because of the complications in regulating the IDO1 redox activity. The cytotoxicity analysis (MTT assay) of these compounds in MDA-MB-231 cells showed a low level of toxicity under up to the twice of IC₅₀ concentration range (Figure 2.2 (A)). Inclusive, the cellular toxicity and IDO1 inhibitory potency correlated the therapeutic potential of the 1*H*-indazoles derivatives.

Table 2.3 EC₅₀ values of the selected compounds for MDA-MB-231 cells.

Compound	MDA-MB-231 cells EC ₅₀ ^b
2.1i	1.71 ± 0.01
2.1m	0.78 ± 0.01
2.2	0.89 ± 0.01

[b] EC₅₀ values are the mean of three independent assays.

2.2.5. Mode of IDO1 enzyme inhibition by the potent 1*H*-indazole derivatives

The enzyme inhibition studies suggested that 1*H*-indazoles derivatives strongly inhibited IDO1 activity. To investigate the mode of IDO1 inhibition, we performed enzyme kinetics in the presence and absence of these selected compounds.²³ The Lineweaver- Burk plot was used to examine the modes of IDO1 inhibition by the compounds. The enzyme kinetics analysis disclosed that compounds **2.1i**, **2.1m** and **2.2** followed uncompetitive mode of inhibition with respect to the substrate L-Trp.

Table 2.4 Mode of IDO1 inhibition by the potent 1*H*-indazole derivatives.

Compound	Mode of IDO1 inhibition
2.1i	uncompetitive
2.1m	uncompetitive
2.2	uncompetitive

However, these calculated modes of IDO1 inhibition might not be validate their true mode of enzyme inhibition. The several literatures supported the uncompetitive or

non-competitive inhibitors, which strongly bound IDO1 enzyme and perturbed the catalytic activity. The detailed L-Trp catabolism mechanism of IDO1 promoted that the formation of ferric-superoxide intermediate was the primary requirement for this oxidation process. Thus, supplementary kinetic studies with respect to O_2 was required to investigate the actual mode of IDO1 enzyme inhibition by these 1*H*-indazole derivatives, which was beyond the scope of this study.⁹

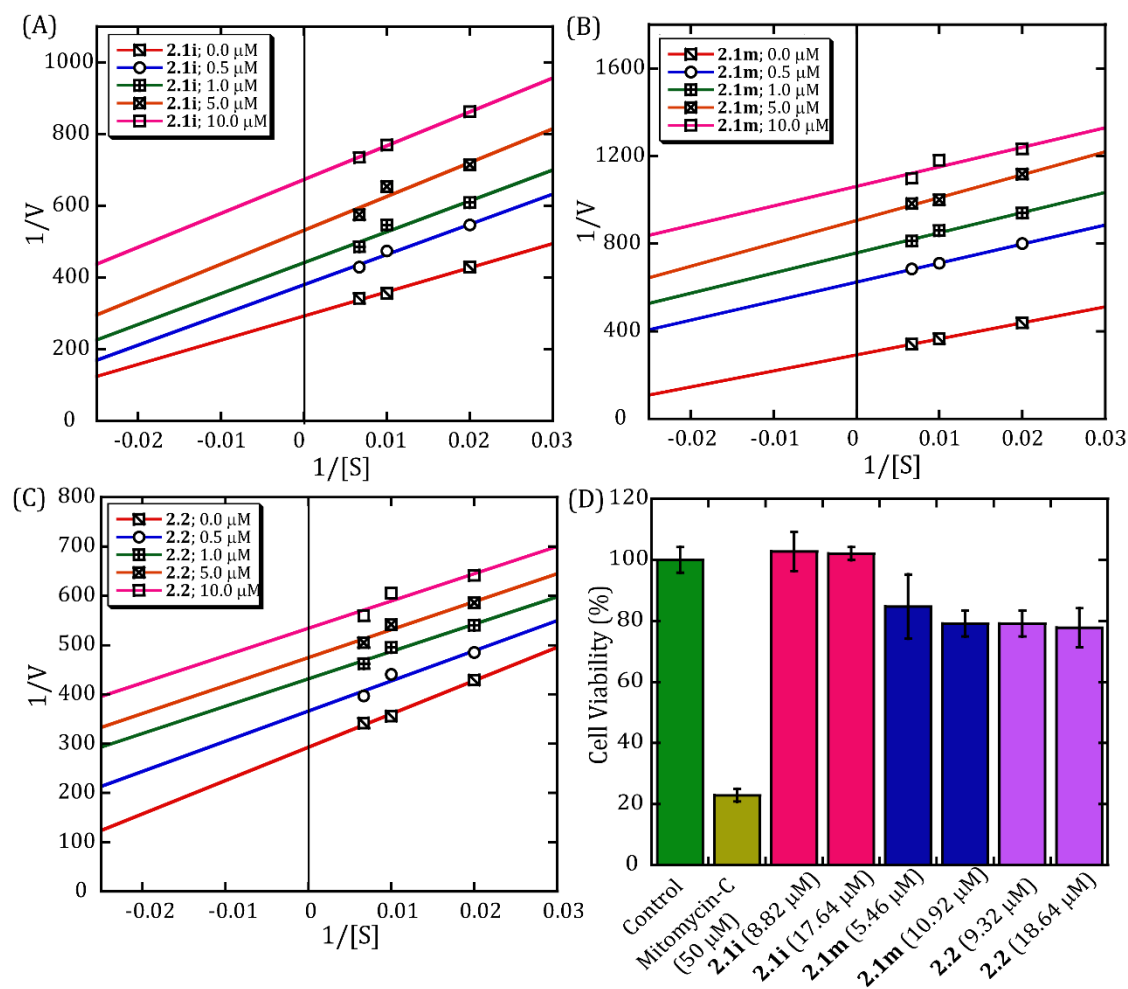


Figure 2.2 Determination of mode of inhibition of the selected compounds. Plot of $1/V$ against $1/[S]$ at different concentration of the compounds **2.1i** (A), **2.1m** (B) and **2.2** (C). Effect of the selected 1*H*-indazole derivatives on the viability of MDA-MB-231 cells (D).

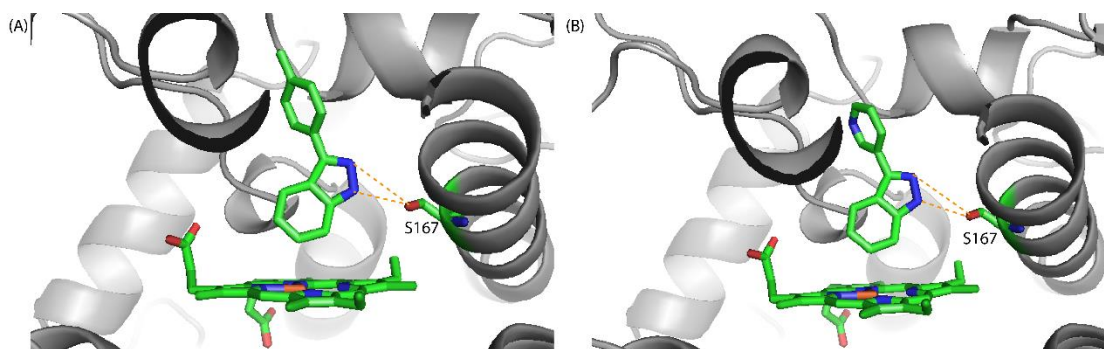


Figure 2.3 Probable mode of interaction of the compounds **2.1i** (A), and **2.2** (B) with of the IDO1 enzyme (4PK5). The model structures were generated using AutoDock 4.2.0. Residues involved in interactions through hydrogen bond formation were shown using dashed lines (yellow). Images were generated using PyMol.

2.2.6. Probable mode of interaction of 1*H*-indazole derivatives with IDO1 enzyme

The IDO1 inhibitory activity and UV-Vis binding analysis revealed that the potent 1*H*-indazoles interact with heme-containing IDO1 active site. To understand their probable mode of interactions, additional molecular docking studies of the potent compounds with IDO1 enzyme were performed (PDB code: 4PK5).²⁴ The interaction energy of compound **2.1i** and **2.2** were -9.09 kcal/mol and -8.58 kcal/mol, respectively. The docked structures and their interaction energies proposed that the 1*H*-indazoles (**2.1i** and **2.2**) preferably interacted with IDO1 enzyme through its “pocket-A” (comprises of Tyr126, Phe163 and Ser167 residues). The 1*H*-indazole ring and the 3- aryl moiety could be participated in interaction with Tyr126, Phe163, Phe164 residues and others present in “pocket-A” through hydrophobic and pi-stacking interactions (Figure 2.3). The 1*H*-indazole ring could be interacted with the Ser167 residue in hydrogen bonding interaction. However, the model structure suggests that 1*H*-indazole derivatives unsuccessful to interact directly with heme group. Though the UV-Vis binding analysis suggested weak ferric heme binding properties of the potent 1*H*-indazole compounds. This differences in the mode of interaction of compounds could be due to their effectiveness in proper fitting into the binding pocket, which was considered as rigid-body during docking analysis. In solution the IDO1 binding pocket might have different conformation(s), which might possibly explain true binding into the active pocket. The interaction energies were in accordance with their inhibitory potency. Overall, hydrogen

bonding, hydrophobic interactions and pi-stacking interaction and molecular volume were facile the substrate L-Trp alike 1*H*-indazole derivatives binding into IDO1 active pockets.

Table 2.5 Inhibitory activity of the selected compounds against purified human IDO1 and TDO enzymes.

Compound	IDO1 inhibition (IC ₅₀ / μ M)) ^a	TDO inhibition (IC ₅₀ / μ M)) ^a	Selectivity ratio ^b
2.1i	8.82 $\pm$ 0.72	95.30 $\pm$ 4.89	11
2.1m	5.46 $\pm$ 0.56	86.81 $\pm$ 2.14	16
2.2	9.32 $\pm$ 0.26	78.24 $\pm$ 3.21	8

[a] IC₅₀ values were the mean of three independent assays against purified enzymes.

[b] Selectivity ratio was calculated as (IC₅₀ value of TDO)/ (IC₅₀ value of IDO1).

Table 2.6 HPLC-based TDO inhibition assays of the selected compounds.

Compound	TDO inhibition (IC ₅₀ / μ M) ^a
2.1i	187.51 $\pm$ 11.19
2.1m	135.24 $\pm$ 3.26
2.2	102.58 $\pm$ 7.43

[a] IC₅₀ values calculated by HPLC method (were the mean of three independent assays).

2.2.7. IDO1 enzyme selectivity of 1*H*-indazole derivatives against purified TDO enzyme

In addition, we analysed TDO enzyme inhibition activities in the presences and absences of the potent 1*H*-indazole compounds. TDO enzyme also participates in kynurenine mediated L-Trp catabolism pathway. TDO also controls the cellular L-Trp level. Hence, to regulate the selectivity of these potent 1*H*-indazole compounds, inhibition activity of TDO enzyme was also measured. TDO activity studies presented that these 1*H*-indazoles compounds, **2.1i** and **2.1m** preferentially inhibited IDO1 (>11-fold) over TDO enzyme under the similar experimental conditions (Table 2.5). Additional HPLC-based TDO inhibitory activities also exhibited analogous inhibition

potency of these selected compounds (Table 2.6). Overall, IDO enzyme inhibitory activity of these 1*H*-indazoles derivatives suggested that these compounds might also targeted variable enzyme inhibition potencies as the designed compound was alike the substrate L-Trp.

2.3. Summary

In this chapter, we synthesized a small series of 3-substituted 1*H*-indazole derivatives and investigated their IDO1 inhibitory efficiencies. Structural modifications including the installation of 3-aryl ring were explored for IDO1 inhibition activity. Couple of 3-aryl 1*H*-indazole derivatives exhibited low-micromolar level inhibitory activity against purified human IDO1 enzyme. The presence of 1*H*-indazole ring and suitable substitution at the C3 position of the parent scaffold played a key role for their moderate *in vitro* inhibitory activities. Spectroscopic-based binding studies indicated that the 1*H*-indazoles preferably interacted with active pocket residues but failed the significant binding with heme group. The slight shifting of ferric-IDO1 Soret peak and docking analysis were not impressive for the potent 1*H*-indazole derivatives. Apart from this, the lower toxicity and moderate activity for IDO1 enzyme supported the requirement for further development of 1*H*-indazole derivatives as potent IDO1 inhibitor. Overall, C3-substitution in the 1*H*-indazole ring might interrupt further radical reaction with Fe³⁺-superoxide intermediate leading to a better approach in designing mechanism-based IDO1 inhibitors.

2.4. Experimental section

2.4.1. Synthesis of the compounds

2.4.1.1. General information

All reagents were purchased from different commercial sources and used directly without further purification. Reactions were monitored by TLC on silica gel 60 F254 (0.25 mm). ¹H NMR and ¹³C NMR were recorded at 400 and 100 MHz, respectively with Varian AS400 spectrometer and 600 and 151 MHz, respectively with Bruker spectrometer, using TMS as an internal standard with CDCl₃, DMSO-*d*₆ and D₂O. The *J* values and δ_{ppm} were reported in Hz and ppm, respectively. Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), m (multiplet)

and br (broadened). HRMS were recorded at Agilent Q-TOF mass spectrometer with Z-spray source using built-in software for analysis of the recorded data. Melting point data were recorded at Buchi Melting Point B-540 apparatus.

2.4.1.2. General procedure for the synthesis of substituted *N*-tosyl aryl hydrazine

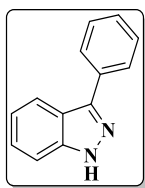
To a stirring solution of the substituted aromatic aldehydes (2 mmol) in methanol (25 mL), was added the desired amount of *p*-toluenesulfonyl hydrazide (2.2 mmol) and the mixture was refluxed at 60 °C for 1.5 hours. After completion of the reaction (monitored by TLC), the excess solvent was removed under reduced pressure and the crude product was obtained as precipitate. The precipitate was washed with petroleum ether and dried under reduced pressure to obtain the desired product with sufficient purity that was directly used for the next step without further purification.²⁵

2.4.1.3. General procedure for the synthesis of substituted 1*H*-indazole from the corresponding *N*-tosyl aryl hydrazine

The 1*H*-indazoles were synthesized according to the reported procedure with slight modifications.¹⁴ Substituted *N*-tosyl arylhydrazone (1.0 mmol) and sodium hydride (2.0 mmol) were added to a double necked round bottomed flask fitted with a condenser and stirred in dry DMF (5.0 mL) for 10 minutes. Then a solution of nitrobenzene (1.1 mmol) in dry DMF was added dropwise under continuous stirring condition and the reaction mixture was refluxed at 60 °C for 4-6 hours. Extent of completion of the reaction was monitored by TLC. After completion of the reaction, excess solvent was removed under reduced pressure and the crude mixture was washed with water. The aqueous layer was then extracted with ethyl acetate (3 × 30 mL) and the combined organic layer was dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. Purification by column chromatography with silica gel and a gradient solvent system of EtOAc to hexane (10 – 25%) provided the desired product with moderate to good yield.

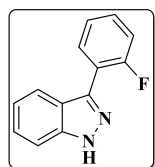
2.4.1.4. Characterization of synthesized compounds

3-Phenyl-1H-indazole (2.1a): brown semi-solid (85%); ^1H NMR (600MHz, CDCl_3)



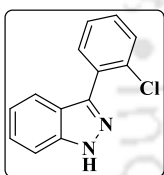
δ_{ppm} 11.46 (br, s, 1H), 8.05-8.01 (m, 3H), 7.56-7.53 (m, 2H), 7.47-7.44 (m, 1H), 7.39-7.37 (m, 1H), 7.33-7.31 (m, 1H), 7.26-7.21 (m, 1H); ^{13}C NMR (151 MHz, CDCl_3) δ_{ppm} 145.9, 141.9, 133.8, 129.2, 128.4, 127.9, 127.1, 121.6, 121.4, 121.2, 110.5; HRMS (ESI) calcd for $\text{C}_{13}\text{H}_{10}\text{N}_2$ $[\text{M} + \text{H}]^+$ 195.0917, found 195.0918.

3-(2-Fluoro-phenyl)-1H-indazole (2.1b): yellow semi-solid (62%); ^1H NMR



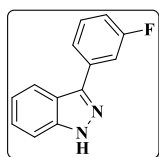
(600MHz, CDCl_3) δ_{ppm} 11.70 (br, s, 1H), 7.79-7.75 (m, 2H), 7.38-7.34 (m, 1H), 7.30-7.27 (m, 1H), 7.23-7.19 (m, 3H), 7.13-7.11 (m, 1H); ^{13}C NMR (151 MHz, CDCl_3) δ_{ppm} 161.2, 159.6, 141.4, 131.4, 130.3, 127.2, 124.6, 122.1, 121.8, 121.5, 116.5, 110.3; HRMS (ESI) calcd for $\text{C}_{13}\text{H}_9\text{FN}_2$ $[\text{M} + \text{H}]^+$ 213.0823, found 213.0823.

3-(2-Chloro-phenyl)-1H-indazole (2.1c): orange semi-solid (81%); ^1H NMR



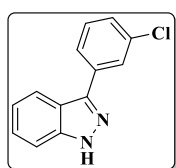
(400MHz, CDCl_3) δ_{ppm} 7.33-7.30 (m, 2H), 7.28-7.21 (m, 4H), 7.19-7.15 (m, 2H); ^{13}C NMR (100 MHz, CDCl_3) δ_{ppm} 144.3, 141.0, 133.6, 132.4, 131.9, 130.4, 130.3, 130.0, 129.5, 127.1, 121.9, 121.4, 110.4; HRMS (ESI) calcd for $\text{C}_{13}\text{H}_9\text{ClN}_2$ $[\text{M} + \text{H}]^+$ 229.0527, found 229.0527.

3-(3-Fluoro-phenyl)-1H-indazole (2.1d): yellow semi-solid (57%); ^1H NMR (600MHz, CDCl_3) δ_{ppm} 10.81 (br, s, 1H), 7.96 (d, 1H, $J = 12$ Hz), 7.72 (d, 1H, $J = 12$ Hz),



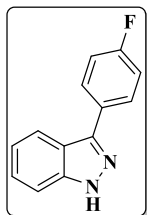
7.64 (d, 1H, $J = 12$ Hz), 7.43-7.34 (m, 3H), 7.20-7.17 (m, 1H), 7.06-7.03 (m, 1H); ^{13}C NMR (151 MHz, CDCl_3) δ_{ppm} 162.6, 144.8, 141.9, 135.9, 130.7, 127.3, 123.4, 122.0, 121.1, 115.3, 115.2, 114.7, 110.4; HRMS (ESI) calcd for $\text{C}_{13}\text{H}_9\text{FN}_2$ $[\text{M} + \text{H}]^+$ 213.0823, found 213.0823.

3-(3-Chloro-phenyl)-1H-indazole (2.1e): yellow semi-solid (68%); ^1H NMR (600MHz, CDCl_3) δ_{ppm} 11.36 (br, s, 1H), 7.93-7.90 (m, 2H), 7.80 (d, 1H, $J = 12$ Hz), 7.53



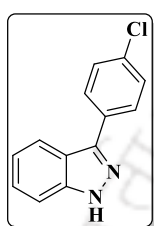
(s, 1H), 7.35-7.30 (m, 4H); ^{13}C NMR (151 MHz, CDCl_3) δ_{ppm} 144.6, 141.9, 135.4, 135.0, 130.4, 129.2, 128.5, 127.8, 126.6, 125.9, 122.1, 121.1, 110.5; HRMS (ESI) calcd for $\text{C}_{13}\text{H}_9\text{ClN}_2$ $[\text{M} + \text{H}]^+$ 229.0527, found 229.0527.

3-(4-Fluoro-phenyl)-1H-indazole (2.1f): brown solid (77%), m.p. 112-114 °C; ¹H



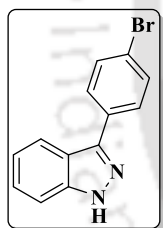
NMR (400MHz, CDCl₃) δ_{ppm} 11.7 (br, s, 1H), 7.89-7.86 (m, 3H), 7.30-7.28 (m, 1H), 7.19-7.11 (m, 4H); ¹³C NMR (100 MHz, CDCl₃) δ_{ppm} 161.8, 145.0, 141.8, 129.6, 129.5, 127.2, 121.7, 121.1, 120.0, 116.3, 116.1, 110.5; HRMS (ESI) calcd for C₁₃H₉FN₂ [M +H]⁺ 213.0823, found 213.0823.

3-(4-Chloro-phenyl)-1H-indazole (2.1g): yellow semi-solid (71%); ¹H NMR



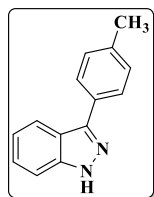
(600MHz, CDCl₃) δ_{ppm} 10.66 (br, s, 1H), 7.91 (d, 1H, *J* = 6 Hz), 7.86 (d, 2H, *J* = 12 Hz), 7.42 (d, 2H, *J* = 12 Hz), 7.37-7.33 (m, 2H), 7.18-7.16 (m, 1H); ¹³C NMR (151 MHz, CDCl₃) δ_{ppm} 144.9, 141.9, 134.3, 132.2, 129.3, 129.0, 127.3, 121.9, 121.1, 110.4; HRMS (ESI) calcd for C₁₃H₉ClN₂ [M +H]⁺ 229.0527, found 229.0527.

3-(4-Bromo-phenyl)-1H-indazole (2.1h): yellow solid (73%), m.p. 118-120 °C; ¹H



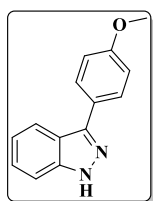
NMR (400MHz, CDCl₃) δ_{ppm} 7.91 (d, 1H, *J* = 8 Hz), 7.79 (d, 2H, *J* = 8 Hz), 7.58 (d, 2H, *J* = 8 Hz), 7.35-7.29 (m, 2H), 7.18-7.14 (m, 1H); ¹³C NMR (100 MHz, CDCl₃) δ_{ppm} 144.8, 141.8, 132.6, 132.3, 129.3, 127.3, 122.5, 121.9, 121.1, 120.9, 110.5; HRMS (ESI) calcd for C₁₃H₉BrN₂ [M +H]⁺ 273.0022, found 273.0020.

3-*p*-Tolyl-1H-indazole (2.1i): yellowish semi-solid (87%); ¹H NMR (400MHz,



CDCl₃) δ_{ppm} 7.94 (d, 1H, *J* = 8 Hz), 7.83 (d, 2H, *J* = 8 Hz), 7.30-7.19 (m, 4H), 7.14-7.10 (m, 1H), 2.37 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ_{ppm} 146.0, 141.9, 138.3, 130.8, 130.2, 129.9, 127.8, 127.0, 121.4, 121.2, 116.0, 110.4, 21.6; HRMS (ESI) calcd for C₁₄H₁₂N₂ [M +H]⁺ 209.1073, found 209.1075.

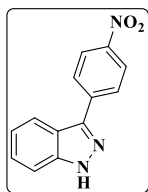
3-(4-Methoxy-phenyl)-1H-indazole (2.1j): yellow solid (62%), m.p.203-205 °C; ^1H



NMR (400MHz, $\text{CDCl}_3 + \text{MeOD-}d_4$) δ_{ppm} 8.38 (br, s, 1H), 8.01-7.89 (m, 2H), 7.80-7.76 (m, 2H), 7.35-7.31 (m, 1H), 7.15-7.11 (m, 1H), 7.02-6.97 (m, 2H), 3.82 (s, 3H); ^{13}C NMR (100 MHz, $\text{CDCl}_3 + \text{MeOD-}d_4$) δ_{ppm} 160.1, 159.6, 146.7, 128.9, 126.9, 122.0, 121.2, 115.6, 114.6, 114.4, 110.4, 55.5;

HRMS (ESI) calcd for $\text{C}_{14}\text{H}_{12}\text{N}_2\text{O}$ $[\text{M} + \text{H}]^+$ 225.1022, found 225.1025.

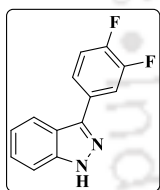
3-(4-nitrophenyl)-1H-indazole (2.1k): yellow solid (51%), m.p.180-182 °C; ^1H



NMR (400MHz, $\text{CDCl}_3 + \text{MeOD-}d_4$) δ_{ppm} 8.23-8.22 (m, 2H), 8.02-7.98 (m, 2H), 7.88-7.86 (m, 1H), 7.45-7.42 (m, 1H), 7.32-7.28 (m, 1H), 7.15-7.10 (m, 1H); ^{13}C NMR (100 MHz, $\text{CDCl}_3 + \text{MeOD-}d_4$) δ_{ppm} 147.0, 142.5, 142.0, 140.4, 127.7, 127.0, 124.2, 112.2, 120.7, 120.4, 110.9; HRMS (ESI) calcd

for $\text{C}_{13}\text{H}_9\text{N}_3\text{O}_2$ $[\text{M} + \text{H}]^+$ 240.0763, found 240.0771.

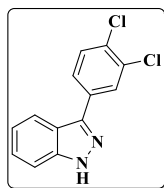
3-(3,4-difluoro-phenyl)-1H-indazole (2.1l): brown semi-solid (53%); ^1H NMR



(600MHz, CDCl_3) δ_{ppm} 10.51 (br, s, 1H), 7.91 (d, 1H, $J = 6$ Hz), 7.75-7.71 (m, 1H), 7.66-7.63 (m, 1H), 7.44 (d, 1H, $J = 6$ Hz), 7.38-7.36 (m, 1H), 7.25-7.18 (m, 2H); ^{13}C NMR (151 MHz, CDCl_3) δ_{ppm} 151.7, 150.1, 144.1, 141.9, 130.8, 127.6, 123.7, 122.1, 120.9, 118.0, 117.9, 116.7, 110.4;

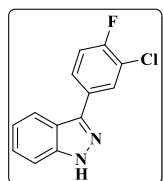
HRMS (ESI) calcd for $\text{C}_{13}\text{H}_8\text{F}_2\text{N}_2$ $[\text{M} + \text{H}]^+$ 231.0728, found 231.0727

3-(3,4-dichloro-phenyl)-1H-indazole (2.1m): yellow semi-solid (58%); ^1H NMR



(600MHz, CDCl_3) δ_{ppm} 7.71-7.70 (m, 2H), 7.46 (d, 2H, $J = 6$ Hz), 7.33-7.31 (m, 3H); ^{13}C NMR (151 MHz, CDCl_3) δ_{ppm} 143.7, 142.1, 133.6, 133.5, 131.1, 130.2, 130.1, 129.4, 127.6, 126.9, 122.4, 120.9, 110.5; HRMS (ESI) calcd for $\text{C}_{13}\text{H}_8\text{Cl}_2\text{N}_2$ $[\text{M} + \text{H}]^+$ 263.0137, found 263.0121

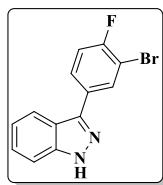
3-(3-Chloro-4-fluoro-phenyl)-1H-indazole (2.1n): yellow solid (67%), m.p.246-



248 °C; ^1H NMR (600MHz, CDCl_3) δ_{ppm} 10.58 (br, s, 1H), 7.97-7.95 (m, 1H), 7.90 (d, 1H, $J = 6$ Hz), 7.79-7.76 (m, 1H), 7.41-7.35 (m, 2H), 7.22-7.18 (m, 2H); ^{13}C NMR (151 MHz, CDCl_3) δ_{ppm} 159.1, 157.4, 143.9, 141.9, 131.1, 129.8, 127.4, 122.1, 121.8, 120.9, 117.3, 110.4; HRMS (ESI) calcd

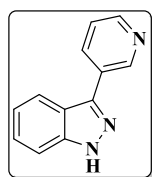
for $\text{C}_{13}\text{H}_8\text{ClFN}_2$ $[\text{M} + \text{H}]^+$ 247.0433, found 247.0432.

3-(3-Bromo-4-fluoro-phenyl)-1H-indazole (2.1o): orange solid (65%), m.p.147-



149 °C; ^1H NMR (400MHz, CDCl_3) δ_{ppm} 8.12-8.10 (m, 1H), 7.90 (d, 1H, J = 8 Hz), 7.84-7.80 (m, 1H), 7.43-7.35 (m, 2H), 7.22-7.17 (m, 2H); ^{13}C NMR (100 MHz, CDCl_3) δ_{ppm} 160.4, 157.9, 143.7, 141.8, 132.7, 131.4, 128.2, 127.4, 122.1, 120.9, 117.2, 117.0, 110.4; HRMS (ESI) calcd for $\text{C}_{13}\text{H}_8\text{BrFN}_2$ $[\text{M} + \text{H}]^+$ 290.9928, found 290.9927.

3-Pyridin-3-yl-1H-indazole (2.2): yellow solid (61%), m.p.175-177 °C; ^1H NMR



(600MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 12.55 (br, s, 1H), 9.21-9.20 (m, 1H), 8.56-8.55 (m, 1H), 8.25-8.22 (m, 1H), 7.94-7.90 (m, 1H), 7.53-7.49 (m, 1H), 7.38-7.29 (m, 2H), 7.18-7.15 (m, 1H); ^{13}C NMR (151 MHz, CDCl_3 + $\text{DMSO}-d_6$) δ_{ppm} 148.6, 148.3, 141.8, 141.6, 134.5, 130.2, 126.4, 123.8, 121.4, 120.7, 120.3, 110.8; HRMS (ESI) calcd for $\text{C}_{12}\text{H}_9\text{N}_3$ $[\text{M} + \text{H}]^+$ 196.0869, found 196.0867.

2.4.2. Purification of the compounds by HPLC analysis

Before performing in vitro enzyme activity, cellular activity, cell viability assays all the synthesized compounds were purified by analytical-HPLC analysis by using Waters 600E HPLC system with an Ascentis® express C18, 2.7 μm HPLC column at a flow rate of 0.5 mL/minute. A solution of 60% methanol and 40% water (isocratic mode) was used as the mobile phase. All the compounds (~1 mg) were dissolved in 1 mL of 60% methanol and 40% water solvent system (total run time was 10 min). The purity levels of the compounds were ≥ 93 -98 %. Analyses were performed by injecting 20 μL of the compound solutions using a UV-detector at 300 nm and the fractions were collected. It was repeated for more than 10-times. The entire collected fractions for a particular compound were dried under reduced pressure and finally the compounds were verified by HRMS analysis.

2.4.3. Expression and purification of recombinant human IDO1 and TDO enzymes

The cDNA of human IDO1 (in the vector pQE30) and TDO (in the vector pET28a) were used for expression of recombinant human IDO1 and TDO enzymes. Both the enzymes were purified according the reported procedures with minor

modifications.²⁶ Briefly, in the protein expression, isopropyl IPTG, hemin and PMSF were added to the culture at the final concentration of 1 mM, 7 μ M and 1 mM, respectively. The cells were grown for 3 hours at 25 °C and 120 rpm. After this incubation period, EDTA was added to the culture at a final concentration of 1 mM and cells were grown for another 12 hours. Cells were collected by centrifugation at 5000 rpm for 10 minutes at 4 °C. The cell pellet was re-suspended in 20 mL of ice-cold PBS containing 1 mM PMSF, 1 mM EDTA and then centrifuged at 15000 rpm for 15 minutes at 4 °C. The pellet was further re-suspended in 20 mL of ice-cold PBS buffer containing 1 mM PMSF and centrifuged at 15,000 rpm for 10 minutes at 4 °C for the removal of EDTA. The pellet was stored at -80 °C. The washed pellet, obtained as described above, was re-suspended in 20 mL of ice-cold 50 mM KPB at pH 6.5 containing 300 mM KCl, 10 mM imidazole, 10 mM MgCl₂, protease inhibitors (complete EDTA free) and DNase (<1mg). The cell lysate was centrifuged at 20,000 rpm for 30 minutes at 4 °C, followed by filtering the supernatant through 0.22 μ m filter. After that, 1 mL of nickel nitrilotriacetic acid resin (Qiagen) was added to the clear supernatant and incubated on ice for 2 hours at 80 rpm. Then, the mixture was poured into the column filled with 50 mM KPB at pH 6.5 containing 300 mM KCl and 10 mM imidazole. For IDO1 enzyme column was sequentially washed with 10 mL of KPB at pH 6.5 containing 300 mM KCl and 20- and 30 mM imidazole, respectively to remove the non-specifically bound protein. The IDO1 protein was eluted with 50 mM KPB at pH 6.5 containing 300 mM KCl and 190 mM imidazole. For TDO enzyme column was sequentially washed with 10 mL of KPB at pH 8.0 containing 300 mM KCl and 20- and 30 mM imidazole, respectively, to remove the non-specifically bound protein. The TDO protein was eluted with 50 mM KPB at pH 8.0 containing 300 mM KCl and 190 mM imidazole. Both IDO1 and TDO proteins were then buffer exchanged by 50 mM Tris-buffer at pH 7.4, 50 mM Tris buffer at pH 8.0, respectively, using Sephadex-G25 column. The purity of the enzyme was confirmed by Coomassie-blue stained SDS-PAGE analysis and it showed 85-90 % purity. The ratio of absorbance of the purified IDO1 and TDO enzyme at 404 nm to that at 280 nm was around 1.3 and 1.2, respectively.

2.4.4. IDO1 and TDO inhibition assay by spectrometric method

Both, IDO1 and TDO inhibition assays were performed according to the earlier reported procedures.^{19, 27} The solubility of the compounds in water was either moderate or poor. Hence, stock solution of the compounds was prepared by first dissolving in DMSO and then diluted with buffer. In the assay system the minimum and maximum amount of DMSO were 0.02% and 2%, respectively. The standard reaction mixture (500 μ L) contained KPB (100 mM, pH 6.5 for IDO1 enzyme and 50 mM, pH 8 for TDO enzyme), sodium ascorbate (20 mM), methylene blue (10 μ M), catalase (240 nM, from brovin liver), L-Trp (150 μ M), purified enzyme (40 nM for IDO1 and 25 nM for TDO), DMSO (0.05 %, v/v), triton-X 100 (0.01 %, v/v) and inhibitors. The reaction was quenched using 100 μ L of 30 % (w/v) TCA. The amount of kynurenine formation was quantified using 2 % (w/v) *p*DMAB in acetic acid. The absorbance of the reaction mixture was recorded by at 480 nm. All these experiments were repeated for three times for each compound.

2.4.5. IDO1 and TDO inhibition assay by HPLC analysis

HPLC-based enzymatic assay for IDO1 and TDO were performed according to the reported procedures.¹⁷ The reaction mixture (250 μ L) contained KPB (100 mM, pH 6.5 for IDO1 and 50 mM, pH 8 for TDO), sodium ascorbate (20 mM), methylene blue (10 μ M), catalase (240 nM, from brovin liver, Sigma), L-Trp (150 μ M), purified enzyme (40 nM for IDO1 and 25 nM for TDO), DMSO (0.05 %, v/v), triton-X 100 (0.01 %, v/v) and inhibitors. The concentration of the compounds was varied from 500 nM to 10 μ M for IDO1 and 1 μ M to 100 μ M for TDO. The reaction mixture was incubated at 37 °C for 1 hour in the absence and presence of the synthesized compounds. The reaction was then quenched using 50 μ L of 30 % (w/v) trichloroacetic acid and incubated at 65 °C for additional 15 minutes to allow complete hydrolysis of *N*-formylkynurenine to kynurenine. Then the reaction mixture was centrifuged at 10,000 rpm for 10 minutes and 100 μ L of the clear reaction mixture was transferred to another tube for HPLC analysis. Then, 20 μ L of the solution was injected through Ascentis® express C18, 2.7 μ m HPLC column with a flow rate of 0.5 mL/minute and mobile phase, containing 50 % sodium citrate buffer (40 mM, pH 2.25) and 50% methanol (v/v) with 400 μ M SDS. The area under the curve at 365 nm corresponds to the kynurenine formation in the absence, presence of inhibitors was recorded, and

the IC₅₀ values were calculated using a standard curve prepared with pure kynurenine (from Sigma) under similar experimental conditions.

2.4.6. Binding analysis by spectroscopic measurement

The efficacy of the compound's direct binding to the enzyme active site was measured by UV-Vis spectroscopic analysis.¹⁹ The spectra were recorded at room temperature using a Perkin Elmer Lambda-25 UV-Vis spectrophotometer. All the measurement was performed using 50 mM Tris-HCl buffer (pH 8.0), IDO1 enzyme (5 μ M) and selected compounds (70 μ M). The deoxy-reaction system was generated by injecting sodium diethionite (~ 10-fold excess) into the solution pre-purged with N₂ gas.

2.4.7. Cell viability analysis

The MTT dye was used to measure cell viability analysis. For this experiment 10,000 cells were seeded in 0.2 mL DMEM/F12 complete medium in 96 well plates and cell culture grade PBS were used to wash the cells after 12 hours incubation. After that, the compounds, at a concentration of IC₅₀ and 2 $\times$ IC₅₀, respectively were incubated into the incomplete medium for 48 hours. Only the cells treated with incomplete medium were considered as 100% viable. 100 μ L of MTT (0.5 mg/mL in PBS) was added into the each well and incubated for 4 hours at 37 °C with 5% CO₂. Then the MTT solution was removed and the formazan crystal was dissolved in 100 μ L cell culture grade DMSO. The absorbance was determined using spectrophotometer (Spectromax M2) at 570 nm and 600 nm.²⁸

2.4.8. Cellular IDO1 activity assay

For the *in vitro* cellular activity assay, MDA-MB-231 breast cancer cells were selected. The cells were treated with human IFN- γ (20 ng/mL) in DMEM/F12 complete media for 48 hours to allow the over expression of IDO1 enzyme in the cells. After that, the compounds (500 nM to 50 μ M) were incubated for a period of 4 hours and 150 μ M of L-L-Trp was incubated for further 5 hours. Cells stimulated with IFN- γ alone served as negative control while cells stimulated with 150 μ M L-Trp served as positive control. Then, the cells were washed with sterile cell-culture grade PBS and were trypsinized followed by centrifugation at 1000 rpm. The cell pellet was re-

dissolved in sterile PBS and centrifuged at 1000 rpm. Then the pellet was lysed in 10 mM HEPES buffer by passing through a sterile syringe. The lysate was used for standard IDO1 assay as mentioned earlier and the IC₅₀ values were determined for each compound.²²

2.4.9. Determination of mode of enzyme inhibition

IDO1 enzyme inhibition mode of the selected compounds was measured according to the reported procedure using spectrophotometric method. The assay was performed by varying L-Trp concentration from 50 to 150 μ M and compound concentrations from 500 nM to 10 μ M. The formation of NFK was recorded at different time interval. The mode of enzyme inhibition was determined from the plot of 1/V against 1/[S], where V was the initial rate of the enzymatic reaction and [S] was the L-Trp concentration.

2.4.10. Molecular docking analysis

AutoDock4.2.0 was used for molecular docking analysis of the compounds with IDO1 enzyme (PDB code: 4PK5).²⁴ The apo-protein was generated and processed by energy minimization. The three-dimensional structure of the ligands was prepared by using the Automated Topology Builder (ATB) and Repository server (<http://atb.uq.edu.au/>). The occupied position of the ligand (in the crystal structure) was used as the centre of docking site (radius: 10 Å; and centre: x = 61, y = 51, z = 19) and the other parameters were set default during docking analysis. Two hundred docked structures were generated in each docking run for an individual ligand and energetically favoured docked conformations were evaluated based on the binding scores (docking score-based on energy function such as a force field with repulsive and attractive van-der Waals terms and electrostatic term). After that, the docking poses were analysed using PyMOL software (The PyMOL Molecular Graphics System, Version 1.0r1, Schrödinger, LLC).

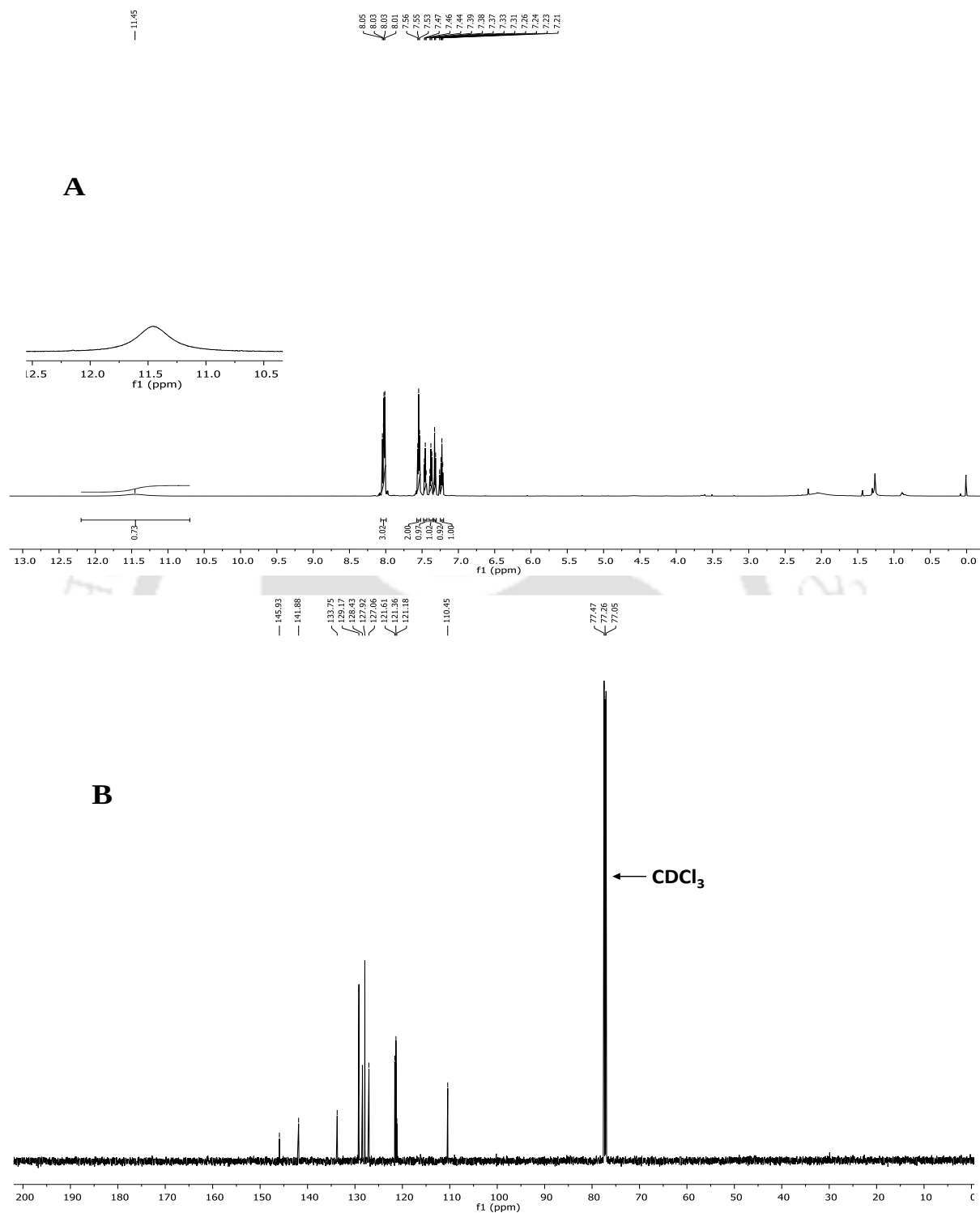
2.5. References

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2.6. ^1H NMR and ^{13}C NMR of the compounds

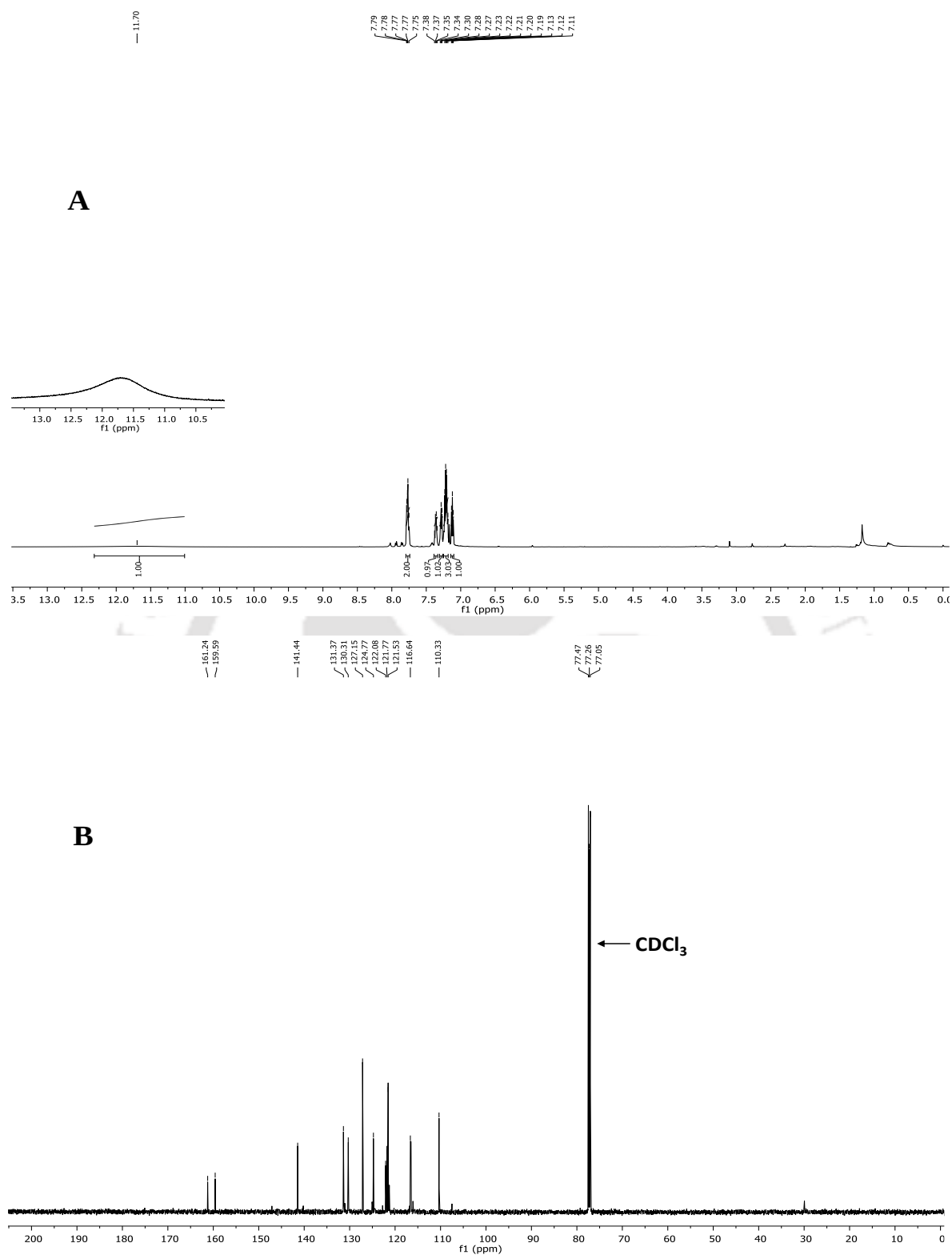


Figure 2.5 ^1H spectra (A) and ^{13}C spectra (B) of compound **2.1b**.

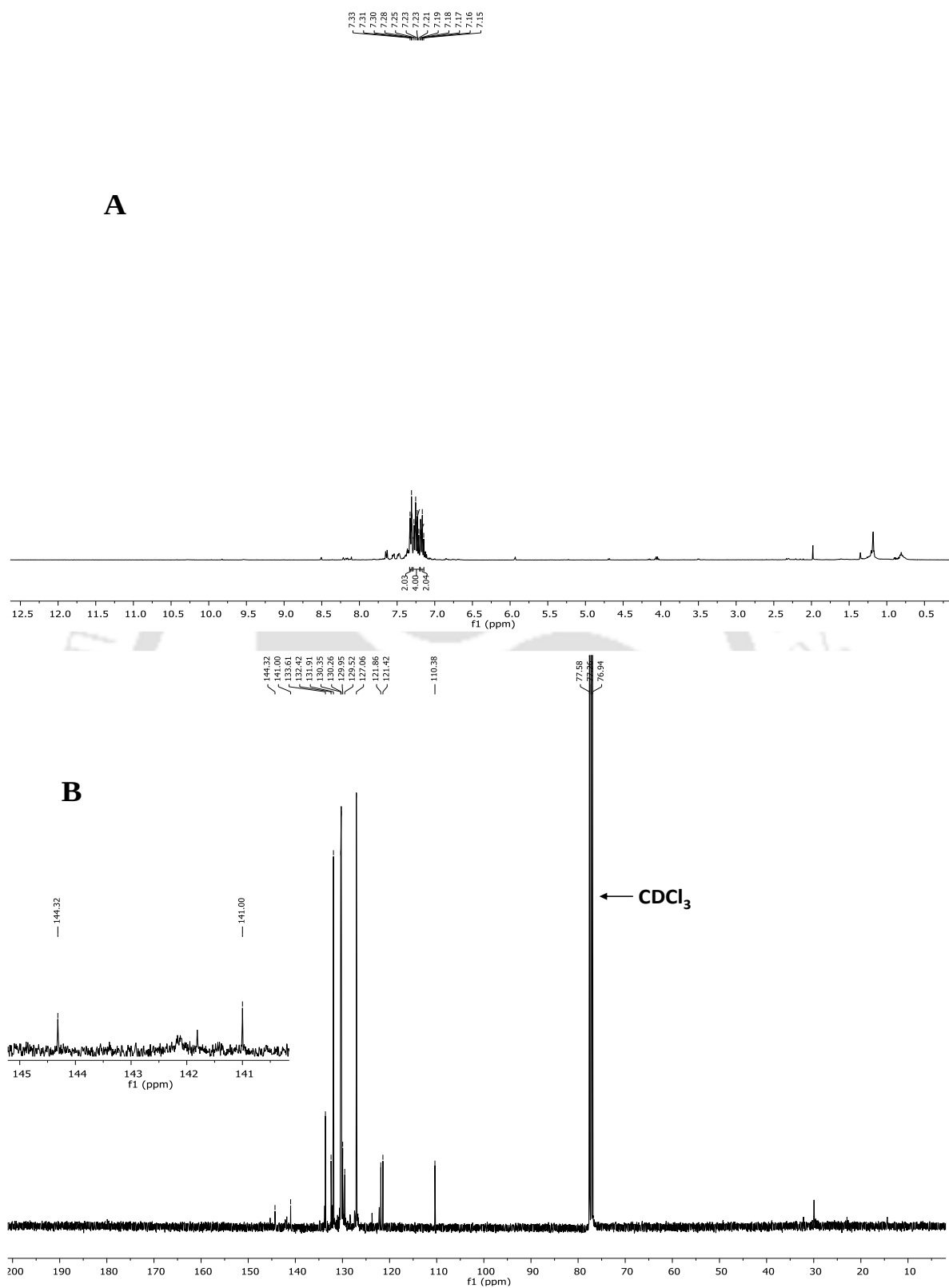


Figure 2.6 ^1H spectra (A) and ^{13}C spectra (B) of compound **2.1c**.

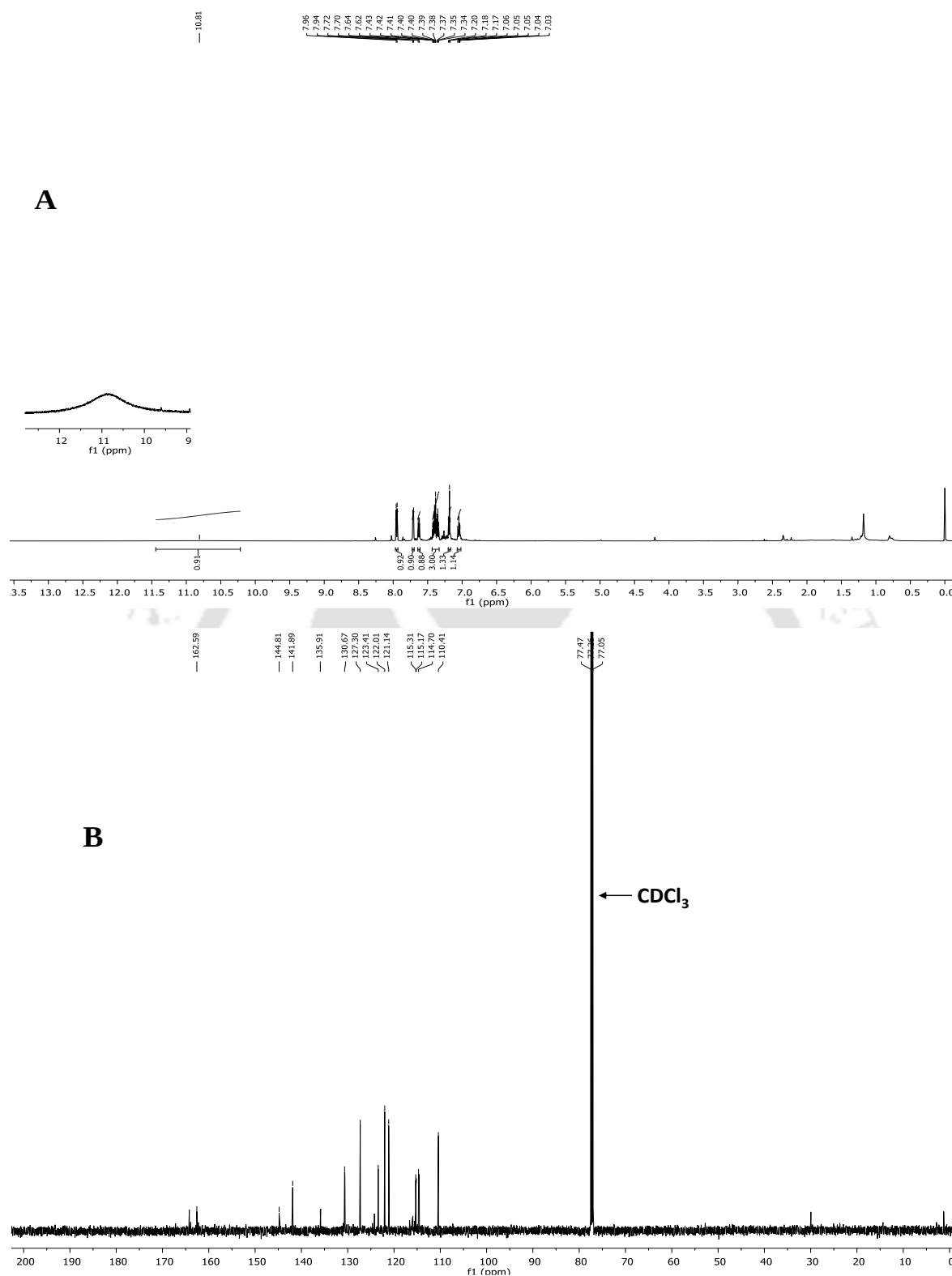


Figure 2.7 ¹H spectra (A) and ¹³C spectra (B) of compound **2.1d**.

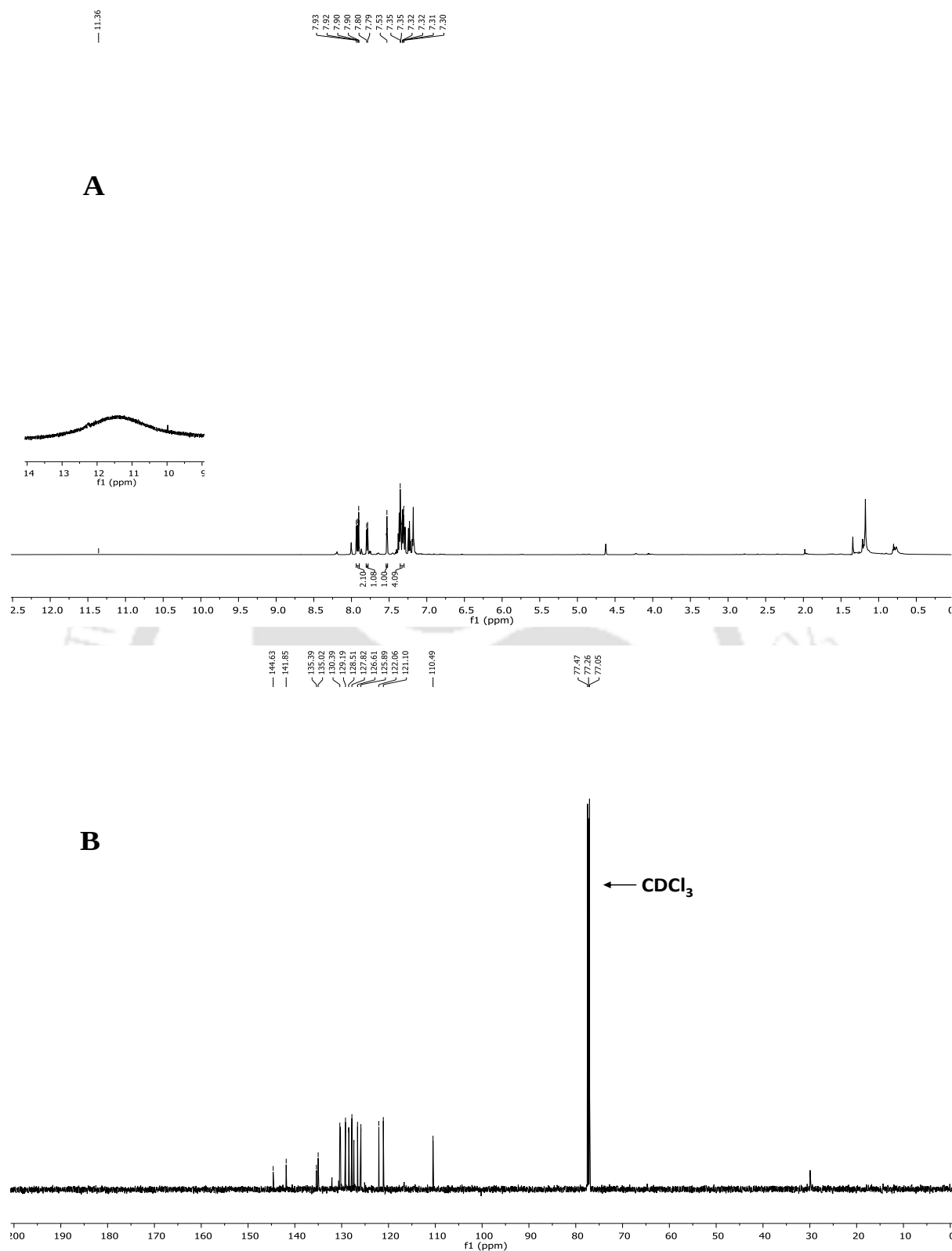


Figure 2.8 ¹H spectra (A) and ¹³C spectra (B) of compound **2.1e**.

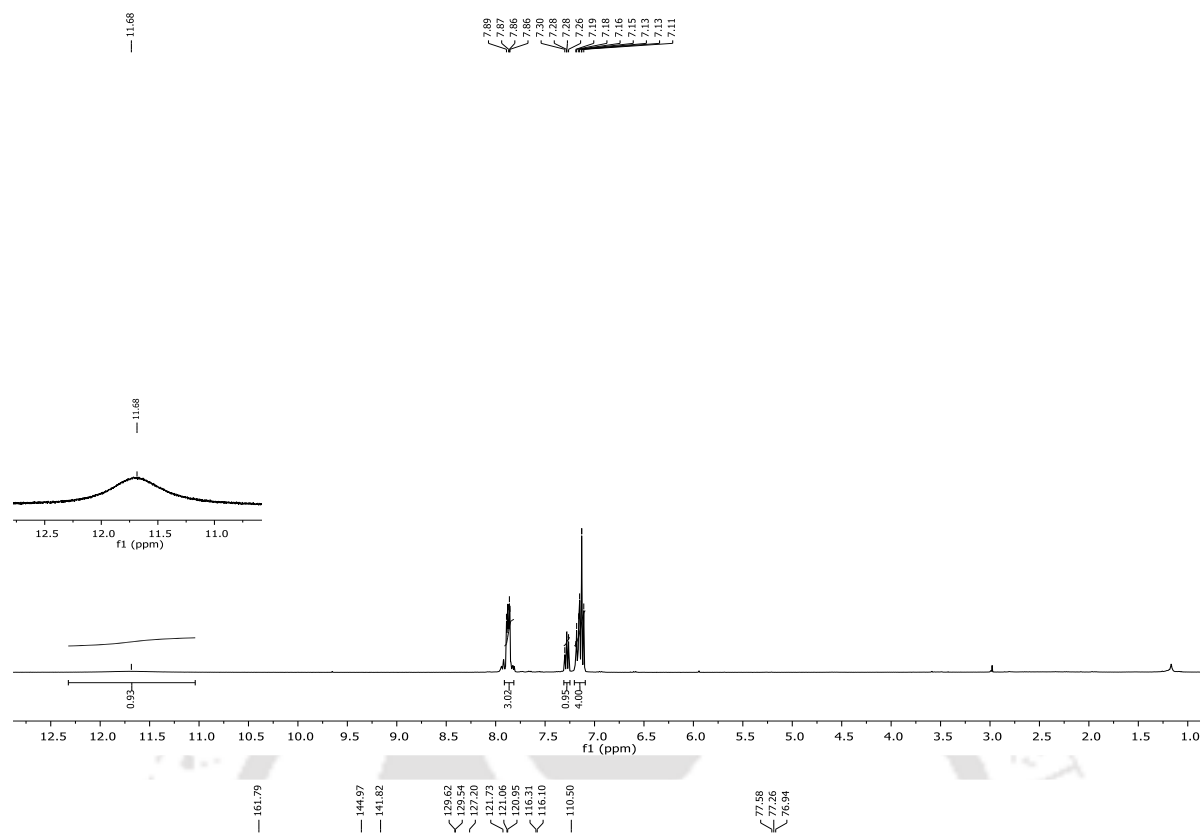
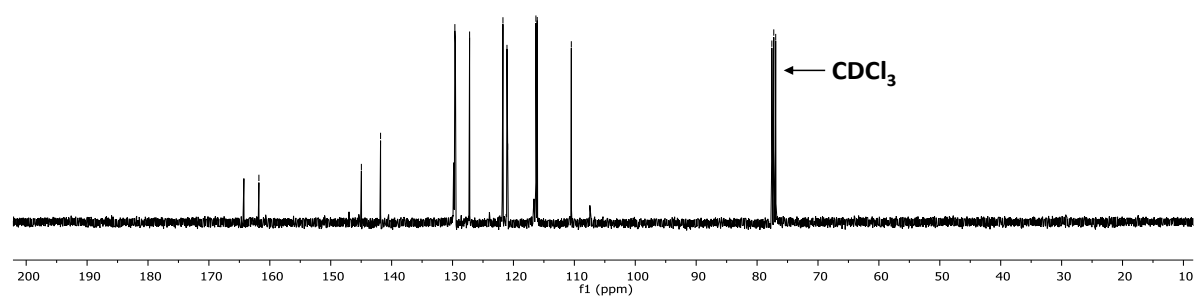
**B**

Figure 2.9 ¹H spectra (A) and ¹³C spectra (B) of compound 2.1f.

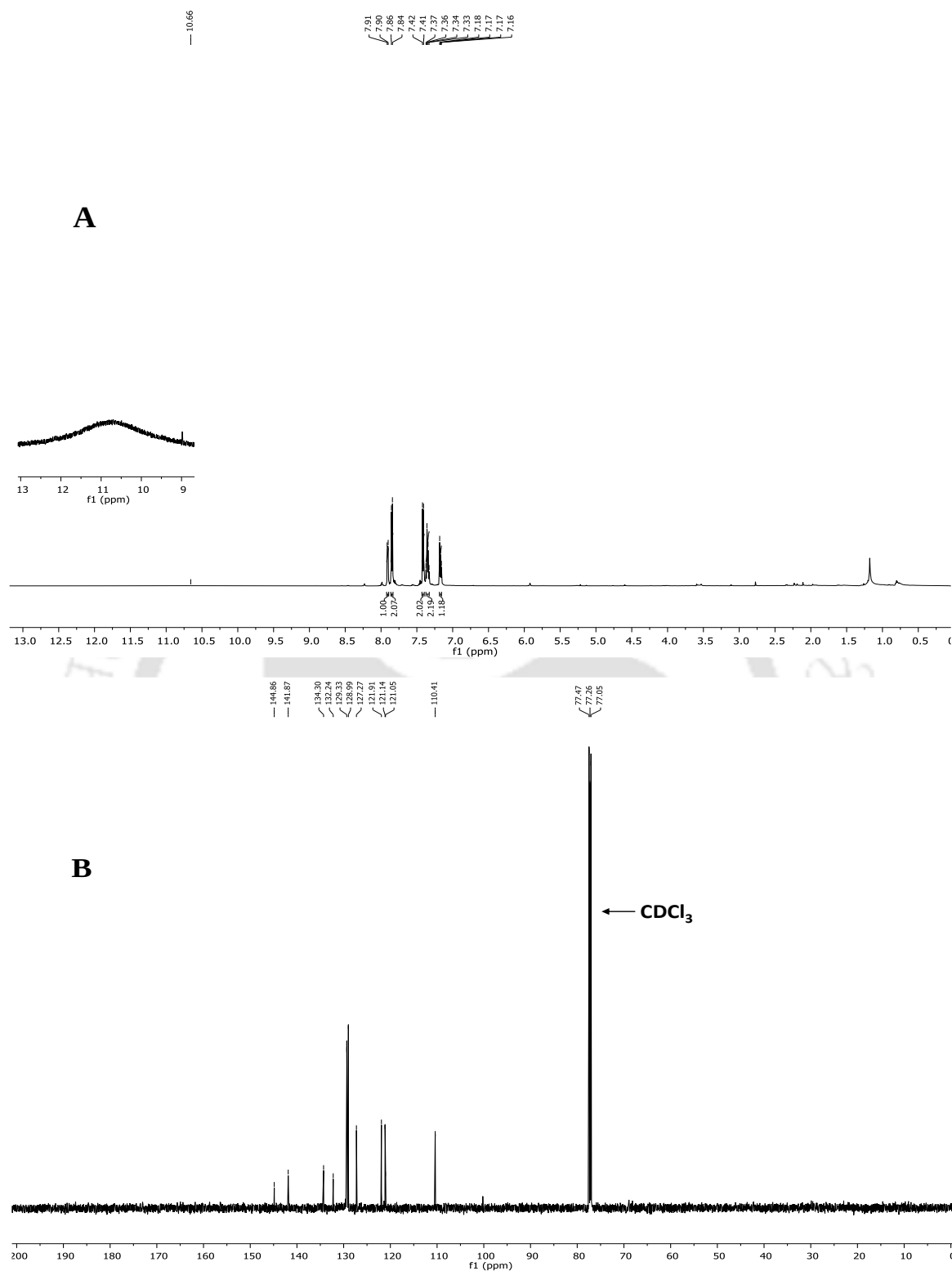


Figure 2.10 ^1H spectra (A) and ^{13}C spectra (B) of compound **2.1g**.

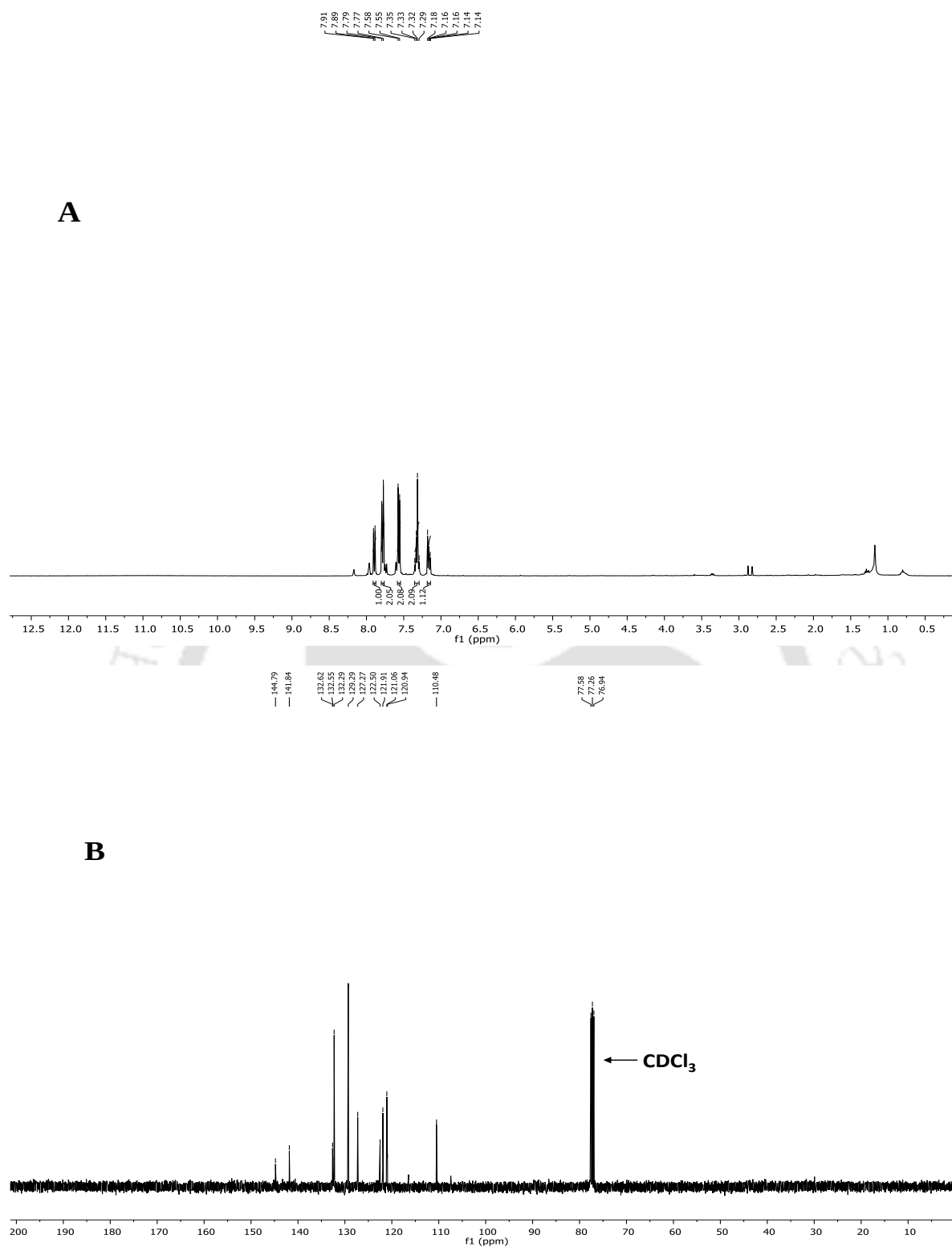


Figure 2.11 ¹H spectra (A) and ¹³C spectra (B) of compound **2.1h**.

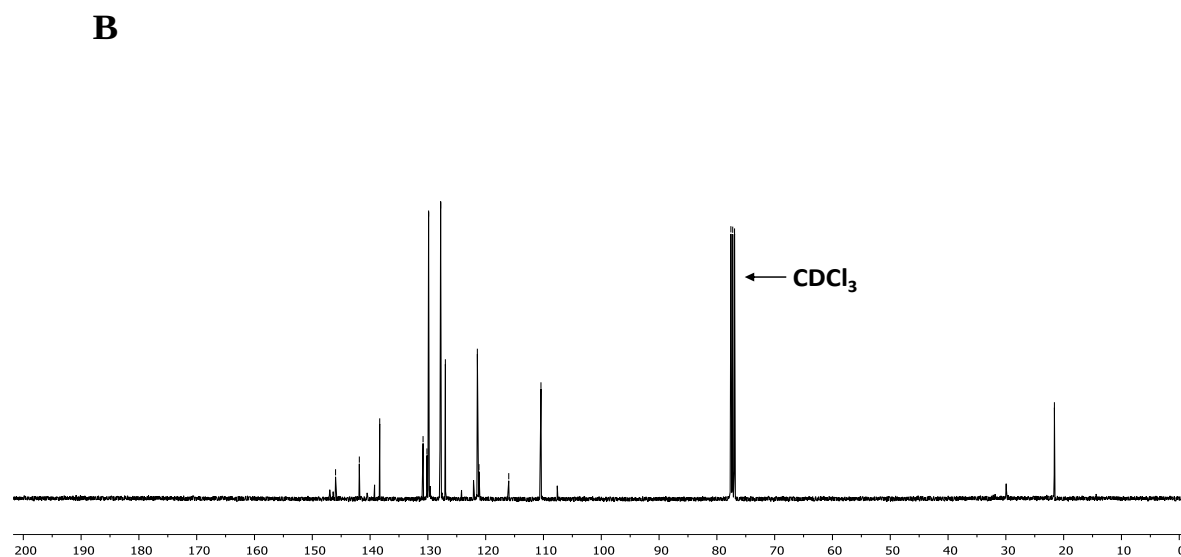
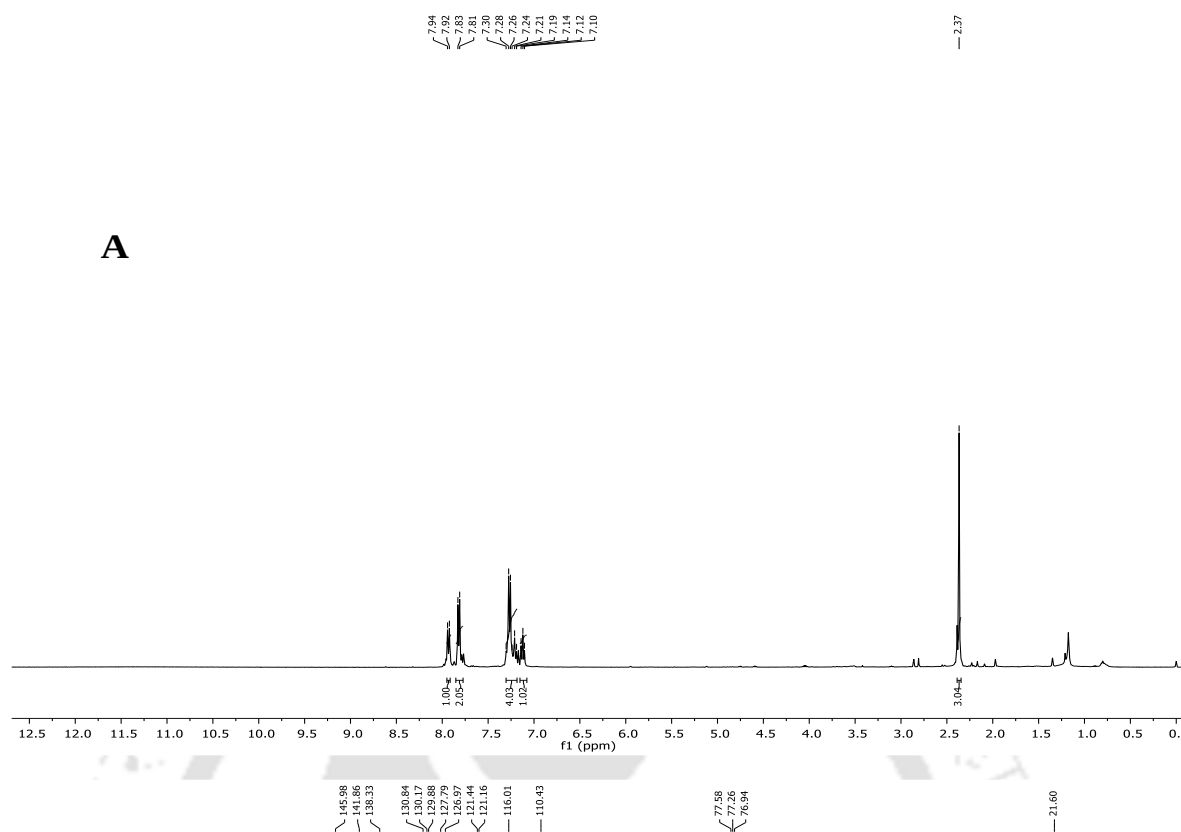


Figure 2.12 ^1H spectra (A) and ^{13}C spectra (B) of compound 2.1i.

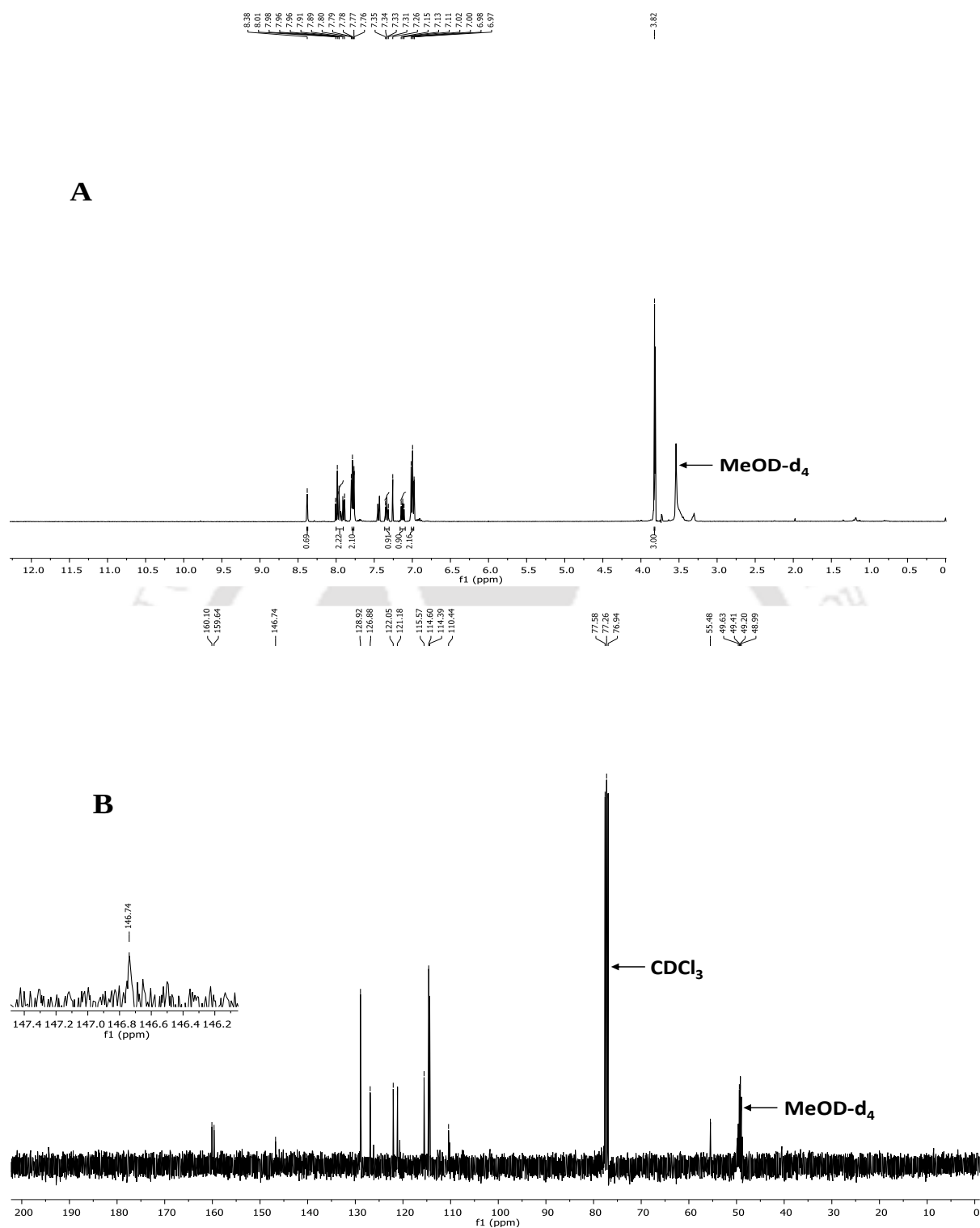


Figure 2.13 ¹H spectra (A) and ¹³C spectra (B) of compound **2.1j**.

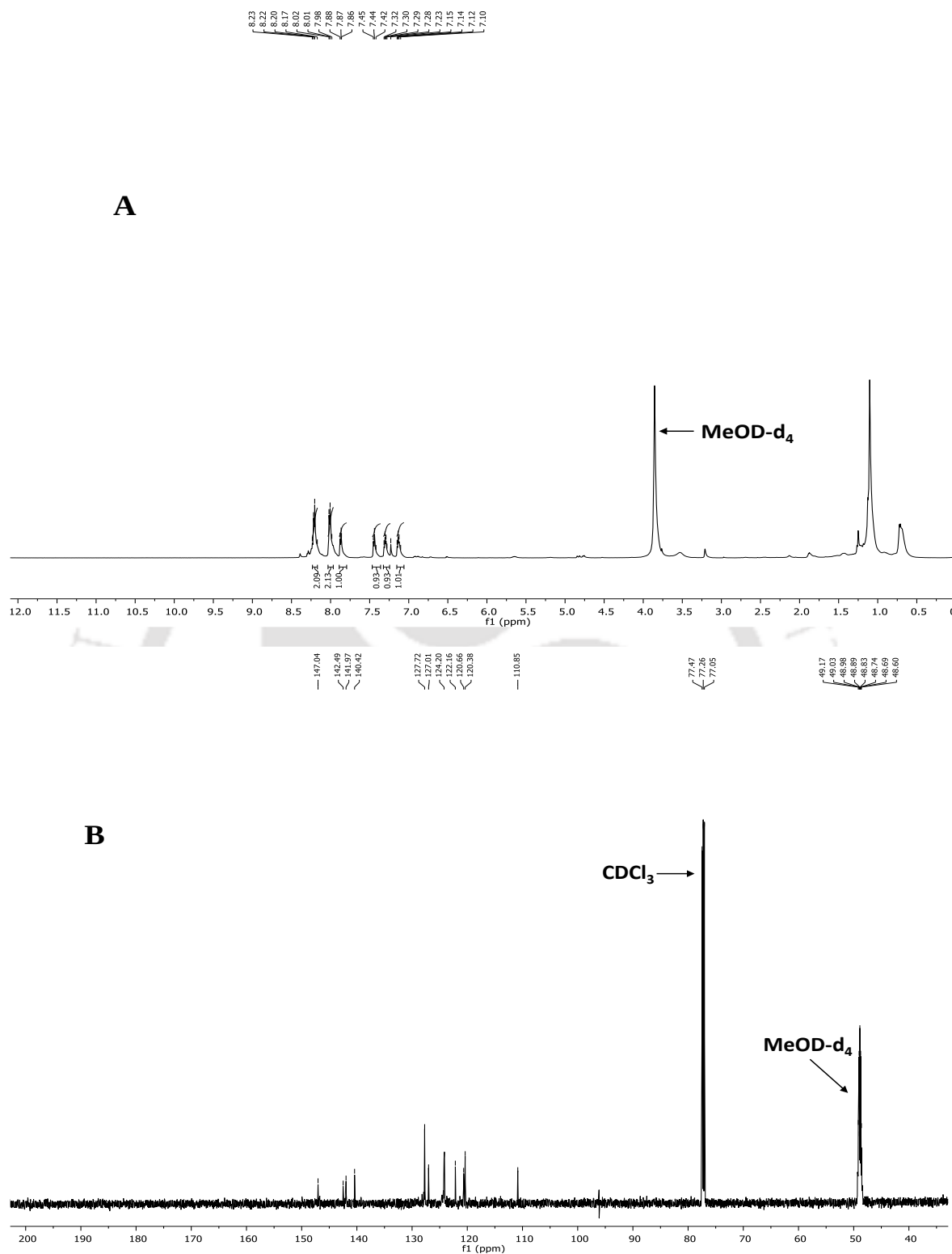


Figure 2.14 ¹H spectra (A) and ¹³C spectra (B) of compound 2.1k.

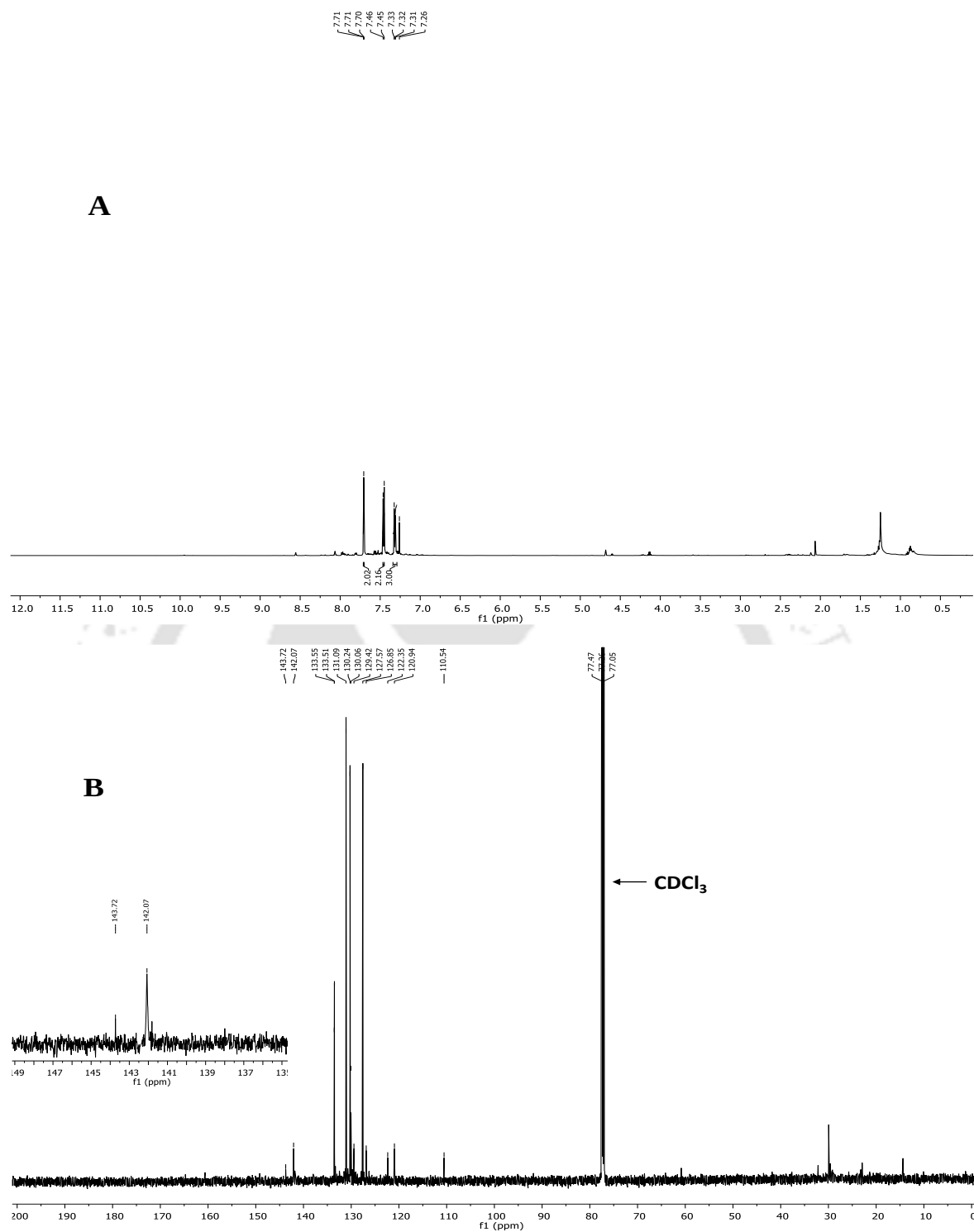


Figure 2.16 ^1H spectra (A) and ^{13}C spectra (B) of compound 2.1m.

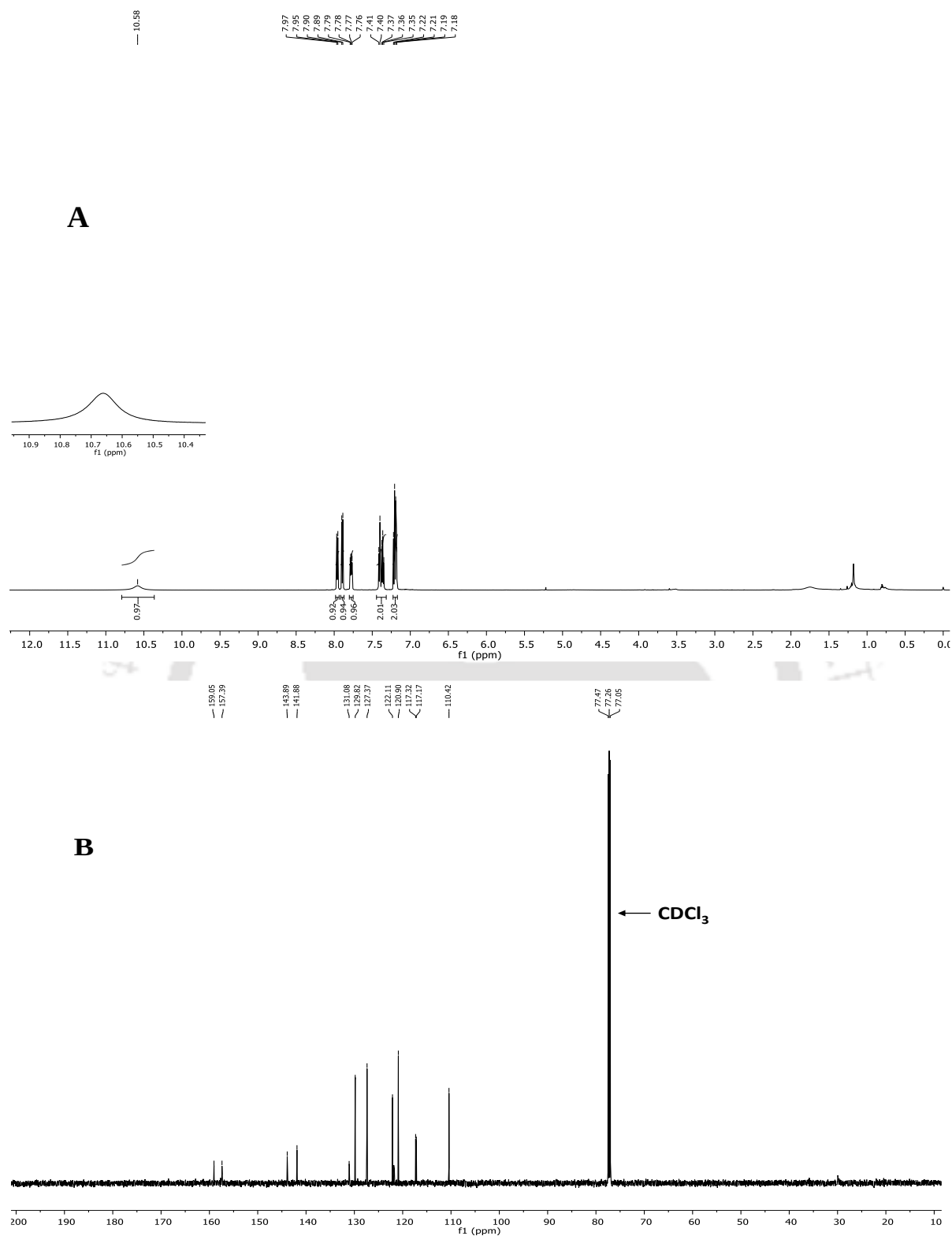


Figure 2.17 ¹H spectra (A) and ¹³C spectra (B) of compound **2.1n**.

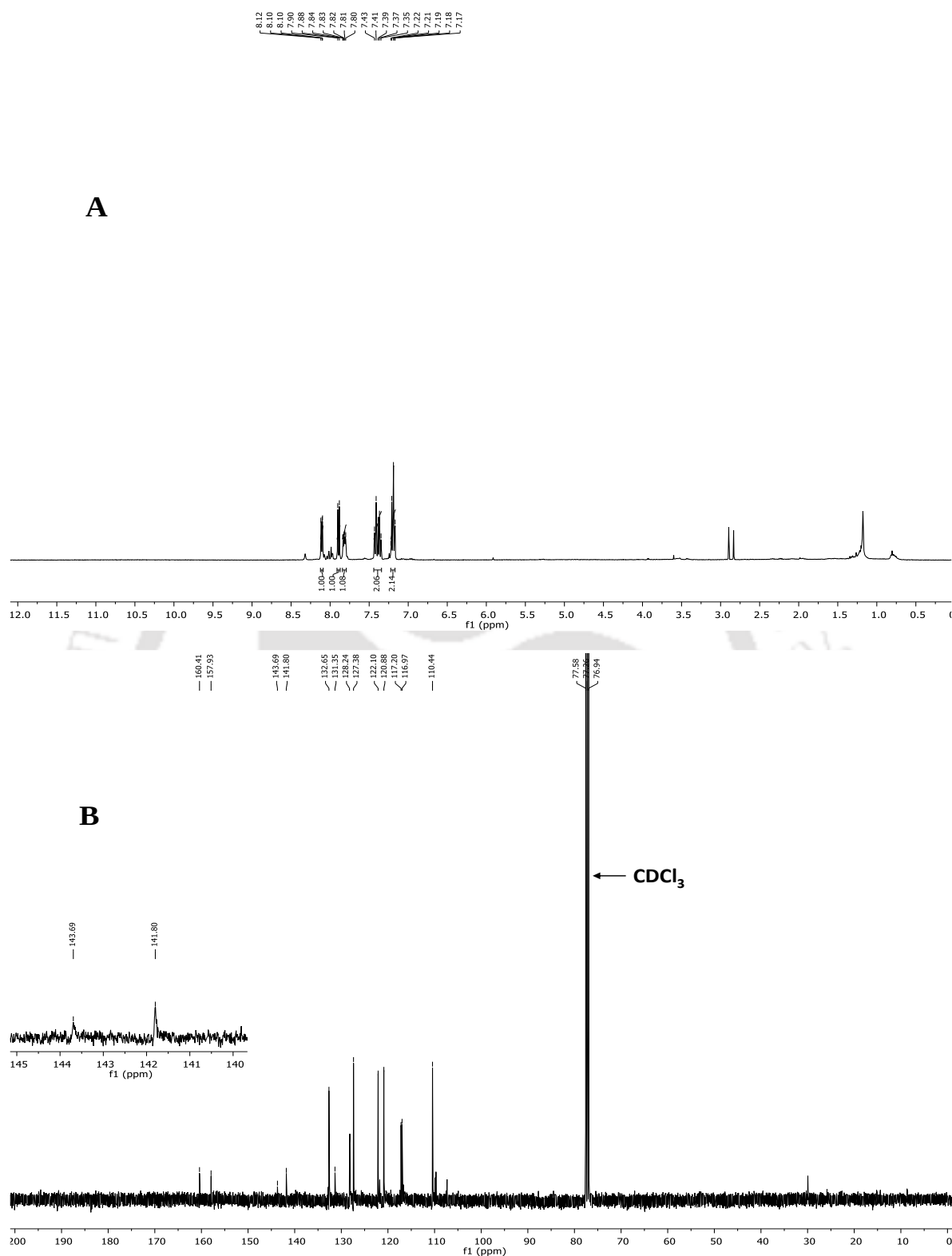
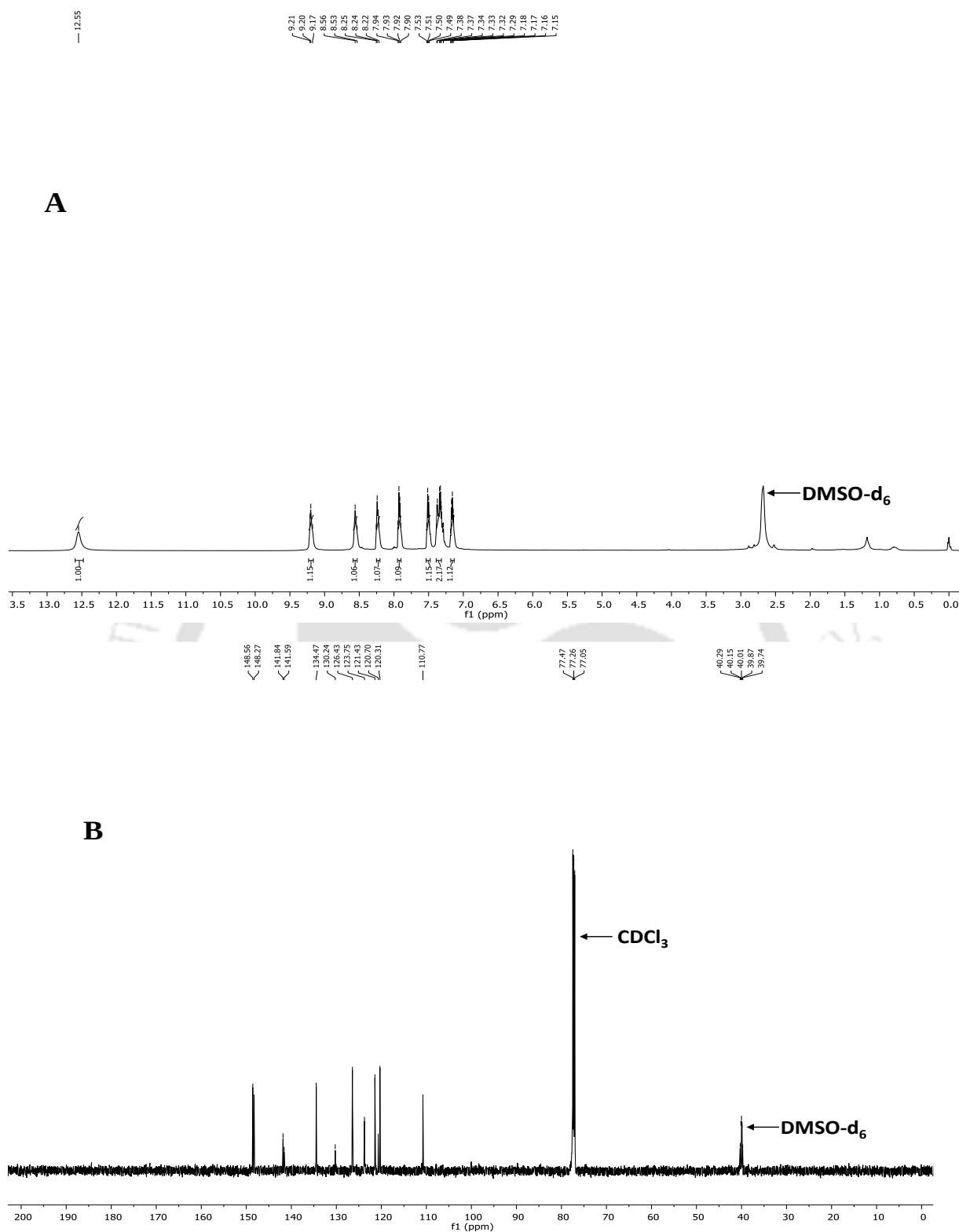


Figure 2.18 ^1H spectra (A) and ^{13}C spectra (B) of compound 2.10.



Chapter 3

4,5-Disubstituted 1,2,3-triazoles Regulates T cell Activity and Mitigates Tumor Growth through Effective IDO1 Inhibition



3.1. Background and objective of present work

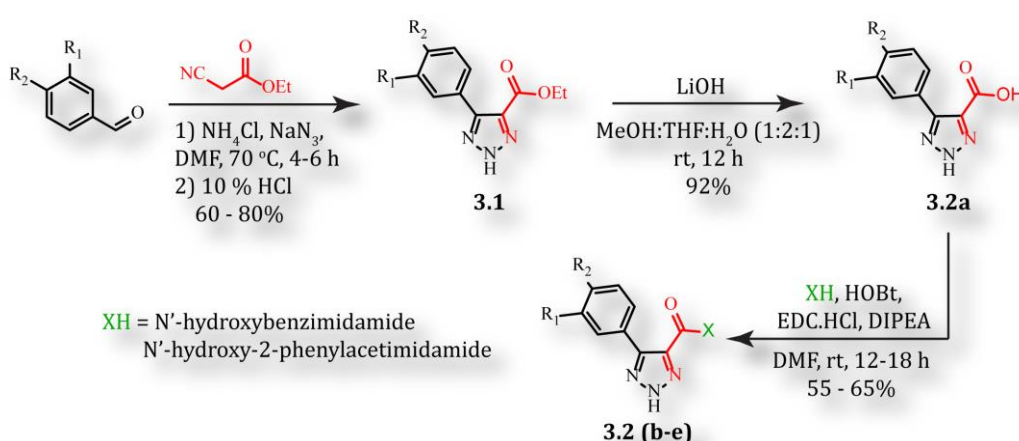
The improvement of body's own immune system was considered one of the safest approaches to fight against cancer and several other diseases. Excessive catabolism of the essential amino acid, L-Trp assisted the cancer cells to escape normal immune obliteration.¹⁻³ Overexpression of IDO1 enzyme was directly related to the induction of L-Trp catabolism mediated immunosuppressive mechanisms and represented an important therapeutic target which was previously discussed in first chapter.⁴⁻⁸ Several classes of small molecule-based IDO1 inhibitors had been already reported, but only few compounds were currently being evaluated in various stages of clinical trials as adjuvants or in combination with chemo and radiotherapies.⁹ In the quest for novel structural class(s) of IDO1 inhibitors, first we developed a series of 1*H*-indazole derivatives which was discussed in previous chapter. The details investigation supported 1*H*-indazole as a moderate IDO1 inhibitors. Though, 1*H*-indazole-based inhibitors bound IDO1 active pocket to inhibit the enzyme, the heme binding properties of the developed compounds was not impressive. Hence, in an endeavour to identify effective IDO1 inhibitor we focussed to develop a new class of IDO1 inhibitors.

The 1,2,3-triazole scaffold had been used as an alternative to imidazole scaffold for its efficacy in providing better specificity for IDO1 enzyme over other heme-containing proteins TDO. Rationally designed 1,2,3-triazole derivative 4-chloro-2-(1*H*-1,2,3-triazol-5-yl)-phenol (MMG-0358) showed nanomolar IDO1 inhibitory activities in both enzymatic and cellular assays. MMG-0358 also showed low cytotoxicity and higher selectivity for IDO1 over TDO enzyme.^{10, 11} The *N*-(2-Ethylphenyl)-1*H*-1,2,3-triazol-5-amine also showed nanomolar-level cellular IDO1 inhibitory activities.⁹ Such higher potency of the 1*H*-1,2,3-triazol derivatives supported 1,2,3-triazol scaffold as the potent therapeutic moiety for inhibition of IDO1 enzyme.

We were focused on finding the simple scaffold for a quick and contaminant-free compound library synthesis, the 1,2,3-triazole scaffold was our interest. We hypothesize that the 4,5-disubstituted 1,2,3-triazole could provide higher inhibitory potency and specificity for the IDO1 enzyme because of its probable interaction with both heme-group and Ser167, a crucial residue in substrate/inhibitor binding to the active site of IDO1 enzyme. Besides, apposite occupation of the active site by the 4,5-

disubstituted 1,2,3-triazole derivatives could be advantageous for their higher selectivity and better inhibitory activities for the IDO1 enzyme. Whereas, MMG-0358 and other triazole-based compounds, which partially occupy the active-site might lack the selectivity and inhibitory activities for the IDO1 enzyme in comparison with the other heme-containing proteins.

The present studies described the design, synthesis of 4,5-disubstituted 1,2,3-triazole derivatives in IDO1 inhibition. The comprehensive biochemical and biophysical studies identified 4,5-disubstituted 1,2,3-triazole compounds as the potent and selective inhibitors IDO1 enzyme. Additionally, this studies also demonstrated controlled regulation of T cell activity to display excellent *in vivo* antitumor efficacy in female Swiss albino mice by the potent 4,5-disubstituted 1,2,3-triazole compounds. Overall this study described the first example of 4,5-disubstituted 1,2,3-triazole-based IDO1 inhibitors with potential applications for immunotherapeutic studies.



Scheme 3.1 Synthesis of 4-carboxylate-5-aryl-2H-1,2,3-triazole derivatives (**3.1**) and 4-carboxamide-5-aryl-2H-1,2,3-triazole derivatives (**3.3**).

3.2. Results and discussions

3.2.1. Synthesis of 4,5-disubstituted 1,2,3-triazole derivatives

To explore the structure-based *in silico* design of IDO1 inhibitors we planned two extensive modifications of the core-structure of the compounds: the 5-aryl substitution of 2H-1,2,3-triazole ring and alteration of 4,5-substitutions of the 2H-1,2,3-triazole ring. We first synthesized halogen substituted 4-carboxylate-5-aryl-2H-1,2,3-triazoles according to the reported procedures.¹² Condensation of cyanoethyl acetate with aromatic aldehyde in the presence of sodium azide and

ammonium chloride allowed the formation of 4-carboxylate-5-aryl-2*H*-1,2,3-triazoles with moderate to excellent yield.¹³ We also synthesized 4-carboxamide-5-aryl-2*H*-1,2,3-triazole derivatives by introducing *N'*-hydroxyamidine groups to explore the nature of C4 substituent in IDO1 inhibition. Hydrolysis of 4-carboxylate-5-aryl-2*H*-1,2,3-triazoles in the presence of LiOH in MeOH:THF:H₂O (1:2:1) solution resulted 5-aryl-2*H*-1,2,3-triazole-4-carboxylic acid. Coupling of 5-aryl-2*H*-1,2,3-triazole-4-carboxylic acid with *N'*-hydroxybenzimidamide, *N'*-hydroxy-2-phenylacetimidamide groups in the presence of HOBt and EDC.HCl in DMF solvent resulted the formation of 4-carboxamide-5-aryl-2*H*-1,2,3-triazole derivatives (Scheme 3.1).¹⁴ These short-step synthetic approaches were highly beneficial for large-scale productions of several 2*H*-triazoles derivatives with rapid structural alterations.

Table 3.1 Inhibitory activity of the 4-carboxylate-5-aryl-2*H*-1,2,3-triazoles against purified human IDO1 enzyme.

Compound	% of Enzyme inhibition ^a					hIDO1 IC ₅₀ (μM)
	R ₁	R ₂	32 nM	4 μM	100 μM	
3.1a	H	H	15	41	81	14.39 ± 3.36
3.1b	H	Me	10	54	84	1.70 ± 0.06
3.1c	F	H	11	35	83	31.44 ± 4.21
3.1d	Cl	H	9	53	77	2.55 ± 0.03
3.1e	Br	H	9	27	40	2.98 ± 0.01
3.1f	H	F	29	49	89	8.09 ± 0.21
3.1g	H	Cl	10	51	63	0.39 ± 0.08
3.1h	H	Br	24	35	61	18.45 ± 1.68
3.1i	F	F	9	70	91	0.55 ± 0.03
3.1j	Cl	Cl	17	69	84	0.16 ± 0.03
3.1k	Cl	F	10	63	87	1.69 ± 0.10
3.1l	Br	F	5	50	79	3.03 ± 0.09

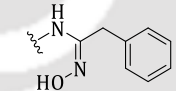
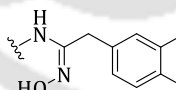
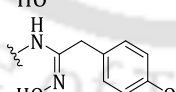
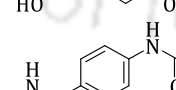
^a % of enzyme inhibition values are the mean of three independent assays.

3.2.2. IDO1 inhibitory activities of 4,5-disubstituted 1,2,3-triazoles

The IDO1 inhibitory activities of 4,5-disubstituted 1,2,3-triazoles were initially evaluated using standard spectrophotometric protocol.^{11, 15-18} Absorption spectra of the compounds with in concentration range 10 nM to 100 μ M showed no or negligible interference with this spectrophotometric method. To improve the IDO1 inhibitory potency of the 1,2,3-triazoles, both the 5-aryl substitution and alteration of the 4,5-substitutions of the 1,2,3-triazole ring were investigated. Firstly, we explored the IDO1 enzyme inhibitory activity of 5-aryl substitution on 1,2,3-triazole ring. We hypothesize that the presence of halogen substituted aryl ring in the compound's core structure could play a vital role in their IDO1 inhibitory efficiencies. The inhibitory activities of the 4-carboxylate-5-aryl-2*H*-1,2,3-triazoles compounds suggest that not only the presence of 5-aryl ring, but also electronic properties of the aryl ring play a crucial role in their inhibitory efficiency against the purified hIDO1 enzyme. In comparison with the ethyl 5-phenyl-2*H*-1,2,3-triazole-4-carboxylate (**3.1a**, IC₅₀ of 14.39 μ M) compound, ethyl 5-(3,4-difluorophenyl)-2*H*-1,2,3-triazole-4-carboxylate (**3.1i**, IC₅₀ of 0.55 μ M) and ethyl 5-(3,4-dichlorophenyl)-2*H*-1,2,3-triazole-4-carboxylate (**3.1j**, IC₅₀ of 0.16 μ M) gave rise to 26- and 88-fold increase in inhibitory activity, respectively (Table 3.1). The IC₅₀ value implied that the 3,4-dihalogen-substituted 5-aryl ring of 1,2,3-triazole scaffold might be adequate in maintaining the IDO1 inhibitory activity. Not only the halogen substituted aryl ring could be involved in interactions with the hydrophobic residues present within the 'pocket-A' but also the 'van der Waals' interaction of carboxylate group in the 'pocket-B' of the IDO1 enzyme was quite advantageous for their enzyme inhibitory activity. Such stronger IDO1 inhibitory activities of the halogen substituted mono aryl ring containing 1,2,3-triazoles could be because of the hydrophobic interactions, halogen bonding with the Lewis bases and /or π -stacking interaction with aromatic amino acids present within the active site. In our previous reports, we successfully demonstrated that halogen substitution at the meta- and /or para-positions of the aryl ring augmented the IDO1 inhibitory efficiencies. Other research groups also confirmed such assistance of halogen-substituted aryl ring on the IDO1 inhibitory efficiencies. To augment the IDO1 inhibitory efficiencies of the 1,2,3-triazoles, we extended our activity studies with 4-carboxylic acid- and *N*-substituted 4-carboxamide-5-aryl-2*H*-1,2,3-triazoles. We hypothesize that the presence of 4-

carboxylic acid group or *N*-substituted moiety in the core structural unit of the 1,2,3-triazoles could play a major role in their IDO1 inhibitory efficiencies because of its interaction with the polar residues present within the 'pocket B', towards the entrance of the active site of the IDO1 enzyme. Structural modifications including the 4-carboxylic acid (**3.2a**) and installation of *N*'-hydroxyamidine (**3.2b-3.2e**) in the 4-carboxamide moiety of the compounds also explored by IDO1 activity studies. Among the tested compounds **3.2d** showed high-potency in IDO1 inhibition with IC₅₀ value 94 nM but installation of 4-carboxylic acid groups of the 1,2,3-triazole scaffold were ineffective against IDO1 activity (Table 3.2). Hence, the inhibitory activities of the 1,2,3-triazole derivatives suggest that not only the presence of 1,2,3-triazole ring but also the nature and electronic properties of the substituents at the C4 and C5 positions of the triazole ring play a crucial role in their inhibitory efficiency against the purified hIDO1 enzyme.

Table 3.2 Inhibitory activity of 4-carboxylic acid/carboxamide-5-aryl-2*H*-1,2,3-triazoles against purified human IDO1 Enzyme.

	Compound			% of Enzyme inhibition ^a			hIDO1 IC ₅₀ (μM)
	R ₁	R ₂	X	32 nM	4 μM	100 μM	
3.2a	F	F		7	22	47	17.42 ± 0.92
3.2b	F	F		8	70	84	0.88 ± 0.02
3.2c	F	F		8	28	40	3.81 ± 0.24
3.2d	F	F		17	86	93	0.09 ± 0.01
3.2e	F	F		17	63	91	2.36 ± 0.31

^a % of enzyme inhibition values are the mean of three independent assays.

The HPLC-based IDO1 inhibitory activity of the selected 1,2,3-triazoles were also measured for additional validation of the inhibitory potency of potent compounds. The amount of kynurenine generation from L-Trp in the presence of

IDO1 enzyme with potent 1,2,3-triazoles were calculated for the measurement of IC_{50} values of the compounds. The results revealed that the IDO1 inhibitory activities of the selected 1,2,3-triazoles were in accordance with the spectrophotometric method. However, the differences in the inhibitory potency between the spectrophotometric and HPLC methods could be because of the reactivity of the *p*DMAB towards the kynurenine under the experimental reaction conditions. The HPLC-based assay also demonstrated that compounds **3.3c** displayed stronger inhibitory activity (IC_{50} = 115 nM) between all the examined 1,2,3-triazoles (Table 3.3).

Table 3.3 HPLC-based IDO1 inhibition assays of the selected compounds.

Compound	% Of inhibition for IDO1 enzyme ^a			hIDO1 IC_{50} (μ M)
	32 nM	4 μ M	100 μ M	
3.1a	26	61	81	2.789 $\pm$ 0.066
3.1i	21	60	80	0.396 $\pm$ 0.010
3.2a	11	30	65	8.944 $\pm$ 0.087
3.2d	13	68	84	0.114 $\pm$ 0.010

^a % of enzyme inhibition values are the mean of three independent assays.

Overall, both the inhibitory activities revealed that the inhibitory potencies of 1,2,3-triazoles were sensitive to di-halogenated aryl-substitution at the C5-position of triazole ring, possibly because of the constrained space in 'pocket-A' of the IDO1 enzyme. Both the electronic properties of the C4 and C5-substituted 1,2,3-triazoles scaffold and interaction of 1,2,3-triazole moiety with the heme-group of IDO1 enzyme might be reflected on the inhibitory potency.

3.2.3. Binding analysis of 1,2,3-triazole derivatives with IDO1 enzyme

The inhibitory potency of the 1,2,3-triazoles demonstrated their aptitude in inhibiting IDO1 activity, but failed to offer any direct evidence of inhibitors binding to the IDO1 protein. In this regard, the binding efficiency of the selected 1,2,3-triazoles with IDO1 enzyme were examined by both UV-Vis and SPR analysis.^{15, 19, 20} The absorption spectra of the heme-group of IDO1 was highly sensitive to the local

environment. As a result, the absorption spectra of heme group provided substantial evidence of the ligand binding aptitude to the active pocket of IDO1 enzyme. The UV-Vis spectra of ferric-IDO1 and deoxy-ferrous-IDO1 in the absence and presence of the selected 1,2,3-triazoles were recorded. However, for both ferric-IDO1 and deoxy-ferrous-IDO1 enzyme, the Soret band at 404 nm got red-shifted by 5–8 nm in the presence of **3.1i** and **3.2d** (Figure 3.1 and Table 3.4). For deoxy-ferrous-IDO1 enzyme there was the Q-band (at 551 nm for only enzyme) got shifted by 10–13 nm in the presence of the compounds. Hence, these UV-Vis spectral properties indicated direct binding of the 1,2,3-triazoles to both ferric-IDO1 and deoxy-ferrous-IDO1 enzyme. Additionally, the UV-Vis spectra of 1,2,3-triazoles in phosphate buffer did not show any spectral overlap in this region.

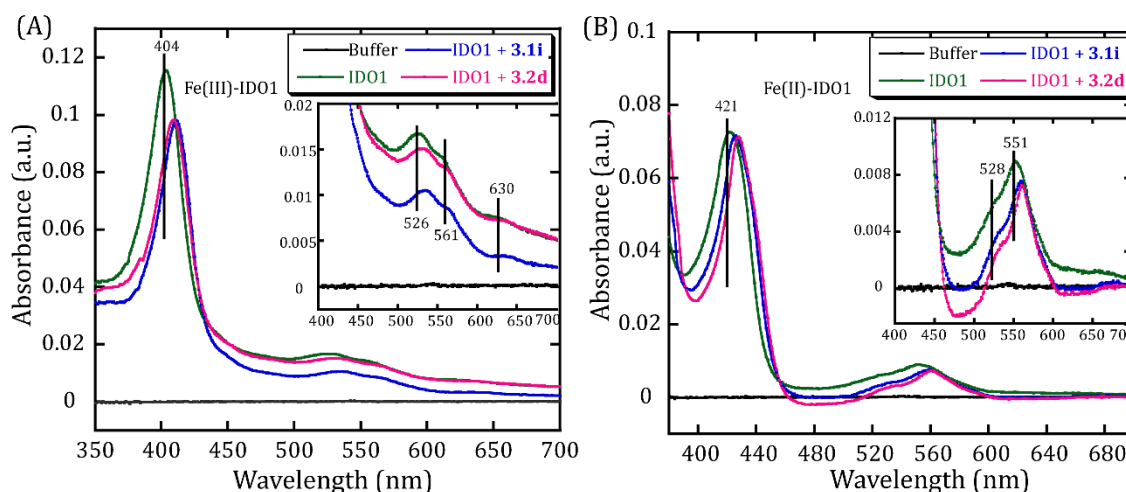


Figure 3.1 Absorption spectra of ferric-IDO1 (A) and deoxyferrous-IDO1 enzyme (B) in the absence and presence of the compounds (20 μM) in 100 mM phosphate buffer at pH 6.5. [IDO1] = 0.65 μM . The ferrous-deoxy reaction environment was generated by adding $\text{Na}_2\text{S}_2\text{O}_4$ to the solution under N_2 atmosphere

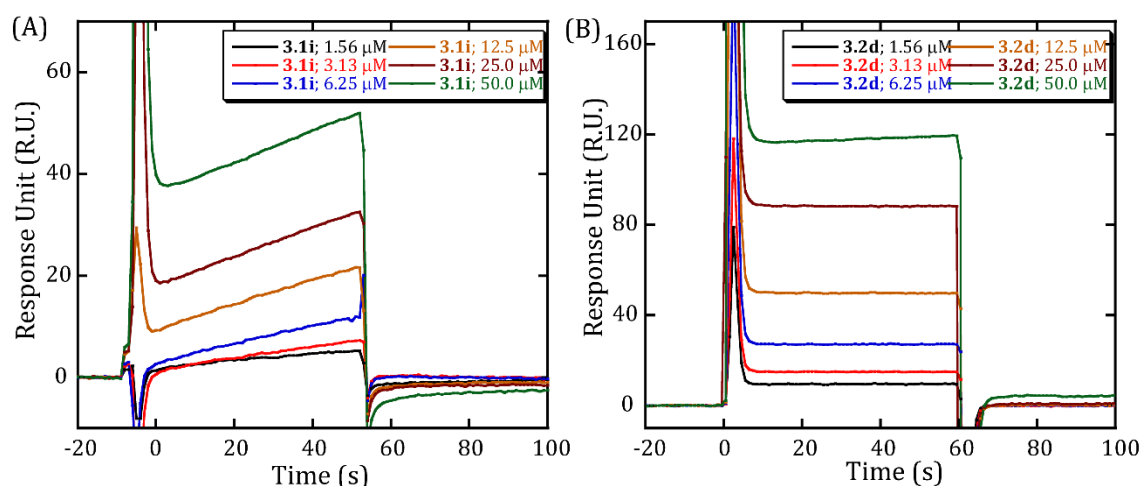
Additional SPR analysis was also performed to measure direct interaction between the selected 1,2,3-triazoles and IDO1 enzyme. The K_d between 1,2,3-triazoles, **3.2d** and IDO1 was 40.23 μM (Table 3.5, Figure 3.2), which validated their binding activity to the IDO1 protein. Hence, both UV-Vis spectral analysis and SPR measurements undoubtedly ascertain the binding of compounds to the IDO1 enzyme.

Table 3.4 Characteristic peaks from the UV-Vis measurements of IDO1 enzyme and potent compounds.

	λ_{max} (nm)	λ_{max} (nm)
	Fe ³⁺ state binding	Fe ²⁺ state binding
Only IDO1	404, 526, 561, 630	421, 528, 551
IDO1 + 3.1i	411, 535, 568, 645	427, 532, 561
IDO1 + 3.2d	409, 537, 566, 645	428, 531, 564

[Compound] = 20 μM ; [IDO1] = 0.65 μM

To understand whether the compound binding induces any structural changes of the IDO1 enzyme, we also performed CD study.^{21, 22} The presence of secondary structural elements of the IDO1 enzyme were in good agreement with the previous studies. The CD results showed that binding of the compounds to the IDO1 enzyme causes no/negligible changes to its secondary structural elements.

**Figure 3.2** Measurements of compound **3.1i** (A) and **3.2d** (B) binding affinity with human IDO1 enzyme by equilibrium SPR analysis. Binding isotherm was generated from the R_{eq} (average of triplicate measurements) versus the concentration of human IDO1 enzyme plot. K_d values determined by nonlinear least-squares analysis of the isotherm using the following equation: $R_{eq} = R_{max}/(1 + K_d/P)$.**Table 3.5** IDO1 binding affinity of potent 1,2,3-triazoles determined from equilibrium SPR analysis.

Compounds	K_d (μM) for IDO1 enzyme ^a
3.1i	61.65 $\pm$ 6.07
3.2d	40.23 $\pm$ 4.99

^a Values represent the mean from triplicate measurements.

Table 3.6 CD analysis of IDO1 enzyme in the presence of selected 1,2,3-triazole compounds.

Compounds	Concentration (μM)	% of Secondary structural elements ^a			
		Helix	Beta	Turn	Random
3.1i	0	29.5	9.5	30.5	30.5
	0.25	28	0	34.1	37.9
	0.5	28.7	0	35.2	36.1
	1	30.2	0	36.3	33.5
	2	30.5	0	35.7	33.8
	4	30.5	0	33.8	35.7
3.2d	0	29.5	9.5	30.5	30.5
	0.25	27.8	0.7	33.9	37.6
	0.5	28	0	33.9	38.1
	1	27.6	0	31.8	40.6
	2	29.8	0	27.6	42.6
	4	31.8	0	29.4	38.8

^a % of the secondary structures of the IDO1 enzyme was analysed using the inbuilt software of the instrument.

3.2.4. Probable mode of interaction of 1,2,3-triazole derivatives with IDO1 enzyme

The enzyme inhibition potencies of 1,2,3-triazoles described that the interaction of the compounds with IDO1 protein. SPR analysis also supported the protein binding properties of the compounds. The spectroscopic analysis in the absence and the presence of the 1,2,3-triazoles clearly demonstrated the direct interaction of the compounds with heme-group present in the active site of IDO1 and

altered its L-Trp catabolism. To understand the molecular determinants which regulated the inhibitory activities of these potent 1,2,3-triazoles, molecular docking analysis were also performed using the co-crystal structure of IDO1 complex i.e. PDB code: 4PK5.²³ The outcome of this docking analysis suggested that compound **3.1i** and **3.2d** had a very similar interaction pattern with IDO1 enzyme. The aryl ring at C5 position of these 1,2,3-triazoles interacted with the protein through its hydrophobic “pocket A” in the distal heme site. The halogen substituted C5-aryl ring assisted the 1,2,3-triazole derivatives to interact with the amino acids present in “pocket A” through hydrophobic, halogen bonding and π -stacking interactions. The triazole ring also involved in interaction with the heme-group and Ser167 amino acid residue with the active IDO1 pocket. This suggested mode of interaction was aligned with the spectroscopic-based binding analysis. Additionally, the C4-substituted moiety of these triazoles interacted slightly differently through the hydrophilic “pocket B” of the IDO1 protein. The carbonyl group of the 4-carboxylate moiety of both the compound **3.1i** and **3.2d** participated in H-bonding through the backbone NH-proton of Ala264 residue. The *N'*-hydroxy-2-(4-hydroxyphenyl)acetimidamide moiety of **3.2d** interacted with the backbone carbonyl group of Phe226, Gly262, 7-propionate of the porphyrin ring and the NH-proton of Ala264 residue through H-bonding (Figure 3.3). The non-covalent interactions such as H-bonding, halogen bonding, π -stacking, and hydrophobic interactions played a decisive role in stronger binding of these potent triazoles to IDO1 protein through its active site. Overall, the modes of interactions of the 1,2,3-triazole derivatives with IDO1 were very similar. However, the variations in inhibitory potencies suggested that their true-mode of interaction could be diverse than these probable ones obtained from the molecular docking analysis. In addition to the substitution on the triazole scaffold reflected their overall molecular volume and pattern of interactions which could be also critical for their interactions under physiological conditions.

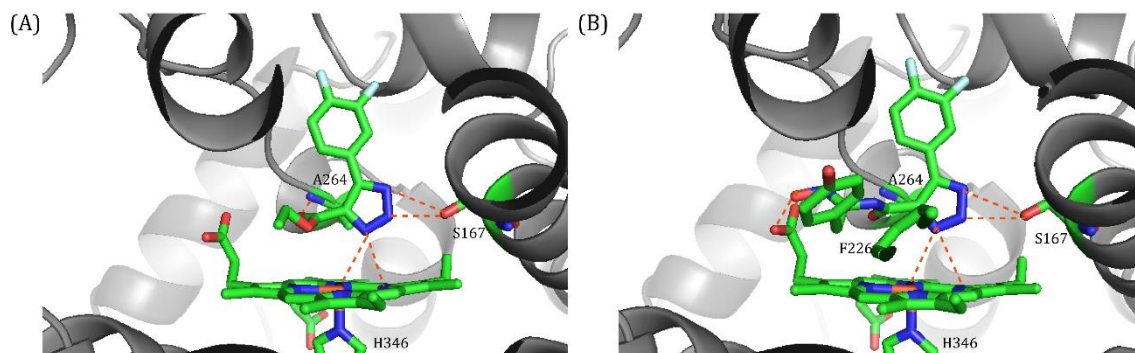


Figure 3.3 Probable mode of interaction of the compounds **3.1i** (A) and **3.2d** (B) with the active site of the IDO1 enzyme (4PK5).

Table 3.7 Docking parameters for the interaction of IDO1 enzyme with 1,2,3-triazoles.

Compound	Rerank score (kJ/mol) ^a	Interaction (kJ/mol) ^b	Internal (kJ/mol) ^c	H-Bond (kJ/mol) ^d	LE1 ^e	LE3 ^f
3.1i	-100.53	-149.78	7.08	-6.79	-7.93	-5.59
3.2d	-90.20	-134.81	3.66	-5.04	-8.20	-5.64

^a The rerank score is a linear combination of E-inter (steric, Van der Waals, hydrogen bonding, electrostatic) between the ligand and the protein, and E-intra. (torsion, sp²-sp², hydrogen bonding, Van der Waals, electrostatic) of the ligand weighted by pre-defined coefficients (kJ/mol). ^b The total interaction energy between the pose and the protein (kJ/mol). ^c The internal energy of the pose (kJ/mol). ^d Hydrogen bonding energy (kJ/mol). ^e Ligand efficiency 1: MolDock scores divided by heavy atoms count. ^f Ligand efficiency 3: Rerank scores divided by heavy atoms count.

3.2.5. Mode of enzyme inhibition of the selected 1,2,3-triazole derivatives

The IDO1 inhibition and binding experiments of the selected triazoles described that the compounds bound to the active site of IDO1 enzyme and inhibited L-Trp catabolism. To understand the mode of enzyme inhibition of these selected triazoles, we performed the standard enzyme kinetics experiments in presence and absence of these triazoles compounds. The Lineweaver-Burk plot was used to investigate the modes of IDO1 enzyme inhibition. The plots of 1/V against 1/[S] showed that

compounds **3.1i** and **3.2d** followed competitive inhibition modes with respect to the substrate, L-Trp (Figure 3.4 and Table 3.7). [S] and V represented the substrate concentration and the initial reaction rate,¹⁹ respectively. Nevertheless, this calculated mode of IDO1 inhibition by the selected 1,2,3-triazole might not exhibit their actual-mode of inhibition. Detailed mechanistic studies of the IDO1 mediated L-Trp catabolism demonstrated that the ferric-superoxide intermediate generation was the prime necessity for the subsequent oxidation process. Hence, both O₂ and L-Trp were the substrate for IDO1 enzyme. Henceforth, supplementary enzyme kinetic studies regarding O₂ were required to recognize the precise mode of IDO1 enzyme inhibition.

Table 3.8 Enzyme kinetics parameters of the IDO1 enzyme in the presence of selected triazoles.

Compound	Mode of IDO1 inhibition	K_i (μM) ^a
3.1i	Competitive	0.24 ± 0.04
3.2d	Competitive	0.26 ± 0.02

^a $K_m = 59.76 \mu\text{M}$ and $K_{cat} = 6.18 \text{ sec}^{-1}$

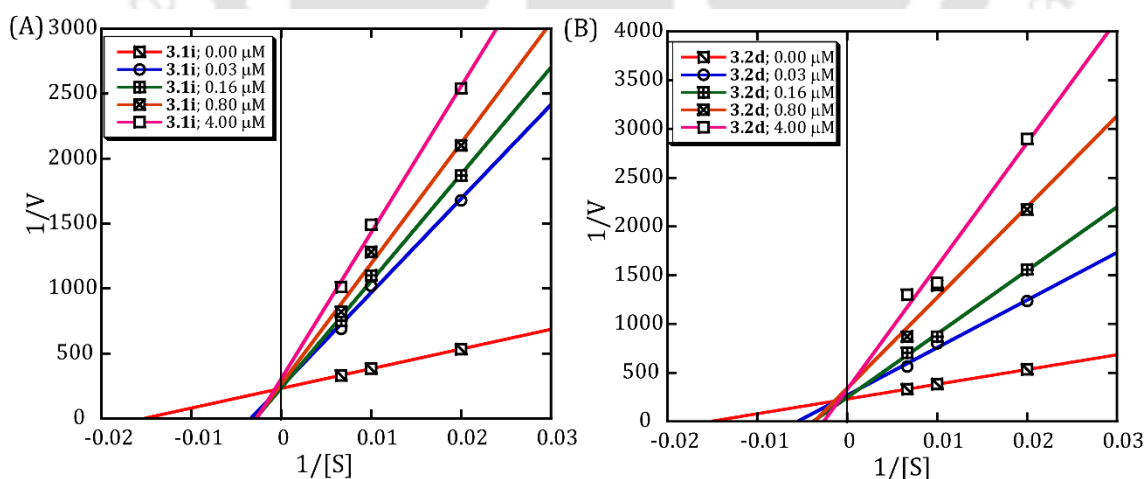


Figure 3.4 Determination of mode of IDO1 inhibition of the selected compounds. Plot of $1/V$ against $1/[S]$ at different concentration of the compounds **3.1i** (A) and **3.2d** (B).

3.2.6. Selective IDO1 inhibitory activity of triazole compounds comparison with purified TDO enzyme.

To investigate the IDO1 inhibition selectivity of these triazoles, we also analysed the inhibitory activity of the selected triazole compounds against TDO enzyme, which was also a member of the same enzyme family as IDO1. TDO enzyme also catalysed L-Trp through the kynurenine pathway. However, TDO was a homotetrameric enzyme and the active site of TDO enzyme was highly specific for L-Trp and indole derivatives.¹¹ The activity studies of TDO enzyme in presence of 1,2,3-triazole derivatives revealed that these selected triazoles had moderate to poor inhibition potencies (Table 3.8).

Table 3.9 Inhibitory activity of the 1,2,3-triazoles against purified human TDO enzyme.

Compound	% of Enzyme inhibition ^a		
	32 nM	4 μ M	100 μ M
3.1a	8	23	34
3.1i	2	15	28
3.2a	4	18	30
3.2d	4	24	55

^a % of enzyme inhibition values are the mean of three independent assays.

The HPLC-based TDO inhibitory activity of the selected 1,2,3-triazoles were also measured for additional validation of the inhibitory potency of potent compounds. TDO inhibitory activity assays in both spectrophotometric and HPLC-based assays showed that these 1,2,3-triazole compounds had only moderate to poor inhibitory potencies (Table 3.9). Under the similar experimental conditions, the potent compounds **3.1i** and **3.2d** showed higher inhibitory activity for IDO1 over TDO enzyme. The IC₅₀ values of these selected compounds against TDO enzyme could not be measured properly because of weaker and/or non-specific inhibitions (Table 3.10).

Table 3.10 HPLC-based TDO inhibition assays of the selected 1,2,3-triazole compounds.

Compound	% of Enzyme inhibition ^a		
	32 nM	4 μ M	100 μ M
3.1a	9	64	73
3.1i	34	65	83
3.2a	6	38	54
3.2d	15	50	73

^a % of enzyme inhibition values are the mean of three independent assays.

Table 3.11 Inhibitory activity of the selected compounds against purified human IDO1 and TDO enzymes.

Compound	% of Enzyme inhibition ^a					
	32 nM		4 μ M		100 μ M	
	IDO1	TDO	IDO1	TDO	IDO1	TDO
3.1a	15	8	41	23	81	34
3.1i	9	2	57	15	80	28
3.2a	7	4	22	18	47	30
3.2d	17	4	71	24	93	55

^a % of enzyme inhibition values are the mean of three independent assays.

The IDO2 was a paralogue of IDO1 enzyme, and it also participated in the L-Trp catabolism process through the kynurenine pathway. However, the IDO2 showed a low L-Trp affinity and low turnover rate. The cellular expression of IDO2 enzyme did not influence the tryptophan/kynurenine ratio at physiological tryptophan concentration. Until recently, there was no report that IDO2 reduced tumor volume. Therefore, neither IDO2 inhibitors were systematically developed nor its biological activities investigated. The structural investigation also revealed that the hIDO2 differs from hIDO1 by four amino acid side chain: Tyr126, Cys129, Phe164, and Ser167 residues of hIDO1 were replaced by His130, Leu133, Ile168 and Thr171 residues of hIDO2. Overall, IDO2 shared only 44% sequence homology with IDO1. As a result, the volume of 'pocket A' was around 15% larger in IDO2 than that in IDO1,

which facilitated binding of larger (in size) inhibitors leading to the decrease in IDO2 selectivity. A recent study showed that 4-aryl-1,2,3-triazole derivatives moderately inhibit the hIDO2 activity ($IC_{50} > 50 \mu M$) and had only 2-fold selectivity over hIDO1 enzyme. The structural investigation of the synthesized 4,5-disubstituted 1,2,3-triazoles derivatives revealed that few of these compounds could also inhibit the activity of IDO2 enzyme. However, due to lack of therapeutic value of IDO2 its inhibitory activities in the presence of our synthesized compounds were not investigated.

3.2.7. Plasma protein interaction of the selected 1,2,3-triazoles derivatives

In addition, to explore whether the plasma protein would bind to these aqueous soluble compounds and regulate its biological activities, the serum binding properties of these potent compounds were qualitatively investigated using Trp-fluorescence analysis.²⁴ The extent of Trp-fluorescence quenching ability of these compounds suggested that compounds **3.1i** interacted more strongly with the human serum proteins than compounds **3.2d** (Figure 3.5). The outcomes of this binding analysis suggested that the serum proteins could assist the transport of the compounds via blood to the sites of action throughout the body.

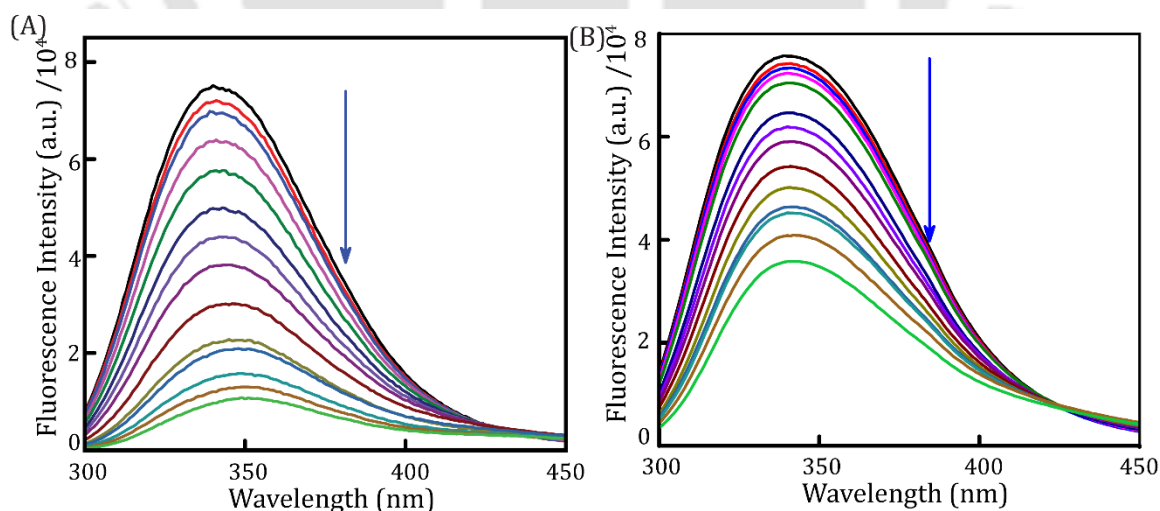


Figure 3.5 Tryptophan-fluorescence emission measurements of human serum in the presence of the compounds **3.1i** (A) and **3.2d** (B).

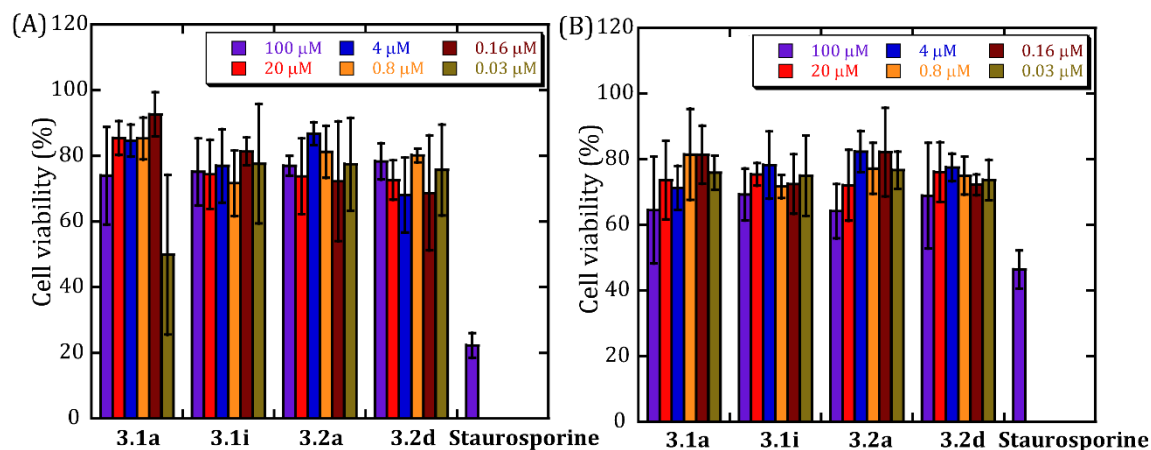


Figure 3.6 Effect of the selected potent 1,2,3-triazole derivatives on the viability of HEK-293 (A) and MDA-MB-231 (B) cells after 48 h.

Table 3.12 EC₅₀ Values of the Selected Compounds in MDA-MB-231 Cells

Compound	EC ₅₀ (μM) ^a
3.1a	3.263 ± 0.045
3.1i	0.162 ± 0.015
3.2a	1.231 ± 0.021
3.2d	0.088 ± 0.010

^a EC₅₀ values are the mean of three independent assays.

3.2.8. Cellular IDO1 inhibitory activities and toxicity of 1,2,3-triazole derivatives

The biological activities of these selected 4,5-disubstituted 1,2,3-triazoles were also investigated under cellular environment. The cytotoxicity of the compounds was performed in both HEK-293 and MDA-MB-231 cells. The MTT assay of the potent triazole compounds showed low level of cytotoxicity of the compounds both in HEK-293 and MDA-MB-231 cells up to the concentration of 100 μM (Figure 3.6). IFN-γ was known to induce the expression of the native IDO1 enzyme in the MDA-MB-231 cells from its mRNA. Hence, IDO1 inhibitory activities of the 4,5-disubstituted 1,2,3-triazoles were measured in MDA-MB-231 cells using IFN-γ as external stimuli.²⁵ The IDO1 inhibitory activities under the cellular environments followed a comparable pattern as that was found against the purified IDO1 enzyme. The measured EC₅₀

values of the potent **3.1i** and **3.2d** were 162 and 88 nM, respectively (Table 3.11). The compound, 4-amino-*N*-(3-chloro-4-fluorophenyl)-*N'*-hydroxy-1,2,5-oxadiazole-3-carboximidamide and MMG-0358, showed EC₅₀ values of 63 and 120 nM, under similar experimental conditions, which were aligned with the reported values. However, smaller variations in IDO1 inhibitory activities of the compounds between the two-activity assay, against the purified enzyme and under the cellular conditions, could be primarily due to the obstacles in regulating the redox activity of the enzyme and/or environmental effect on the assay systems. Overall, a good data correlation between these assays corroborated the IDO1 inhibitory potencies of these selected triazoles.

3.2.9. Effect of potent 1,2,3-triazoles on proliferation and functions of T cells

The above experimental evidences confirmed that the selected 4,5-disubstituted 1,2,3-triazole derivatives were potent inhibitors of the IDO1 enzyme. It was well documented that the immune-regulatory enzyme IDO1 promotes immunologic tolerance in tumor microenvironment.²⁶ The macrophages or dendritic cells present in PBL also produced IDO1 enzyme that also inhibited the T cell activation and proliferation.²⁷ In the tumor-microenvironment the immune suppression followed several paths. The CD8 + T cells were one of the critical immune cells that promoted tumor rejection.²⁸ However, dysfunction of cytotoxic CD8 + T cells was known to elevate the immune evasion of tumor cells. The IDO1-mediated Trp metabolites were reported to be protective against T cell-mediated fetal rejection. The IDO1 inhibition had shown protective effect and boosted up CD8 + T cells activity against host influenza viral infection.²⁹ However, the effect of IDO1 inhibition on rejuvenation of CD8 + T cells in the tumor micro-environment of the breast cancer cells had never studied before. Hence, to explore the therapeutic potential of these selected IDO1 inhibitors, we explored the activity of the cytotoxic T cells in breast tumor micro-environment. Initially, freshly isolated PBLs were cultured in RPMI with 10% FBS and treated with IDO1 inhibitor for 48 hours. The compound treatments showed significant up-regulation in PBL cells than the control group (Figure 3.7A; $P < 0.05$). Since, IDO1-mediated Trp metabolism promoted dampening of CD8 + T cell responses; we sought to check whether IDO1 inhibitor treatment elevated proliferation of cytotoxic T cells in the tumor micro-environment.

The PBLs were cultured in 20% conditioned medium of human metastatic breast cancer cell line MDA-MB-231 for 48 hours. Prior cultures the cells were allowed to be stimulated with PMA and ionomycin. The outcome of the FACS analysis showed that the treatment of IDO1 inhibitors significantly induced CD8 + T cell proliferation (Figure 3.7B and 3.8; $P < 0.001$). The increase in total CD8 + T cell number inspired us to investigate whether the potent IDO1 inhibitors were regulating the effector memory phenotype of cytotoxic T cells or not. The CD8 + T cells were phenotype for CCR7 and CD45RA expression, where CCR7 low CD45RA low phenotype was designated as effector memory phenotype. Two days' post-treatment with IDO1 inhibitors a remarkable aggrandize effector memory phenotype of cytotoxic T cells (Figure 3.7C and 3.9; $P < 0.001$) was observed. These results supported IDO1 mediated abrogation of effector memory CD8 + T cell production. Tumor cells evade cytotoxic T cells mediated rejection due to the enhanced dysfunction in the tumor-reactive T cells. Germination of T cell dysfunction was believed to be mediated by the elevation in surface protein PD-1 in cytotoxic T cells. Detailed studies demonstrated that the binding of surface protein PD-L1 of the tumor cells to PD-1 or B7.1 of the T cells conveys an inhibitory signal that decreased the proliferation of antigen-specific T-cells in lymph nodes. The activation of cytotoxic T cells by PD-1/PD-L1 blockade therapy had astonishing responses in different cancer patients. Surprisingly, in breast cancer the effect of PD-1/PD-L1 blockade therapy was not so encouraging. The germination of malfunctioned cytotoxic T cells in breast cancer could be one of reasons. In this study, we also investigated whether IDO1 inhibition attenuates the boosted PD-1 expression of CD8 + T cells in breast tumor micro environment. The outcome of the studies showed that the IDO1 inhibition prodigiously suppressed the PD-1 level of cytotoxic T cells in the tumor micro-environment ($P < 0.001$), suggesting that these compounds may rejuvenate the cytotoxic T cells in breast cancer (Figure 3.7D and 3.10). Hence, the results from this study depicted that IDO1 regulation by these potent inhibitors could elevate aspects of adaptive immune response against breast cancer.

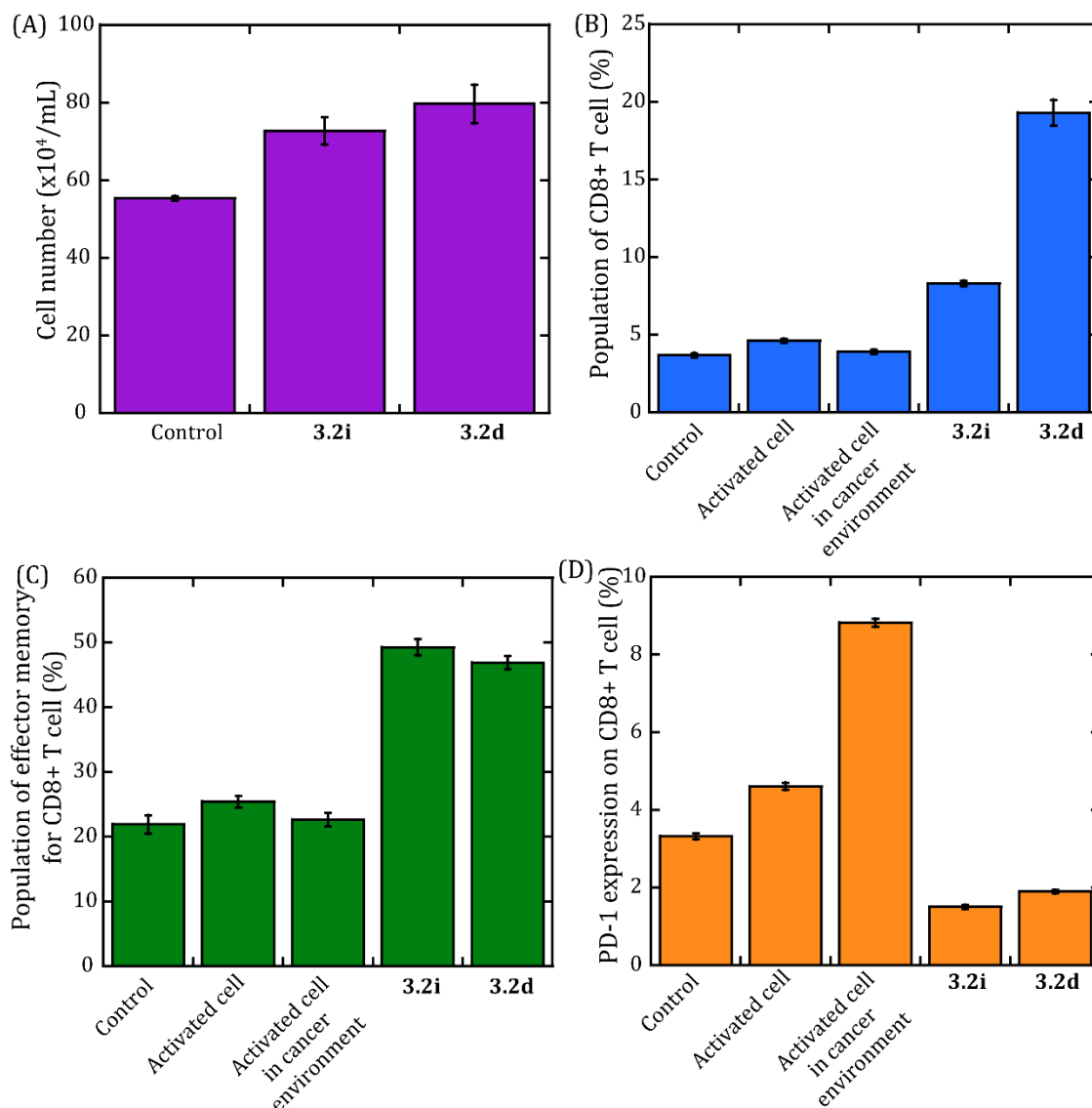


Figure 3.7 T cell activity studies in the presence of the compounds. The proliferation of the PBLs ($P = 0.0075$) in the presence of the compounds. (A) Regulation of the CD8+ T cells in the tumor micro-environment ($P < 0.0001$) in the presence of the compounds. (B) Regulation of effector memory CD8+ T cells in tumor micro-environment ($P < 0.0001$) in the presence of the compounds. (C) Regulation of the PD-1 expression on CD8+ T cells in tumor micro-environment ($P < 0.0001$) in the presence of the compounds. (D) The results were found statistically significant post statistical analysis with one-way ANOVA.

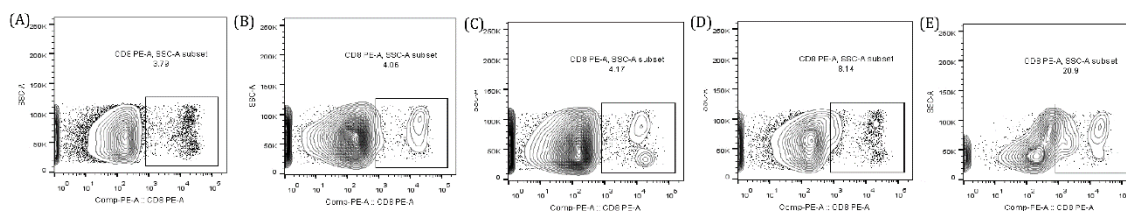


Figure 3.8 Up-regulation of CD8+T cells in tumor micro-environment post-treatment. Only cell (A), activated cell (B), control cell in tumor micro-environment (C), for **3.1i** (D), and **3.2d** (E). The concentration of the compounds was 50 μ M for the T cell activity studies.

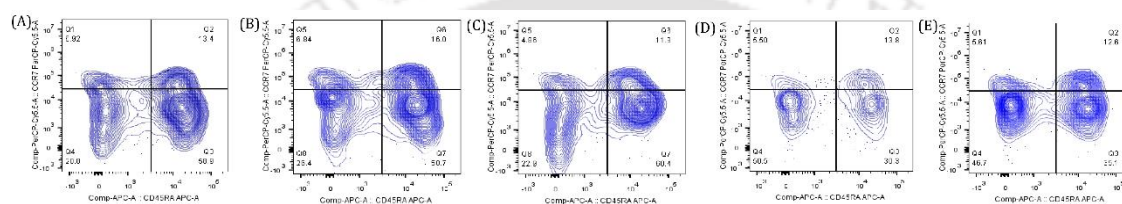


Figure 3.9 Up-regulation of effector memory CD8+T cells in tumor micro-environment post-treatment. Only cell (A), activated cell (B), control cell in tumor micro-environment (C), for **3.1i** (D) and **3.2d** (E). The concentration of the compounds was 50 μ M for the T cell activity studies.

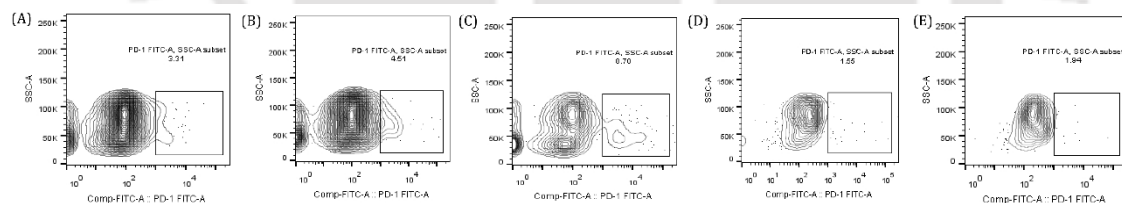


Figure 3.10 Down-regulation of PD-1 expression on CD8+T cells in tumor micro-environment post-treatment. Only cell (A), activated cell (B), control cell in tumor micro-environment (C), for **3.1i** (D) and **3.2d** (E). The concentration of the compounds was 50 μ M for the T cell activity studies.

3.2.10. Antitumor efficacy of the potent triazole compound

These IDO1 inhibitors promoted enhancement of CD8+ T cell activities inspired us to investigate the *in vivo* antitumor efficacy in female Swiss albino mice.³⁰ For the *in vivo* experiments the EAC solid tumor model was used to understand the effect of IDO1 inhibition on tumor burden. The EAC solid tumor model was popular and well

recognized *in vivo* tumor model for anti-tumor therapy.³¹⁻³³ The treatment with compounds **3.2d** showed remarkable regression in tumor growth (Figure 3.11A, B). The compound was effective in attenuating tumor growth with TGI = 80%. Post-treatment tumor tissues were found to have high infiltration of CD8+ T cells (Figure 3.11 C, D and E).³⁴

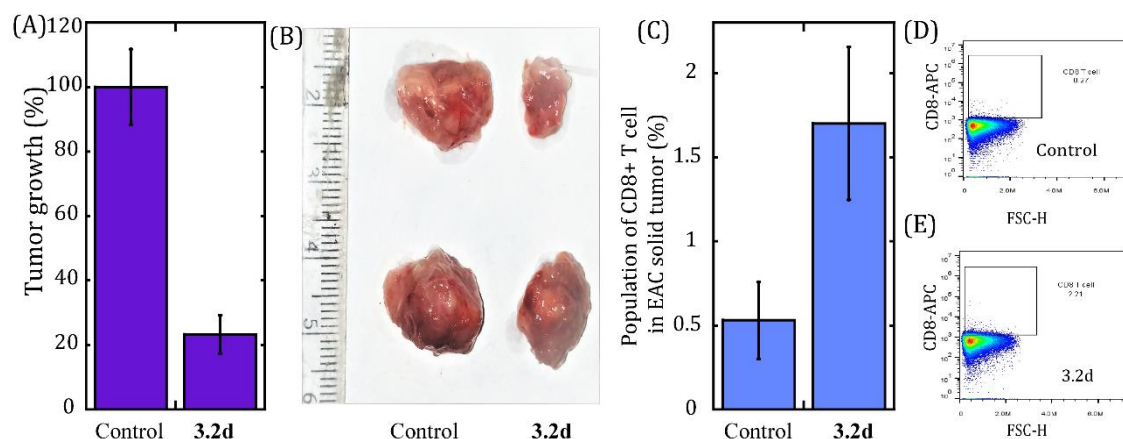


Figure 3.11 The effect of compounds (5 mg/kg body weight) on the growth of EAC solid tumor model in female Swiss albino mice (A, B). The compounds were injected intravenously at alternate days from the 5th day of the tumor implant. CD8+ T cell population in *in vivo* solid tumor (C, D and E).

3.3. Summary

This study described the design and synthesis of 4,5-disubstituted 1,2,3-triazoles as potent IDO1 enzyme inhibitor. Consequential modification of the 1,2,3-triazole scaffold allowed to pinpoint the potent IDO1 inhibitors under the *in vitro* conditions. Both, spectrophotometric and HPLC-based inhibition assays revealed that the di-halogen substitution at C5 aryl ring of these triazoles was important for lower inhibitory potencies. The spectroscopic and SPR-based binding analysis confirmed that the selected triazoles interact with the IDO1 enzyme. Molecular docking studies also proposed that the C4- and halogen-substituted aryl ring at the C5- position of the triazole scaffold assist these compounds in binding to the IDO1 enzyme through non-covalent interactions including hydrogen bonding, halogen bonding, hydrophobic and pi-stacking interactions. Under similar experimental conditions, these compounds showed stronger IDO1 inhibitory activity than the TDO

enzyme. The MTT and cellular IDO1 activity of these selected 1,2,3-triazoles also showed trivial cytotoxicity and low-nanomolar level inhibitory activities. Until now, limited triazole-based compounds had been described with such selective and stronger IDO1 enzyme inhibitory activities. The T cell activity studies identified that the IDO1 enzyme had an immune-damping effect in breast tumor micro-environment, which could be reversed by the treatment with IDO1 inhibitor. These potent IDO1 inhibitor treatment uplifted the total CD8 + T cell number in tumor micro-environment and elevated its effector memory phenotype. The triazoles compounds also attenuated PD-1 expression on cytotoxic T cells, which could help reverse the immune dormancy of these CD8 + T cells to show their effector function. Intravenous treatment of the compounds showed excellent *in vivo* antitumor efficacy in the female Swiss albino mice.

These results suggested that 4,5-disubstituted 1,2,3-triazole derivatives represent a promising class of IDO1 inhibitors, but further structural modifications were required to enhance the *in vivo* antitumor efficacy. It was important to mention that, although we had chemically synthesized and characterized a series of 4,5-disubstituted 2*H*-1,2,3-triazole but multiple tautomers of 2*H*-triazoles might exist in aqueous medium, and same was applicable for other reported 1*H*-triazoles. Overall, these findings suggest that suitably substituted 4,5-disubstituted 1,2,3-triazole derivatives were potent inhibitors of IDO1 enzyme and could be of interest as drug targets in cancer and other life-threatening diseases.

3.4. Experimental section

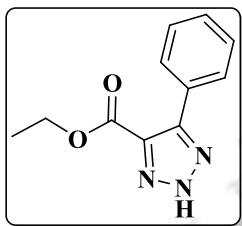
3.4.1. Synthesis of the compounds

3.4.1.1. General information

Described at section 2.4.1.1.

3.4.1.2. Synthesis and characterization of 2*H*-1,2,3-triazole compounds

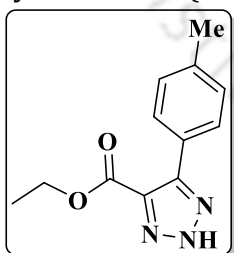
Ethyl 5-phenyl-2H-1,2,3-triazole-4-carboxylate (3.1a)³⁵ –To a stirring solution of benzaldehyde (180 μ L, 1.7 mmol) and ethyl 2-cyanoacetate (176 μ L, 1.87 mmol) in DMF (1.5 mL) was added NH_4Cl (315 mg, 5.95 mmol) and stirred for 10 minutes. Then, NaN_3 (332 mg, 5.1 mmol) was added to the resultant reaction mixture and allowed to stir under reflux at 70 $^\circ\text{C}$ for 6 h. The progress of the reaction was monitored by TLC technique. After completion the reaction, the reaction mixture was



cooled down to room temperature and neutralized by 10% dilute HCl solution. Then, the reaction mixture was diluted with cold water and ethyl acetate. The organic layer was extracted and washed with brine and dried over anhydrous Na_2SO_4 . The organic solvent was removed under reduced pressure.

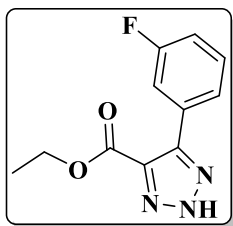
Purification by column chromatography using EtOAc/Hexane (15-40%) solvent gradient produced the target compound 223 mg (63% yield, time = 6 h) of **3.1a** as white solid, Mp: 107 -109 $^\circ\text{C}$ (in lit: 109-111 $^\circ\text{C}$) ; ^1H NMR (600 MHz, CDCl_3) δ_{ppm} 7.78 – 7.76 (m, 2H), 7.40 – 7.39 (m, 3H), 4.36-4.32 (m, 2H), 1.28-1.26 (m, 3H); ^{13}C NMR (100 MHz, CDCl_3) δ_{ppm} 161.3, 146.4, 134.2, 129.9, 129.4, 128.5, 127.9, 61.9, 14.2; FT-IR (KBr) 3072, 2980, 1736, 1520, 1434, 1117 cm^{-1} ; HRMS (ESI) calcd. for $\text{C}_{11}\text{H}_{11}\text{N}_3\text{O}_2$ $[\text{M} + \text{H}]^+$: 218.0885, found: 218.0885.

Ethyl 5-(p-tolyl)-2H-1,2,3-triazole-4-carboxylate (3.1b) – Synthesized as compound **3.1a** using 4-methylbenzaldehyde (170 μ L, 1.44 mmol), ethyl 2-cyanoacetate (160 μ L, 1.58 mmol), NH_4Cl (267 mg, 5.04 mmol) and NaN_3 (281 mg,



4.32 mmol). The reaction provided 185 mg (60% yield, time = 6 h) of **3.1b** as white solid, Mp: 139-141 $^\circ\text{C}$; ^1H NMR (600 MHz, CDCl_3) δ_{ppm} 7.67 (d, J = 12.0 Hz, 2H), 7.20 (d, J = 6 Hz, 2H), 4.34 (q, J = 7.1 Hz, 2H), 2.35 (s, 3H), 1.28 (t, J = 7.1 Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3) δ_{ppm} 161.3, 140.1, 129.3 (d, J = 2.7 Hz), 124.8, 61.8, 21.6, 14.3; FT-IR (KBr) 3045, 2983, 1740, 1521, 1424, 1107 cm^{-1} ; HRMS (ESI) calcd. for $\text{C}_{12}\text{H}_{13}\text{N}_3\text{O}_2$ $[\text{M} + \text{H}]^+$: 232.1041, found: 232.1041.

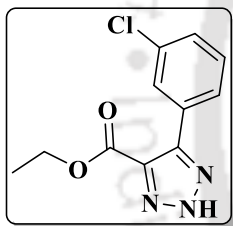
Ethyl 5-(3-fluorophenyl)-2H-1,2,3-triazole-4-carboxylate (3.1c) – Synthesized as compound **3.1a** using 3-fluorobenzaldehyde (165 μ L, 1.56 mmol), ethyl 2-cyanoacetate (182 μ L, 1.71 mmol), NH_4Cl (289 mg, 5.46 mmol) and NaN_3 (304 mg,



4.68 mmol). The reaction provided 263 mg (72% yield, time = 5 h) of **3.1c** as white solid Mp: 132-134 $^{\circ}\text{C}$; ^1H NMR (600 MHz, CDCl_3) δ_{ppm} 7.64 (d, J = 7.7 Hz, 1H), 7.60 (d, J = 9.8 Hz, 1H), 7.39 (dd, J = 14.0, 8.0 Hz, 1H), 7.14 – 7.11 (m, 1H), 4.42 (q, J = 7.1 Hz, 2H), 1.35 (t, J = 7.1 Hz, 3H).; ^{13}C NMR (100 MHz, CDCl_3) δ_{ppm} 163.9,

161.5, 161.0, 130.1 (d, J = 8.3 Hz), 125.2 (d, J = 3.0 Hz), 116.7 (dd, J = 22.3, 7.1 Hz), 62.2, 14.3; FT-IR (KBr) 3082, 2975, 1733, 1521, 1435, 1127 cm^{-1} ; HRMS (ESI) calcd. for $\text{C}_{11}\text{H}_{10}\text{FN}_3\text{O}_2$ $[\text{M} + \text{H}]^+$: 236.0791, found: 236.0791.

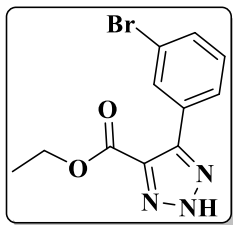
Ethyl 5-(3-chlorophenyl)-2H-1,2,3-triazole-4-carboxylate (3.1d) – Synthesized as compound **3.1a** using 3-chlorobenzaldehyde (130 μ L, 1.15 mmol), ethyl 2-cyanoacetate (135 μ L, 1.58 mmol), NH_4Cl (213 mg, 4.03 mmol)



and NaN_3 (224 mg, 3.45 mmol). The reaction provided 219 mg (76% yield, time = 4 h) of **3.1d** as white solid; Mp: 130-132 $^{\circ}\text{C}$; ^1H NMR (600 MHz, CDCl_3) δ_{ppm} 7.87 (s, 1H), 7.75 (d, J = 7.4 Hz, 1H), 7.41 – 7.36 (m, 2H), 4.42 (q, J = 7.1 Hz, 2H), 1.37 (t, J = 7.1 Hz, 3H).;

^{13}C NMR (150 MHz, CDCl_3) δ_{ppm} 161.1, 146.3, 134.4 (d, J = 6.8 Hz), 130.3, 129.8, 129.7, 129.6, 127.7, 62.2, 14.2; FT-IR (KBr) 3062, 2978, 1738, 1523, 1424, 1119 cm^{-1} ; HRMS (ESI) calcd. for $\text{C}_{11}\text{H}_{10}\text{ClN}_3\text{O}_2$ $[\text{M} + \text{H}]^+$: 253.0432, found: 253.0432.

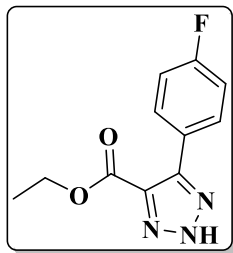
Ethyl 5-(3-bromophenyl)-2H-1,2,3-triazole-4-carboxylate (3.1e) – Synthesized as compound **3.1a** using 3-bromobenzaldehyde (100 μ L, 0.85 mmol), ethyl 2-cyanoacetate (100 μ L, 0.94 mmol), NH_4Cl (158 mg, 2.98 mmol)



and NaN_3 (166 mg, 2.55 mmol). The reaction provided 176 mg (70% yield, time = 5 h) of **3.1e** as pale yellow gummy solid; ^1H NMR (600 MHz, CDCl_3) δ_{ppm} 8.02 (s, 1H), 7.80 (d, J = 7.7 Hz, 1H), 7.55 (d, J = 7.9 Hz, 1H), 7.31 (t, J = 7.9 Hz, 1H), 4.41 (q, J = 7.1 Hz,

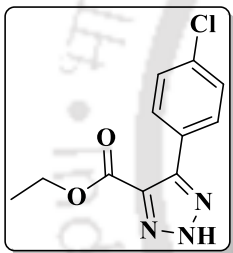
2H), 1.37 (t, J = 7.1 Hz, 3H).; ^{13}C NMR (100 MHz, CDCl_3) δ_{ppm} 161.1, 132.7, 132.4, 130.7, 130.0, 128.1, 122.4, 62.2, 14.3; FT-IR (KBr) 3065, 2981, 1735, 1521, 1437, 1115 cm^{-1} ; HRMS (ESI) calcd. for $\text{C}_{11}\text{H}_{10}\text{BrN}_3\text{O}_2$ $[\text{M} + \text{H}]^+$: 296.0029, found: 296.0029.

Ethyl 5-(4-fluorophenyl)-2H-1,2,3-triazole-4-carboxylate (3.1f) – Synthesized as compound **3.1a** using 4-fluorobenzaldehyde (130 μ L, 1.21 mmol), ethyl 2-cyanoacetate (142 μ L, 1.33 mmol), NH_4Cl (224 mg, 4.24 mmol) and NaN_3 (236 mg,



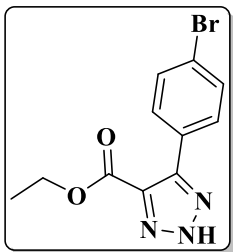
3.63 mmol). The reaction provided 201 mg (71% yield, time = 4 h) of **3.1f** as red solid; Mp: 130 - 132 $^{\circ}\text{C}$; ^1H NMR (600 MHz, CDCl_3) δ_{ppm} 7.85 - 7.81 (m, 2H), 7.12 - 7.06 (m, 2H), 4.41-4.37 (m, 2H), 1.34-1.31 (m, 3H); ^{13}C NMR (100 MHz, CDCl_3) δ_{ppm} 165.0, 162.5, 161.1, 141.6, 131.5 (d, $J = 8.5$ Hz), 131.0, 124.9, 119.1, 115.7, 115.5, 62.1, 14.3; FT-IR (KBr) 3071, 2986, 1743, 1523, 1431, 1121 cm^{-1} ; HRMS (ESI) calcd. for $\text{C}_{11}\text{H}_{10}\text{FN}_3\text{O}_2$ $[\text{M} + \text{H}]^+$: 236.0791, found: 236.0791.

Ethyl 5-(4-chlorophenyl)-2H-1,2,3-triazole-4-carboxylate (3.1g) – Synthesized as compound **3.1a** using 4-chlorobenzaldehyde (125 mg, 0.89 mmol), ethyl 2-cyanoacetate (103 μ L, 0.97 mmol), NH_4Cl (165 mg, 3.12 mmol) and NaN_3 (174 mg,



2.67 mmol). The reaction provided 179 mg (80% yield, time = 4 h) of **3.1g** as white solid; Mp: 165 - 167 $^{\circ}\text{C}$ (in lit. 164-167 $^{\circ}\text{C}$); ^1H NMR (600 MHz, CDCl_3) δ_{ppm} 7.82 (d, $J = 8.5$ Hz, 2H), 7.42 (d, $J = 8.5$ Hz, 2H), 4.41 (q, $J = 7.1$ Hz, 2H), 1.37 (t, $J = 7.1$ Hz, 3H); ^{13}C NMR (100 MHz, CDCl_3) δ_{ppm} 161.2, 146.3, 136.0, 134.3, 130.8, 130.7, 128.8, 126.7, 62.2, 14.3; FT-IR (KBr) 3074, 2987, 1744, 1512, 1429, 1120 cm^{-1} ; HRMS (ESI) calcd. for $\text{C}_{11}\text{H}_{10}\text{ClN}_3\text{O}_2$ $[\text{M} + \text{H}]^+$: 253.0432, found: 253.0432.

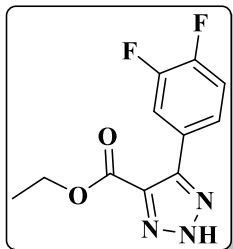
Ethyl 5-(4-bromophenyl)-2H-1,2,3-triazole-4-carboxylate (3.1h) – Synthesized as compound **3.1a** using 4-bromobenzaldehyde (120 mg, 0.65 mmol), ethyl 2-cyanoacetate (76 μ L, 0.72 mmol), NH_4Cl (121 mg, 2.28 mmol) and NaN_3 (127 mg, 1.95



mmol). The reaction provided 130 mg (68% yield, time = 6 h) of **3.1h** as pale yellow solid; Mp: 180-182 $^{\circ}\text{C}$; ^1H NMR (600 MHz, CDCl_3) δ_{ppm} 7.75 (d, $J = 8.2$ Hz, 2H), 7.58 (d, $J = 8.5$ Hz, 2H), 4.41 (q, $J = 7.1$ Hz, 2H), 1.37 (t, $J = 7.1$ Hz, 3H); ^{13}C NMR (100 MHz, $\text{DMSO}-d_6$) δ_{ppm} 160.9, 131.2 (d, $J = 10.1$ Hz), 122.8, 60.9, 14.0; FT-IR (KBr) 3071, 2977, 1747, 1511, 1437, 1123 cm^{-1} ; HRMS (ESI) calcd. for $\text{C}_{11}\text{H}_{10}\text{BrN}_3\text{O}_2$ $[\text{M} + \text{H}]^+$: 296.0029, found: 296.0030.

Ethyl 5-(3,4-difluorophenyl)-2H-1,2,3-triazole-4-carboxylate (3.1i) –

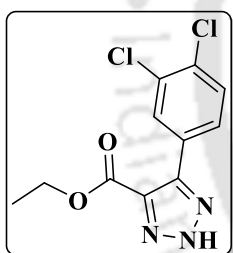
Synthesized as compound **3.1a** using 3,4-difluorobenzaldehyde (140 μ L, 1.26 mmol), ethyl 2-cyanoacetate (148 μ L, 1.39 mmol), NH_4Cl (233 mg, 4.41 mmol) and NaN_3 (246 mg, 3.78 mmol). The reaction provided 230 mg (71% yield, time = 5 h) of **3.1i** as pale



yellow solid; Mp: 128-130 $^{\circ}\text{C}$; ^1H NMR (600 MHz, CDCl_3) δ_{ppm} 7.77-7.73 (m, 2H), 7.14-7.11 (m, 1H), 4.43 (q, $J = 7.1$ Hz, 2H), 1.41-1.38 (m, 3H).; ^{13}C NMR (100 MHz, CDCl_3) δ_{ppm} 161.0, 155.7, 153.2, 145.9, 134.1, 129.3 (d, $J = 11.1$ Hz), 126.0 (d, $J = 3.4$ Hz), 121.0, 118.8 (d, $J = 19.2$ Hz), 118.0, 117.8, 117.4 (d, $J = 17.9$ Hz), 62.3, 14.2; FT-IR (KBr) 3073, 2975, 1739, 1517, 1431, 1119 cm^{-1} ; HRMS (ESI) calcd. for $\text{C}_{11}\text{H}_9\text{F}_2\text{N}_3\text{O}_2$ $[\text{M} + \text{H}]^+$: 254.0696, found: 254.0691.

Ethyl 5-(3,4-dichlorophenyl)-2H-1,2,3-triazole-4-carboxylate (3.1j) –

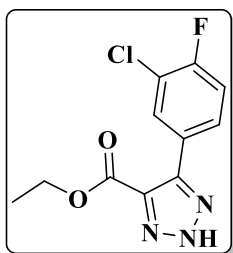
Synthesized as compound **3.1a** using 3,4-dichlorobenzaldehyde (115 mg, 0.66 mmol), ethyl 2-cyanoacetate (77 μ L, 0.72 mmol), NH_4Cl (122 mg, 2.31 mmol) and NaN_3 (129 mg, 1.98 mmol). The reaction provided 154 mg (82% yield, time = 4 h) of



3.1j as white solid; Mp: 140-142 $^{\circ}\text{C}$; ^1H NMR (600 MHz, CDCl_3) δ_{ppm} 8.04 (s, 1H), 7.76 (d, $J = 8.3$ Hz, 1H), 7.51 (d, $J = 8.4$ Hz, 1H), 4.44 (q, $J = 7.1$ Hz, 2H), 1.39 (t, $J = 7.1$ Hz, 3H).; ^{13}C NMR (100 MHz, CDCl_3) δ_{ppm} 160.9, 146.4, 134.0, 132.7, 131.4, 130.5, 128.8, 62.5, 14.3; FT-IR (KBr) 3071, 2976, 1745, 1518, 1433, 1109 cm^{-1} ; HRMS (ESI) calcd. for $\text{C}_{11}\text{H}_9\text{Cl}_2\text{N}_3\text{O}_2$ $[\text{M} + \text{H}]^+$: 286.0072, found: 286.0070.

Ethyl 5-(3-chloro-4-fluorophenyl)-2H-1,2,3-triazole-4-carboxylate (3.1k) –

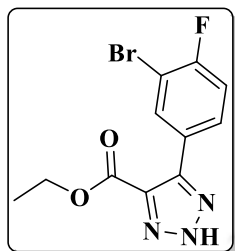
Synthesized as compound **3.1a** using 3-chloro-4-fluorobenzaldehyde (120 μ L, 0.74 mmol), ethyl 2-cyanoacetate (86 μ L, 0.81 mmol), NH_4Cl (137 mg, 2.59 mmol) and



NaN_3 (144 mg, 2.22 mmol). The reaction provided 143 mg (72% yield, time = 5 h) of **3.1k** as pale off-white solid; Mp: 110-112 $^{\circ}\text{C}$; ^1H NMR (600 MHz, CDCl_3) δ_{ppm} 7.99 (d, $J = 1.7$ Hz, 1H), 7.89 – 7.88 (m, 1H), 7.25 (s, 1H), 4.43 (q, $J = 7.1$ Hz, 2H), 1.39 (t, $J = 7.1$ Hz, 3H).; ^{13}C NMR (100 MHz, CDCl_3) δ_{ppm} 160.6, 146.7, 138.5, 131.7, 129.0, 126.7, 125.0, 119.6, 114.9, 62.3, 14.4; FT-IR (KBr) 3073, 2974, 1741, 1519,

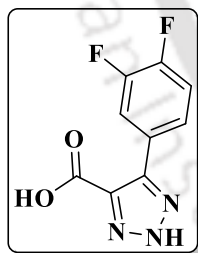
1417, 1112 cm^{-1} ; HRMS (ESI) calcd. for $\text{C}_{11}\text{H}_9\text{ClFN}_3\text{O}_2$ $[\text{M} + \text{H}]^+$: 270.0367, found: 270.0367.

Ethyl 5-(3-bromo-4-fluorophenyl)-2H-1,2,3-triazole-4-carboxylate (3.11) – Synthesized as compound **3.1a** using 3-bromo-4-fluorobenzaldehyde (120 μL , 0.58 mmol), ethyl 2-cyanoacetate (68 μL , 0.64 mmol), NH_4Cl (108 mg, 2.03 mmol) and



NaN_3 (113 mg, 1.74 mmol). The reaction provided 124 mg (68% yield, time = 6 h) of **3.11** as brown solid; Mp: 142-144 $^\circ\text{C}$; ^1H NMR (600 MHz, CDCl_3) δ_{ppm} 8.16 (s, 1H), 7.95 – 7.93 (m, 1H), 7.26 – 7.25 (m, 1H), 4.47-4.44 (m, 2H), 1.42 – 1.39 (m, 3H).; ^{13}C NMR (150 MHz, CDCl_3) δ_{ppm} 160.8, 146.4, 140.0, 134.7, 129.7, 126.9, 119.2, 113.7, 62.3, 14.4; FT-IR (KBr) 3087, 2991, 1737, 1523, 1429, 1121 cm^{-1} ; HRMS (ESI) calcd. for $\text{C}_{11}\text{H}_9\text{BrFN}_3\text{O}_2$ $[\text{M} + \text{H}]^+$: 313.9935, found: 313.9935.

5-(3,4-difluorophenyl)-2H-1,2,3-triazole-4-carboxylic acid (3.2a) – To a stirring solution of ethyl 5-(3,4-difluorophenyl)-2H-1,2,3-triazole-4-carboxylate (**3.1i**, 2 g, 7.9 mmol) in $\text{MeOH}:\text{THF}:\text{H}_2\text{O}$ (1:2:1, 15 mL) was added LiOH (1.9 g, 79 mmol) and allowed to stir at room temperature for 12 h. The progress of the reaction was monitored by TLC technique. After completion of the reaction, it was neutralised by



10% dilute HCl . The obtained solid precipitate was filter and washed the residue with cold water. The solid residue was dried under vacuum to provide the target compound 1.6 g (92% yield, time = 12 h) of **3.2a** as Dutch white solid; Mp: 162-164 $^\circ\text{C}$; ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ_{ppm} 7.84 – 7.82 (m, 1H), 7.72 – 7.70 (m, 1H), 7.41 (s, 1H).;

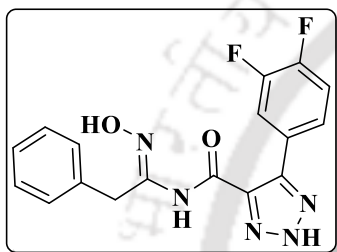
^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) δ_{ppm} 163.1, 154.4, 152.7, 128.4, 126.4, 121.7, 117.7; FT-IR (KBr) 3412, 3045, 1712, 1530, 1316, 1120, 1022 cm^{-1} ; HRMS (ESI) calcd. for $\text{C}_9\text{H}_5\text{F}_2\text{N}_3\text{O}_2$ $[\text{M} - \text{H}]^-$: 224.0277, found: 224.0051.

General procedure for the synthesis of hydroxyamidines derivatives

To a stirring solution of substituted benzonitrile/phenylacetonitrile (4 mmol) and hydroxylamine hydrochloride (6 mmol) in MeOH (5 mL) was added triethylamine (14 mmol), and the mixture was allowed to reflux at 60 $^\circ\text{C}$ for 12 hours. The progress of the reaction was monitored by TLC technique. After completion of the reaction, it

was cooled down to room temperature, and the solvent was removed under reduced pressure. Then the reaction mixture was diluted with ethyl acetate. The organic layer was extracted and washed with brine and dried over anhydrous Na_2SO_4 . The organic solvent was further removed under reduced pressure. The reaction mixture was purified by column chromatography.

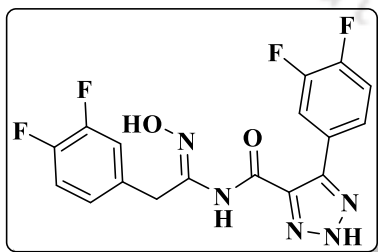
(Z)-5-(3,4-difluorophenyl)-N-(1-(hydroxyimino)-2-phenylethyl)-2H-1,2,3-triazole-4-carboxamide (3.2b) – To a stirring solution of **3.2a** (120 mg, 0.53 mmol), HOBT (158 mg, 1.17 mmol), EDC.HCl (225 mg, 1.17 mmol), *N*-hydroxy-2-phenylacetimidamide (87 mg, 0.58 mmol) and DIPEA (276 μL , 1.59 mmol) provided 118 mg (62% yield, time = 16 h) of **3.2b** as pale yellow solid; Mp: 152-154 °C; ^1H



NMR (400 MHz, $\text{MeOD}-d_4$) δ_{ppm} 7.81 – 7.78 (m, 1H), 7.72 (d, J = 8.2 Hz, 1H), 7.38-7.36 (m, 2H), 7.30 (t, J = 7.4 Hz, 2H), 7.26 – 7.21 (m, 2H), 3.51 (s, 2H); ^{13}C NMR (100 MHz, $\text{MeOD}-d_4$) δ_{ppm} 161.1, 160.5, 156.9, 154.5, 137.5, 130.7 (d, J = 11.1 Hz), 129.9 (d, J = 15.7 Hz), 128.3, 127.2 (d, J = 3.6

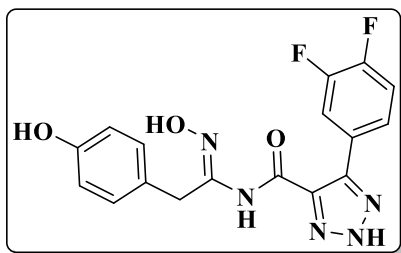
Hz), 122.3, 118.8, 118.6, 38.0; FT-IR (KBr) 3423, 3379, 2942, 1649, 1513, 1425, 1324, 1107 cm^{-1} ; HRMS (ESI) calcd. for $\text{C}_{17}\text{H}_{13}\text{F}_2\text{N}_5\text{O}_2$ $[\text{M} + \text{H}]^+$: 358.1071, found: 358.1069.

(Z)-5-(3,4-difluorophenyl)-N-(2-(3,4-difluorophenyl)-1-(hydroxyimino)ethyl)-2H-1,2,3-triazole-4-carboxamide (3.2c) – To a stirring solution of **3.2a** (125 mg, 0.56 mmol), HOBT (165 mg, 1.22 mmol), EDC.HCl (234 mg, 1.22 mmol), 2-(3,4-difluorophenyl)-*N*-hydroxyacetimidamide (115 mg, 0.62 mmol) and DIPEA (292 μL , 1.68 mmol) provided 142 mg (65% yield, time = 14 h) of **3.2c** as pale yellow solid;



Mp: 135-137 °C; ^1H NMR (400 MHz, $\text{MeOD}-d_4$) δ_{ppm} 7.83 (dd, J = 12.4, 1.9 Hz, 1H), 7.76 – 7.74 (m, 1H), 7.35– 7.27 (m, 2H), 7.23 – 7.19 (m, 2H), 3.50 (s, 2H); ^{13}C NMR (100 MHz, $\text{MeOD}-d_4$) δ_{ppm} 160.5, 157.0, 154.5, 152.7, 152.2, 149.7 (d, J = 12.6 Hz), 135.1, 130.7 (d, J =

11.4 Hz), 127.3 (d, J = 3.6 Hz), 126.5 (dd, J = 6.4, 3.6 Hz), 122.4 (d, J = 1.5 Hz), 119.0 – 118.3 (m), 37.1; FT-IR (KBr) 3419, 3377, 2943, 1654, 1527, 1419, 1329, 1109 cm^{-1} ; HRMS (ESI) calcd. for $\text{C}_{17}\text{H}_{11}\text{F}_4\text{N}_5\text{O}_2$ $[\text{M} + \text{H}]^+$: 394.0882, found: 394.0882.

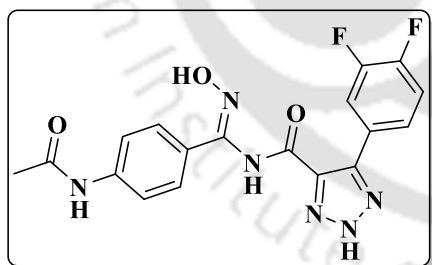
(Z)-5-(3,4-difluorophenyl)-N-(1-(hydroxyimino)-2-(4-hydroxyphenyl)ethyl)-**2H-1,2,3-triazole-4-carboxamide (3.2d)** – To a stirring solution of **3.2a** (135 mg,

0.60 mmol), HOBT (178 mg, 1.32 mmol), EDC.HCl (253 mg, 1.32 mmol), *N*-hydroxy-2-(4-hydroxyphenyl)acetimidamide (120 mg, 0.66 mmol) and DIPEA (312 μ L, 1.8 mmol) provided 123 mg (55% yield, time = 18 h) of **3.2d** as Dutch white solid; Mp: 162-164 $^{\circ}$ C; ^1H NMR (600 MHz, DMSO- d_6)

δ_{ppm} 7.82 (dd, $J = 12.5, 1.8$ Hz, 1H), 7.71 – 7.70 (m, 2H), 7.41 (t, $J = 8.6$ Hz, 2H), 7.13 (d, $J = 8.5$ Hz, 1H), 6.72 – 6.71 (m, 1H), 4.27 (s, 1H), 4.03 (s, 1H), 3.83 (s, 2H); ^{13}C NMR (150 MHz, DMSO- d_6) δ_{ppm} 170.5, 156.3, 154.0, 152.3, 129.9, 128.1 (d, $J = 10.8$ Hz), 126.0 (d, $J = 3.2$ Hz), 125.8 (d, $J = 7.8$ Hz), 125.6, 121.3, 119.1 (d, $J = 18.2$ Hz), 117.4 – 117.1 (m), 115.4, 52.0; FT-IR (KBr) 3421, 3381, 2945, 1654, 1527, 1423, 1104 cm^{-1} ; HRMS (ESI) calcd. for $\text{C}_{17}\text{H}_{13}\text{F}_2\text{N}_5\text{O}_3$ $[\text{M}]^+$: 373.0571, found: 373.0564.

(Z)-N-((4-acetamidophenyl)(hydroxyimino)methyl)-5-(3,4-difluorophenyl)-

2H-1,2,3-triazole-4-carboxamide (3.2e) – To a stirring solution of **3.2a** (130 mg, 0.58 mmol), HOBT (172 mg, 1.27 mmol), EDC.HCl (244 mg, 1.27 mmol), *N*-(4-(*N*-hydroxycarbamimidoyl)phenyl)acetamide (123 mg, 0.64 mmol) and DIPEA (303 μ L, 1.74 mmol) provided 131 mg (57% yield, time = 18 h) of **3.2e** as yellow solid; Mp:



141-143 $^{\circ}$ C; ^1H NMR (600 MHz, DMSO- d_6) δ_{ppm} 9.74 (s, 1H), 8.39 (s, 1H), 7.70 – 7.67 (m, 1H), 7.54 – 7.53 (m, 1H), 7.40 (d, $J = 8.9$ Hz, 2H), 7.28 (d, $J = 8.9$ Hz, 2H), 5.75 (s, 1H), 1.99 (s, 3H); ^{13}C NMR (150 MHz, DMSO- d_6) δ_{ppm} 167.7, 156.0, 135.9, 133.1, 120.1, 119.6, 119.2, 118.1, 23.8; FT-IR (KBr) 3420, 3391, 2931, 1644, 1532, 1422, 1099 cm^{-1} ; HRMS (ESI) calcd. for $\text{C}_{18}\text{H}_{14}\text{F}_2\text{N}_6\text{O}_3$ $[\text{M} + \text{H}]^+$: 401.1129, found: 401.1127.

3.4.2. Purification of the compounds by HPLC analysis

The purity of the synthesized compounds was analysed by analytical HPLC analysis according to earlier mentioned method in section 2.4.1.2.¹⁶

3.4.3. Expression and purification of recombinant human IDO1 and TDO enzymes

Expression and purification of the enzymes were carried out as described previously in the section 2.4.3. with the following minor modifications.^{36, 37} Recombinant human IDO1, and TDO enzymes were expressed by *Escherichia coli* (M15 cell for IDO1 and BL21 (DE3) cell for TDO) using cDNA of human IDO1 (in the vector pQE30 and pREP4 plasmid) and TDO (in the vector pET 28a) respectively.

3.4.4. IDO1 and TDO inhibition assay by the spectrometric method

Both IDO1 and TDO inhibition assays were performed according to the reported procedures as described in section 2.4.4.¹⁶⁻¹⁸

3.4.5. Kynurenine assay by HPLC analysis

HPLC-based IDO1, and TDO inhibition assays were performed according to the earlier reported procedure as described in section 2.4.5.^{11, 16-18}

3.4.6. Binding analysis by spectroscopic measurement

The efficacy of the compound's direct binding to the enzyme active site was measured by UV-Vis spectroscopic analysis according to the mentioned process in section 2.4.6.¹⁶⁻¹⁸

3.4.7. SPR-based Binding analysis of triazole derivatives

The SPR analysis was carried out according to the reported procedure with minor modifications.^{19, 37} In brief, the measurements were performed at 25 °C on the Biacore-X100 instrument using CM5 sensor chip (GE Healthcare). PBS buffer at pH 7.4 was used as running buffer. The surface of the CM5 sensor chip was first modified with Anti-His Antibody using amine-coupling kit at a flow rate of 30 μ L/ minute, followed by IDO1 enzyme (30 μ g/ mL) immobilization using His-capture kit at a flow rate of 10 μ L/minute for 2 minutes. The IDO1 enzyme capture level was 4000 RU. The binding efficacy (association constant; ka) of the compounds was determined by measuring the change in RU of the SPR sensogram in absence or presence of the compounds (0 to 50 μ M).

3.4.8. Circular Dichroism analysis

The CD spectra of the IDO1 enzyme in the absence or presence of compounds were recorded with a Jasco-810-spectropolarimeter according to the reported procedure.²¹ Samples of hIDO1 (2 μ M) were prepared in 10 mM KPB buffer (pH 6.5) in a 1 mm path length quartz cuvette. Spectra were recorded by varying the concentration of the compound (250 nM to 4 μ M) at 17 °C. Also, the spectra were collected at a scan rate 100 nm/sec and 1 nm bandwidth from 200 nm to 260 nm with three times scan for averaging. All final spectra were corrected for buffer background CD signals. The % of the secondary structures of the IDO1 enzyme was analysed using the inbuilt software of the instrument.

3.4.9. Molecular docking analysis

MoleGro Virtual Docker version 6.0 (MoleGro Aps, Aarhus, Denmark) was used for molecular docking analysis of the compounds with IDO1 enzyme (PDB code: 4PK5) as mentioned earlier in section 2.4.10.²³

3.4.10. Determination of mode of enzyme inhibition

The mode of IDO1 enzyme inhibition was measured according to the reported spectrophotometric method as mentioned earlier section, 2.4.9.^{17, 18}

3.4.11. Analysis of the serum binding ability of the potent compounds

Tryptophan fluorescence measurements of the human serum were performed in the presence of compounds on a Fluoromax-4 spectrofluorometer at room temperature.³⁸ For fluorescence measurements, 30 μ L of 100% human serum and varying concentrations (0-220 μ M) of compounds were incubated in 10 mM PBS buffer, pH 7.4. The solution was excited at 290 nm, and emission spectra were recorded from 300 to 560 nm. The resulting tryptophan emission spectra for each concentration of the compounds were plotted.

3.4.12. Cell viability analysis

Cell viability analysis was performed in HEK-293 and MDA-MB-231 cells by using MTT dye according to the similar protocol mentioned in section 2.4.7.¹⁶

3.4.13. Cellular activity assay

The cellular enzyme activity assay was performed according to the reported procedure as mentioned earlier in section 2.4.8.³⁹

3.4.14. T cell activity studies

T cell activity study was designed to evaluate the effects of IDO1 inhibition on cytotoxic T cells present in the breast tumor micro-environment. To mimic breast tumor micro-environment, 20% conditioned medium of human metastatic breast cancer cell line MDA-MB-231 with fresh RPMI and 10% FBS was used. Freshly isolated PBL cells were stimulated with PMA and ionomycin prior to culture in 20% conditioned medium and compounds at selected concentrations. Drug treatment was done for 48 hours followed by immunophenotyping by flow cytometry. For cell proliferation experiments freshly isolated PBLs were cultured in RPMI with 10% FBS and inhibitors for 48 hours. After that the cells were counted with hemocytometer. All the experiments were repeated thrice before analysing data. The concentration of the compounds was 50 μ M for all the T cell activity studies. All the T cell culture related experiments were conducted in the designated biosafety level facility available at the University of Calcutta.

3.4.14.1. PBL isolation and culture

PBL was isolated from freshly isolated blood samples of healthy volunteers in a previously described protocol.⁴⁰ Briefly, blood was diluted with equal volume of sterile PBS and allowed in density gradient centrifugation. Isolated PBLs were washed and cultured in RPMI medium with 10% FBS. PBLs were isolated from fresh blood of healthy volunteers and cultured in RPMI medium with or without compound at selected concentration for 48 hours. Treatment of the compound uplifted cell proliferation. To evaluate whether 4,5-disubstituted 1,2,3-triazole regulate T cell activity, PBL was isolated and cultured at 20% conditioned medium of human metastatic breast cancer cell line MDA-MB-231. To perform this experiment PBL was isolated from three healthy volunteers and three independent experiments were performed. All the volunteers signed informed consent form.

3.4.14.2. T cell activation with PMA and ionomycin

The T cells were activated according to standardized protocol published elsewhere.⁴¹ PMA (100 ng/mL) and ionomycin (1 µg/mL) were used to stimulate the T cells for 2 hrs. PBLs freshly cultured in RPMI with 10% FBS was added in the culture plate at 2×10^5 cells/ well and cultured at 37 °C with 5% CO₂ for the duration of activation.

3.4.14.3. Immunophenotyping by flowcytometry

After treatment for given period of time the lymphocytes were analysed for alteration of protein expression by flow cytometry according to previously published protocols.⁴² Briefly the cells were pelleted down resuspended in cell staining buffer. After cell counting cells were stained against PE-CD8 (Miltenyi Biotek.), PerCP-Cy 5.5-CCR-7 (Miltenyi Biotek.), APC-CD45RA (Miltenyi Biotek.), VioBright-FITC-PD-1 (Miltenyi Biotek.) antibodies as per the manufacturer recommended concentration and incubated in ice for given period of time. Post incubation, the cells were washed, suspended in cell staining buffer before data was acquired using FACS-Verse flow cytometer. The results were analyzed with FlowJo software (v X.0.7). The experiments were repeated thrice prior data analysis.

3.4.15. *In vivo* activity studies

3.4.15.1. Animals and ethical statement

Female Swiss albino mice of age 6 weeks (weight = 20 gm) were selected for the experiments. Animals were housed in animal facility room, Department of Zoology, University of Calcutta at 25 ± 2 °C, relative humidity of nearly $45 \pm 5\%$ with alternative 12 hours day/ night cycle with access of food and water. All the experiments were carried out as per guideline of the CPCSEA, Government of India (Registration No: 885/GO/Re/S/05/CPCSEA) and approved by the Institutional animal ethical committee (IAEC), University of Calcutta.

3.4.15.2. Tumor cell preparation and transplantation

EAC cells were maintained in the ascetic form in female Swiss albino mice by intraperitoneal transplantation every 10th day on each mouse. The ascetic fluid was drawn on 7th day on implantation according to previously described protocol.⁴³

Isolated cells were diluted in sterile PBS. 2×10^6 cells were then implanted on thigh muscle of all the animals selected for experiment.

3.4.15.3. Experiment design

Female Swiss albino mice were divided into two groups ($n = 6$ each group). Post inoculation of the cells in thigh muscle on day 0 mice in their respective group was allowed to treat for 5 days on every alternate day at treatment of 5 mg/ kg body weight described below. All the mice were sacrificed on day 10. 'Group A' was placebo control group (5% DMSO at 10 mL/kg) and 'Group B' was for compound **3.2d**.

3.4.15.4. CD8+ T cell populations *in vivo* solid tumors

Tumor tissues post sacrifice were digested in collagenase IV medium for 2 hours. The tissues were passed through wire mesh and single-cell strainer. Then the cells were washed in PBS, and immunophenotyping was performed, and cells were stained against APC-CD8 for finding infiltrated CD8+ T cell populations by flow cytometry analysis.

3.5. References

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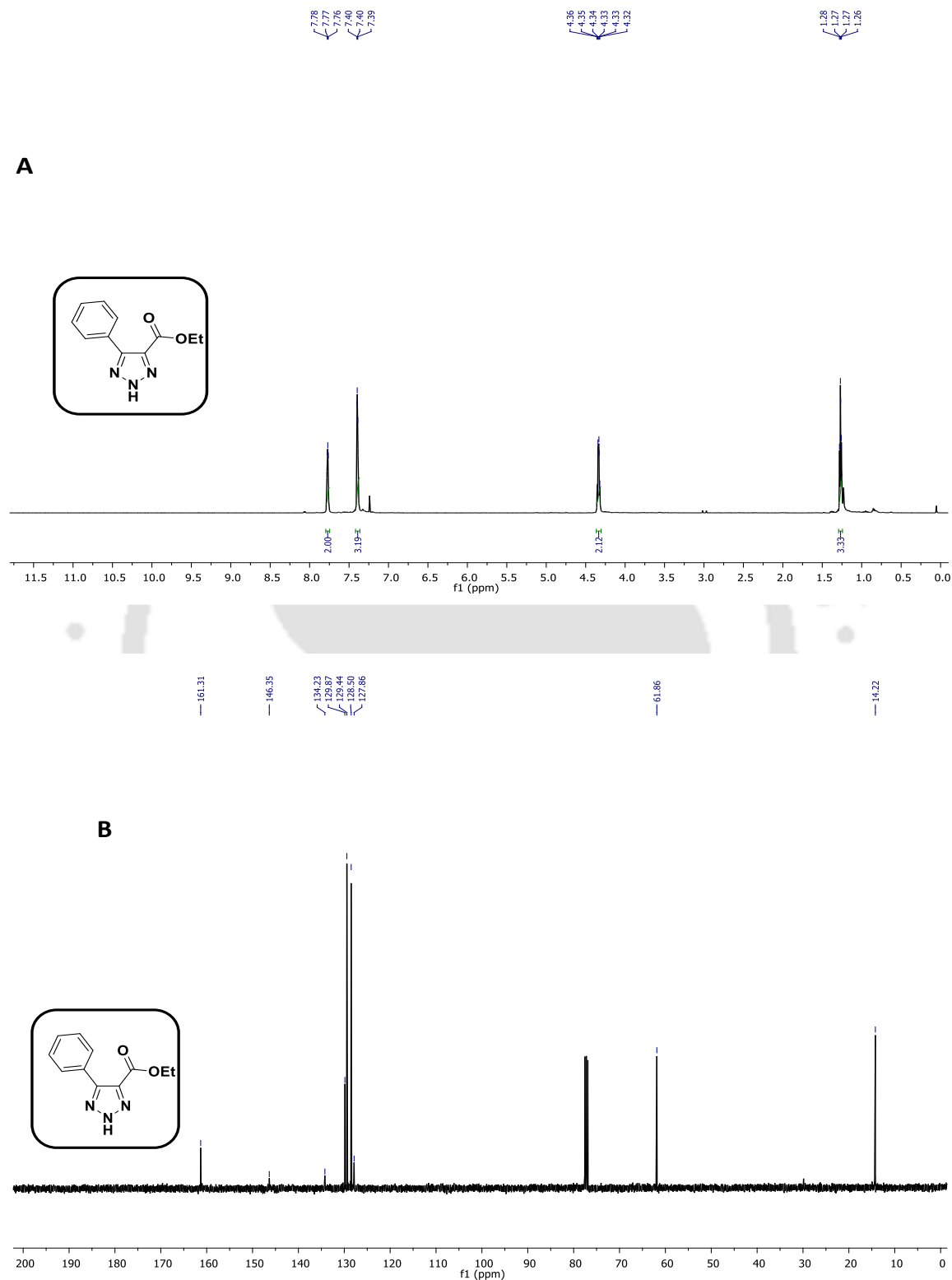
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3.6. ^1H and ^{13}C NMR spectra of synthesized compoundsFigure 3.12 ^1H (A) and ^{13}C (B) NMR of compound 3.1a.

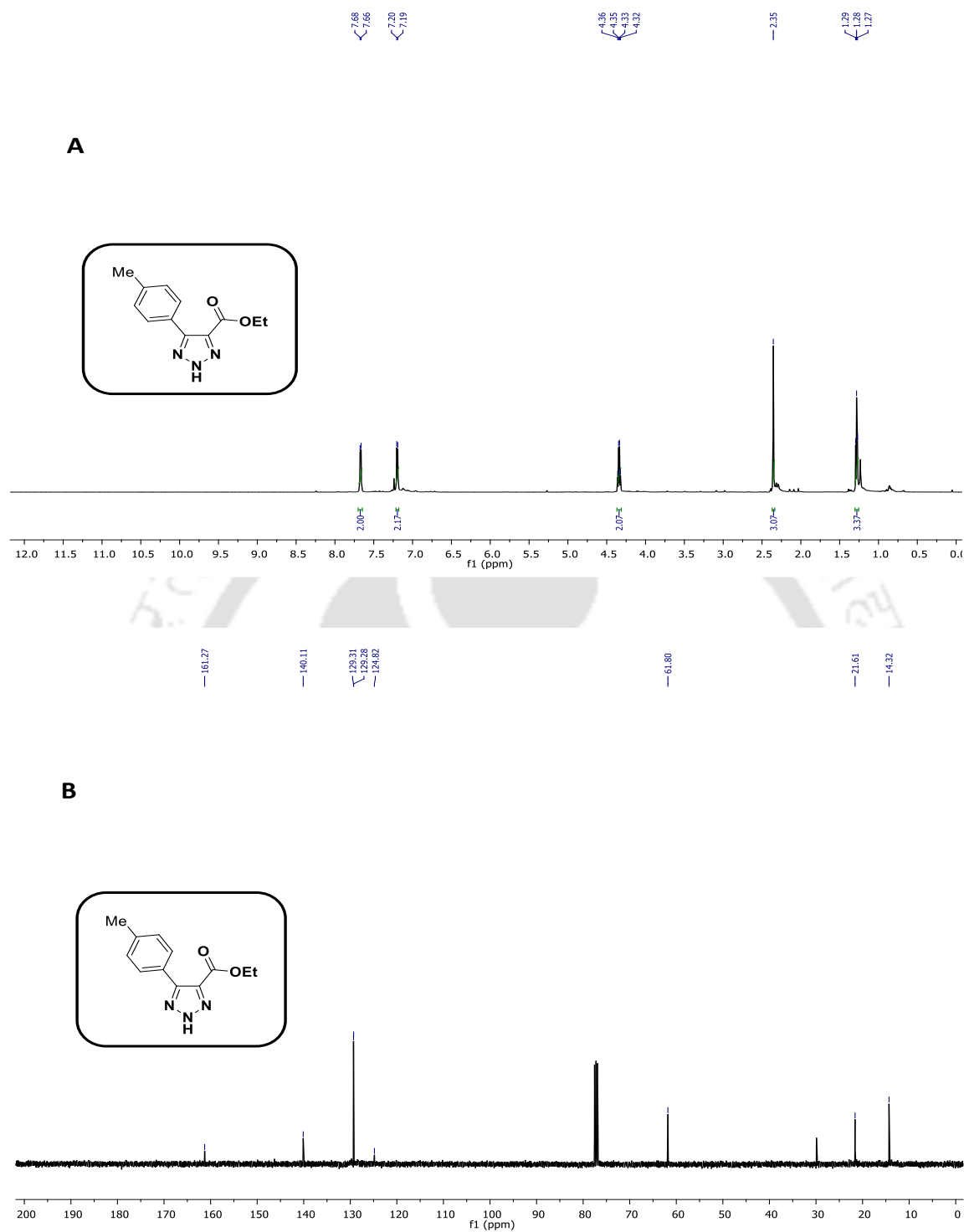


Figure 3.13 ^1H (A) and ^{13}C (B) NMR of compound 3.1b.

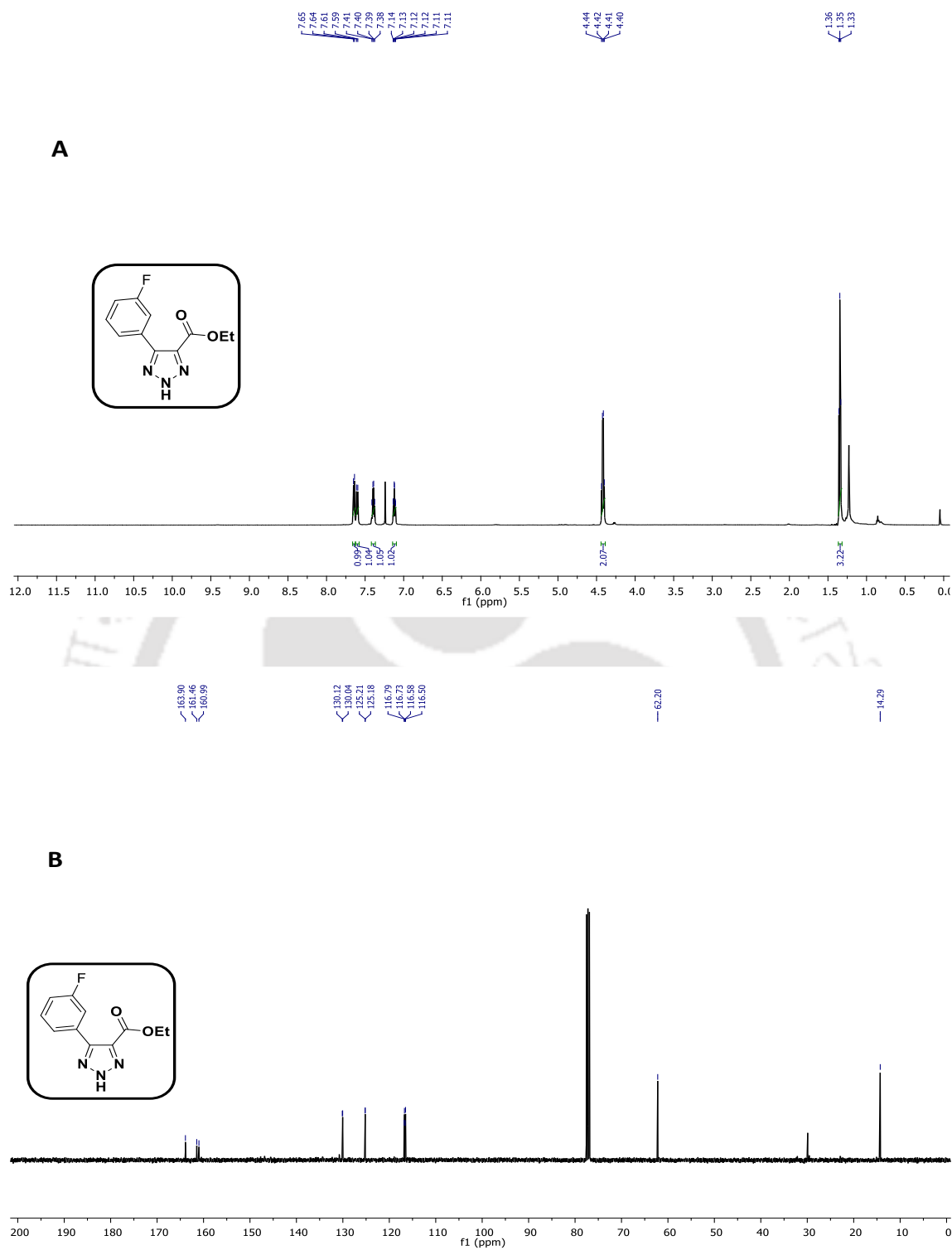


Figure 3.14 ¹H (A) and ¹³C (B) NMR of compound **3.1c**.

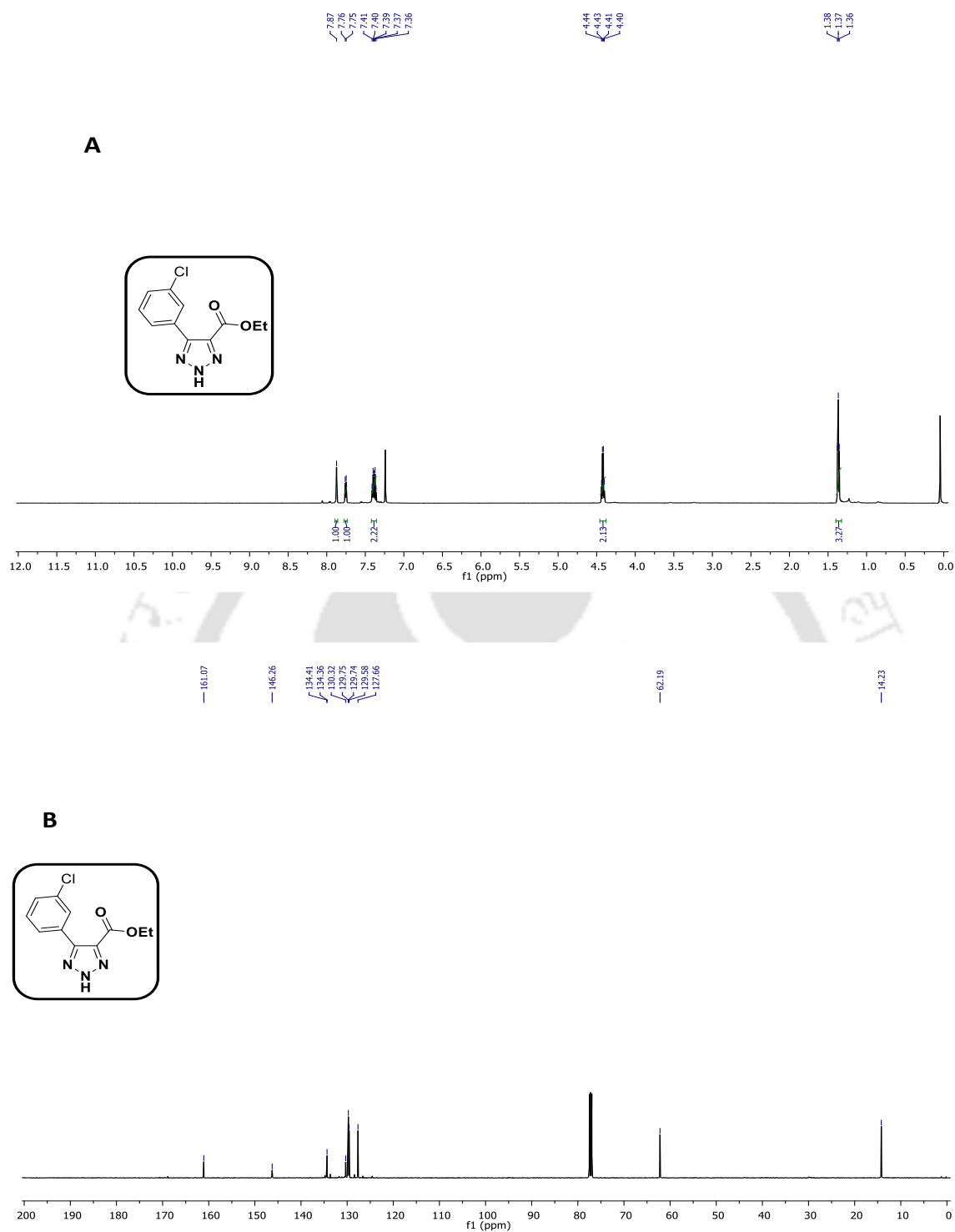


Figure 3.15 ^1H (A) and ^{13}C (B) NMR of compound **3.1d**.

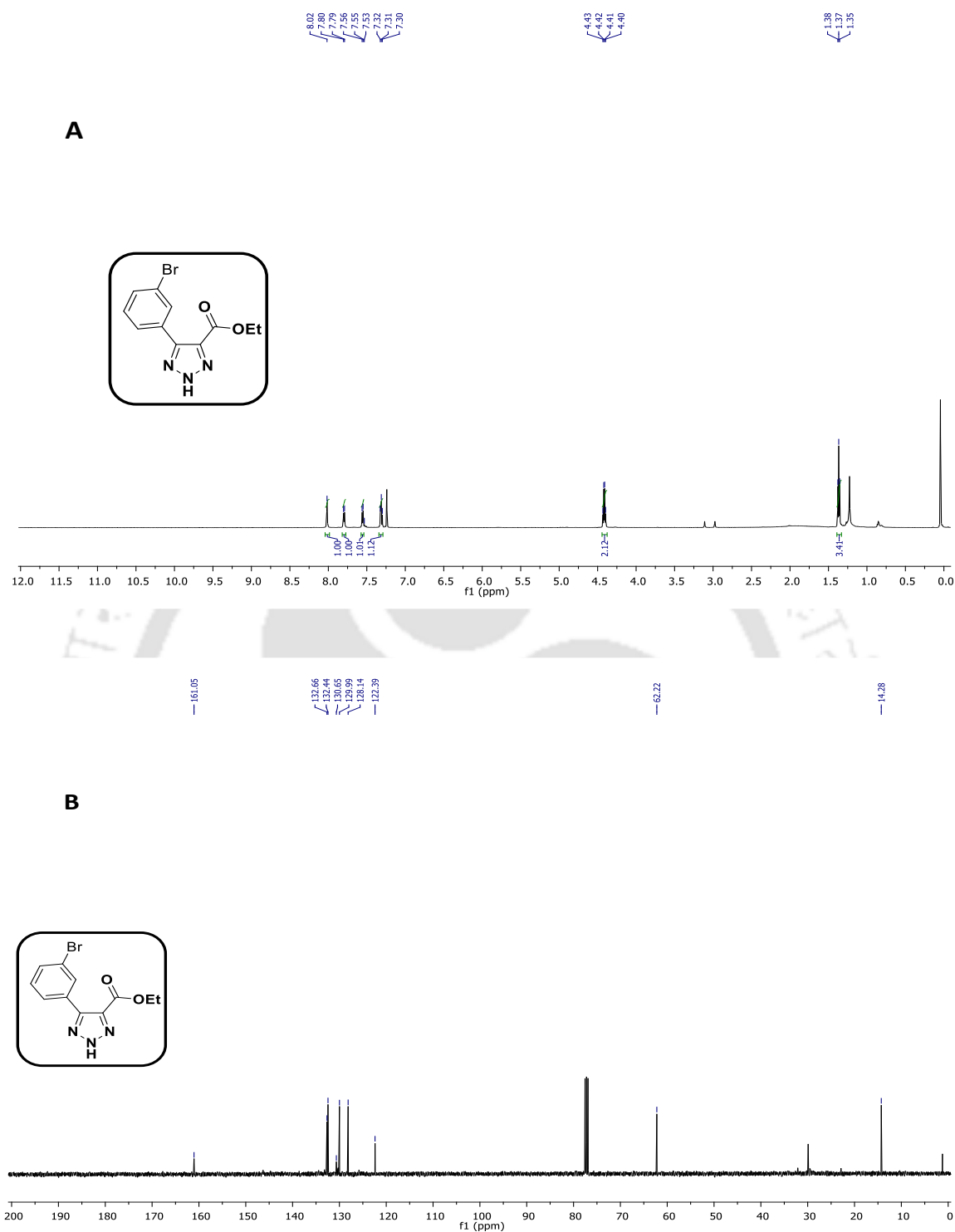


Figure 3.16 ^1H (A) and ^{13}C (B) NMR of compound 3.1e.

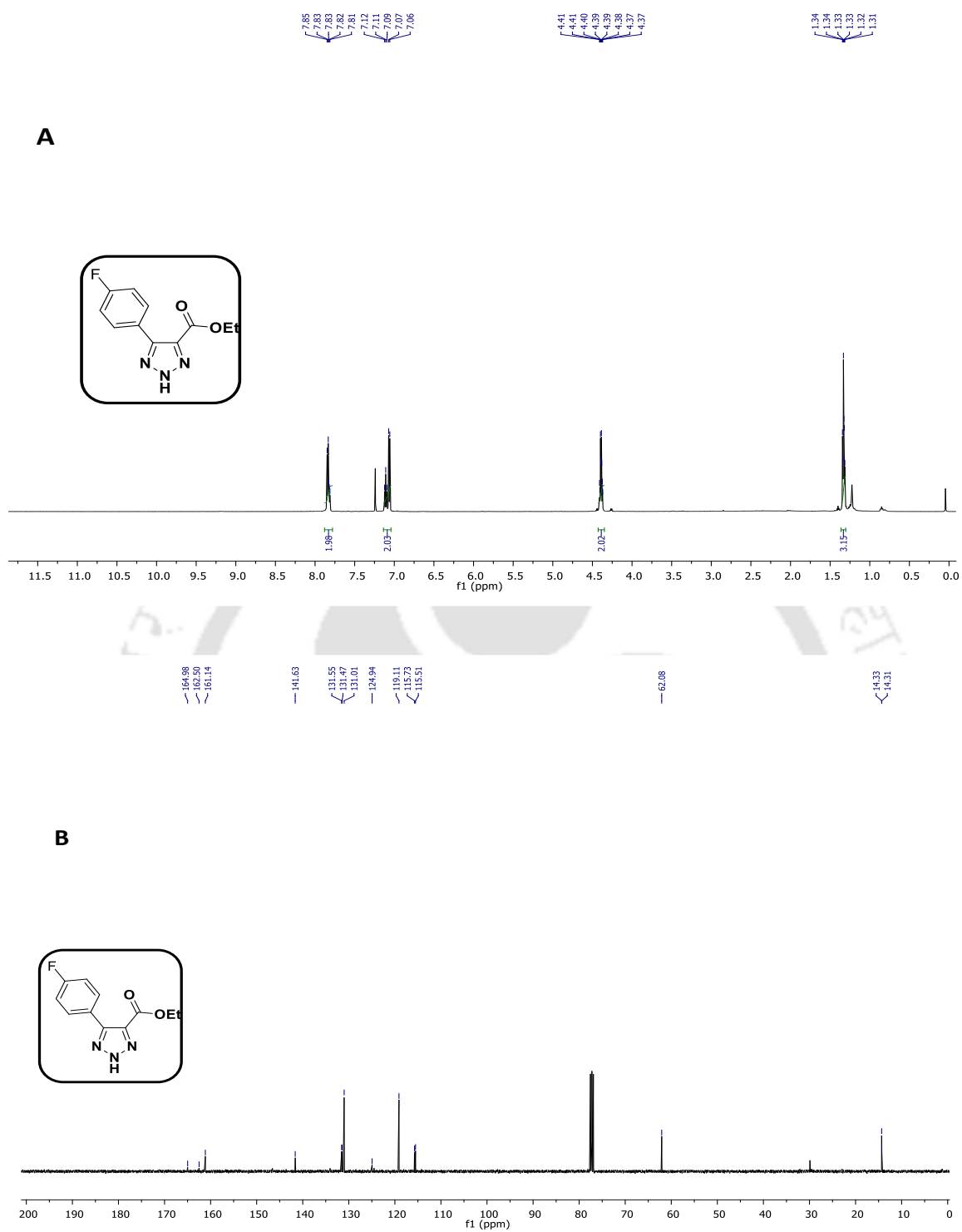


Figure 3.17 ^1H (A) and ^{13}C (B) NMR of compound 3.1f.

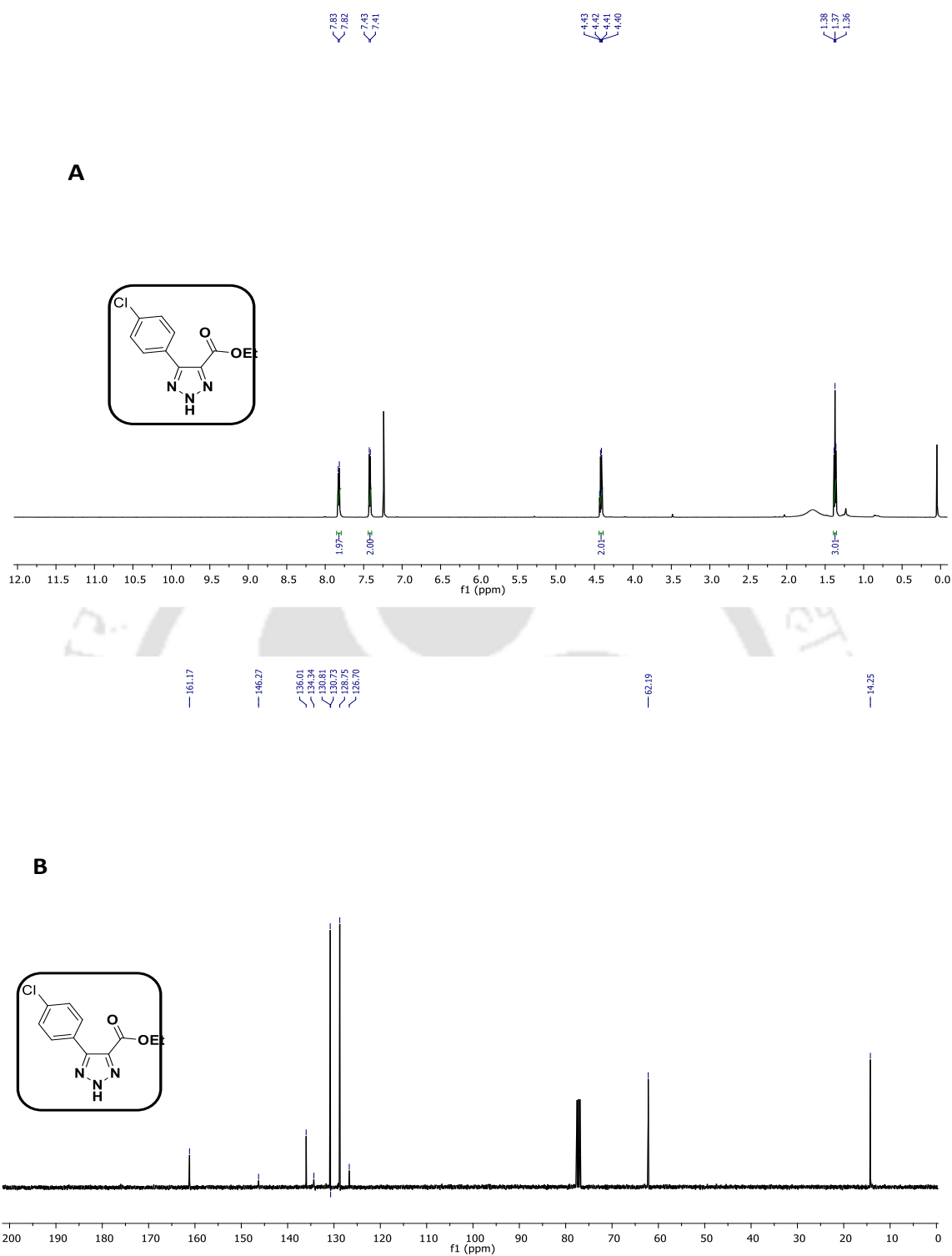


Figure 3.18 ^1H (A) and ^{13}C (B) NMR of compound **3.1g**.

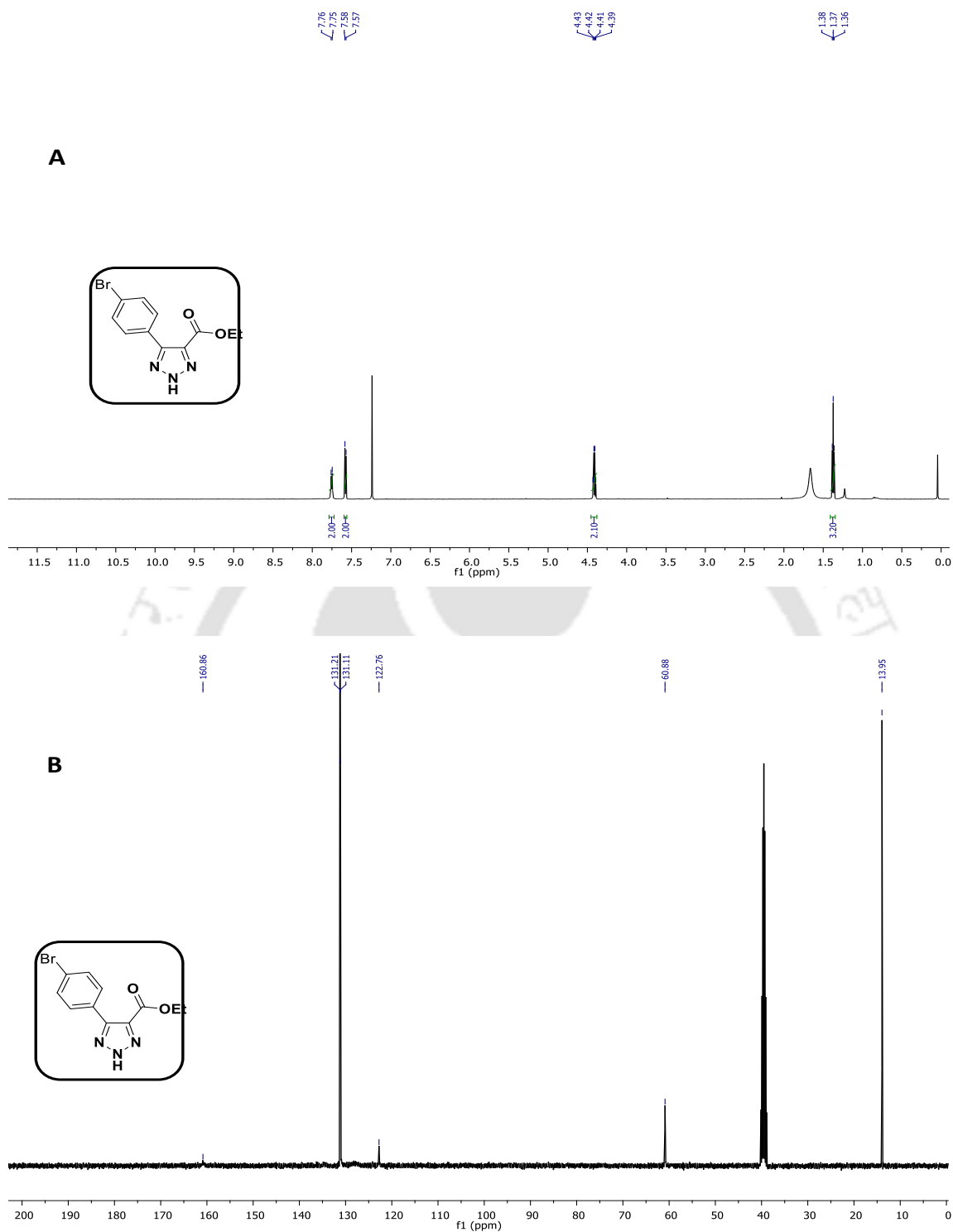


Figure 3.19 ^1H (A) and ^{13}C (B) NMR of compound **3.1h**.

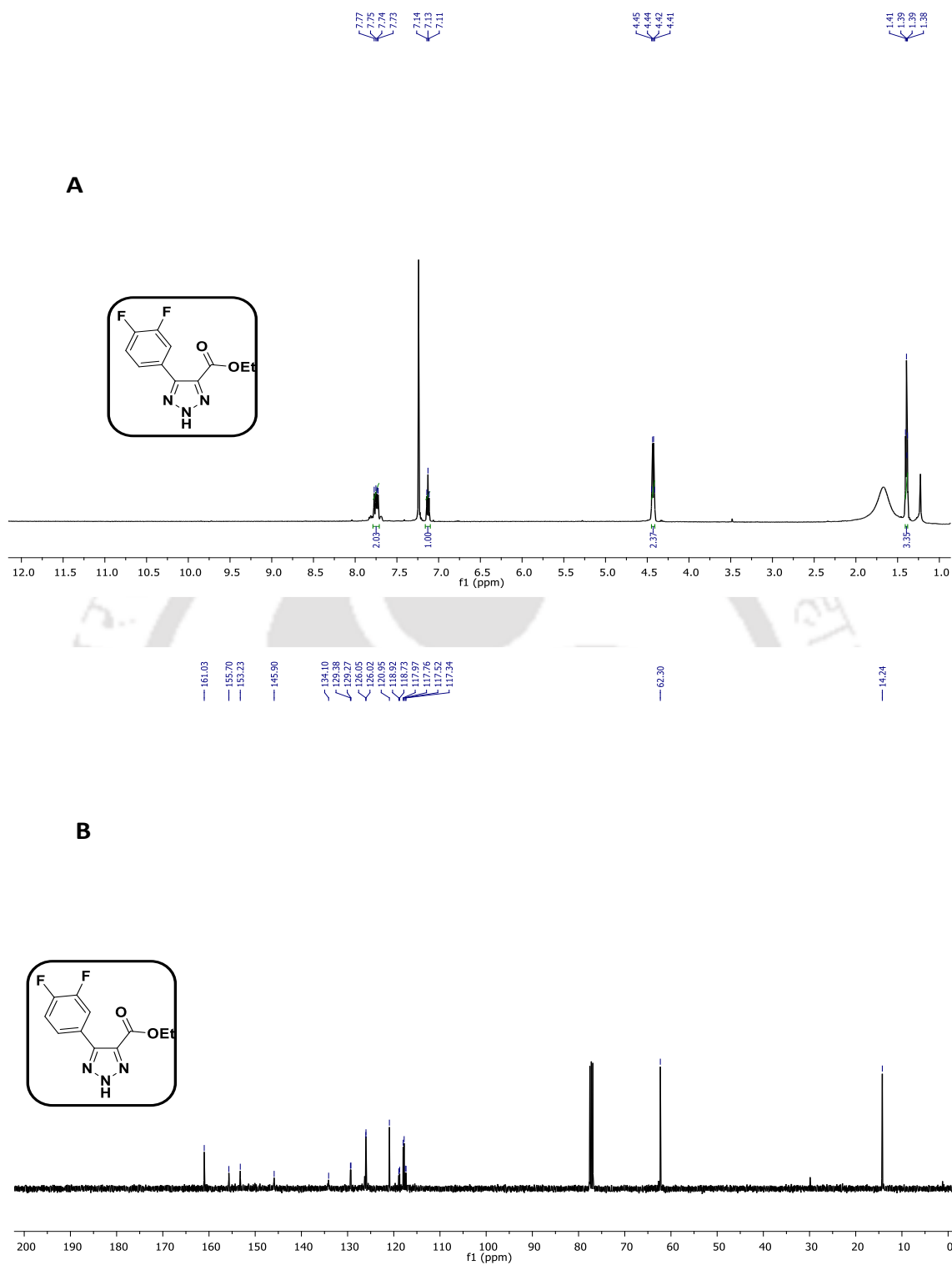


Figure 3.20 ^1H (A) and ^{13}C (B) NMR of compound 3.1i.

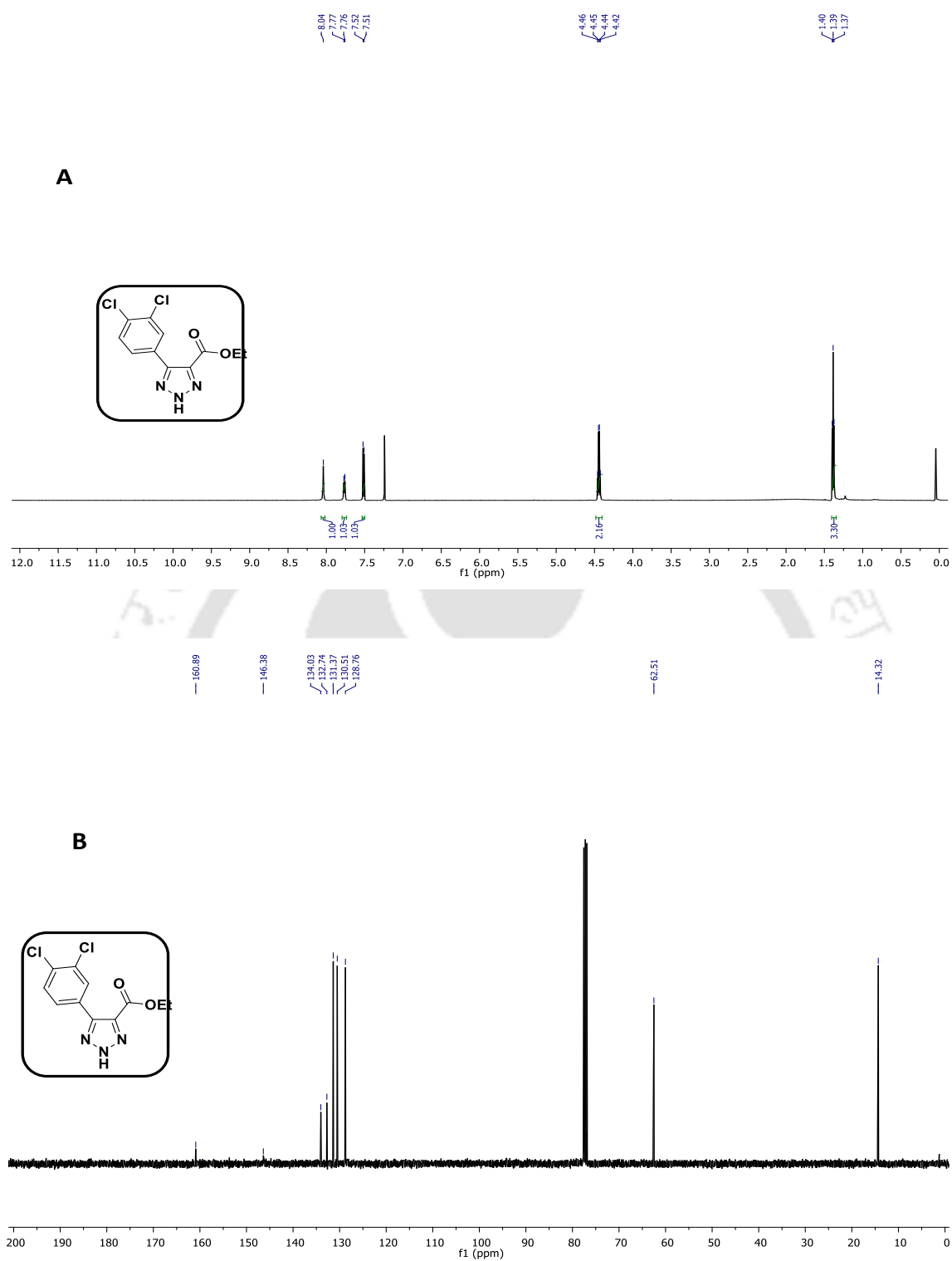


Figure 3.21 ^1H (A) and ^{13}C (B) NMR of compound **3.1j**.

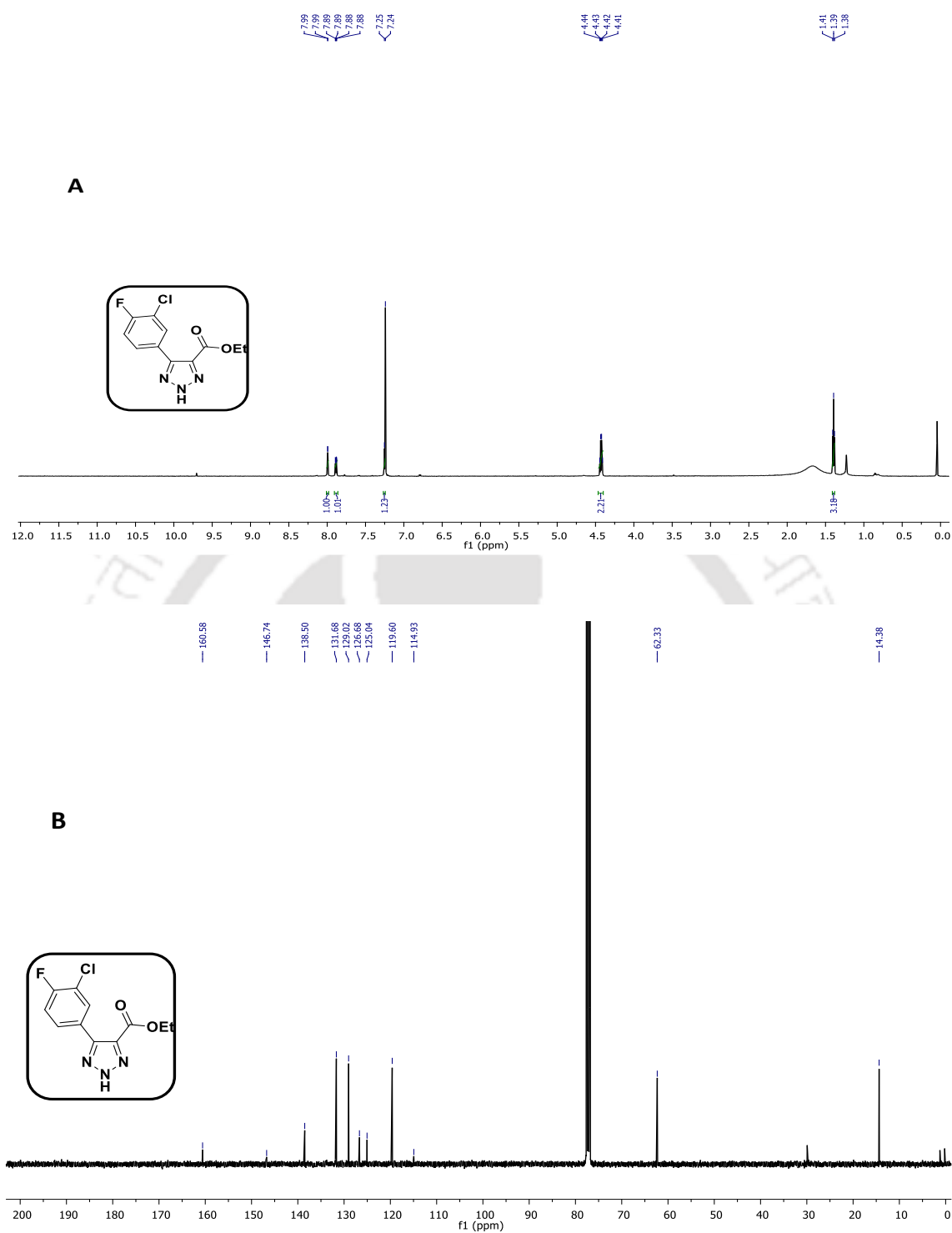


Figure 3.22 ^1H (A) and ^{13}C (B) NMR of compound **3.1k**.

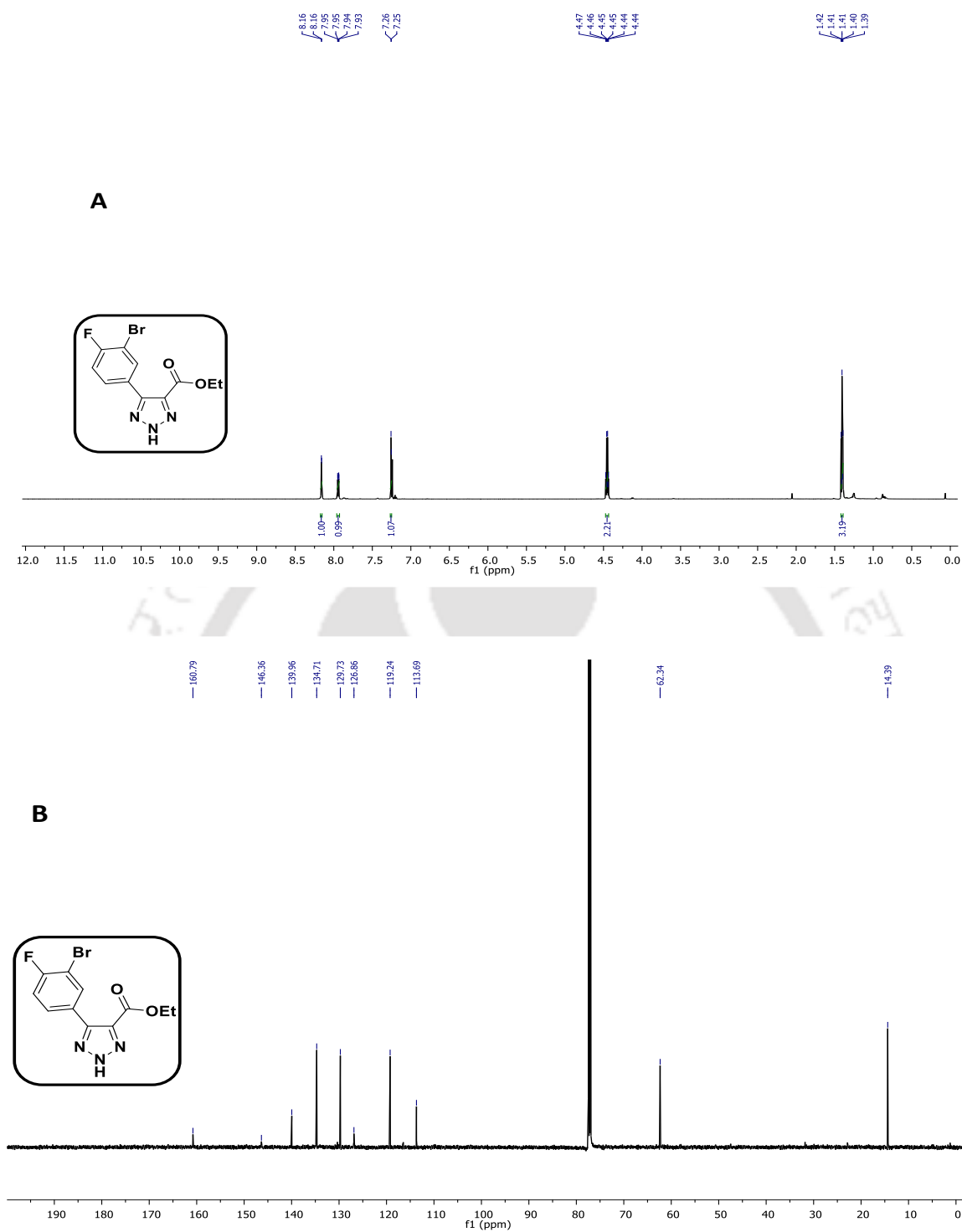


Figure 3.23 ^1H (A) and ^{13}C (B) NMR of compound 3.1l.

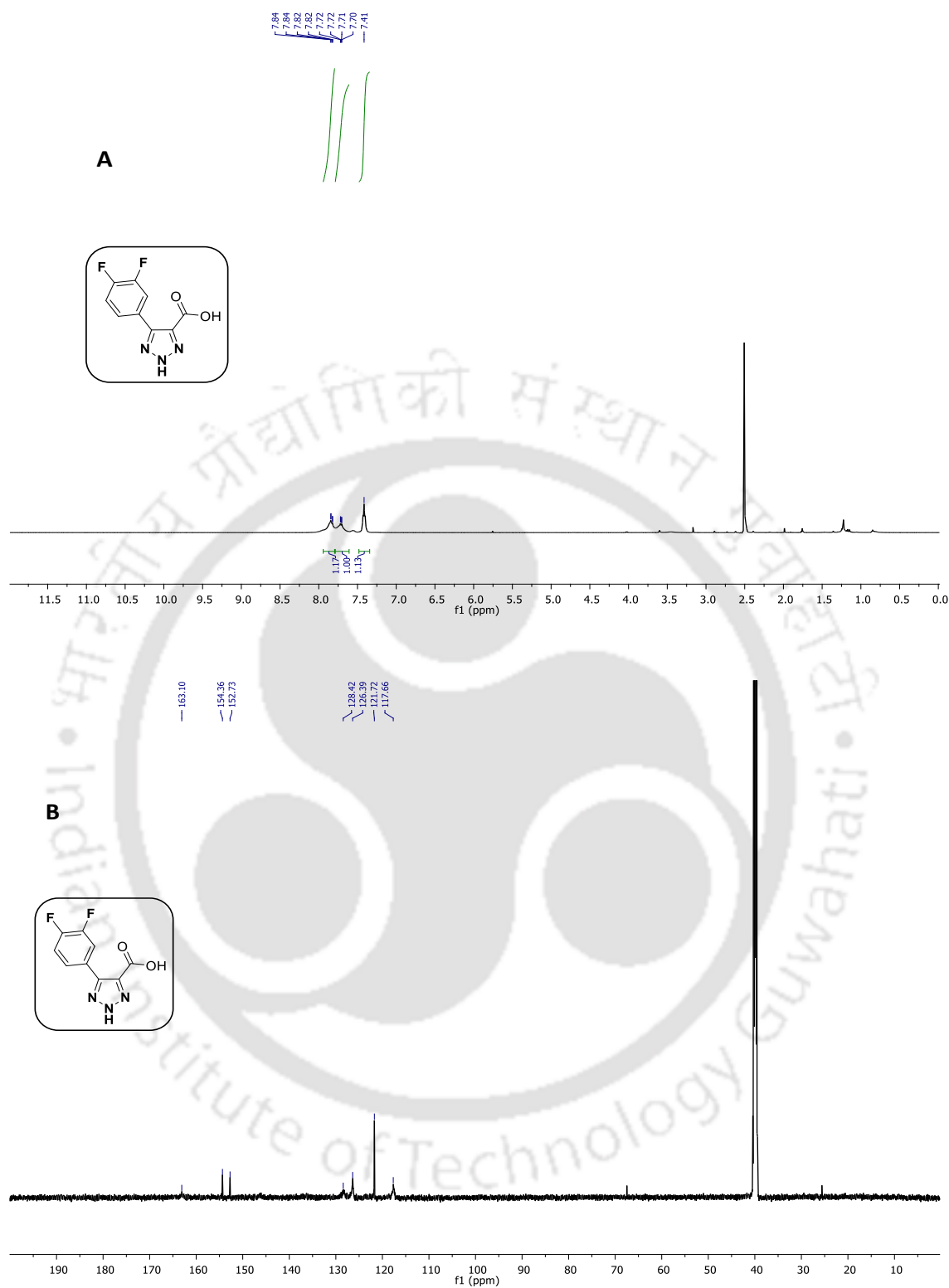


Figure 3.24 ^1H (A) and ^{13}C (B) NMR of compound **3.2a**.

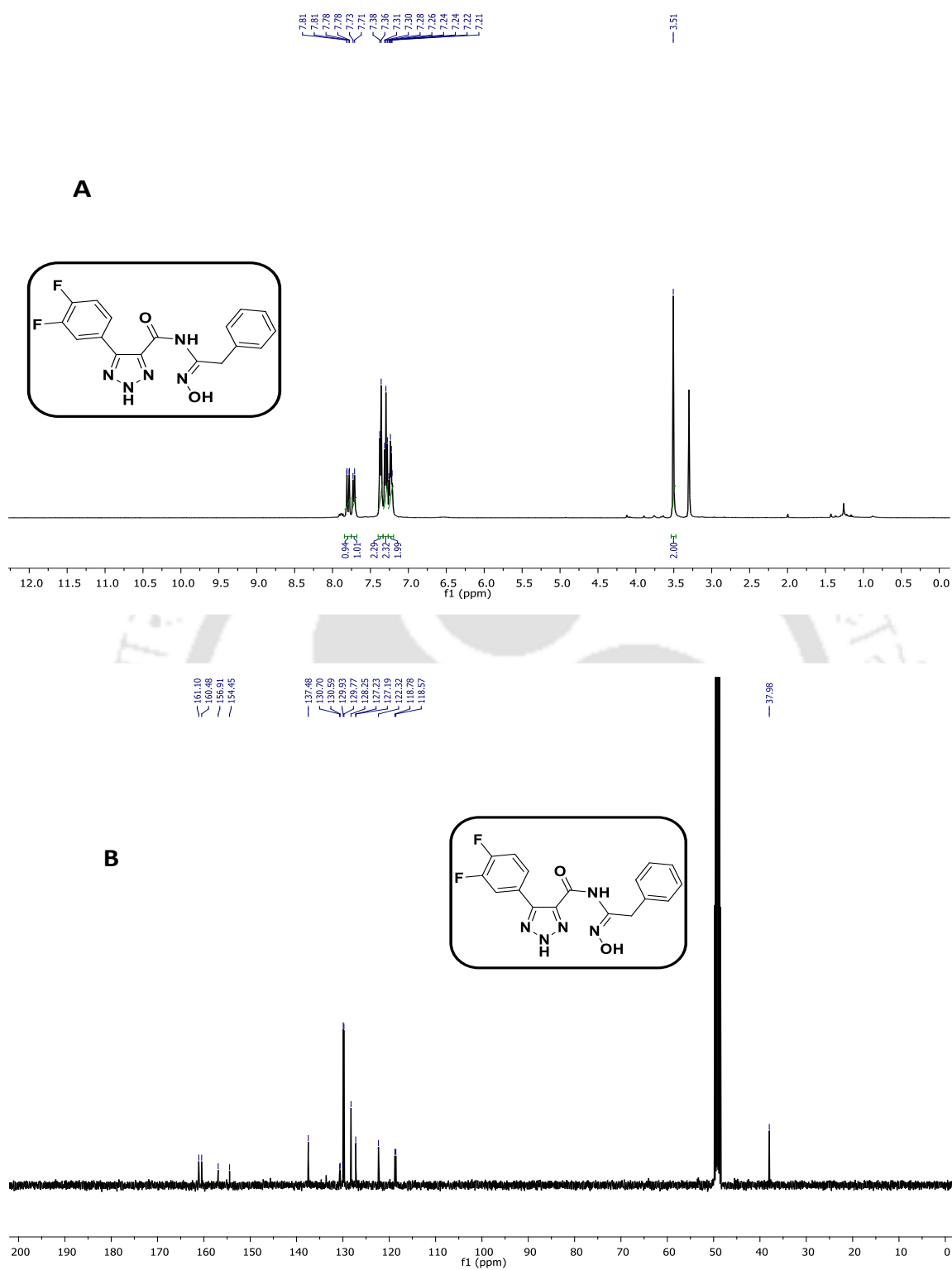


Figure 3.25 ^1H (A) and ^{13}C (B) NMR of compound 3.2b.

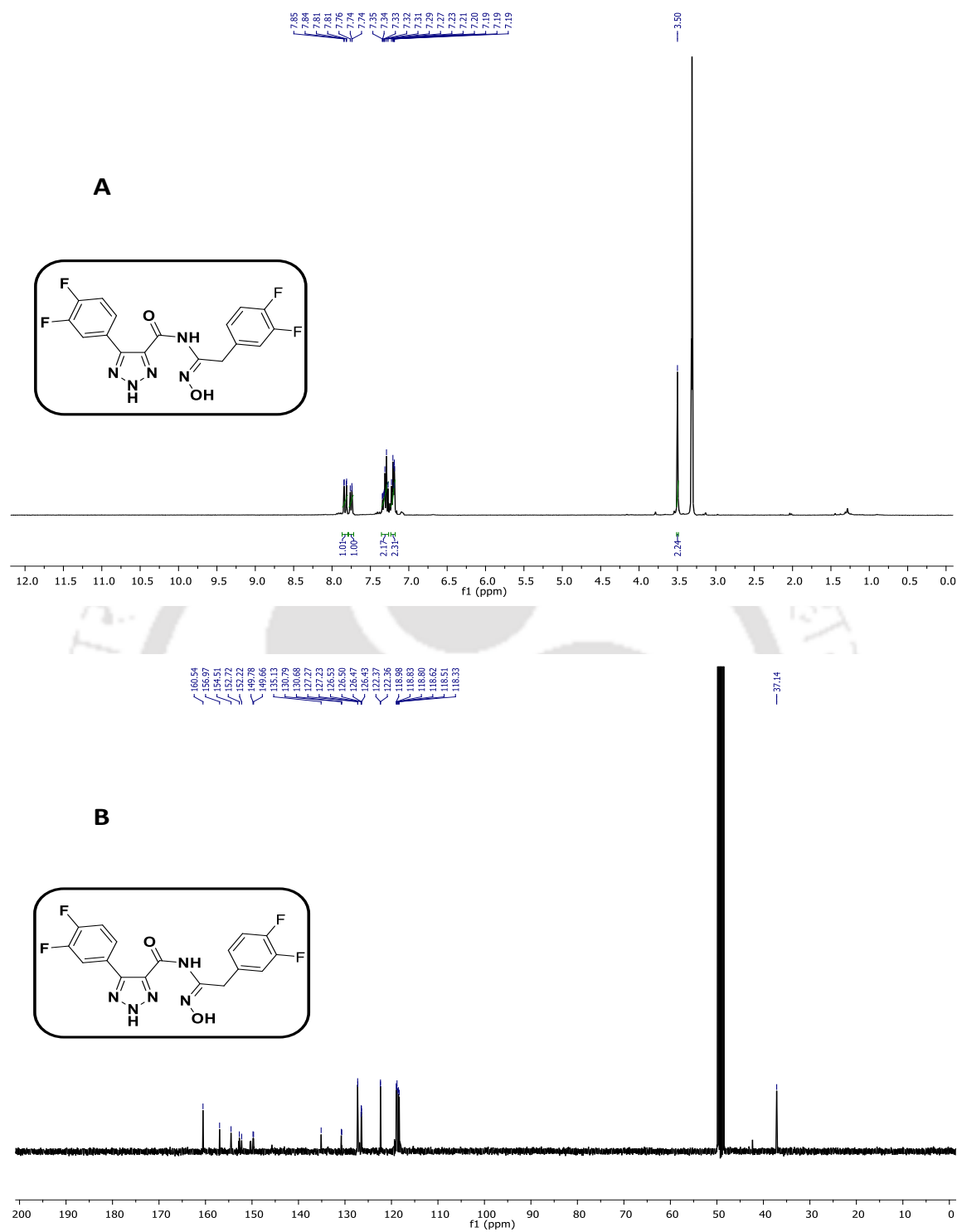


Figure 3.26 ^1H (A) and ^{13}C (B) NMR of compound 3.2c.

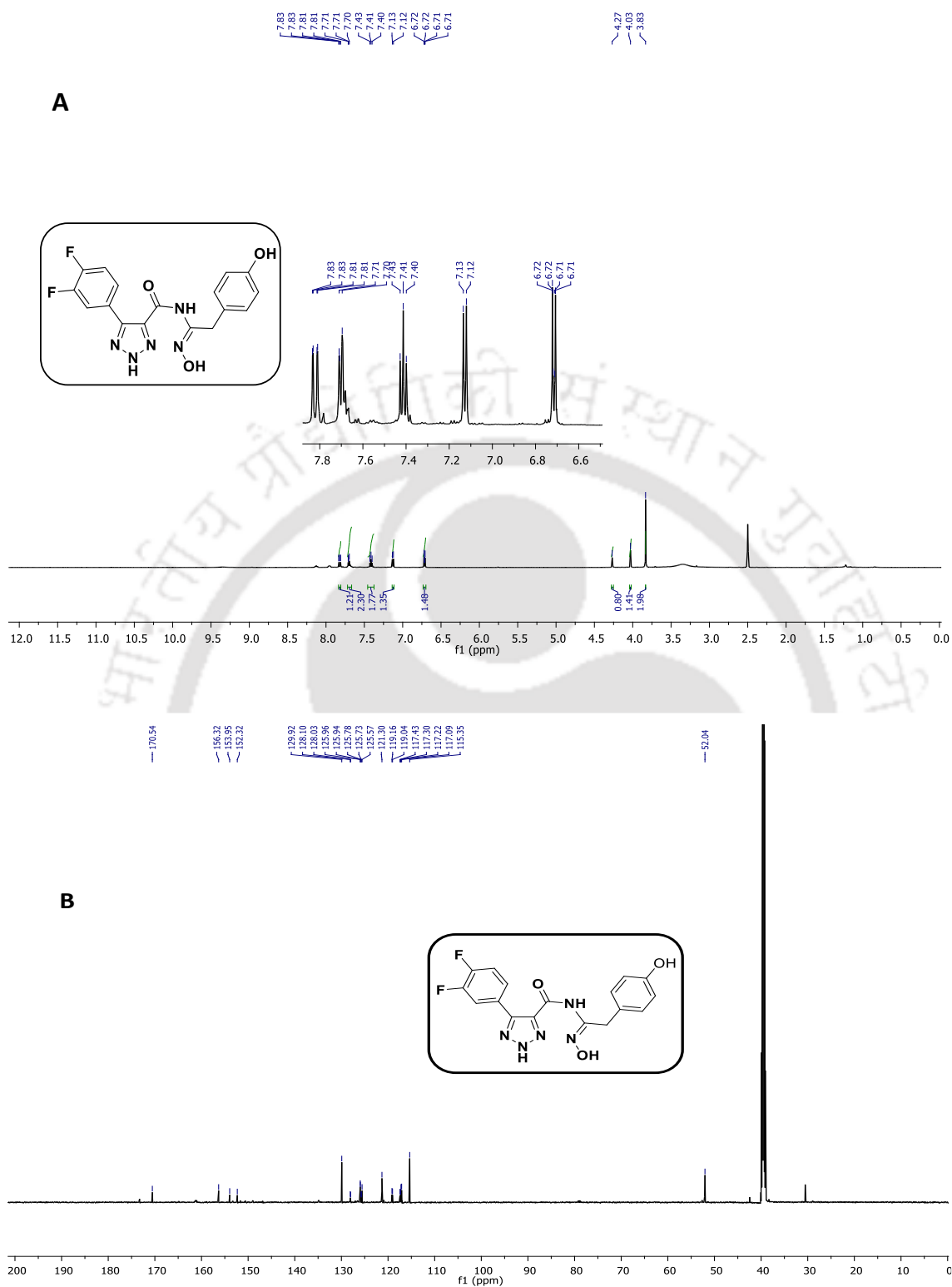


Figure 3.27 ^1H (A) and ^{13}C (B) NMR of compound 3.2d.

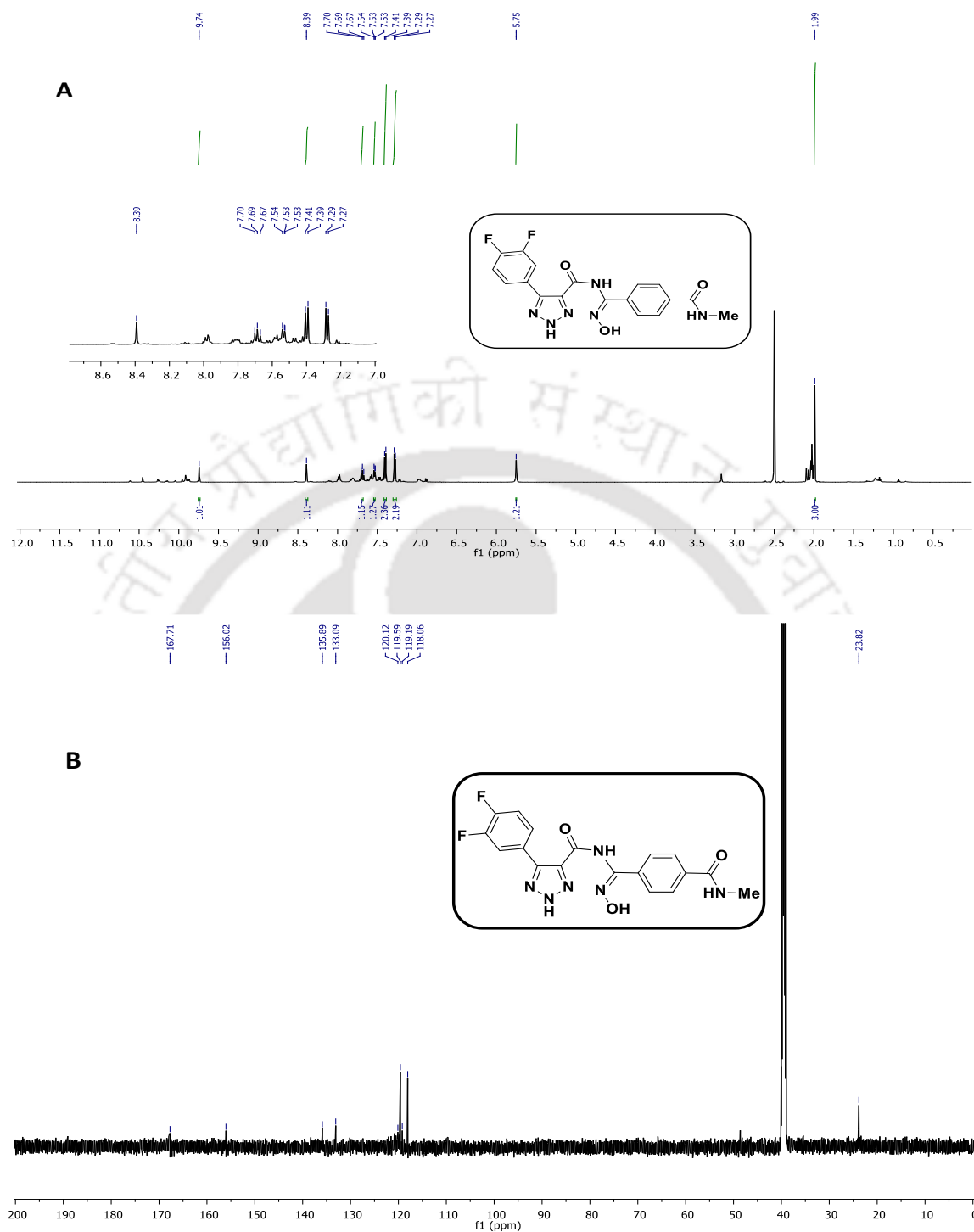


Figure 3.28 ^1H (A) and ^{13}C (B) NMR of compound 3.2e.



Chapter 4

Inhibition of Immunosuppressive IDO1 Enzyme by Targeting the Heme and Apo-form

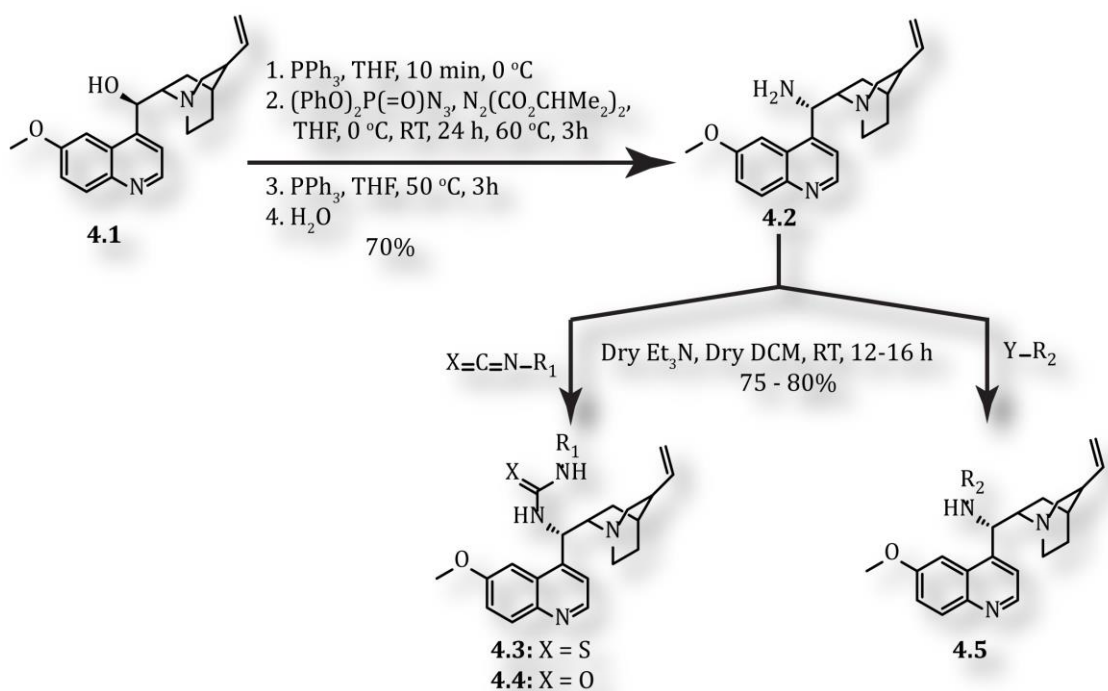


4.1. Background and objective of present work

Cancerous cells are known to arrest the immune system and suppress tumoricidal immunity. Positive modulation of the immune response is considered as one of the innovative approaches for the treatment of cancer and other diseases. The use of immune checkpoint inhibitors displayed therapeutic potential against cancer.^{1, 2} However, the outcome is not as impressive as expected; therefore, finding an alternative approach to rejuvenate the immune system and make cancer immunotherapy more effective has become imperative.³ Among the various combinatorial strategies that minimize the tumor immunosuppression, the inhibition of IDO1 activity has taken a leading role. Various studies already confirmed that the inhibition of IDO1 enzyme activity promotes the efficiency of chemotherapeutic and radio-therapeutic treatments.^{4, 5} In this regard, IDO1 is considered a potential target for the treatment of cancer. The inhibition of IDO1 activity could reinstate the aptitude of the immune cells to remove the cancerous cells on its own or in combination with immune checkpoint inhibitors, which was also previously discussed in first chapter. Different IDO1 inhibitors, in combination with immune checkpoint inhibitors, are currently being explored for the possible treatment of different types of cancer. We have also developed 1*H*-indazole, 1,2,3-triazole derivatives which was discussed in previous chapter derivatives which was discussed in previous chapter. Interestingly, the 4,5-disubstituted 1,2,3-triazole derivatives exhibited effective IDO1 inhibition, consequence regulation of T cell activity as well as mitigation of tumor growth. However, the unfortunate failure of the ECHO-301 phase III clinical trial of epacadostat/keytruda combination against metastatic melanoma came as a complete shock to immunotherapy-related research on cancer.⁶ Although clinical trials with the combination of IDO1 inhibitors/immune checkpoint inhibitors have been reinitiated, the reinvestigation of the IDO1 inhibition mechanism has become obligatory to accomplish apposite clinical benefits.⁷

The IDO1 mediated catabolism of L-Trp requires not only L-Trp but also molecular oxygen and prosthetic heme group. Coordination with heme-iron and inherent binding affinity to the L-Trp binding pocket is the most common IDO1 inhibition mechanism of the reported inhibitors, including the epacadostat.⁵ Recently, it was demonstrated that the heme-binding to the IDO1 enzyme is a

dynamic and reversible process and around 85% of IDO1 exists as the heme-free conformation of the enzyme (apo-IDO1) under the cellular environment.³ Subsequently, the accumulation of free heme-cofactor due to the formation of apo-IDO1 in tumor cells over-activate the other heme-containing proteins, augment the expression of heme exporters and also affect the turnover rate of the IDO1 enzyme, suggesting that not only the heme and its precursor but also the heme biosynthetic pathway is stimulated in cancer cells.^{8,9} The elevated level of the free-heme group is also responsible for the increase in the basal production of ROS, which facilitates the survival of cancer cells.⁸ Hence, the development of apo-IDO1 ligand and reduction of the level of free heme could be advantageous and a practical one in controlling the side effects due to the overexpression of IDO1 and generation of free heme in cancer cells.^{3,8} However, IDO1 inhibitors with such dual activity have not yet been reported.



Scheme 4.1 Synthesis of (1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-vinylquinuclidin-2-yl)methanamine (**4.2**) from quinine (**4.1**). Synthesis of thiourea-based quinine derivatives (**4.3**), urea-based quinine derivatives (**4.4**) and benzenesulfonyl-based derivatives (**4.5**). from **4.2**.

The natural alkaloid, quinine, has antimalarial, antipyretic with analgesic and anti-inflammatory properties. The quinine and its derivatives are known to bind the heme via its quinoline moiety and prevent hemozoin formation during malaria.¹⁰ In this work, inspired by the heme-binding ability of the quinine, we developed a series of quinine derivatives apposite for binding to the apo-IDO1. Our detailed mechanistic studies revealed that the potent quinine derivatives compete with the heme for binding to the apo-IDO1 and perturb its reversible binding propensity to the apo-IDO1 via the formation of the heme-inhibitor complex.

4.2. Results and discussions

4.2.1. Synthesis of quinine derivatives

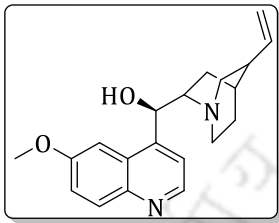
We hypothesize that the replacement of the alcoholic group with a substituted amine group will allow the quinine derivatives to bind strongly with the apo-IDO1. The quinine derivatives can also bind to the heme group through its quinoline moiety, which might affect the rebinding activity of free heme. In this regard, the quinine (**4.1**) was converted to 9(S)-amino-9-deoxyepiquinine (**4.2**) using the Mitsunobu inversion–reduction reaction protocol (Table 4.1).¹¹ Further modification of the amine group of **4.2** with various aryl isocyanate, isothiocyanates, and sulfonyl derivatives provide a series of thiourea (**4.3**), urea (**4.4**), and sulfonamide (**4.5**)-based derivatives of quinine (Table 4.1).

4.2.2. Selective IDO1 inhibitory activities of quinine derivatives

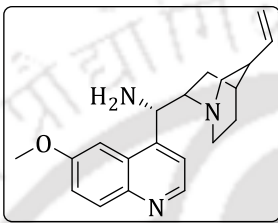
The initial screening (Table 4.1) revealed that the quinine derivatives **4.4g**, **4.5a**, and **4.5b** strongly inhibit the IDO1 mediated catabolism of L-Trp to kynurenine ($IC_{50} = 0.70\text{--}3.51$ nM; Table 4.1 and Table 4.2).³ The reported epacadostat analogue (**5I**) also showed IDO1 inhibitory activity and the calculated IC_{50} value (83 nM) is following the reported ones.¹² However, the potent compounds showed no significant inhibitory activity against the TDO enzyme, a member of the same enzyme family as that of IDO1 (Table 4.3). The TDO preferably binds to L-Trp and indole derivatives, whereas the active site of IDO1 is more receptive and can accommodate various types of small molecules.^{12, 13} Therefore, the potent quinine derivatives selectively inhibit the activity of the IDO1 enzyme. The IDO1 mediated oxidation of L-Trp was also strongly inhibited in the presence of these quinine derivatives under the

cellular environments (EC_{50} = 5.22 – 14.22 nM; Table 4.2 and Fig. 4.2).^{3, 12, 13} The MTT assay of these compounds revealed their negligible toxicity in both HeLa and BHK-21 cells, suggests that these compounds are suitable candidates for the IDO1 inhibitory studies (Fig. 4.1).

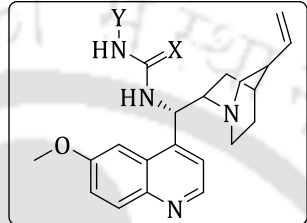
Table 4.1 Inhibitory activity of the synthesized quinine derivatives against purified human IDO1 enzyme.



4.1

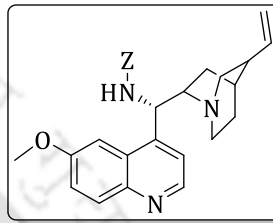


4.2

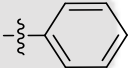
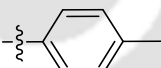
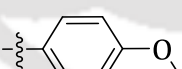
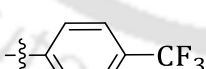
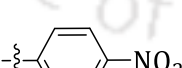
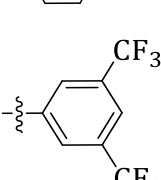
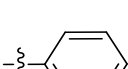


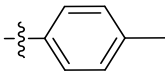
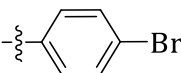
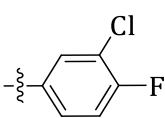
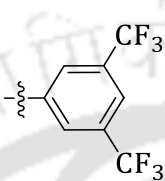
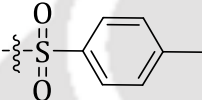
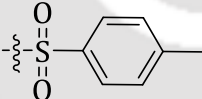
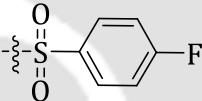
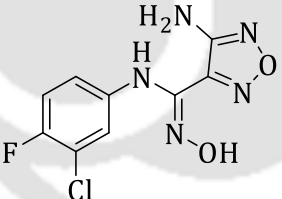
4.3; X = S

4.4: X = O



4.5

Compound			% of IDO1 inhibition ^a		
	X	Y/Z	50 nM	250 nM	1250 nM
4.1	-	-	16	26	42
4.2	-	-	12	16	33
4.3a	S		4	9	18
4.3b	S		14	28	50
4.3c	S		12	23	34
4.3d	S		14	22	33
4.3e	S		10	22	38
4.3f	S		8	19	33
4.4a	O		16	36	48

4.4b	0		9	35	55
4.4c	0		5	20	30
4.4d	0		10	32	43
4.4e	0		6	10	27
4.4f	0		10	22	44
4.4g	0		21	51	91
4.5a	-		16	54	92
4.5b	-		16	58	94
51^b			20	53	89

^a hIDO1 inhibition values are the mean of three independent assays. ^b Compound **51** is epacadostat analogue.

4.2.3. Selective IDO1 inhibition mechanism of quinine derivatives

The robust IDO1 inhibition by the compounds **4.4g**, **4.5a**, and **4.5b**, prompted us to explore the mechanism of IDO1 inhibition. These quinine derivatives are devoid of any significant similarity to the substrate L-Trp or any reported IDO1 inhibitor that can elucidate their mode of action. The IDO1 inhibitory activities of these compounds were first investigated with different pre-incubation times at 37 °C.³ The result showed low IDO1 inhibitory activities of the compounds with 10 or 20

minutes' pre-incubation time with IDO1. However, an increase in inhibitory activities was observed with the rise in pre-incubation time, and maximum inhibitory activities (>90%) were attained with 60 minutes (Fig. 4.3). Meanwhile, the temperature-dependent activity assay showed an increase in IDO1 inhibitory activities from 20 °C to 37 °C, suggesting the likelihood of loss of heme-cofactor in the presence of the compounds (Fig. 4.3B).³ The proximity of the potent quinine derivatives to the solvent-exposed surface area might lead to the partial unfolding of the holo-IDO1, promoting the release of heme-cofactor.¹⁴

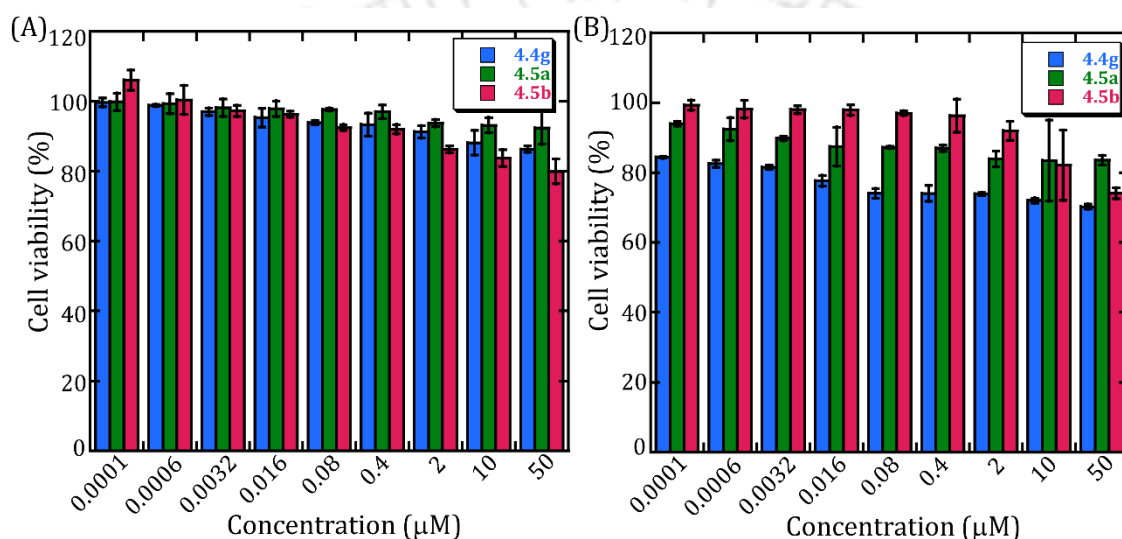
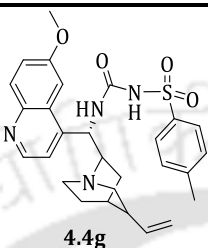
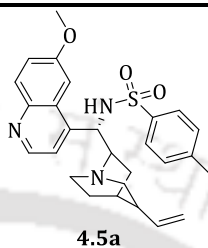
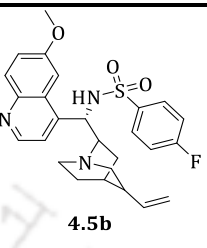


Figure 4.1 Effect of the selected compounds on the viability of BHK-21 (A) and HeLa (B) cells. The cells were treated with the indicated concentrations of the compounds for 48 h. The MTT assay determined cell viability. Cell viability (%) are averaged with standard deviation (error bars) from three independent experiments.

To investigate whether the loss of heme-cofactor from the IDO1 active site as the crucial initiatory step of inhibition, the UV-Vis spectral analysis was performed in the absence and presence of these compounds.³ The depletion of Soret-peak suggested the loss of heme-cofactor from the IDO1 (Fig. 4.4A). The epacadostat analogue (**51**) showed a red-shift of the Soret-peak, indicating the coordination of hydroxyamidine moiety with the heme-cofactor (Fig. 4.4A).¹² However, there was no significant change in the Soret-peak of the TDO enzyme. The UV-Vis spectral analysis of other heme-containing proteins such as hemoglobin and myoglobin revealed no

loss or binding of heme-cofactor, suggesting that the loss of heme could be more selective to IDO1 (Fig. 4.4).

Table 4.2 Inhibitory activity of the potent quinine derivatives against human IDO1 enzyme.

			
	4.4g	4.5a	4.5b
IC ₅₀ (nM) ^a	3.51 ± 0.29	0.70 ± 0.37	1.49 ± 0.36
EC ₅₀ (nM) ^b	21.78 ± 3.89	1.47 ± 0.36	5.38 ± 1.83
EC ₅₀ (nM) ^c	14.22 ± 2.98	5.22 ± 0.33	7.79 ± 1.11

^aInhibitory activities against purified IDO1 enzymes after incubation for 60 minutes.

^b *p*DMAB-based inhibitory activities under the cellular environment ^cHPLC-based inhibitory activities under the cellular environment.

Table 4.3 Inhibitory activity of the quinine derivatives against purified human TDO enzyme using *p*DMAB.

Compound	% of inhibitory activity ^a					
	50 nM		250 nM		1250 nM	
	IDO1	TDO	IDO1	TDO	IDO1	TDO
4.4g	21	5	51	6	91	13
4.5a	16	7	54	10	92	16
4.5b	16	5	58	9	94	12
5l	20	3	53	13	89	14

^a inhibition values are the mean of three independent assays.

To further explore whether these quinine derivatives promote the release of heme-cofactor from IDO1, the PpIX quenching assay was performed.¹⁵ The quenching of the PpIX fluorescence signal in the presence of heme suggested the interaction of PpIX with heme (Fig. 4.5).

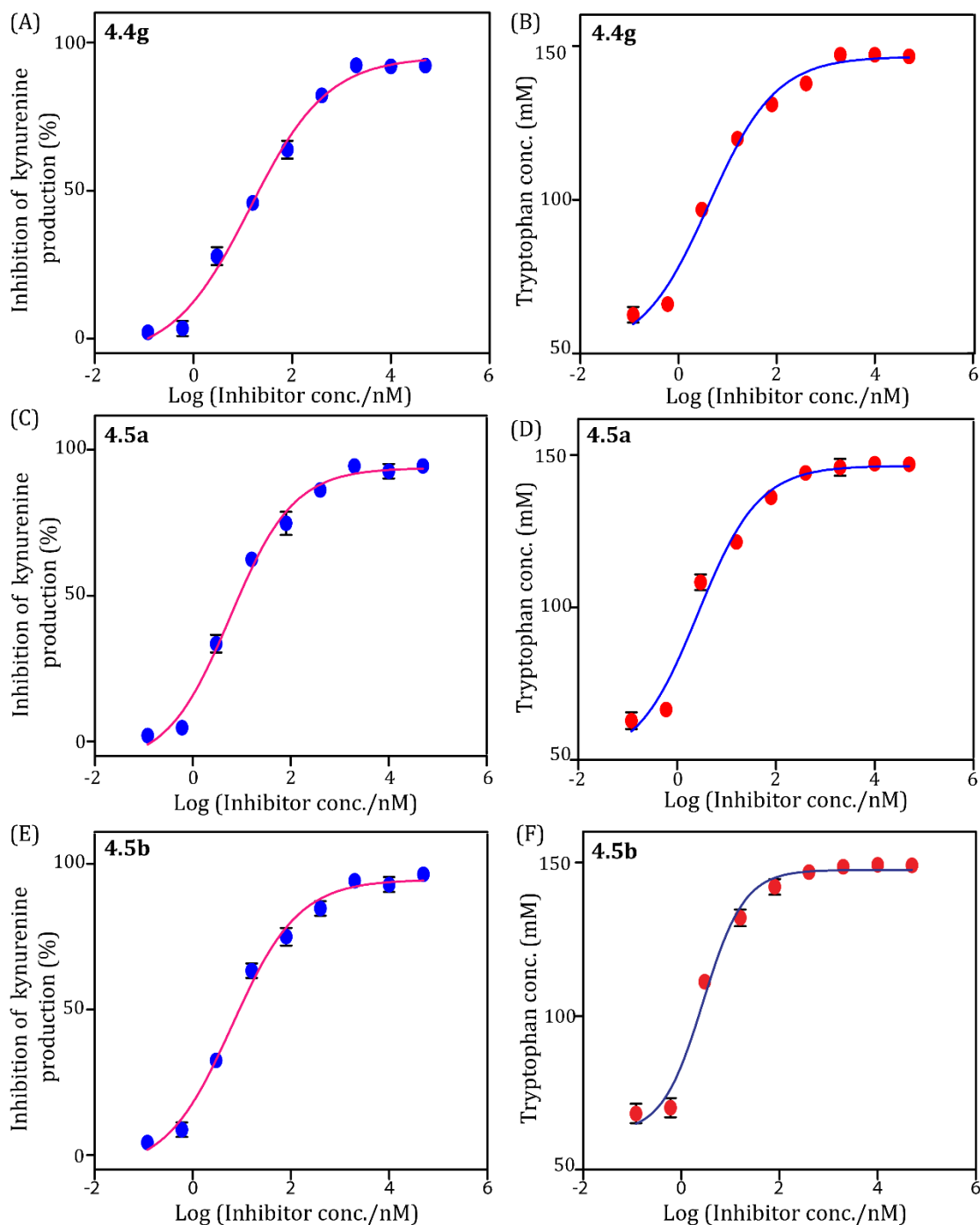


Figure 4.2 Plots of concentration of kynurenine (A, C, E) and tryptophan (B, D, F) in the absence and presence of different concentration of compounds. HPLC-based cellular (HeLa) IDO1 inhibitory assay.

However, there was no change in the PpIX fluorescence signal in the presence of the only compounds. Therefore, the UV-Vis spectroscopic and PpIX fluorescence analyses suggest the possibility of compound-mediated release

of heme-cofactor from IDO1 (Fig. 4.5B).¹² The loss of heme-cofactor and no significant change in CD spectra of IDO1 before and after the incubation period suggest that the compound mediated release heme-cofactor from the IDO1 active site is not due to the alteration of its structural integrity (Fig. 4.6 and Table 4.4).

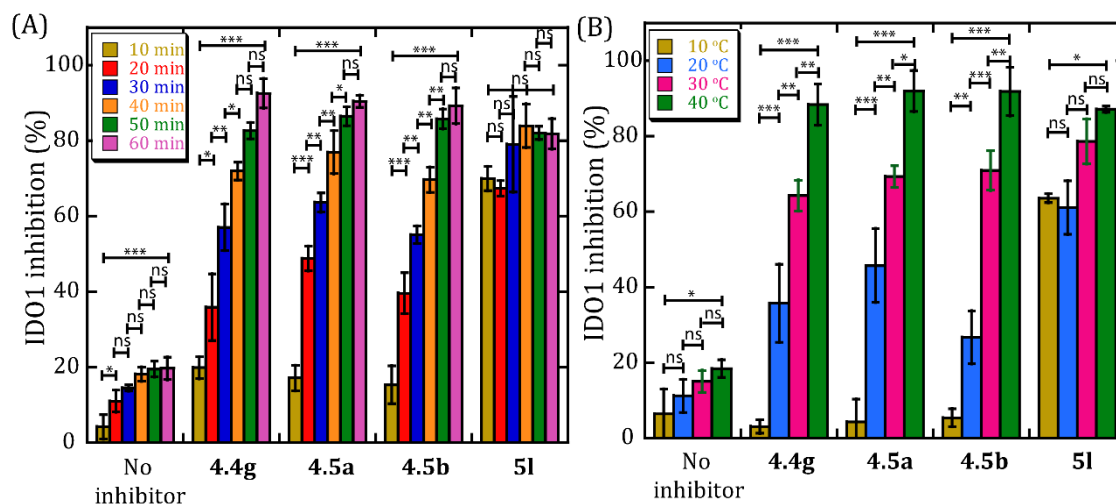


Figure 4.3 Inhibitory activities of IDO1 (200 nM) in the absence and presence of compounds (6.25 μ M) at different times (A) and temperature (B). The inhibition assay was performed at 37 $^{\circ}$ C for 60 minutes.

4.2.4. Rate limiting process of IDO1 inhibition by quinine derivatives

The compound mediated reduction of Soret-peak of IDO1 and reduction of PpIX fluorescence in the presence of IDO1 suggest that the loss of heme-cofactor plays a crucial role in the IDO1 inhibition process. These studies also indicate that compound mediated inhibition is due to the formation of apo-IDO1 from the holo-IDO1, not due to the inhibition of holo-IDO1 activity. Therefore, to understand the rate-limiting process of IDO1 inhibition, we performed the concentration-dependent activity assay.³ The pre-incubation of IDO1 with 0 to 25-folds concentrated compounds for 60 minutes, followed by IDO1 activity assay in the presence of L-Trp, showed a non-linear relationship between the IDO1 activity and concentration of the compounds, suggesting that the detachment of heme-cofactor in the presence of compounds and consequent generation of apo-IDO1 is the slowest process of the IDO1

inhibition in the presence of the compounds (Fig. 4.7A). Similarly, the concentration-dependent IDO1 inhibition assay was performed by pre-incubating IDO1 with various concentrations of inhibitors in the presence of L-Trp and the reducing agents (ascorbate, methylene blue, and catalase). The identical non-linear inhibition profile supported that inhibition was independent of IDO1 turnover (Fig. 4.7B), supporting that the apo-IDO1 formation via heme dissociation as the rate-limiting step of the inhibition mechanism of these potent compounds.

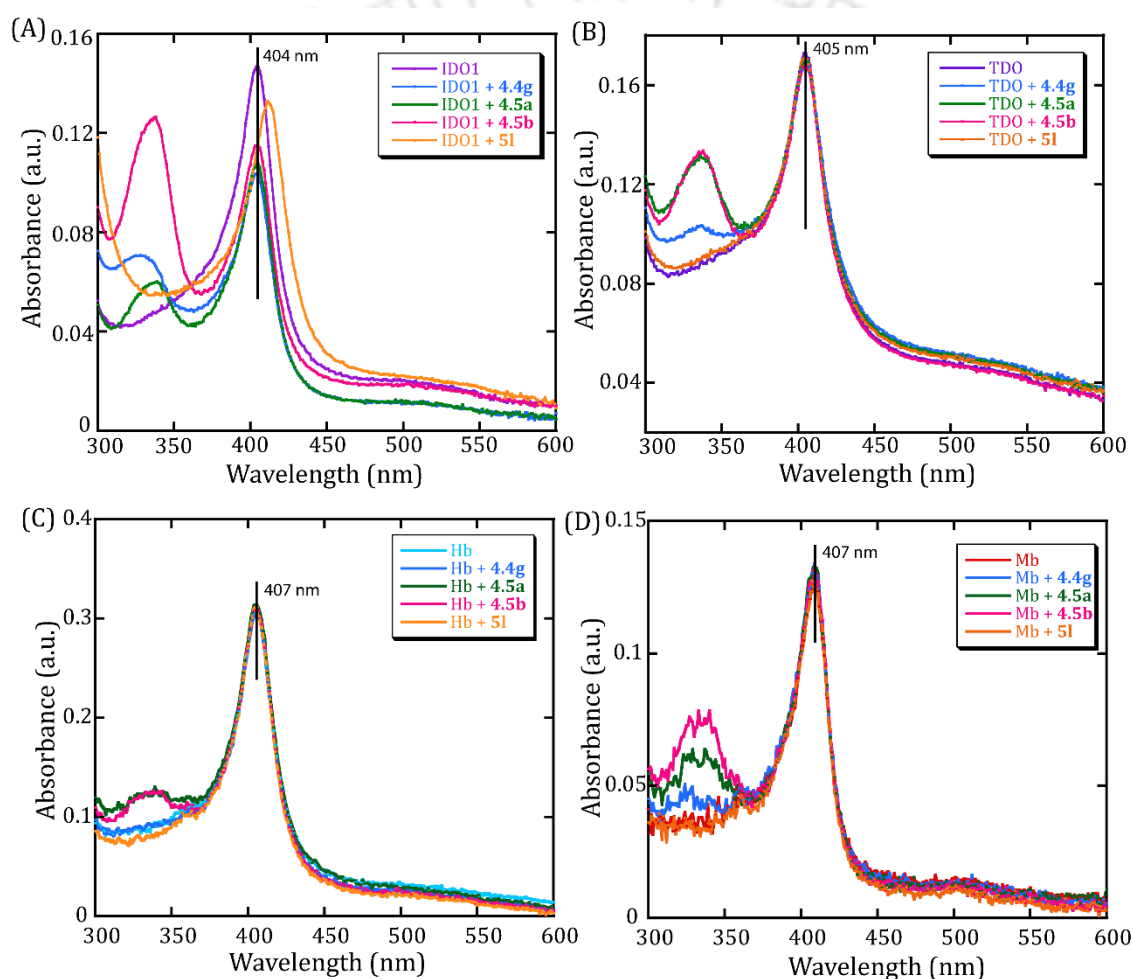


Figure 4.4 Absorption spectra of the ferric-IDO1 enzyme (A), ferric-TDO enzyme (B), hemoglobin (C), and myoglobin (D) (0.9 μM) in the absence and presence of the compounds (10 μM) in 100mM phosphate buffer at pH 6.5 after 15 min incubation at 37 $^{\circ}\text{C}$.

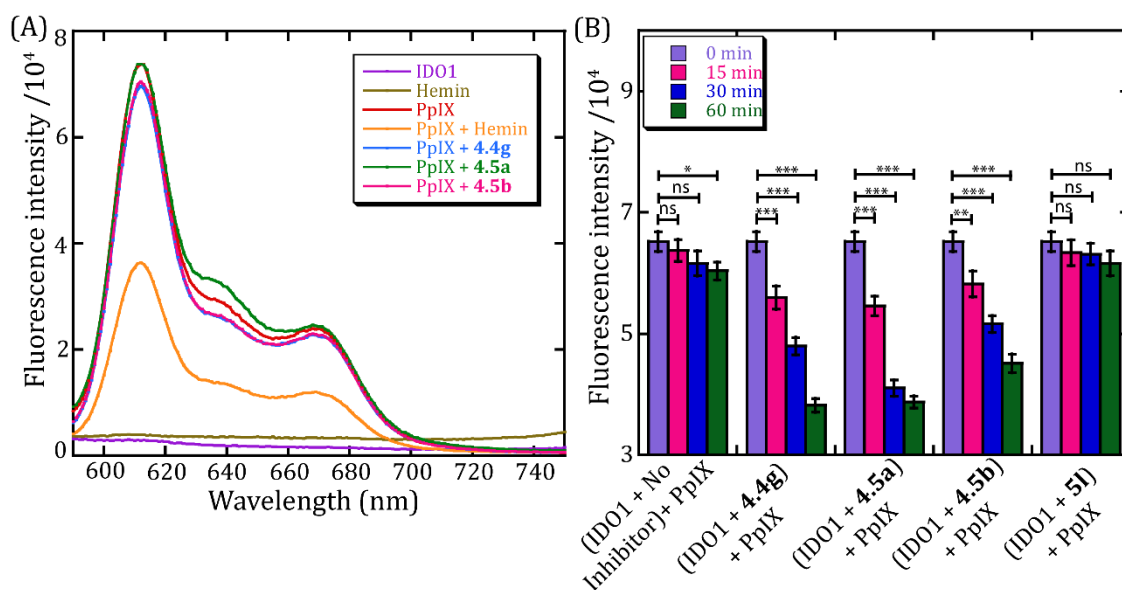


Figure 4.5 Fluorescence spectra of PpIX (5 μ M) in the presence of hemin (0.5 μ M) and other compounds (25 μ M) (A). The change in PpIX fluorescence upon the addition of incubated IDO1 (0.75 μ M) in the presence and absence of compounds (25 μ M) at various times (B).

Table 4.4 Circular dichroism analysis of apo-IDO1 and IDO1 in the absence and presence of compounds.

Compound	% of secondary structural elements			
	Helix	Beta	Turn	Random
IDO1 + No inhibitor	52.4	0	0	47.6
IDO1 + 4.4g	56.8	0	0	43.2
IDO1 + 4.5a	58.9	0	0	41.1
IDO1 + 4.5b	59	0	0	41
IDO1 + 5I	52.3	0	0	47.7
Apo-IDO1	57.2	0	0	42.8

^aSecondary structural elements were analysed using inbuilt software

4.2.5. Redox state dependent heme release from IDO1 active pocket by quinone derivatives

The concentration-dependent activity study showed that the redox state of iron in the heme-cofactor might influence the heme release aptitude of IDO1. Henceforth,

we performed the UV-Vis-based assay, where both inactive and active IDO1 were incubated with potent compounds.³ The depletion of Soret-peak suggested that the release aptitude of heme-cofactor was slower (2.1 to 4.9-folds) for catalytically active IDO1 than the catalytically inactive IDO1 (Table 4.5 and Fig. 4.8).

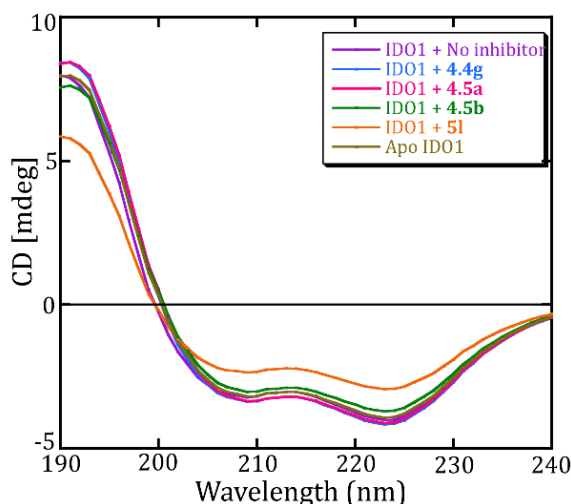


Figure 4.6 CD spectra of apo-IDO1 and IDO1 in the absence and presence of compounds.

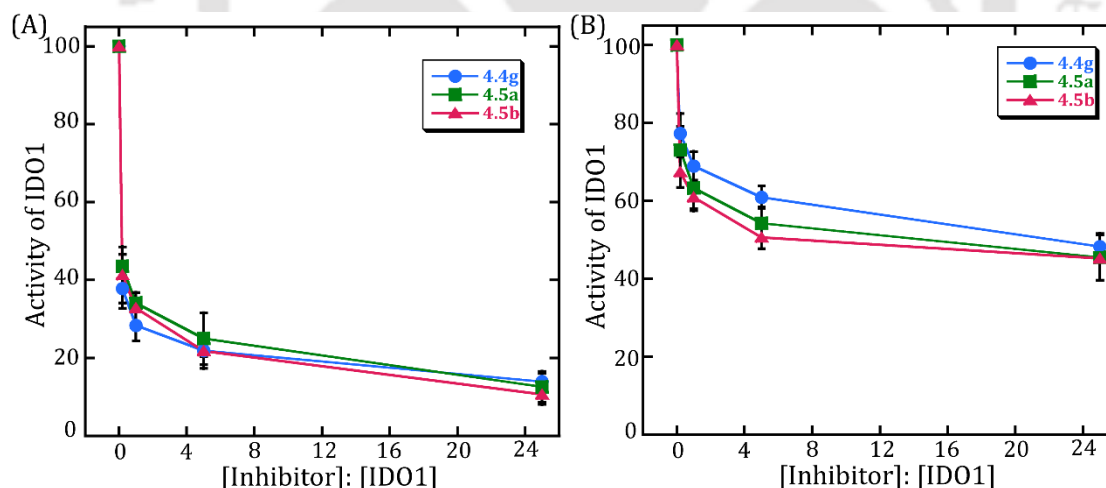


Figure 4.7 Purified IDO1 (200 nM) was incubated with 0 to 25-fold concentrated compounds at 37 °C for 60 minutes in absence (A) /presence (B) of L-Trp, ascorbate, methylene blue, and catalase and inhibition was investigated using the spectrophotometric-based activity assay.

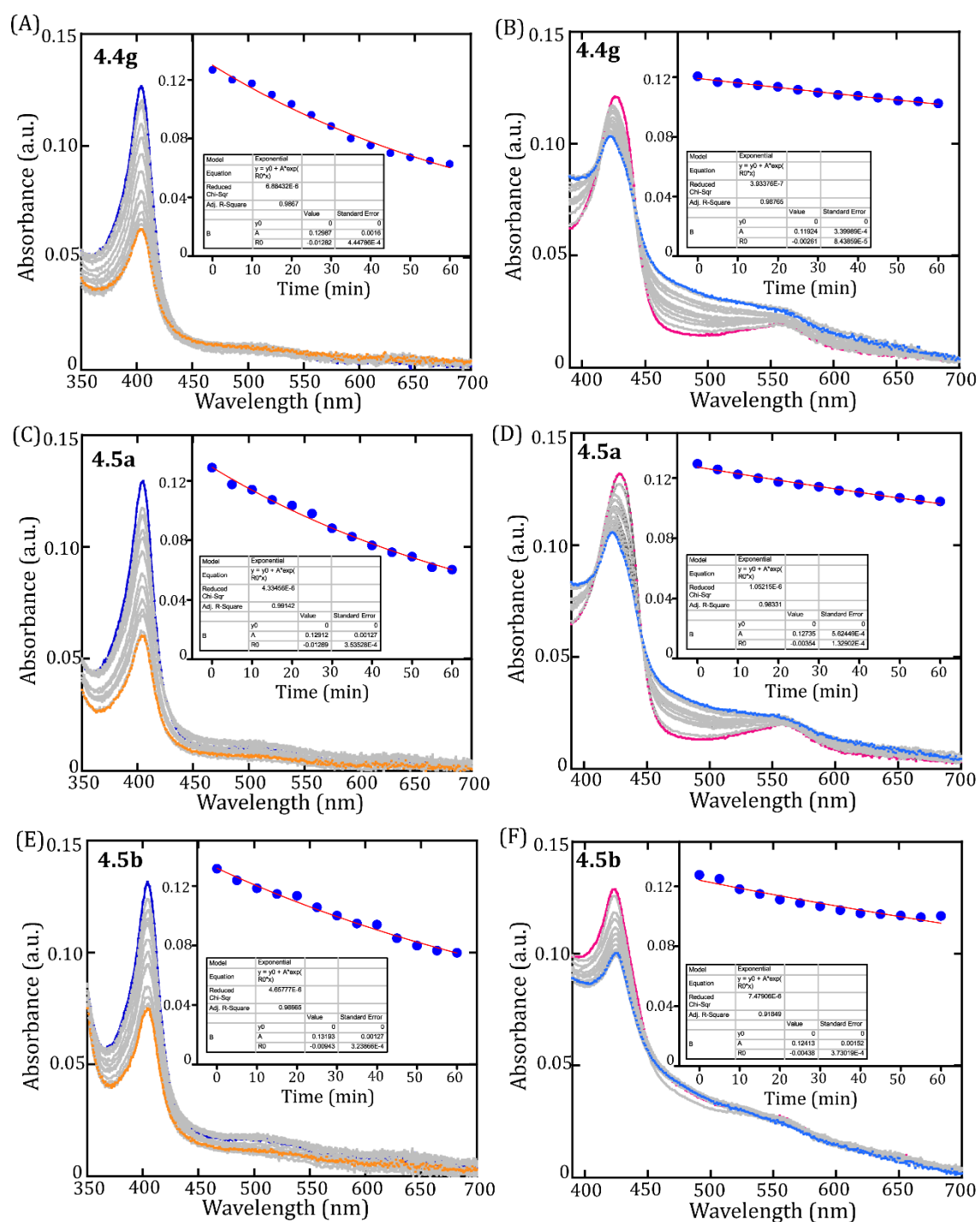


Figure 4.8 Time-dependent heme dissociation from IDO1 (0.75 μM) in the presence of compound (25 μM) at 37 $^{\circ}\text{C}$. Absorbance spectra of ferric-IDO1 (A), (C), (E) and ferrous-IDO1 (B), (D), (F) in presence of **4.4g**, **4.5a**, **4.5b** respectively. Inset shows the loss in absorbance for ferric-IDO1 as a function of time. The ferrous-deoxy reaction environment was generated by adding $\text{Na}_2\text{S}_2\text{O}_4$ to the solution under an N_2 atmosphere. Heme dissociation from IDO1 was monitored by the loss of absorbance of the Soret peak: 404 nm for ferric-IDO1 and 425 nm for ferrous-IDO1.

Table 4.5 The rate of Soret-peak reduction in the presence of inhibitors

Compounds	Heme dissociation rate (min ⁻¹) ^a		Heme dissociation rate (ferric / ferrous IDO1)
	Ferric-IDO1	Ferrous-IDO1	
4.4g	0.0128 ± 0.0004	0.0026 ± 0.0001	4.9
4.5a	0.0127 ± 0.0004	0.0035 ± 0.0001	3.6
4.5b	0.0094 ± 0.0003	0.0044 ± 0.0004	2.1

^aHeme dissociation rates were calculated using Origin software.

4.2.6. Binding studies of apo-IDO1 with quinine derivatives

The activity studies of the apo-IDO1 in the presence of external hemin revealed the recurrence of catalytic activity (Fig. 4.9A), and the CD analysis revealed that the structural integrity of the IDO1 protein was retained (Fig. 4.6). As expected, the apo-IDO1 equilibrated with the potent compounds failed to show any significant activity on further addition of external hemin.

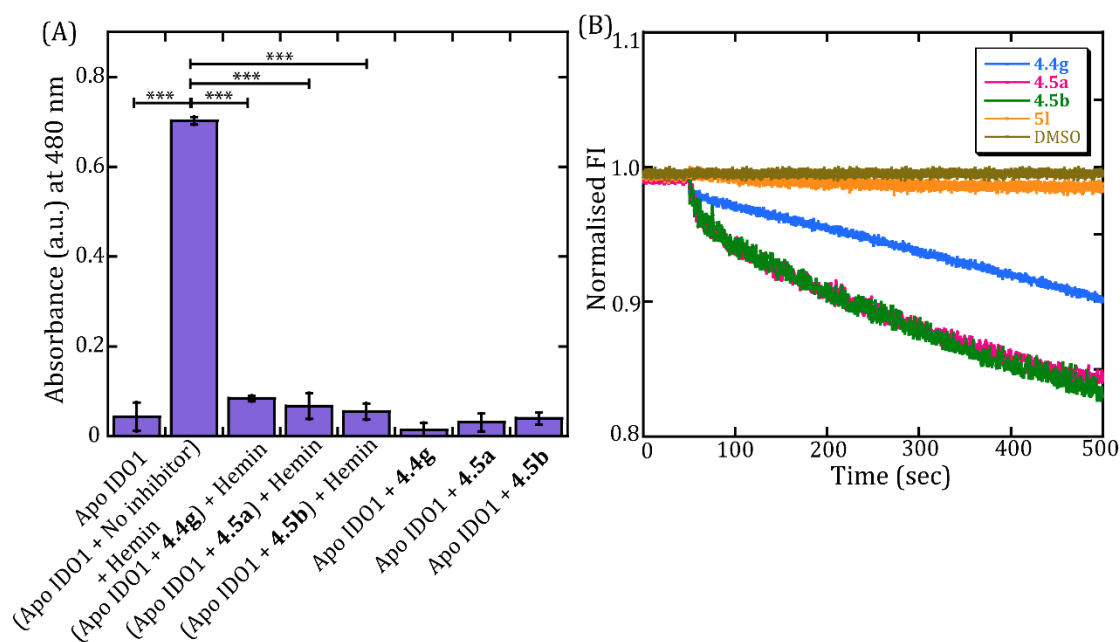


Figure 4.9 The activity of apo-IDO1 in the absence and presence of the compound and/or hemin (A). The binding of quinine derivatives (10 μ M) to the apo-IDO1 was investigated by time-dependent quenching of intrinsic Trp fluorescence assay (B).

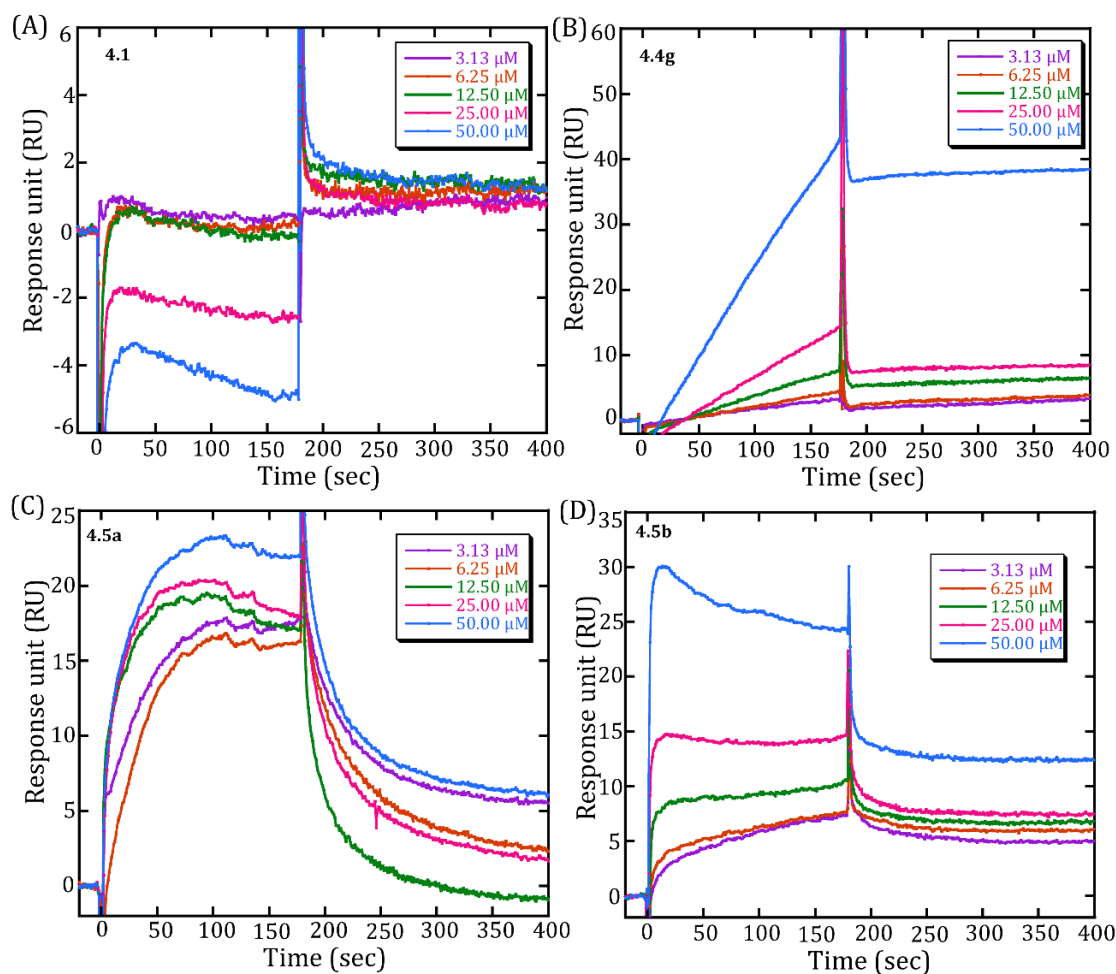


Figure 4.10 Measurements of the compound binding affinity of the human apo-IDO1 enzyme by equilibrium SPR analysis. (A) for **4.1**, (B) for **4.4g**, (C) for **4.5a** and (D) for **4.5b**.

Table 4.6 The binding affinity of the compounds to apo-IDO1.

Analyte	K_a (1/Ms) ^a	K_d (1/s) ^a	K_D (μM) ^a
4.4g	9.017	3.196×10^{-6}	0.354
4.5a	2.661×10^4	8.104×10^{-3}	0.305
4.5b	4.334×10^4	2.962×10^{-3}	0.068

^aRate was calculated using the inbuilt Biacore Insight Evaluation software.

The SPR-based real-time binding analysis (Fig. 4.10) revealed that these selected quinine derivatives strongly bind (K_D = 68-354 nM) to the apo-IDO1 (Table 4.6).¹⁶ The time-dependent Trp-quenching assay also showed that the quinine

derivatives bind to the apo-IDO1 (Fig. 4.9B). The competition of quinine derivatives and heme-cofactor for the same binding site also explains that the dissociation of heme from the IDO1 is the rate-limiting step of their inhibitory mechanism. The molecular docking analysis with the apo-IDO1 protein (PDB ID: 6AZW) revealed that the resulting top poses of the potent compounds are located within the binding site of the apo-IDO1 (Fig. 4.11). The MD simulations studies revealed that the compounds remain strongly bounded within the binding site of the apo-IDO1 (Fig. 4.12).^{17, 18} The hydrophobic and hydrogen bond interactions could be the driving force for the binding of the compounds with the apo-IDO1 protein (Fig. 4.13 and 4.14).

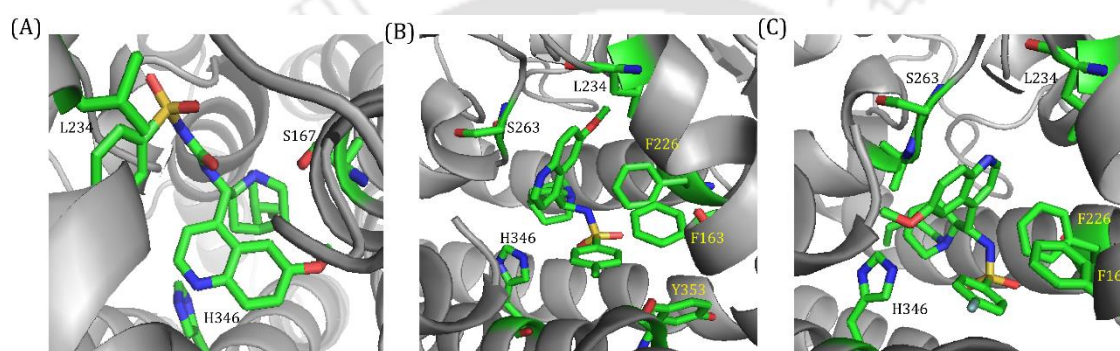


Figure 4.11 Probable mode of interactions of **4.4g** (A), **4.5a** (B) and **4.5b** (C) with the apo-IDO1 (6AZW).

4.2.7. Binding studies of aqueous heme and quinine derivatives

The previous experiments revealed that the selected quinine derivatives compete with heme to bind apo-IDO1 (Fig. 3A).³ Recent studies also suggested that free heme is essential for maintaining homeostasis and various cellular functions. However, excess accumulation of free heme in the cytoplasm and dysregulation of heme metabolism promotes tumorigenesis.^{8, 9} The DFT calculations demonstrate that the potent compounds might interact with the heme-iron through quinoline moiety (Fig. 4.15B, 4.19, and Table 4.9). The concentration-dependent diminution of the Soret-peak revealed that quinine derivatives interact with the free heme-cofactor. The binding affinities of these quinine derivatives are 1.7-7.7-fold lower than the quinine (Fig. 4.15C, and Table 4.7). The time-dependent quenching of PpIX fluorescence in the presence of only heme confirm the interaction of PpIX with heme.¹⁵ However,

the presence of quinine derivatives in the solution of heme significantly affects the extent of PpIX fluorescence quenching, suggesting their possible interactions (Fig. 4.15D, and Table 4.8). The quinoline-based drug molecules are known to bind heme.⁹ Hence, the binding studies suggest that the quinine derivatives might partially suppress the activity of overproduced labile heme.

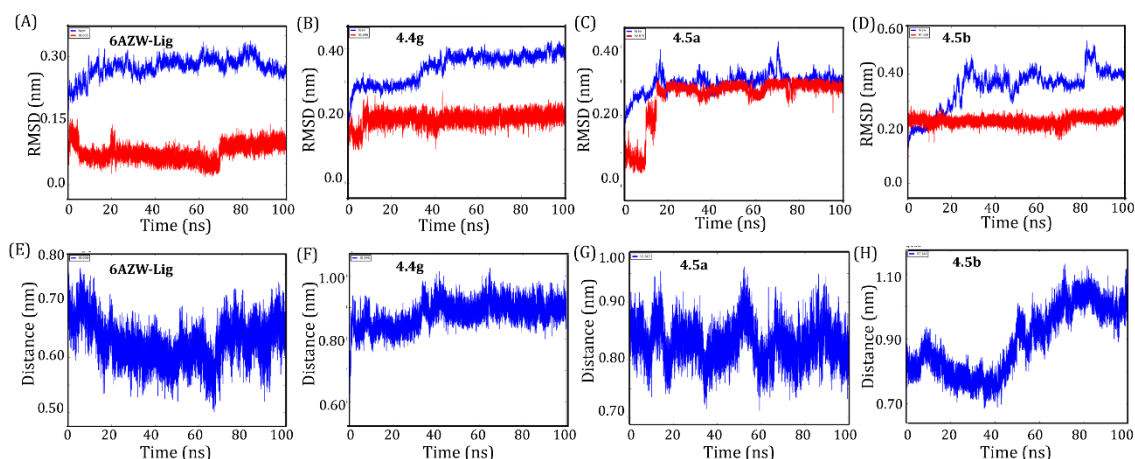


Figure 4.12 Evolution of the RMSD against initial MD step versus time for the ligand (blue plot) and the protein (red plot) (A to D). **6AZW-Lig** (A, crystal ligand of 6azw protein model), **4.4g** (B), **4.5b** (C) and **4.5a** (D). Evolution of distances versus time from each ligand to the centre of the protein's mass (E to H). **6AZW-Lig** (E, crystal ligand of 6azw protein model), **4.4g** (F), **4.5b** (G) and **4.5a** (H).

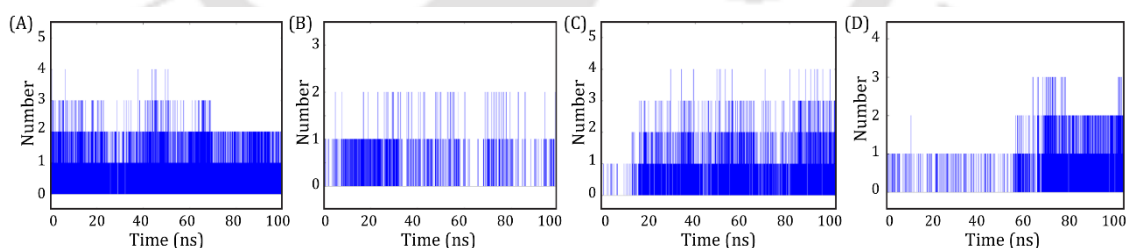


Figure 4.13 Evolution of the number of established hydrogen bonds versus time between each ligand and the protein. **6AZW-Lig** (A, crystal ligand of 6azw protein model), **4.4g** (B), **4.5b** (C) and **4.5a** (D).

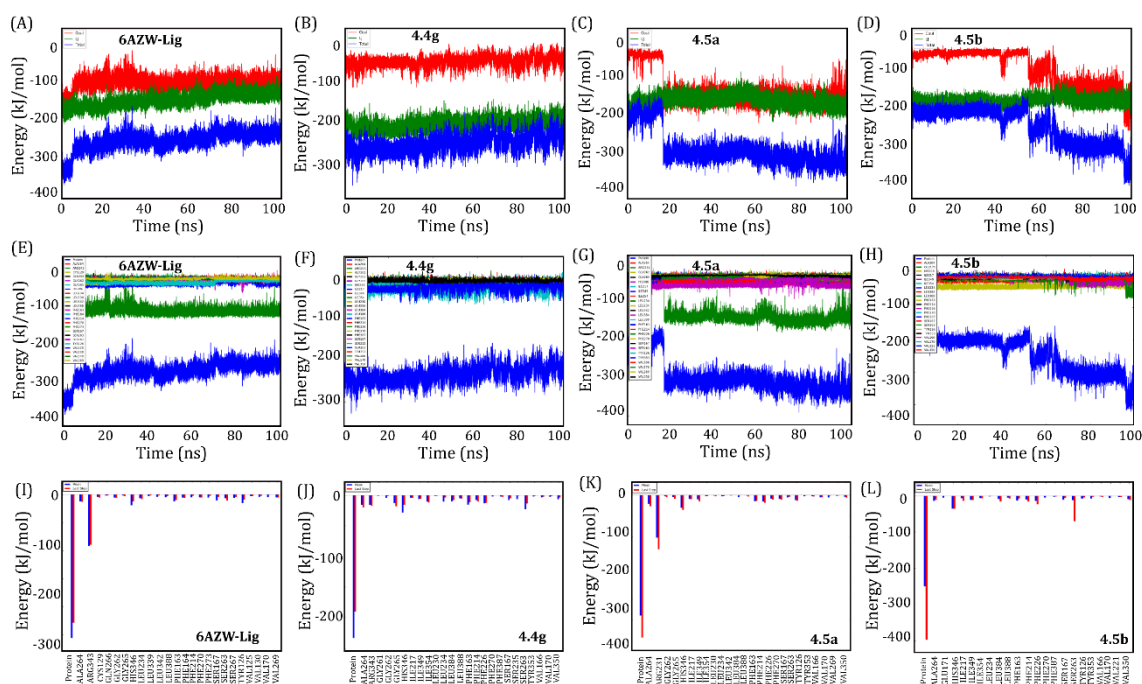


Figure 4.14 Evolution of interaction energies versus time between each ligand and the protein and the blue plot represents total interaction energy while red, and green plots represent Coulomb and Lennard-Jones interactions (A to D). **6AZW-Lig** (A, crystal ligand of 6azw protein model), **4.4g** (B), **4.5b** (C) and **4.5a** (D). Evolution of interaction energies (in KJ/mol) versus time (picoseconds) between each ligand and the protein decomposed in a per residue scheme (E to H) and the blue plot represents total interaction energy with the protein, while the rest of the colours are referred to as specific residues in the sub-box. **6AZW-Lig** (E, crystal ligand of 6azw protein model), **4.4g** (F), **4.5b** (G) and **4.5a** (H). Decomposition of interaction energies between each ligand and top interacting protein residues (I to L) and blue bars represent mean energy value during the MD trajectory, while red bars represent interaction energy values at the MD trajectory's last step. **6AZW-Lig** (I, crystal ligand of 6azw protein model), **4.4g** (J), **4.5b** (K) and **4.5a** (L).

4.2.8. Inhibition studies of IDO1 by heme-inhibitor complex

The cellular IDO1 activity studies in the presence of IFN- γ and external heme showed a marked increase in IDO1 activity, suggesting the presence of a significant amount of apo-IDO1 under the cellular environment. However, heme equilibrated with the potent compounds (in the molar ratio of 1:5) showed a substantial reduction in IDO1 activity under similar experimental

conditions suggesting that the complexation of heme with the potent compounds could disrupt the rebinding of heme to the apo-IDO1 (Fig. 4.16).

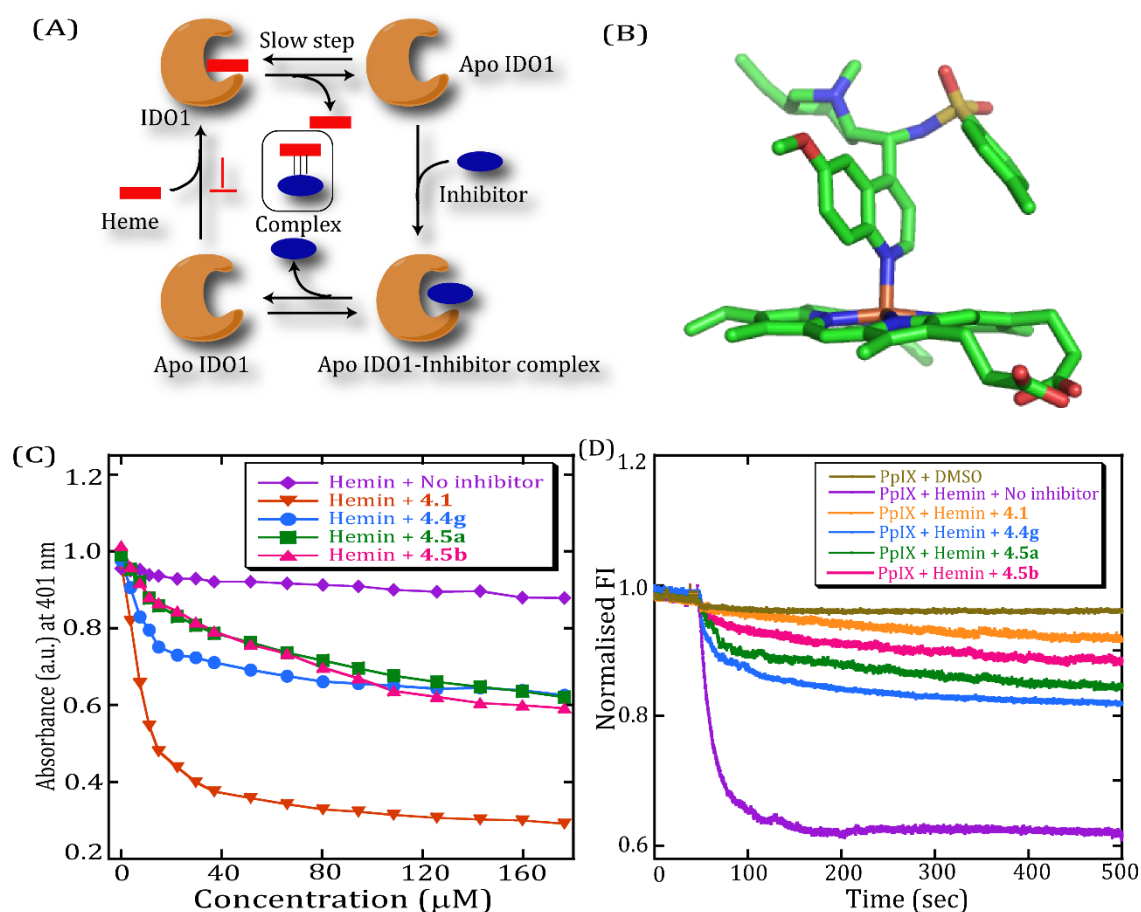


Figure 4.15 schematic diagram depicts the mode of IDO1 inhibition in the presence of quinone derivatives (A). DFT-optimized structures of the compound 4.5a in complexation with heme (B). The plot of absorbance (at 401 nm) of heme (5 μM) in the presence of different concentrations (0-175 μM) of compounds (C). The plot of PpIX fluorescence (5 μM) in the absence or presence of only heme or solution of heme (1.5 μM) equilibrated with compound (30 μM) (D).

Meanwhile, the western blot analysis confirms similar expressions of the IDO1 protein across the different treatments, suggesting that the activity difference is not due to the protein expression level (Fig. 4.17). The direct measurements of heme content in IDO1 purified from the IFN- γ -activated cells by the MALDI-TOF and HPLC analysis of the immune-precipitated IDO1

protein confirmed that the potent quinine derivatives inhibit the intracellular IDO1 activity by increasing the levels of IDO1 apo-protein (Fig. 4.16, 4.17B and 4.18).¹⁹ Therefore, the cellular IDO1 activity is being hampered by both compound bindings to the apo-IDO1 and the formation of the heme-compound complex.

Table 4.7 The binding constant of hemin and selected quinine derivatives

Compound	Heme binding constant (M^{-1})
4.1	1.400×10^5
4.4g	8.176×10^4
4.5a	2.013×10^4
4.5b	1.828×10^4

Binding constant was quantified using Bindfit v 0.5 software

Table 4.8 The inhibitor-hemin affinity constant in the aqueous medium

Compound	PpIX fluorescence quenching rate (sec^{-1}) ^a
Hemin	0.0550 ± 0.00018
4.1	0.0043 ± 0.00005
4.4g	0.0147 ± 0.00012
4.5a	0.0069 ± 0.00006
4.5b	0.0087 ± 0.00011

^a Rate of PpIX fluorescence quenched are calculated using Origin software

4.3. Summary

In summary, we synthesized a series of quinine derivatives that manifest selective and robust IDO1 enzyme inhibitory activity both under *in vitro* and cellular conditions. The biophysical and cellular experiments demonstrate that the potent quinine derivatives compete with the heme-cofactor for the same site and perturbs the rebinding of heme to the active site of IDO1, confirming the inhibition of its catalytic activity. Overall, this new mechanism of IDO1 inhibition due to the binding of the compound to both apo-IDO1 protein and

heme-cofactor could help in designing superior inhibitor or provide an inimitable advantage over other reported IDO1 inhibitors.

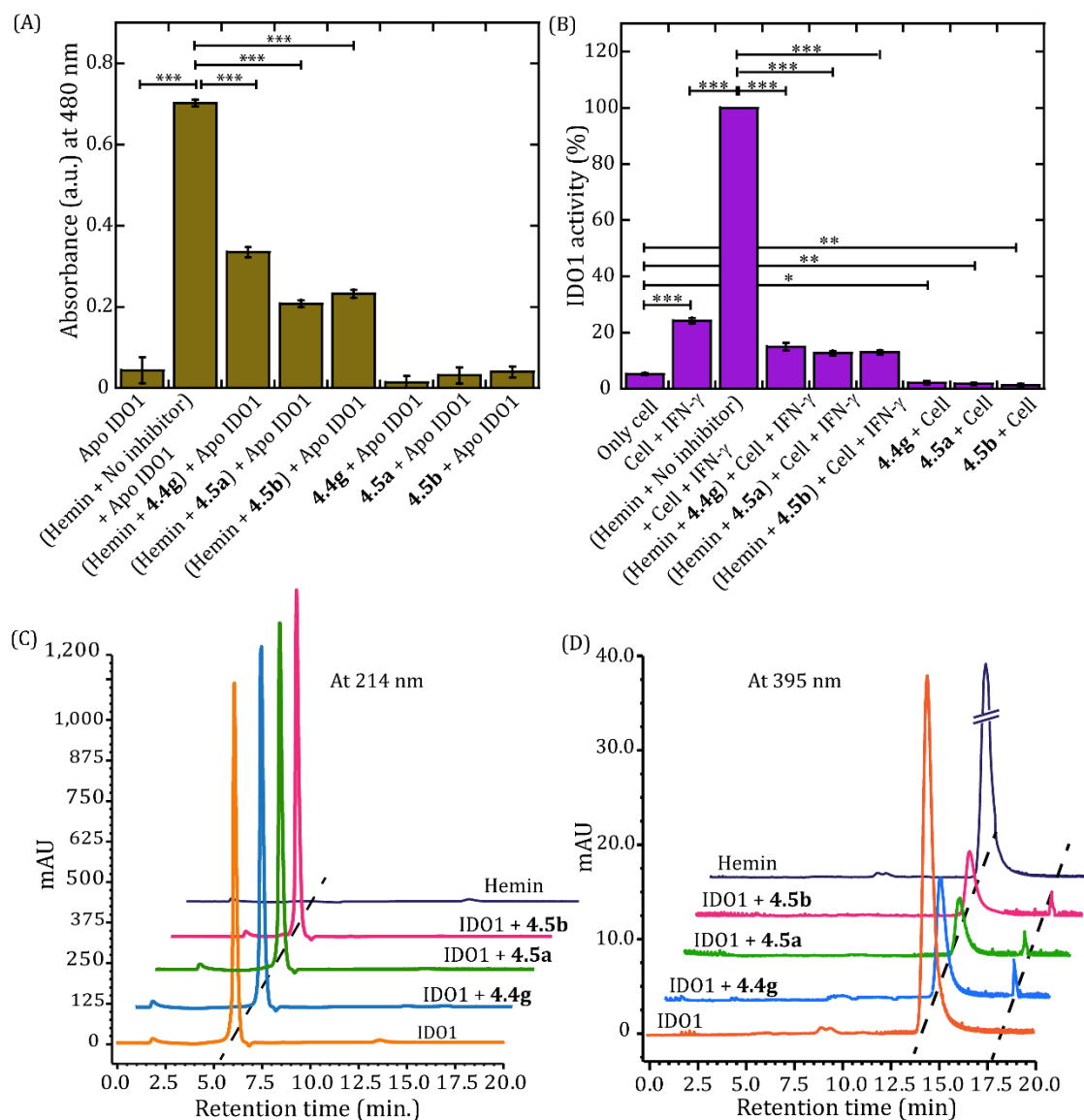


Figure 4.16 The activity of hemin incubated apo-IDO1(A). HPLC-based IDO1 activity assay under the cellular environment (HeLa) (B). The HPLC assay for the direct measurement of heme content in IDO1 purified from the IFN- γ activated cells via immunoprecipitation of IDO1 protein.

4.4. Experimental section

4.4.1. Synthesis of the compounds

4.4.1.1. General information

Described at section 2.4.1.1.

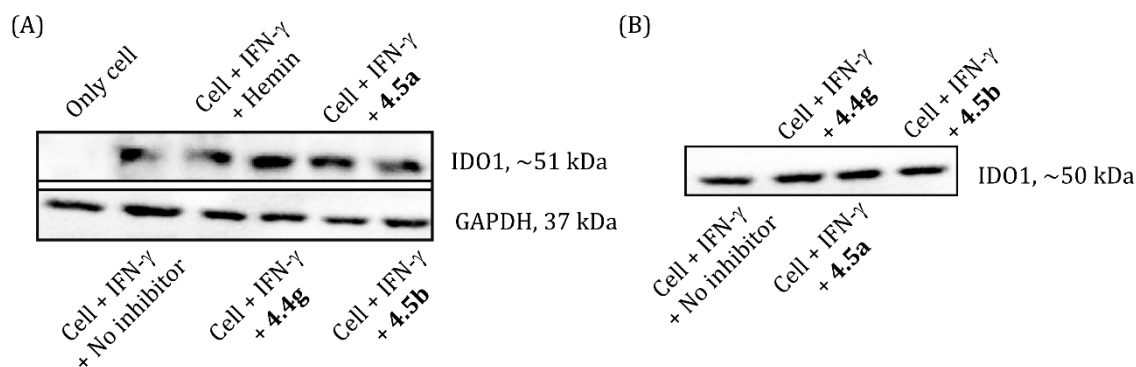


Figure 4.17 The expression levels of IDO1 in HeLa cells across different treatments (A). The western blots to confirm that a similar amount of IDO1 protein was immune-precipitated from each sample prior to mass spectrometry or HPLC analysis (B). Antibodies against the IDO1 enzyme was used to develop the blot. The concentration of IFN- γ was 50 ng/ mL; concentrations of hemin and compounds were 5 μ M.

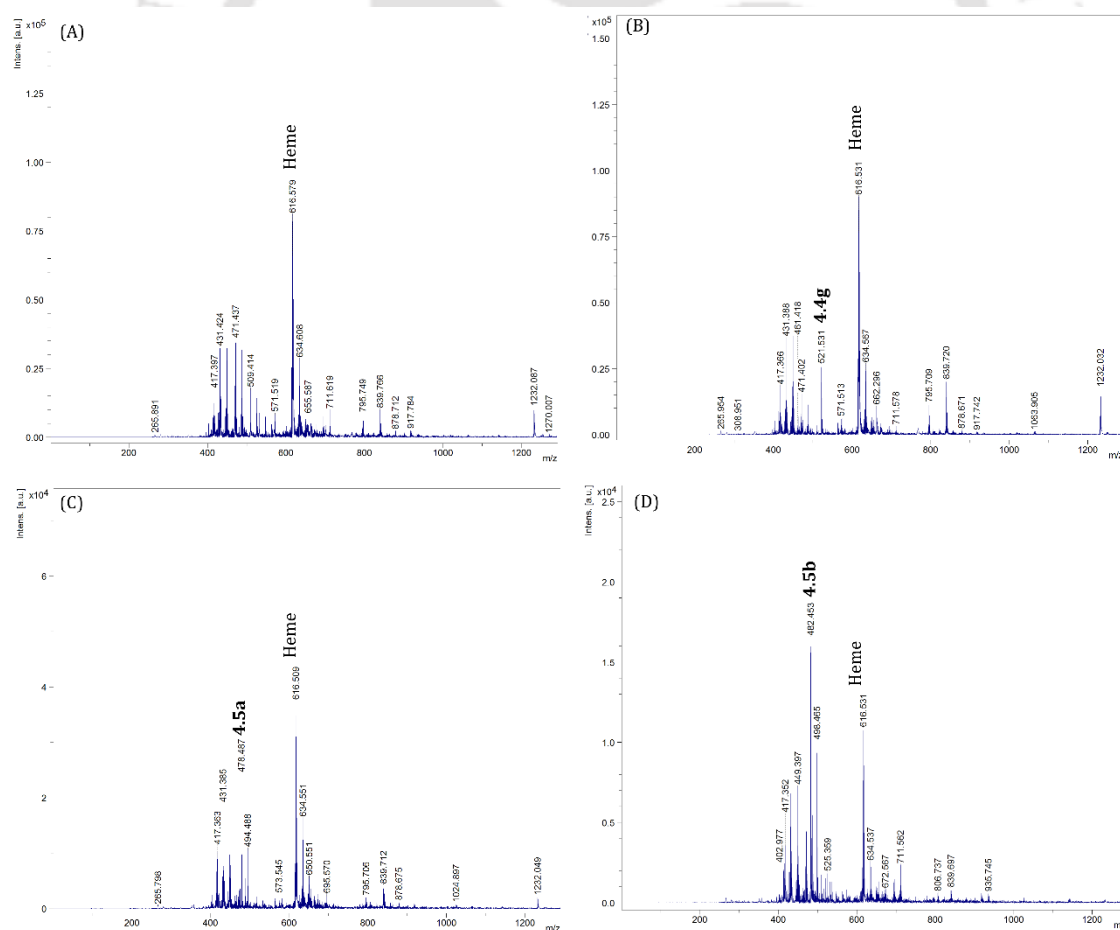


Figure 4.18 The MALDI-TOF analysis for the direct measurement of heme content in IDO1 purified from the IFN- γ activated cells via

immunoprecipitation of IDO1 protein. The molecular weight of heme, **4.4g**, **4.5a**, and **4.5b** is 616.5, 520, 477, and 481.

4.4.1.2. Synthesis of quinine derivatives

4.4.1.2.1. Synthesis of (1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-vinylquinuclidin-2-yl)methanamine ²⁰

To a stirring solution of quinine (**4.1**, 6.2 mmol, 1.0 equiv.) in dry THF (10 mL) at 0 °C was added triphenylphosphine (7.4 mmol, 1.2 equiv.) solution in dry THF under N₂ atmosphere and stirring was continued for 10 minutes at room temperature. After that, the solution was cooled down to 0 °C, and DIAD (7.4 mmol, 1.2 equiv.), followed by diphenyl phosphoryl azide (PhO)₂P(O)N₃ (7.4 mmol, 1.2 equiv.) in dry THF was added gently (dropwise). Then, the reaction mixture was stirred at room temperature for 24 hours, and additional stirring for 3 hours was also continued at 60 °C. Next, the solution of triphenylphosphine (7.4 mmol, 1.2 equiv.) in dry THF was then added slowly to the reaction mixture, and stirring was continued at 50 °C for 3 hours and then the reaction mixture was cooled down to room temperature. Then distilled H₂O (700 µL) was added to quench the reaction, and again, the reaction mixture was stirred for another 18 hours. After completion of the reaction, the solvent was removed under reduced pressure. Then, DCM (30 mL) and HCl (10%, 30 mL) were added to the reaction mixture. After the DCM addition, the organic layer was separated, and the aqueous layer was extracted with DCM (3 × 30 mL). The aqueous layer was basified with NH₄OH (30-33%) until the solution's pH was adjusted to 12 and subsequently extracted with DCM (3 × 30 mL). The combined organic solution was then dried over anhydrous Na₂SO₄, filtered, and the solvent was removed under reduced pressure. The crude reaction mixture was purified through the column chromatography using a solvent gradient system of MeOH/ EtOAc (0-10%) to give a light yellow liquid compound **4.2** with a 70% yield.

4.4.1.2.2. Synthesis of the thiourea-based quinine derivatives

To a stirring solution of (1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-vinylquinuclidin-2-yl)methanamine (**4.2**, 1 equiv.) and triethylamine (2 equiv.) in DCM was added to a solution containing the substituted aryl isothiocyanates (1.1 equiv.) in DCM. The whole reaction mixture was stirred for 10-12 hours at room

temperature. After the 10-12 h, the progress of the reaction was monitored by TLC. After completing the reaction, the mixture was diluted with water and extracted with EtOAc (3 × 30 mL). The separated organic layer was then washed with brine solution and dried over anhydrous Na₂SO₄. The organic solvent was removed under reduced pressure. The purification was done through the column chromatography using MeOH/DCM's solvent system to get the pure product as a crystalline solid.

4.4.1.2.3. Synthesis of the urea-based quinine derivatives

To a stirring solution of (1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-vinylquinuclidin-2-yl)methanamine (1 equiv.) and triethylamine (2 equiv.) in DCM was added to a solution containing the substituted aryl isocyanates (1.1 equiv.) in DCM. The whole reaction mixture was stirred for 10-12 hours at room temperature. After the 10-12 h, the progress of the reaction was monitored by TLC. After completing the reaction, the mixture was diluted with water and extracted with EtOAc (3 × 30 mL). The separated organic layer was then washed with brine solution and dried over anhydrous Na₂SO₄. The organic solvent was removed under reduced pressure. The purification was done through the column chromatography using a solvent system of MeOH/DCM to get the pure product as a crystalline solid.

4.4.1.2.4. Synthesis of the 4-methylbenzenesulfonyl isocyanate-based derivative

To a stirring solution of (1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-vinylquinuclidin-2-yl)methanamine (1 equiv.) and triethylamine (2 equiv.) in DCM was added to a solution containing the substituted 4-methylbenzenesulfonyl isocyanate (1.1 equiv.) in DCM, and the whole reaction mixture was stirred for 10-12 hours at room temperature. After the 10-12 h, the progress of the reaction was monitored by TLC. After completing the reaction, the mixture was diluted with water and extracted with EtOAc (3 × 30 mL). The separated organic layer was then washed with brine solution and dried over anhydrous Na₂SO₄.

4.4.1.2.5. Synthesis of the benzenesulfonyl-based derivatives

To a stirring solution of (1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-vinylquinuclidin-2-yl)methanamine (1 equiv.) and triethylamine (2 equiv.) in DCM

was added to a solution containing the substituted benzenesulfonyl Chloride derivatives (1.1 equiv.) in DCM. The whole reaction mixture was stirred for 10-12 hours at room temperature. After the 10-12 h, the progress of the reaction was monitored by TLC. After completing the reaction, the mixture was diluted with water and extracted with EtOAc (3 × 30 mL). The separated organic layer was then washed with brine solution and dried over anhydrous Na₂SO₄. The organic solvent was removed under reduced pressure. The purification was done through the column chromatography using MeOH/DCM's solvent system to get the pure product as a crystalline solid.

4.4.1.3. Characterization of the synthesized quinine derivatives

1-((1S)-(6-methoxyquinolin-4-yl))((1S,2S,4S)-5-vinylquinuclidin-2-yl)methyl)-3-phenylthiourea(4.3a) — Following the general procedure as mentioned in section 4.4.1.2.2., the reaction between (9 S)-9-amino(9-deoxy)epiquinine (100 mg, 0.3091) and isothiocyanatobenzene (41 µL, 0.340 mmol) provided the colorless solid compound **4.3a** as 80% yield (113.34 mg). The product was scrutinized by ¹H NMR, ¹³C NMR and HRMS analyses which matched with the reported literatures.²¹ ¹H NMR (600 MHz, DMSO-*d*₆) δ_{ppm} 9.71 (s, 1H), 8.71 (d, *J* = 6.0 Hz, 1H), 8.34 (brs, 1H), 7.93-7.91 (m, 2H), 7.56-7.55 (m, 1H), 7.42 – 7.40 (m, 3H), 7.25 (t, *J* = 7.7 Hz, 2H), 7.03 (t, *J* = 7.3 Hz, 1H), 5.99 (s, 1H), 5.83-5.74 (m, 1H), 4.99-4.89 (m, 2H), 3.92 (s, 3H), 3.22-3.16 (m, 2H) 2.69-2.66 (m, 2H), 2.27 (s, 1H), 1.57 (s, 3H), 1.20 (s, 2H), 0.82 (s, 1H). ¹³C NMR (150 MHz, DMSO-*d*₆) δ_{ppm} 180.2, 170.8, 157.5, 148.3, 144.6, 142.3, 139.9, 131.7, 129.0, 128.5, 124.3, 122.8, 121.7, 114.7, 103.6, 66.8, 60.2, 59.8, 56.1, 55.5, 41.4, 27.8, 27.5, 25.9, 21.2, 14.5. HRMS (ESI) calcd. for C₂₇H₃₀N₄OS (M+ H)⁺: 459.2213, found: 459.2217.

1-((1S)-(6-methoxyquinolin-4-yl))((1S,2S,4S)-5-vinylquinuclidin-2-yl)methyl)-3-(p-tolyl)thiourea(4.3b) — Following the general procedure as mentioned in section 4.4.1.2.2., the reaction between (9 S)-9-amino(9-deoxy)epiquinine (100 mg, 0.3091) and 1-isothiocyanato-4-methylbenzene (0.147 mL, 0.340 mmol) provided the colorless solid compound **4.3b** as 80% yield (113.34 mg). The product was scrutinized by ¹H NMR, ¹³C NMR and HRMS. ¹H NMR (600 MHz, DMSO-*d*₆) δ_{ppm} 11.24 (brs, 1H), 11.06 (brs, 1H), 8.71 (d, *J* = 4.6 Hz, 1H), 7.93 (d, *J* = 9.1 Hz, 1H), 7.69-7.65

(m, 1H), 7.42-7.40 (m, 3H), 7.01 (d, $J = 8.1$ Hz, 2H), 6.22 (s, 1H), 5.84-5.78 (m, 1H), 5.00 (d, $J = 17.2$ Hz, 1H), 4.92 (d, $J = 10.3$ Hz, 1H), 3.95 (s, 3H), 3.40 – 3.30 (m, 3H), 3.13-3.09 (m, 1H), 2.68-2.62 (m, 2H), 2.22 (s, 3H), 1.56-1.48 (m, 3H), 1.26 – 1.22 (m, 2H), 1.18-1.13 (m, 2H), 0.70-0.64 (m, 1H). ^{13}C NMR (150 MHz, DMSO- d_6) δ_{ppm} 180.1, 175.5, 157.4, 148.2, 144.4, 138.4, 132.4, 131.5, 128.9, 122.8, 121.7, 114.6, 103.9, 56.1, 55.5, 41.6, 40.4, 28.0, 27.6, 25.9, 24.7, 20.8. HRMS (ESI) calcd. for $\text{C}_{28}\text{H}_{32}\text{N}_4\text{OS}$ ($\text{M} + \text{H}$) $^+$: 473.2330, found: 473.2340.

1-(4-methoxyphenyl)-3-((1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5

vinylquinuclidin-2-yl)methyl)thiourea (4.3c)^{22, 23} — Following the general procedure as mentioned in section 4.4.1.2.2., the reaction between (9S)-9-amino(9-deoxy)epiquinine (100 mg, 0.3091) and 1-isothiocyanato-4-methoxybenzene (56 mg, 0.340 mmol) provided the colorless solid compound **4.3c** as 80% yield (120.76 mg). The product was scrutinized by ^1H NMR, ^{13}C NMR and HRMS analyses which matched with the reported literatures. ^1H NMR (600 MHz, CDCl_3) δ_{ppm} 8.43 (s, 1H), 7.97 (d, $J = 9.2$ Hz, 1H), 7.82 (s, 1H), 7.36-7.34 (m, 1H), 7.16 – 7.09 (m, 3H), 6.90-6.89 (m, 3H), 5.95 (s, 1H), 5.67-5.62 (m, 1H), 4.95 – 4.90 (m, 2H), 4.14-4.09 (m, 1H), 3.95 (s, 3H), 3.83 (s, 3H), 3.35 (s, 1H), 3.11-3.07 (m, 1H), 2.69 – 2.58 (m, 2H), 2.27 – 2.23 (m, 1H), 2.05 (s, 1H), 1.68 – 1.56 (m, 3H), 1.28 – 1.25 (m, 1H), 0.95 – 0.91 (m, 1H). ^{13}C NMR (150 MHz, CDCl_3) δ_{ppm} 180.9, 171.2, 158.3, 157.7, 147.5, 144.6, 140.9, 131.5, 130.3, 128.3, 121.9, 114.7, 114.6, 102.4, 60.4, 55.7, 55.5, 55.2, 41.5, 39.3, 29.7, 27.7, 27.3, 25.7, 22.7, 21.1, 14.2. HRMS (ESI) calcd. for $\text{C}_{28}\text{H}_{32}\text{N}_4\text{O}_2\text{S}$ ($\text{M} + \text{H}$) $^+$: 489.2319, found: 489.2328.

1-((1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-vinylquinuclidin-2-yl)methyl)-

3-(4-(trifluoromethyl)phenyl)thiourea (4.3d)²⁴ — Following the general procedure as mentioned in section 4.4.1.2.2., the reaction between the (9S)-9-amino(9-deoxy)epiquinine (100 mg, 0.3091) and 1-isothiocyanato-4-(trifluoromethyl)benzene (69 mg, 0.340 mmol) provided the colorless solid compound **4.3d** as 80% yield (130 mg). The product was scrutinized by ^1H NMR, ^{13}C NMR and HRMS analyses which matched with the reported literatures. ^1H NMR (600 MHz, DMSO- d_6) δ_{ppm} 10.08 (s, 1H), 8.75 (d, $J = 6.0$, 1H), 8.71 (brs, 1H), 7.96 (d, $J = 9.2$ Hz, 1H), 7.93 (s, 1H), 7.75 (d, $J = 8.3$ Hz, 2H), 7.66 – 7.60 (m, 3H), 7.45-7.43 (m, 1H),

6.02 (s, 1H), 5.86-5.80 (m, 1H), 5.02-4.93 (m, 2H), 3.97 (s, 3H), 3.25-3.21 (m, 2H), 2.73-2.71 (m, 2H), 2.28 (brs, 1H), 1.63 – 1.55 (m, 3H), 1.23 (s, 2H), 0.85 (brs, 1H). ^{13}C NMR (150 MHz, DMSO- d_6) δ_{ppm} 180.0, 157.5, 148.1, 144.6, 143.9, 142.3, 131.7, 129.2 (d, $J = 12.1$ Hz), 128.4, 127.6, 126.1 (d, $J = 3.8$ Hz), 125.77, 124.3 – 122.9 (m), 122.2, 60.2, 56.1, 55.5, 41.4, 29.5, 27.8, 27.5, 25.8, 22.5, 21.2, 14.5, 14.4. HRMS (ESI) calcd. for $\text{C}_{26}\text{H}_{29}\text{F}_3\text{N}_4\text{OS}$ ($\text{M} + \text{H}$) $^+$: 527.2087, found: 527.2093.

1-((1S)-(6-methoxyquinolin-4-yl))((1S,2S,4S)-5-vinylquinuclidin-2-yl)methyl)-3-(4-nitrophenyl)thiourea(4.3e). – Following the general procedure as mentioned in section 4.4.1.2.2., the reaction between the (9S)-9-amino(9-deoxy)epiquinine (100 mg, 0.3091) and 1-isothiocyanato-4-nitrobenzene (171 mg, 0.340 mmol) provided the colorless solid compound **4.3e** as 80% yield (130 mg). The product was scrutinized by ^1H NMR, ^{13}C NMR and HRMS analyses which matched with the reported literatures ^1H NMR (600 MHz, DMSO- d_6) δ_{ppm} 8.95 (s, 1H), 8.74 (d, $J = 4.5$ Hz, 1H), 8.15 (d, $J = 9.0$ Hz, 2H), 7.96 (d, $J = 9.2$ Hz, 1H), 7.90 (s, 1H), 7.83 (d, $J = 8.8$ Hz, 2H), 7.60 (d, $J = 4.5$ Hz, 1H), 7.47- 7.44 (m, 1H), 5.86-5.80 (m, 1H), 5.01 (d, $J = 17.2$ Hz, 1H), 4.94 (d, $J = 10.4$ Hz, 1H), 3.97 (s, 3H), 3.26-3.22 (m, 1H), 2.73-2.71 (m, 2H), 2.31-2.29 (m, 1H), 1.63-1.53 (m, 3H), 1.27-1.23 (m, 2H), 0.86-0.84 (m, 1H). ^{13}C NMR (150 MHz, DMSO- d_6) δ_{ppm} 180.1, 169.4, 163.1, 158.4, 148.3, 145.5, 144.70 143.3, 141.98, 132.0, 126.0, 122.4, 118.3, 114.9, 56.1, 40.4, 27.7, 26.4, 14.5. HRMS (ESI) calcd. for $\text{C}_{27}\text{H}_{29}\text{N}_5\text{O}_3\text{S}$ ($\text{M} + \text{H}$) $^+$: 504.2025, found: 504.2031.

1-(3,5-bis(trifluoromethyl)phenyl)-3-((1S)-(6-methoxyquinolin-4-yl))((1S,2S,4S)-5-vinylquinuclidin-2-yl)methyl)thiourea (4.3f)²⁵ – Following the general procedure as mentioned in section 4.4.1.2.2., the reaction between the (9 S)-9-amino(9-deoxy)epiquinine (100 mg, 0.3091) and 1-isothiocyanato-3,5-bis(trifluoromethyl)benzene (92 mg, 0.340 mmol) provided the colorless solid compound **4.3f** as 80% yield (146.96 mg). The product was c scrutinized by ^1H NMR, ^{13}C NMR and HRMS analyses which matched with the reported literatures. ^1H NMR (600 MHz, DMSO- d_6) δ_{ppm} 8.94 (s, 1H), 8.71 (d, $J = 6.0$ Hz, 1H), 8.12 (s, 2H), 7.94 – 7.91 (m, 2H), 7.67 (s, 1H), 7.58 (d, $J = 4.6$ Hz, 1H), 7.43-7.40 (m, 1H), 6.04 (s, 1H), 5.84-5.76 (m, 1H), 5.00-4.90 (m, 2H), 3.94 (s, 3H), 3.23-3.16 (m, 2H) 2.70 (s, 2H), 2.27 (s, 1H), 1.57 (s, 3H), 1.20 (s, 2H), 0.81 (brs, 1H). ^{13}C NMR (150 MHz, DMSO- d_6) δ_{ppm} 179.9,

157.6, 148.1, 144.5, 142.2, 131.7, 130.8 (q, $J = 32.8$ Hz), 129.21, 28.5, 126.4, 124.6, 122.8, 121.8, 120.9, 116.3, 114.8, 103.5, 59.8, 56.1, 55.5, 41.5, 27.4, 25.7. HRMS (ESI) calcd. for $C_{29}H_{28}F_6N_4OS$ ($M + H$)⁺: 595.1961, found: 595.1969.

1-((1S)-(6-methoxyquinolin-4-yl))((1S,2S,4S)-5-vinylquinuclidin-2-yl)methyl)-3-phenylurea(4.4a) – Following the general procedure as mentioned in section

4.4.1.2.3., the reaction between the (9 S)-9-amino(9-deoxy)epiquinine (100 mg, 0.3091) and isocyanatobenzene (0.137 mL, 0.340 mmol) provided the colorless solid compound **4.4a** as 80% yield (146.96 mg). The product was scrutinized by ¹H NMR, ¹³C NMR and HRMS analyses. ¹H NMR (600 MHz, DMSO-*d*₆) δ_{ppm} 9.48 (s, 1H), 8.69 (d, $J = 4.5$ Hz, 1H), 7.94 (d, $J = 9.1$ Hz, 1H), 7.82 (s, 1H), 7.60 (d, $J = 5.8$ Hz, 1H), 7.55 (d, $J = 4.6$ Hz, 1H), 7.42 (d, $J = 9.2$ Hz, 1H), 7.34 (d, $J = 8.0$ Hz, 2H), 7.13 (t, $J = 7.8$ Hz, 2H), 6.81 (t, $J = 7.4$ Hz, 1H), 5.88-5.82 (m, 1H), 5.00 (d, $J = 17.1$ Hz, 1H), 4.93 (d, $J = 10.3$ Hz, 1H), 3.95 (s, 3H), 3.24 – 3.22 (m, 3H), 3.15 (t, $J = 11.8$ Hz, 1H), 2.70 – 2.60 (m, 2H), 2.26-2.23 (m, 1H), 1.55 (s, 2H), 1.50-1.48 (m, 1H), 1.34-1.29 (m, 1H), 1.22 (s, 1H), 0.77 – 0.74 (m, 1H). ¹³C NMR (150 MHz, DMSO-*d*₆) δ_{ppm} 175.3, 157.5, 155.5, 148.1, 144.5, 142.6, 141.3, 131.6, 128.9, 121.6, 121.1, 117.7, 114.7, 103.1, 56.1, 55.8, 40.9, 40.4, 27.9, 27.7, 26.5, 24.4. HRMS (ESI) calcd. for $C_{27}H_{30}N_4O_2$ ($M + H$)⁺: 443.2402, found: 443.2415.

1-((1S)-(6-methoxyquinolin-4-yl))((1S,2S,4S)-5-vinylquinuclidin-2-yl)methyl)-3-(p-tolyl)urea(4.4b) – Following the general procedure as mentioned in section

4.4.1.2.3., the reaction between (9 S)-9-amino(9-deoxy)epiquinine (100 mg, 0.3091) and 1-isocyanato-4-methylbenzene (155 mg, 0.340 mmol) provided the colorless solid compound **4.4b** as 80% yield (146.96 mg). The product was scrutinized by ¹H NMR, ¹³C NMR and HRMS analysis. ¹H NMR (600 MHz, DMSO-*d*₆): δ_{ppm} 9.33 (d, $J = 7.4$ Hz, 1H), 8.69 (d, $J = 4.5$ Hz, 1H), 7.94 (d, $J = 9.2$ Hz, 1H), 7.81 (d, $J = 2.7$ Hz, 1H), 7.54 (d, $J = 4.6$ Hz, 1H), 7.51-7.47 (s, 1H), 7.41 (dd, $J = 9.2, 2.6$ Hz, 1H), 7.22 (d, $J = 8.2$ Hz, 2H), 6.93 (d, $J = 8.2$ Hz, 2H), 5.88-5.79 (m, 1H), 5.00 (d, $J = 17.1$ Hz, 1H), 4.93 (d, $J = 10.3$ Hz, 1H), 3.94 (s, 3H), 3.26-3.19 (m, 2H), 3.17 – 3.13 (m, 1H), 2.69 – 2.60 (m, 2H), 2.16 (s, 3H), 1.58-1.55 (m, 2H), 1.50-1.48 (m, 1H), 1.33 – 1.29 (m, 1H), 1.25-1.22 (m, 1H), 0.77 – 0.73 (m, 1H). ¹³C NMR (150 MHz, DMSO-*d*₆) δ_{ppm} 174.9, 157.5, 155.5, 148.1, 144.5, 142.6, 138.7, 131.6, 129.7, 129.4, 121.6, 117.8, 114.7, 103.1, 56.1, 55.8,

40.9, 40.4, 27.9, 27.7, 26.5, 20.7. HRMS (ESI) calcd. for $C_{28}H_{32}N_4O_2$ ($M+H$)⁺: 457.2559, found: 457.2567.

1-(4-fluorophenyl)-3-((1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-

vinylquinuclidin-2-yl)methyl)urea(4.4c) – Following the general procedure as mentioned in section 4.4.1.2.3., the reaction between (9 S)-9- amino(9-deoxy)epiquinine (100 mg, 0.3091) 1-fluoro-4-isocyanatobenzene (156 mg, 0.340 mmol) provided the colorless solid compound **4.4c** as 80% yield (146.96 mg). The product was scrutinized by 1H NMR, ^{13}C NMR and HRMS analysis. 1H NMR (600 MHz, $CDCl_3$) δ_{ppm} 8.75 (d, $J = 4.5$ Hz, 1H), 8.04 (d, $J = 9.2$ Hz, 1H), 7.93 – 7.88 (m, 1H), 7.78 (s, 1H), 7.39 (dd, $J = 9.2, 2.7$ Hz, 1H), 7.34 (d, $J = 4.6$ Hz, 1H), 7.11 – 7.04 (m, 2H), 6.84–6.80 (m, 2H), 6.46 (s, 1H), 5.66–5.60 (m, 1H), 4.96 (d, $J = 10.4$ Hz, 1H), 4.86 (d, $J = 17.1$ Hz, 1H), 3.98 (s, 3H), 3.63 (s, 1H), 3.42 (s, 1H), 3.15 (s, 1H), 3.0–2.96 (m, 1H), 2.68–2.62 (m, 1H), 2.39–2.36 (m, 1H), 2.27–2.20 (m, 1H), 1.69–1.62 (m, 2H), 1.59 – 1.51 (m, 2H), 1.31–1.24 (m, 2H), 0.93 – 0.90 (m, 1H). ^{13}C NMR (150 MHz, $CDCl_3$) δ_{ppm} 159.4, 158.2, 157.8, 155.8, 147.4, 144.8, 140.6, 134.9 (d, $J = 2.7$ Hz), 131.6, 128.5, 122.2, 121.4, 115.4, 115.3, 114.9, 101.9, 55.8, 55.4, 41.3, 38.8, 31.9, 29.7, 29.4, 27.4, 27.1, 26.5, 22.7, 14.2. HRMS (ESI) calcd. for $C_{27}H_{29}FN_4O_2$ ($M+H$)⁺: 461.2308, found: 461.2319.

1-(4-bromophenyl)-3-((1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-

vinylquinuclidin-2-yl)methyl)urea(4.4d) – Following the general procedure as mentioned in section 4.4.1.2.3., the reaction between the (9 S)-9- amino(9-deoxy)epiquinine (100 mg, 0.3091) and 1-bromo-4-isocyanatobenzene (177 mg, 0.340 mmol) provided the colorless solid compound **4.4d** as 80% yield (146.96 mg). The product was scrutinized by 1H NMR, ^{13}C NMR and HRMS analysis. 1H NMR (600 MHz, $CDCl_3$) δ_{ppm} 8.65 (d, $J = 4.4$ Hz, 1H), 7.93 (d, $J = 9.2$ Hz, 1H), 7.79 (s, 1H), 7.68 (s, 1H), 7.28 (dd, $J = 9.2, 2.6$ Hz, 1H), 7.23 (d, $J = 4.6$ Hz, 1H), 6.98 – 6.96 (m, 1H), 6.71 (t, $J = 8.6$ Hz, 1H), 6.35 (s, 1H), 5.56 – 5.50 (m, 1H), 4.86 (d, $J = 10.4$ Hz, 1H), 4.76 (d, $J = 17.2$ Hz, 1H), 3.88 (s, 3H), 3.53 (s, 1H), 3.32 (s, 1H), 3.04 (s, 1H), 2.89–2.85 (m, 1H), 2.54 (dd, $J = 19.7, 10.2$ Hz, 1H), 2.27 (d, $J = 11.4$ Hz, 1H), 2.12 (s, 1H), 1.57 (s, 1H), 1.54 (dd, $J = 9.6, 5.9$ Hz, 1H), 1.46 (dd, $J = 21.5, 10.5$ Hz, 1H), 1.16 (s, 1H), 0.84 – 0.78 (m, 1H). ^{13}C NMR (150 MHz, $CDCl_3$) δ_{ppm} 158.8, 157.5, 157.2, 155.2, 146.7, 144.2, 140.0,

134.3, 130.9, 128.0, 121.5, 120.8, 114.8, 114.7, 114.3, 101.3, 59.52 – 59.0, 55.2, 54.8, 40.6, 38.2, 31.3, 29.1, 28.8, 26.8, 26.6, 25.8, 22.1, 13.6. HRMS (ESI) calcd. for $C_{27}H_{29}BrN_4O_2$ ($M+H$)⁺: 522.1453, found: 522.1461.

1-(3-chloro-4-fluorophenyl)-3-((1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-vinylquinuclidin-2-yl)methyl)urea(4.4e) — Following the general procedure as mentioned in section 4.4.1.2.3., the reaction between (9 S)-9- amino(9-deoxy)epiquinine (100 mg, 0.3091) and 2-chloro-1-fluoro-4-isocyanatobenzene (168 mg, 0.340 mmol) provided the colorless solid compound **4.4e** as 80% yield (146.96 mg). The product was scrutinized by ¹H NMR, ¹³C NMR and HRMS analysis. ¹H NMR (600 MHz, DMSO-*d*₆) δ_{ppm} 10.59 (brs, 1H), 8.70 (d, *J* = 4.5 Hz, 1H), 8.54-8.48 (m, 1H), 7.94 (d, *J* = 9.1 Hz, 1H), 7.80 (d, *J* = 2.8 Hz, 1H), 7.75-7.74 (m, 1H), 7.55 (d, *J* = 4.6 Hz, 1H), 7.42-7.40 (m, 1H), 7.25 – 7.22 (m, 1H), 7.18 (t, *J* = 9.1 Hz, 1H), 5.90-5.84 (m, 1H), 5.02 (d, *J* = 17.1 Hz, 1H), 4.95 – 4.93 (m, 1H), 3.95 (s, 3H), 3.30 – 3.26 (m, 2H), 3.15-3.11 (m, 1H), 2.70-2.67 (m, 1H), 2.64-2.59 (m, 1H), 2.25-2.21 (m, 1H), 1.55-1.52 (m, 2H), 1.24-1.22 (m, 2H), 0.72-0.68 (m, 1H). ¹³C NMR (150 MHz, DMSO-*d*₆) δ_{ppm} 175.2, 157.6, 155.6, 152.5, 150.9, 148.2, 144.5, 142.7, 139.2, 131.7, 129.3, 121.6, 120.3, 119.2, 119.1, 118.5, 117.6, 117.6, 117.0, 116.9, 114.6, 103.1, 56.0, 41.0, 40.5, 31.7, 30.3, 29.2, 27.9, 27.7, 26.6, 24.5, 14.4. HRMS (ESI) calcd. for $C_{27}H_{28}ClFN_4O_2$ ($M+H$)⁺: 496.1855, found: 496.1868.

1-(3,5-bis(trifluoromethyl)phenyl)-3-((1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-vinylquinuclidin-2-yl)methyl)ure(4.4f) — Following the general procedure as mentioned in section 4.4.1.2.3., the reaction between (9 S)-9- amino(9-deoxy)epiquinine (100 mg, 0.3091) and 1-isocyanato-3,5-bis(trifluoromethyl)benzene (0.133 mL, 0.340 mmol) provided the colorless solid compound **4.4f** as 80% yield (146.96 mg). The product was scrutinized by ¹H NMR, ¹³C NMR and HRMS analysis. ¹H NMR (600 MHz, DMSO-*d*₆) δ_{ppm} 8.95 (s, 1H), 8.71 (d, *J* = 4.5 Hz, 2H), 8.11 (s, 2H), 7.93 (d, *J* = 9.2 Hz, 1H), 7.91 (s, 1H), 7.68 (s, 1H), 7.59 (d, *J* = 4.6 Hz, 1H), 7.43 – 7.41 (m, 1H), 5.84 – 5.78 (m, 1H), 5.74 (s, 1H), 4.98 (d, *J* = 17.4 Hz, 1H), 4.92 (d, *J* = 10.1 Hz, 1H), 3.94 (s, 3H), 3.21 – 3.17 (m, 1H), 2.69 (s, 2H), 2.59-2.54 (m, 1H), 2.26 (s, 1H), 1.60-1.55 (m, 3H), 1.21 (s, 2H), 0.80 (s, 1H). ¹³C NMR (150 MHz, DMSO-*d*₆) δ_{ppm} 175.4, 157.7, 155.7, 152.6, 151.8, 148.2, 144.6, 142.8, 139.4,

131.7, 129.3, 121.7, 120.4, 120.4, 119.3, 119.2, 118.5, 117.7, 117.7, 117.1, 116.9, 114.7, 103.1, 41.11, 40.54, 31.8, 31.6, 30.3, 29.5, 29.3, 28.0, 27.8, 26.7, 24.6, 14.6. HRMS (ESI) calcd. for $C_{29}H_{28}F_6N_4O_2$ ($M+H$)⁺: 579.2150, found: 579.21562.

***N*-(((1*S*)-(6-methoxyquinolin-4-yl))((1*S*,2*S*,4*S*)-5-vinylquinuclidin-2-yl)methyl)carbamoyl)-4-methylbenzenesulfonamid(4.4g)** —

Following the general procedure as mentioned in section 4.4.1.2.4., the reaction between the (9*S*)-9-amino(9-deoxy)epiquinine (100 mg, 0.3091) and 4-methylbenzenesulfonyl isocyanate (0.137 mL, 0.340 mmol) provided the colorless solid compound **4.4g** as 80% yield (146.96 mg). The product was scrutinized by ¹H NMR, ¹³C NMR and HRMS analysis. ¹H NMR (600 MHz, DMSO-*d*₆) δ_{ppm} 8.69 (d, *J* = 4.4 Hz, 1H), 7.93 (d, *J* = 9.2 Hz, 1H), 7.72 (d, *J* = 8.2 Hz, 2H), 7.52 (d, *J* = 4.5 Hz, 2H), 7.38-7.36 (m, 2H), 7.31 (s, 1H), 5.91 – 5.85 (m, 1H), 5.66 (d, *J* = 5.0 Hz, 1H), 4.99 (d, *J* = 17.2 Hz, 1H), 4.95 (d, *J* = 10.4 Hz, 1H), 3.90 (s, 1H), 3.39 (s, 1H), 3.20 (s, 1H), 3.08 – 3.05 (m, 1H), 2.87-2.83 (m, 1H), 2.44-2.42 (m, 1H), 2.37 (s, 3H), 2.19 (s, 1H), 1.75-1.4 (m, 2H), 1.68-1.62 (m, 1H), 1.44-1.40 (m, 1H). ¹³C NMR (150 MHz, DMSO-*d*₆) δ_{ppm} 160.2, 157.2, 149.8, 147.9, 144.4, 143.0, 142.3, 141.8, 131.6, 129.8, 127.6, 126.1, 121.4, 119.6, 114.5, 102.9, 71.4, 61.1, 56.3, 55.9, 42.2, 27.9, 24.6, 21. HRMS (ESI) calcd. for $C_{28}H_{32}N_4O_4S$ ($M+H$)⁺: 521.2178, found: 521.2187.

***N*-(((1*S*)-(6-methoxyquinolin-4-yl))((1*S*,2*S*,4*S*)-5-vinylquinuclidin-2-yl)methyl)-4-methylbenzenesulfonamide(4.5a)** —

Following the general procedure as mentioned in section 4.4.1.2.5., the reaction between the (9*S*)-9-amino(9-deoxy)epiquinine (100 mg, 0.3091) and 4-methylbenzenesulfonyl chloride (162 mg, 0.340 mmol) provided the colorless solid compound **4.5a** as 80% yield (146.96 mg). The product was scrutinized by ¹H NMR, ¹³C NMR and HRMS analysis. ¹H NMR (600 MHz, CDCl₃) δ_{ppm} 8.61 (d, *J* = 4.5 Hz, 1H), 7.93 (d, *J* = 8.4 Hz, 1H), 7.84 (d, *J* = 9.2 Hz, 1H), 7.57 (d, *J* = 4.5 Hz, 1H), 7.42 (d, *J* = 8.1 Hz, 2H), 7.16-1.14 (m, 1H), 7.07 (d, *J* = 2.7 Hz, 1H), 5.95 (s, 1H), 5.65-5.59 (m, 1H), 4.99 – 4.94 (m, 2H), 3.96 (s, 1H), 3.68 (s, 3H), 3.30-3.26 (m, 1H), 3.22 – 3.19 (m, 1H), 2.90 – 2.81 (m, 2H), 2.50 (s, 3H), 2.46-2.44 (m, 1H), 2.33 (s, 1H), 1.99 – 1.92 (m, 3H), 1.67 – 1.61 (m, 1H), 1.42-1.37 (m, 1H). ¹³C NMR (150 MHz, CDCl₃) δ_{ppm} 158.1, 147.1, 146.8, 143.8, 141.6, 140.4, 138.2, 131.3, 130.2, 128.9, 127.1, 125.9, 125.6, 122.0, 118.7, 116.6, 99.9, 60.2, 56.4,

55.3, 43.9, 37.8, 27.1, 24.9, 21.9, 21.3, 18.8. HRMS (ESI) calcd. for $C_{27}H_{31}N_3O_3S$ ($M+H$)⁺: 478.2120, found: 478.2131.

4-fluoro-*N*-((1*S*)-(6-methoxyquinolin-4-yl)((1*S*,2*S*,4*S*)-5-vinylquinuclidin-2-yl)methyl)benzenesulfonamide(4.5b) — Following the general procedure as mentioned in section 4.4.1.2.5., the reaction between the (9*S*)-9- amino(9-deoxy)epiquinine (100 mg, 0.3091) and 4-fluorobenzenesulfonyl chloride (163 mg, 0.340 mmol) provided the colorless solid compound **4.5b** as 80% yield (146.96 mg). The product was scrutinized by ¹H NMR, ¹³C NMR and HRMS analyses. ¹H NMR (600 MHz, DMSO-*d*₆): δ_{ppm} 8.57 (d, *J* = 4.4 Hz, 1H), 7.88-7.80(m, 1H), 7.45-7.40 (m, 4H), 7.35-7.29 (m, (d, 1H), 7.0-7.69 (m, 2H), 5.81-5.63(m, 1H), 4.96 – 4.86 (m, 2H), 3.95 (s, 3H), 3.11-3.04 (m, 2H), 2.88-2.82 (m, 1H), 2.63 – 2.60 (m, 1H), 2.21-2.17 (m, 1H), 1.53 – 1.43 (m, 3H), 1.25-1.23 (m, 1H), 1.15-1.11 (m, 1H), 0.93 – 0.71 (m, 1H), 0.54-0.50 (m, 1H). ¹³C NMR (150 MHz, DMSO-*d*₆) δ_{ppm} 164.8, 163.1, 157.8, 147.9, 142.4, 131.7, 129.5, 121.7, 120.5, 115.6, 115.5, 114.7, 101.5, 59.7, 55.9, 55.4, 52.5, 27.8, 27.5, 25.6. HRMS (ESI) calcd. for $C_{26}H_{28}FN_3O_3S$ ($M+H$)⁺: 482.1869, found: 482.1878.

4.4.2. Purification of holo-human IDO1 and TDO enzymes

Expression and purification of the enzymes were carried out as described previously in the section 2.4.3. with the following minor modifications.^{12, 13, 26-29} The *N*-terminus 6×histidine-tagged recombinant human IDO1, and TDO enzymes were expressed in *Escherichia coli* (BL21-DE3 strain) cells by transforming the cDNAs of human IDO1 and TDO (with pET 28a vector).

4.4.3. Expression and purification of apo-IDO1 enzyme

Human apo-IDO1 was expressed and purified with a similar procedure, as described earlier, with minor modifications.³⁰ During protein expression, no external heme was added to the culture media. After purification using nickel-nitrilotriacetic acid resin, the protein was dialyzed in 25 mM HEPES, 250 mM NaCl and 100 μ M TCEP at pH 7.2. Then, the dialyzed enzyme was incubated with 150 mM of MESNA by overnight at room temperature to release the heme cofactor. After that, the apo-IDO1 was dialyzed in 25 mM HEPES, 250 mM NaCl at pH 7.2 and buffer exchanged with 50 mM potassium phosphate buffer at pH 6.8 containing 150 mM KCl and 10 % glycerol

using the Sephadex-G25 column. The heme content was investigated by measuring the absorbance ratio of the purified apo-IDO1 enzyme at 404 nm/280 nm (around 0.05).

4.4.4. IDO1 and TDO inhibition assay by the spectrometric method

Both IDO1 and TDO inhibition assays were performed according to the reported procedures as described in section 2.4.4.^{12, 13, 26-29}

4.4.5. Cell viability analysis

Cell viability analysis was performed in HEK-293 and MDA-MB-231 cells by using MTT dye according to the similar protocol mentioned in section 2.4.7.^{12, 13, 26}

4.4.6. Cellular IDO1 activity assay

A. UV-Vis-based cellular IDO1 inhibition assay

The cellular enzyme activity assay was performed according to the reported procedure as mentioned earlier in section 2.4.8. with minor modifications.³ The HeLa cells were selected for this experiment DMEM phenol-red free medium (containing 10% FBS)) was used.

B. HPLC-based cellular IDO1 inhibition assay

The cellular enzyme activity assay was performed according to the reported procedure with minor modifications.³ Cells were cultured in presence and absence of compounds according to the mentioned procedure in section 4.4.6 and incubated for 20 h. After that, clear media was taken for HPLC analysis. 20 μ L of the media was then injected through Ascentis® express C18, 2.7 μ m HPLC column. The mobile phase was 50% sodium citrate buffer (40 mM, pH 2.25) and 50% methanol (v/v) containing 400 μ M SDS with a flow rate of 0.5 mL/minute. The 365 nm and 280 nm chromatograms were monitored to calculate the kynurenine formation and tryptophan depletion, respectively.

4.4.7. UV-Vis spectroscopic measurement

The UV-visible spectra study was performed to explore the binding of the compounds to the enzyme and the heme lability of the enzyme.^{3, 31} The absorbance

spectra were recorded in EVOLUTION 201 spectrophotometer and the spectroscopic analysis were performed according to the mentioned process in section 2.4.6.

4.4.8. Detection of labile heme by fluorescence assay

The fluorescence signal of PpIX was used to detect the free heme in solution.^{15, 32} Fluoromax-4 spectrofluorometers were used experiment. To detect the presence of heme in the solution, the first 5 μ M of IDO1 was incubated in 100 mM KPB at pH 6.5 with 50 μ M of inhibitors for different time (0 min to 60 min) at 37 °C. After that, PpIX (5 μ M) was added to the solution and incubated for 5 min at room temperature. The PpIX fluorescence signal ($\lambda_{\text{ex}} = 400$ nm, $\lambda_{\text{em}} = 615$ nm) was monitored to detect the free heme in solution.

4.4.9. CD analysis

The CD spectra were recorded with a Jasco-810-spectropolarimeter according to the reported procedure in section 3.4.8.

4.4.10. Binding analysis by SPR analysis

The SPR analysis was performed to confirm the binding of the selected inhibitors with apo-IDO1 protein according to the earlier mentioned section 3.4.7.^{33, 34} The equilibrium dissociation constant (K_D) value of the potent compound to apo-IDO1 was calculated using the inbuilt Biacore Insight Evaluation software.

4.4.11. Tryptophan fluorescence-based binding assay

Tryptophan emission fluorescence was also monitored to find the selected compound's binding affinity with apo-IDO1 on a Fluoromax-4 spectrofluorometer at room temperature. Briefly, 1 mL of apo-IDO1 (250 nM) placed into the instrument condition under slow stirring condition. The fluorescence intensity was monitored (at $t = 0$ s) at $\lambda_{\text{em}} = 350$ nm ($\lambda_{\text{ex}} = 280$ nm). The kinetics was initiated by adding the respective compound (10 μ M) to the solution at $t = 50$ s and continued up to 500 s.

4.4.12. Molecular docking and molecular dynamics simulations studies

4.4.12.1. Preparation of protein and ligands

The 3-D coordinates of the IDO1 enzyme were obtained from the Protein Data Bank (PDB ID: 6AZW).³ The protein was prepared using the Maestro program, which includes the addition of the hydrogen atoms, capping of both N and C termini of the protein.³⁵⁻³⁷ Then the protonation states of all residues are defined according to neutral pH. Structures of **4.4g**, **4.5a**, and **4.5b** ligands were built manually, and Gasteiger charges were added.

4.4.12.2. Blind docking

Molecular docking of 6AZW crystal ligand and **4.4g**, **4.5a**, and **4.5b** ligands with the IDO1 protein and the detailed binding energy calculations were performed with Lead Finder docking software using default configuration parameters.³⁸ The docking score produced by Lead Finder was taken as the predicted value of the ligand binding energy. Only the top-ranked poses were used for structural and energy analyses. The scoring function from Lead Finder considers mainly LJ term, H-bonds, electrostatic interactions, hydrophobic stabilization, and the ligand's internal energy. We adopted a blind docking approach where multiple docking runs were started around geometric centres of all sites within the selected threshold.¹⁷ A histogram with the resulting distribution of binding energies and their structural clusters of poses were generated.

4.4.12.3. Molecular dynamics

Molecular dynamics simulations were performed using the GROMACS2016 molecular dynamics package. The topologies of the drug molecules were generated by ACPYPE according to the Amber99sb force field.^{18, 39-43} The structure of the top pose obtained from blind docking was considered as initial protein-ligand conformation in each case. Each complex was then immersed in a dodecahedra box containing TIP3P water molecules.⁴⁴ Periodic boundary conditions were considered for every simulation run. Na⁺ ions were added to neutralize the charge of the system. The LINCS algorithm was chosen to constrain covalent bond lengths. An integration step of 2 fs was applied to simulate the system.⁴⁵ Electrostatic interactions were calculated using the PME method.⁴⁶ Initially, all the systems were energy minimized using steepest-descent algorithm. The minimized systems were then subjected to position restraint dynamics where the complex was restraint by employing a

restraining potential. 200 ps position restrained simulation was performed in the NVT ensemble using the V-rescale thermostat with a coupling constant of $\tau = 0.5$ ps. This step was followed by 800 ps equilibration in the NPT ensemble. Parrinello–Rahman barostat was employed with a time constant for coupling set to 2.0 ps for isotropic pressure coupling.⁴⁷ Temperature was maintained at 300 K by employing the V-rescale thermostats with a coupling constant of $\tau = 0.5$ ps. Final production simulations were performed with a constant temperature of 300 K and a constant pressure (1 atm) using the NPT ensemble. Subsequently, 100 ns of unrestrained NPT MD simulations were performed for each system, generating the production run from which data were collected.

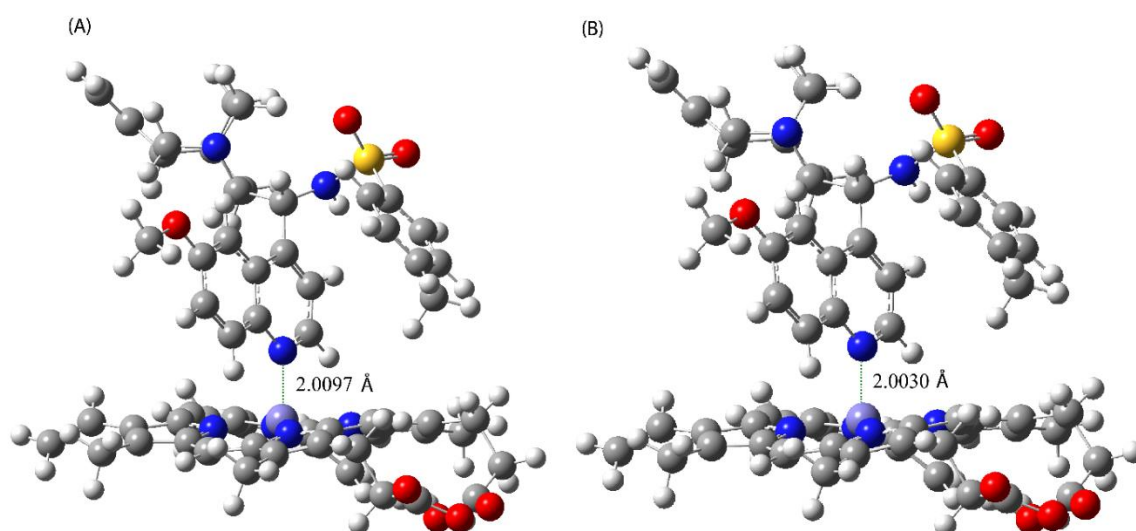


Figure 4.19 Optimized structure of the compound in B3LYP/6-31G (d,p) (A) and in B3LYP/6-31G (d) (B).

4.4.13. DFT studies

The DFT is accepted as one of the most successful approaches for analysing any of the molecules' structural and electronic properties and their interacting systems. Investigation of various non-covalent interactions like hydrogen bond and charge distribution of the molecules or atom is widely used. To find out the interaction pattern of our compound with the free heme, we performed DFT analysis (Gaussian 16 program) using the B3LYP/6-31G (d,p) and B3LYP/6-31G (d) basic set. Initially,

we have separately optimized the structure of the compound and the free heme using the same basic set. After optimizing both structures, we performed a DFT analysis to determine the possible interaction pattern of our compound with the free heme. The result showed the potential interaction between the quinoline ring nitrogen atom with the iron of the free heme (2.0097 Å). The interactions are within the hydrogen bonding distance, which supports our hypothesis that our compound can also bind with a labile free heme group.

Table 4.9 XYZ Cartesian coordinate for interaction

B3LYP/6-31G (d, p)				B3LYP/6-31G (d)			
Energy = -4934.17481073 a.u.				Energy = -4934.07244509 a.u.			
	X	Y	Z		X	Y	Z
N	2.370092	0.057804	1.657875	N	2.359699	0.066005	1.667434
C	3.157229	-0.9147	1.091136	C	3.149185	-0.91146	1.112522
N	0.596841	2.11536	2.435853	N	0.581881	2.129454	2.418824
C	3.508294	-1.93052	2.071627	C	3.491803	-1.92128	2.102025
N	0.89864	3.32716	-0.06974	N	0.900468	3.323194	-0.09331
O	8.619739	-1.96307	-1.7731	O	8.624995	-1.98606	-1.71576
C	2.940199	-1.53303	3.259803	C	2.917476	-1.51419	3.284043
N	2.756473	1.338898	-0.79453	N	2.763872	1.330065	-0.79074
O	7.981692	-1.3547	-3.81535	O	8.023968	-1.36307	-3.76497
C	2.235385	-0.30054	2.982007	C	2.216543	-0.28253	2.993221
C	1.499618	0.405342	3.928758	C	1.47602	0.431466	3.930408
H	1.472295	0.00753	4.935232	H	1.442746	0.040722	4.940086
C	0.747762	1.545357	3.680983	C	0.725462	1.56949	3.669397
C	-0.11078	2.204834	4.659834	C	-0.13895	2.236541	4.637841
C	-0.83934	3.137017	3.958307	C	-0.86394	3.162451	3.924362
C	-0.36267	3.088192	2.593774	C	-0.37908	3.102893	2.56321
C	-0.7485	3.986667	1.605454	C	-0.75925	3.993235	1.565166
H	-1.51655	4.706065	1.864014	H	-1.53035	4.713939	1.813248
C	-0.1142	4.137415	0.382293	C	-0.11599	4.136136	0.345574

C	-0.30868	5.278997	-0.50384	C	-0.30424	5.271617	-0.54967
C	0.651499	5.168862	-1.48336	C	0.663683	5.155573	-1.52079
C	1.384725	3.952365	-1.19754	C	1.395158	3.941292	-1.22132
C	2.417074	3.454217	-1.98538	C	2.433893	3.437966	-1.99753
H	2.736227	4.054763	-2.82918	H	2.760131	4.033261	-2.84311
C	3.050711	2.233181	-1.80333	C	3.065988	2.217861	-1.80286
C	4.065315	1.700941	-2.69613	C	4.086947	1.679761	-2.68463
C	4.371266	0.451054	-2.23345	C	4.389364	0.432807	-2.2116
C	3.561737	0.249304	-1.04078	C	3.570885	0.238802	-1.02385
C	3.672129	-0.8485	-0.20145	C	3.673699	-0.85388	-0.17671
H	4.316746	-1.6562	-0.52847	H	4.32052	-1.66464	-0.49355
C	2.992772	-2.21912	4.592634	C	2.960381	-2.19175	4.622403
H	3.463087	-3.20346	4.518894	H	3.42415	-3.18109	4.556969
H	1.989738	-2.36669	5.008027	H	1.954205	-2.32964	5.036281
H	3.559109	-1.63909	5.331761	H	3.529097	-1.61097	5.360779
C	-0.19536	1.946889	6.095062	C	-0.23146	1.9906	6.074886
H	-1.17891	2.105588	6.537094	H	-1.21793	2.152764	6.510442
C	0.801252	1.583582	6.916053	C	0.761245	1.635652	6.904994
H	1.823351	1.464754	6.571819	H	1.786621	1.514045	6.56899
H	0.620877	1.426197	7.974598	H	0.575284	1.48807	7.96485
C	-1.86331	4.099546	4.477737	C	-1.89207	4.128979	4.430037
H	-2.16022	3.854692	5.500211	H	-2.19792	3.890202	5.452559
H	-2.76783	4.098952	3.859191	H	-2.79282	4.125483	3.804108
H	-1.48116	5.127811	4.489173	H	-1.50985	5.158419	4.438794
C	-1.34609	6.288667	-0.32113	C	-1.34272	6.282918	-0.38128
H	-2.25451	5.943071	0.171651	H	-2.2557	5.940757	0.106608
C	-1.29705	7.575957	-0.69581	C	-1.28937	7.568516	-0.76285
H	-0.4179	8.018211	-1.15142	H	-0.40611	8.008966	-1.21426
H	-2.14297	8.235487	-0.53016	H	-2.13626	8.230645	-0.60733
C	0.89416	6.071783	-2.65315	C	0.914784	6.05212	-2.6946
H	-0.02754	6.586219	-2.93975	H	-0.00696	6.560605	-2.99565
H	1.254916	5.517047	-3.52452	H	1.288274	5.493465	-3.55941

H	1.641145	6.844773	-2.4305	H	1.655915	6.831537	-2.46941
C	4.64141	2.429776	-3.87216	C	4.672576	2.401157	-3.86147
H	5.188435	3.328502	-3.56249	H	5.225525	3.29811	-3.55319
H	3.860371	2.754847	-4.56984	H	3.896162	2.729858	-4.56427
H	5.337235	1.79807	-4.42899	H	5.366876	1.76245	-4.4145
C	5.411255	-0.50174	-2.7477	C	5.433765	-0.52274	-2.71291
H	5.610426	-0.32264	-3.80734	H	5.632589	-0.35628	-3.7755
H	5.047044	-1.53468	-2.68275	H	5.075302	-1.55752	-2.63357
C	6.752606	-0.39135	-1.99004	C	6.774877	-0.39377	-1.95589
H	7.118372	0.640489	-2.06172	H	7.135412	0.639254	-2.04527
H	6.620775	-0.5972	-0.92556	H	6.641173	-0.58269	-0.88768
C	7.824873	-1.28139	-2.61618	C	7.849693	-1.29156	-2.5683
H	8.302171	-1.91319	-0.84909	H	8.298946	-1.9253	-0.7923
C	4.318615	-3.17619	1.822474	C	4.300993	-3.17077	1.866334
C	5.797587	-3.12821	2.304764	C	5.77683	-3.1223	2.358961
C	6.646354	-2.14085	1.52802	C	6.631777	-2.13726	1.584675
O	7.520525	-2.48038	0.754818	O	7.509523	-2.47794	0.816476
O	6.381867	-0.83336	1.709572	O	6.36783	-0.82846	1.764911
H	5.564684	-0.72083	2.226334	H	5.543766	-0.71701	2.277588
H	6.259829	-4.1091	2.179371	H	6.237863	-4.10547	2.24009
H	5.824837	-2.86814	3.369288	H	5.796369	-2.86004	3.423993
H	3.848976	-4.0195	2.341566	H	3.826672	-4.01106	2.387979
H	4.306147	-3.43593	0.759209	H	4.296186	-3.43764	0.803892
Fe	1.473271	1.581736	0.717164	Fe	1.469572	1.583231	0.709849
N	-4.04072	-2.8772	0.576591	N	-4.04565	-2.88093	0.559312
C	-3.89474	-1.70869	-0.29498	C	-3.898	-1.70957	-0.30997
C	-2.56072	-0.98162	-0.14763	C	-2.56258	-0.98426	-0.16202
C	-5.08494	-0.69021	-0.1642	C	-5.08539	-0.68934	-0.17052
C	-2.03571	-0.20007	-1.22863	C	-2.03062	-0.20973	-1.24495
C	-0.78879	0.492502	-1.04086	C	-0.78339	0.482185	-1.05503
N	-0.11383	0.489847	0.1724	N	-0.11506	0.48734	0.162067
C	-0.63695	-0.26176	1.13666	C	-0.64496	-0.25691	1.127996

C	-1.82414	-1.00403	1.012153	C	-1.83253	-0.99881	1.002147
C	-0.24409	1.184368	-2.14242	C	-0.23164	1.165429	-2.15847
H	-0.11041	-0.2832	2.077475	H	-0.12379	-0.27244	2.072085
H	-2.13205	-1.58527	1.874312	H	-2.14598	-1.57272	1.868053
N	-6.09778	-0.9271	-1.21499	N	-6.11695	-0.93484	-1.20046
C	-6.97293	-2.07032	-0.86856	C	-6.98696	-2.07416	-0.83028
C	-7.79756	-1.77738	0.421478	C	-7.78401	-1.77383	0.475437
C	-7.27406	-0.46177	1.028946	C	-7.25282	-0.45111	1.06076
C	-7.54182	0.707661	0.038455	C	-7.54053	0.709529	0.06525
H	-7.76726	-0.2585	1.986522	H	-7.72881	-0.24039	2.026285
C	-6.93444	0.282193	-1.33582	C	-6.95515	0.273734	-1.31578
H	-7.72081	0.048937	-2.05886	H	-7.75227	0.034719	-2.02579
H	-6.32974	1.089138	-1.76359	H	-6.35574	1.077257	-1.75885
C	-5.75135	-0.59458	1.241686	C	-5.72603	-0.57866	1.246686
H	-4.65757	0.287892	-0.40503	H	-4.66182	0.286266	-0.42973
H	-5.56377	-1.49022	1.841991	H	-5.52543	-1.46701	1.855132
H	-5.35531	0.263834	1.796581	H	-5.32146	0.287688	1.784628
C	-8.9925	1.126355	0.029064	C	-8.99299	1.123796	0.077958
H	-6.97854	1.574589	0.419821	H	-6.97198	1.581192	0.429736
C	-9.79908	1.300261	-1.01874	C	-9.8206	1.285298	-0.95607
H	-9.4812	1.141308	-2.04464	H	-9.52334	1.118302	-1.98767
H	-10.8267	1.623692	-0.88304	H	-10.8475	1.606687	-0.80398
H	-9.39951	1.325659	1.022582	H	-9.38193	1.330761	1.07787
H	-7.63033	-2.24787	-1.72576	H	-7.66225	-2.25295	-1.67397
H	-6.35275	-2.96019	-0.75596	H	-6.36529	-2.96453	-0.72709
H	-8.86484	-1.68158	0.195643	H	-8.85738	-1.68502	0.272693
H	-7.69135	-2.59348	1.144538	H	-7.65752	-2.58383	1.203425
C	-2.69552	-0.10346	-2.47681	C	-2.68337	-0.11904	-2.49727
C	-2.14193	0.608601	-3.52705	C	-2.12371	0.585435	-3.54967
C	-0.8905	1.244327	-3.35689	C	-0.87159	1.219095	-3.37699
O	-2.86135	0.636051	-4.6824	O	-2.83778	0.607106	-4.70832
C	-2.34197	1.352867	-5.79434	C	-2.31285	1.316638	-5.82181

H	0.708971	1.665864	-2.02302	H	0.722668	1.645283	-2.0382
H	-3.65927	-0.57302	-2.639	H	-3.64737	-0.58792	-2.66192
H	-0.42548	1.785913	-4.17162	H	-0.40076	1.754668	-4.19318
H	-3.08077	1.244134	-6.58911	H	-3.05021	1.203589	-6.61821
H	-1.38519	0.937563	-6.1343	H	-1.35496	0.896485	-6.15458
H	-2.21289	2.41802	-5.56597	H	-2.18332	2.383148	-5.59717
H	-3.9726	-2.0978	-1.31253	H	-3.97969	-2.09708	-1.32781
S	-3.6887	-4.43302	0.032406	S	-3.68506	-4.43594	0.012111
H	-3.77641	-2.77957	1.54998	H	-3.7721	-2.78182	1.531186
C	-1.97393	-4.47481	-0.49278	C	-1.96968	-4.46998	-0.51267
C	-0.97014	-4.71766	0.44741	C	-0.96476	-4.70781	0.428007
C	-1.66077	-4.25217	-1.83456	C	-1.65631	-4.24723	-1.85462
C	0.359147	-4.71451	0.034835	C	0.364908	-4.70014	0.015835
H	-1.23355	-4.92929	1.478181	H	-1.22656	-4.91948	1.459718
C	-0.32432	-4.25112	-2.22822	C	-0.31941	-4.24185	-2.24783
H	-2.45551	-4.1046	-2.55696	H	-2.45051	-4.10275	-2.57894
C	0.704792	-4.47083	-1.30315	C	0.710371	-4.45686	-1.32234
H	1.142673	-4.91012	0.761826	H	1.14921	-4.89198	0.743956
H	-0.07746	-4.08124	-3.27244	H	-0.07292	-4.07207	-3.29278
O	-4.50225	-4.64307	-1.16744	O	-4.49822	-4.64774	-1.18736
O	-3.81893	-5.26289	1.232831	O	-3.81181	-5.26741	1.211774
H	2.764963	-5.11475	-1.14697	H	2.773904	-5.09522	-1.16425
H	2.265838	-4.67579	-2.78854	H	2.273153	-4.66149	-2.80821
H	2.558496	-3.41469	-1.58775	H	2.563744	-3.39552	-1.60981
C	2.150555	-4.42306	-1.73111	C	2.156727	-4.40583	-1.7504
C	2.150555	-4.42306	-1.73111	C	2.156727	-4.40583	-1.7504

4.4.14. Heme affinity measurement by UV-Vis method

The spectrophotometric method was used to measure heme-inhibitor affinity. First, the hemin was dissolved in DMSO followed by dilution to 5 μ M in 20 mM HEPES buffer, pH 7.2 (containing 40 % DMSO). The stock solution of inhibitors was prepared in DMSO solution. Then, hemin was titrated with an increasing concentration of inhibitors. The change in the absorbance of the Soret peak for heme (401 nm) in the

presence of a growing concentration of inhibitors (0 to 175 μM) was monitored. The heme affinity of the selected inhibitors was quantified using Bindfit software.

4.4.15. PpIX fluorescence-based heme affinity measurement

The absorbance of hemin at 401 nm in the absence and presence of compounds was monitored to investigate the heme-masking ability of the compounds. Freshly prepared hemin (0.5 mM in DMSO) in 20 mM HEPES buffer, pH 7.2 was incubated in the absence and presence of different concentrations of the compound at room temperature for 1 h. The extent of quenching of hemin absorbance at 401 nm was monitored to investigate the compounds' heme-masking ability.

4.4.16. Cellular apo-IDO1 activation assay

Cellular apo-IDO1 activity assay was performed according to the method mentioned above. But, in addition to the compound, external hemin was added to the cell. The hemin was previously incubated with the compounds for 4 hours.

4.4.17. Immunoblot analysis

Initially, the HeLa cells were incubated in the absence and presence of IFN- γ along with various compounds (5 μM) according to the previous procedure. Media was discarded, and cells were lysed by using lysis-buffer containing 1M Tris (pH 6.8), 20 % glycerol, 4 % SDS, 400 mM DTT, and 0.004 % bromophenol blue and stored at $-20\text{ }^{\circ}\text{C}$ for further analysis. Protein extracts were separated by 12 % SDS-PAGE and transferred into nitrocellulose blotting membrane. Then, skimmed milk was used for blocking for 2 hours. Anti-IDO antibody and GAPDH were used in dilutions of 1:500 and 1:5000, respectively for overnight. Protein of interest were detected by HRP-conjugated goat anti- Rabbit antibody in dilution 1:5000 for 2 hours. Further, the visualization was performed using the ECL western blotting substrate.

4.4.18. Direct measurement of heme content in IDO1 purified from the IFN- γ activated cells

Initially, the HeLa cells were incubated in the presence of IFN- γ and L-Trp along with various compounds (50 μM) according to the previous procedure.¹⁹ The media was discarded, and cells were lysed using lysis buffer according to the reported

method. The lysate was incubated with an anti-IDO antibody (2 µg/mL of lysate) and followed by protein G-agarose at 4 °C. The solution was centrifuged and discarded the supernatant. The precipitate was washed with Tris buffer, pH 7.4, and the protein was eluted with 200 mM glycine buffer, pH 2.6. The solution was further neutralized with Tris buffer, pH 8.0, and used for further analysis. The purified protein was denatured with NaOH solution. To verify that the potent quinine derivatives are inhibiting intracellular IDO1 activity by increasing the levels of IDO1 apo-protein compared with that of IDO1-holo-protein, the heme content was directly measured by HPLC and MALDI-TOF analysis. The solution was analysed in HPLC using acetonitrile/water mobile phase with a C18 column

4.5. References

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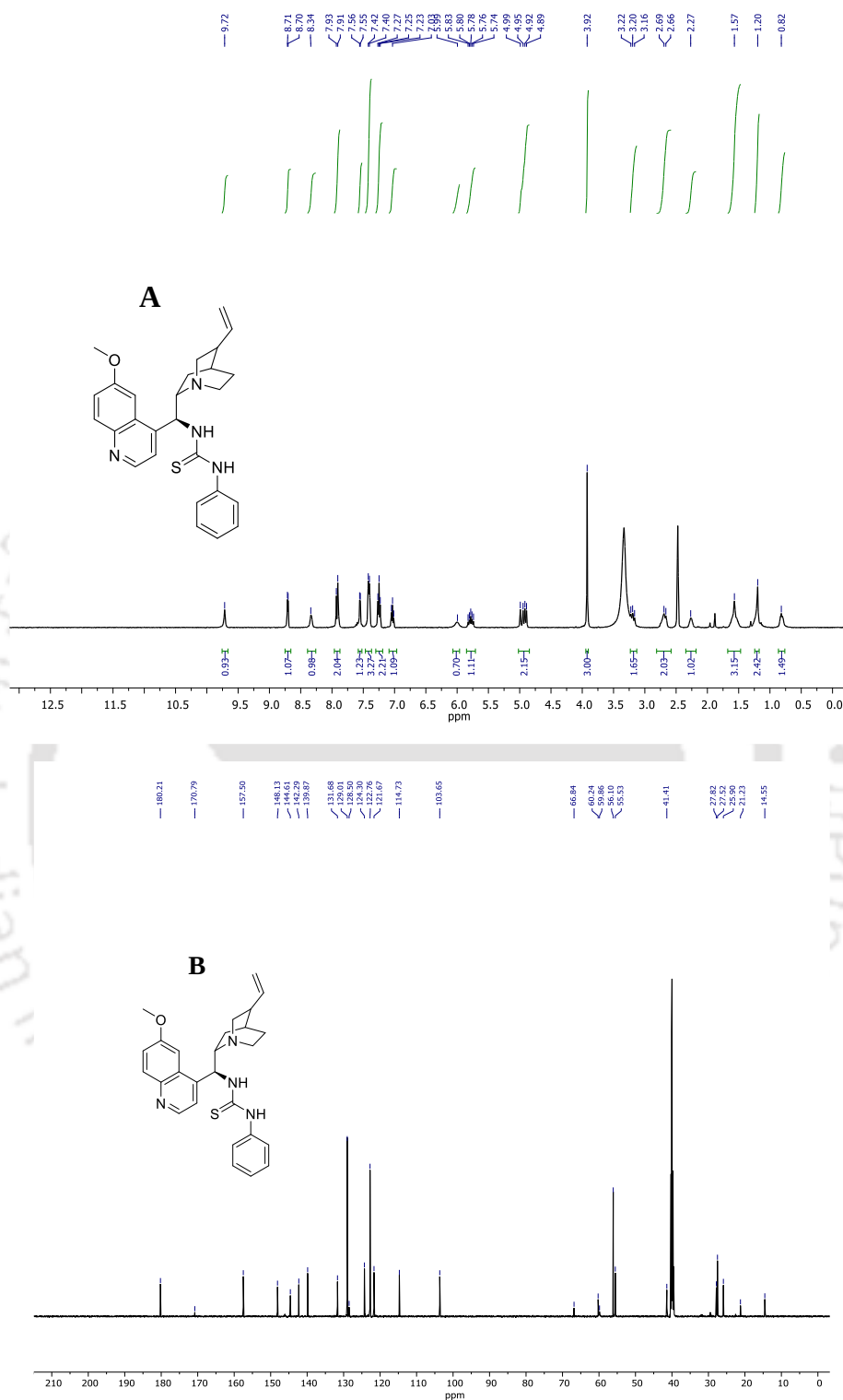
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4.6. NMR spectra of the synthesized compound

Figure 4.20 ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 4.3a.

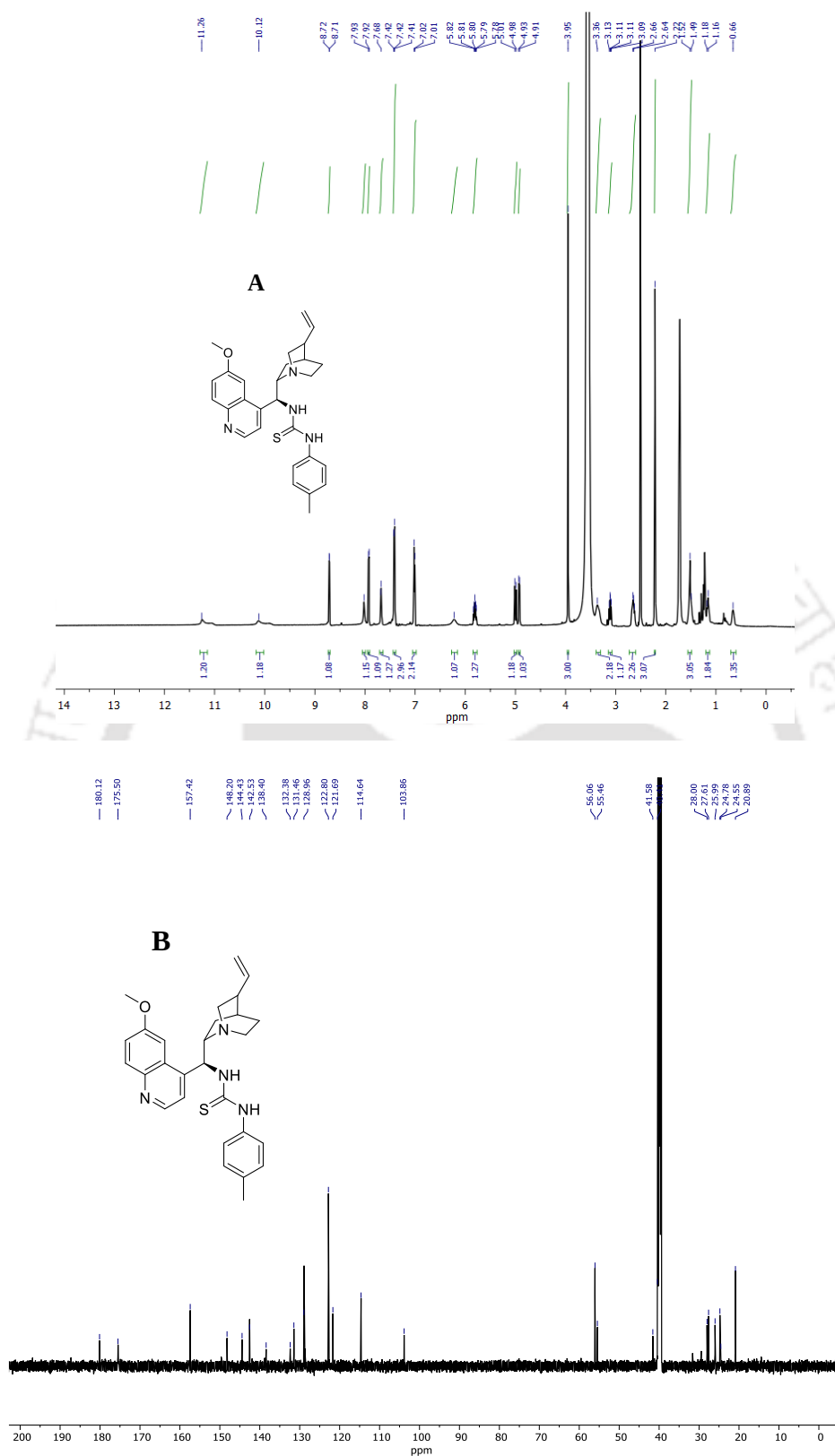
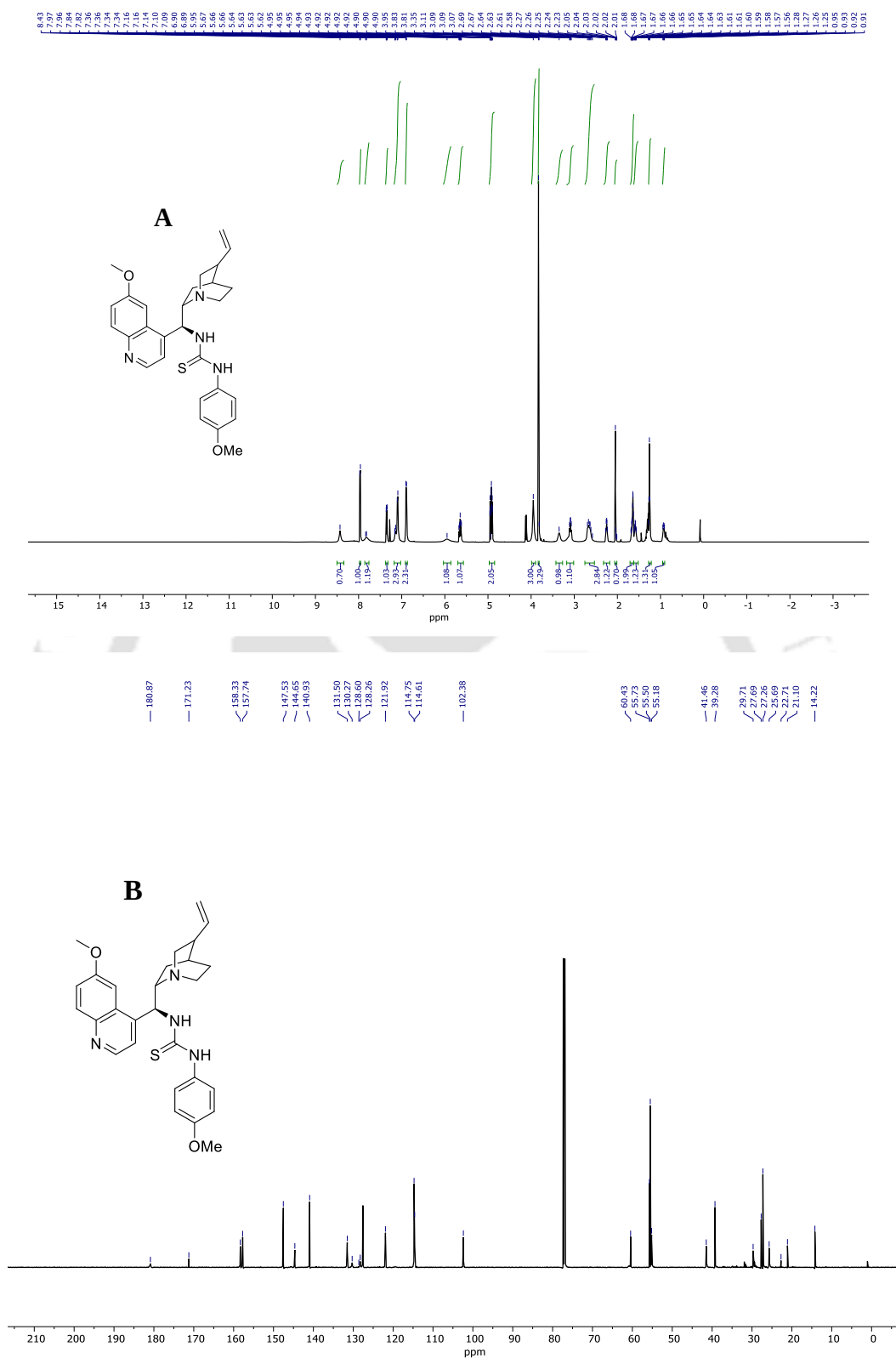


Figure 4.21 ^1H NMR (A) and ^{13}C NMR (B) spectra of compound **4.3b**.



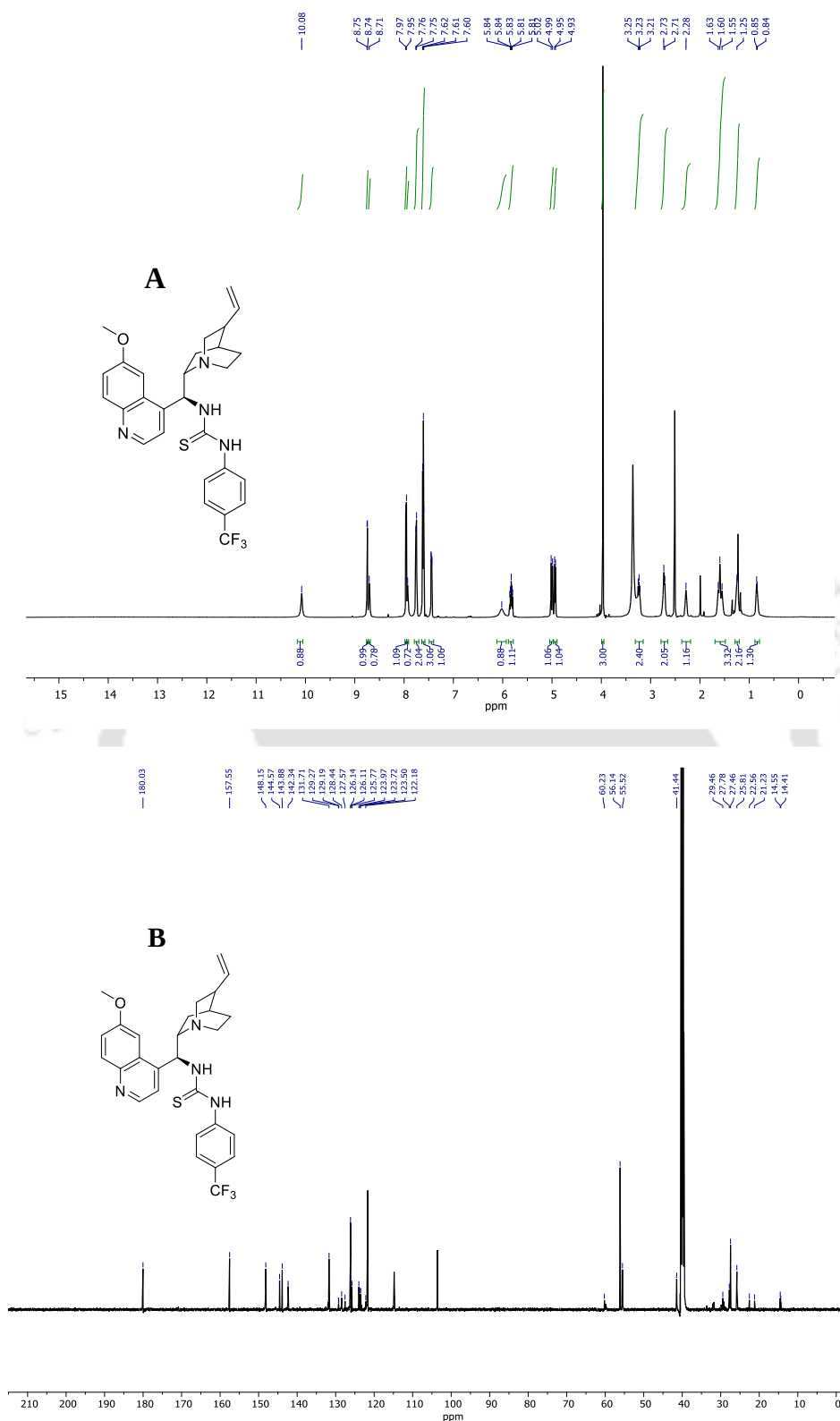


Figure 4.23 ¹H NMR (A) and ¹³C NMR (B) spectra of compound **4.3d**.

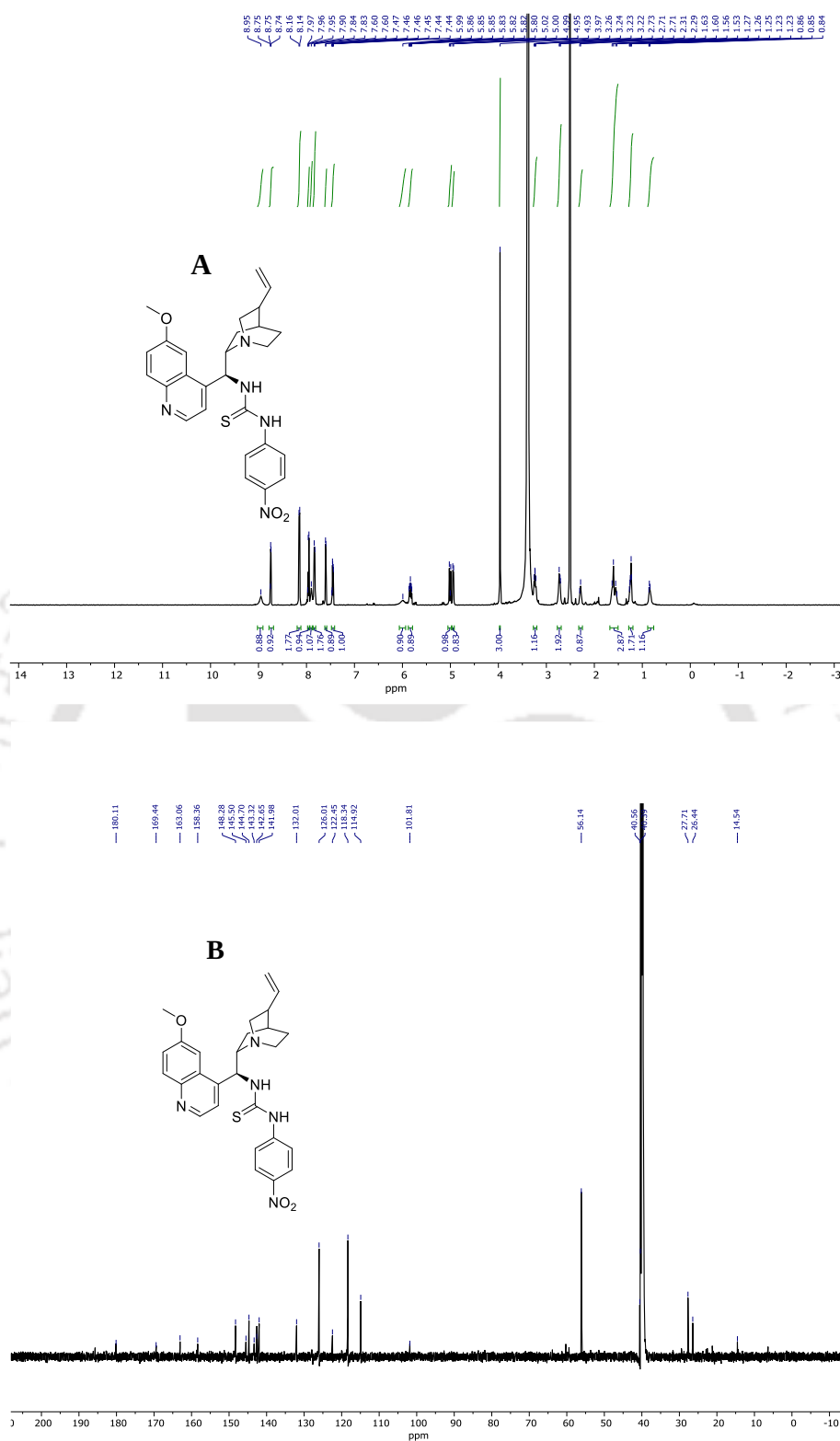
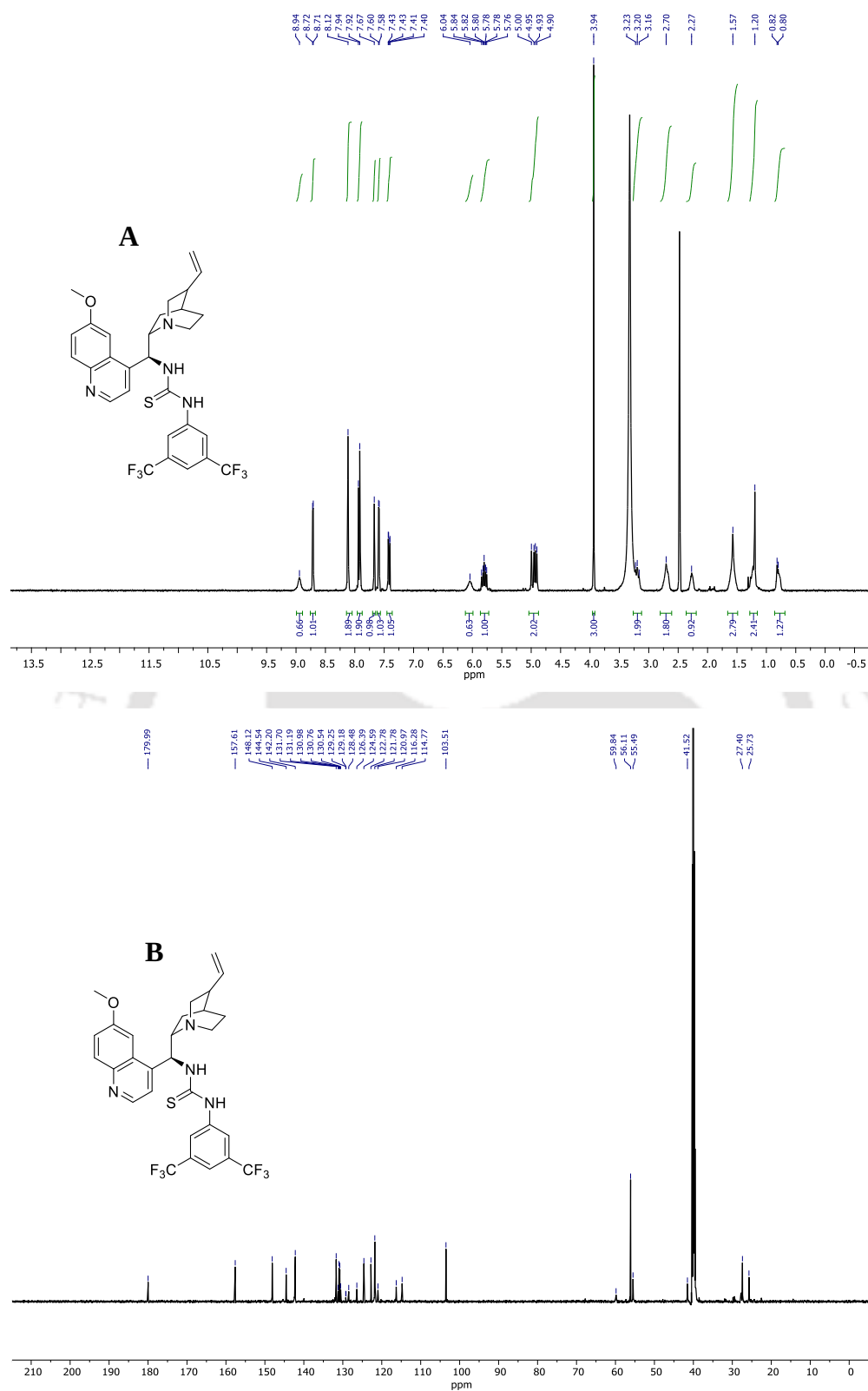


Figure 4.24 ^1H NMR (A) and ^{13}C NMR (B) spectra of compound **4.3e**.



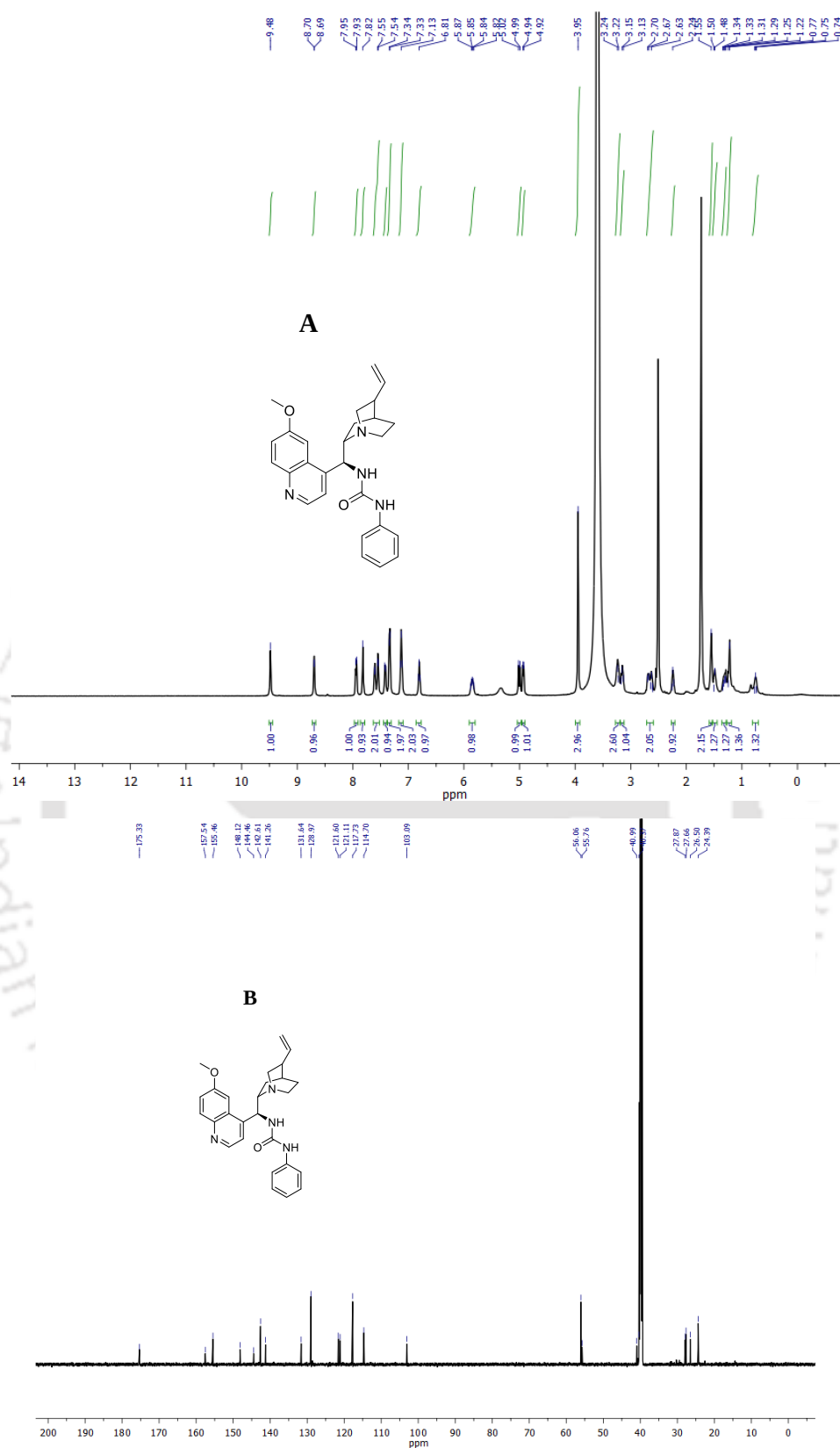
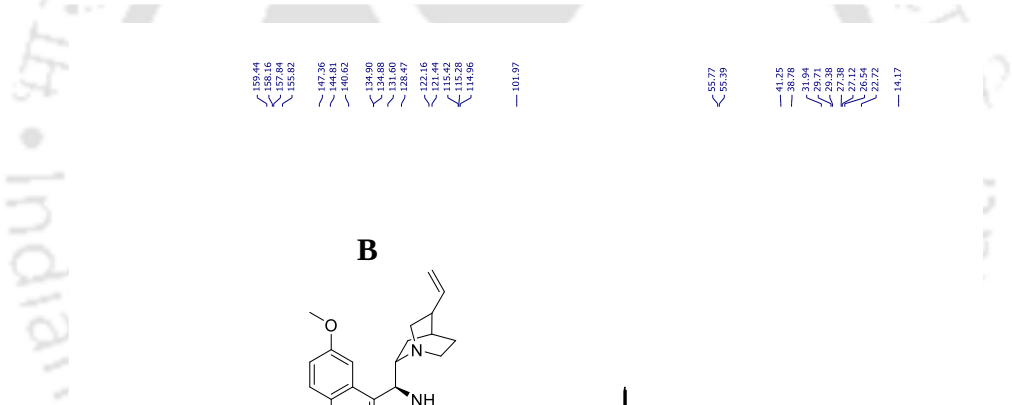


Figure 4.26 ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 4.4a.



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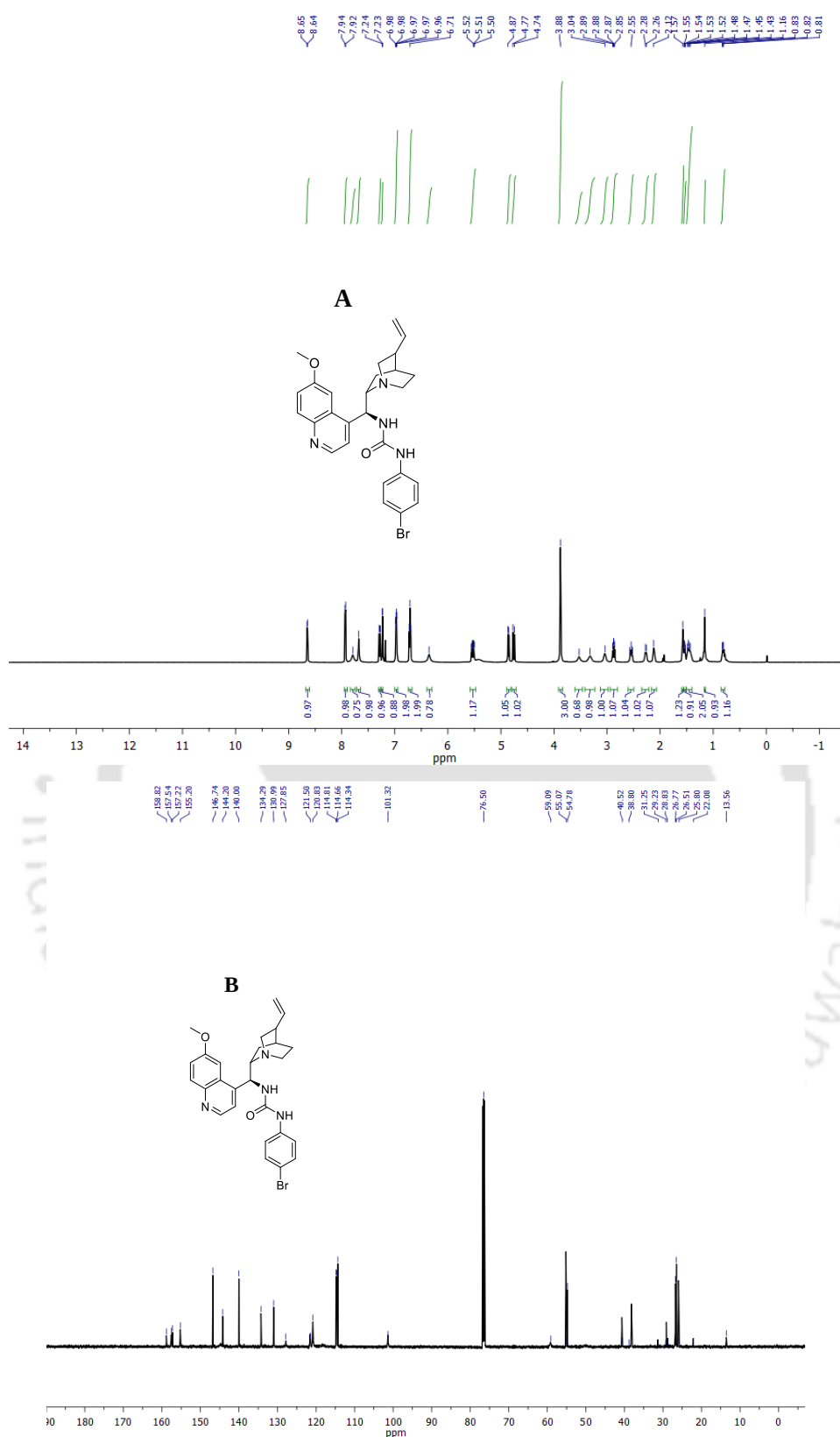
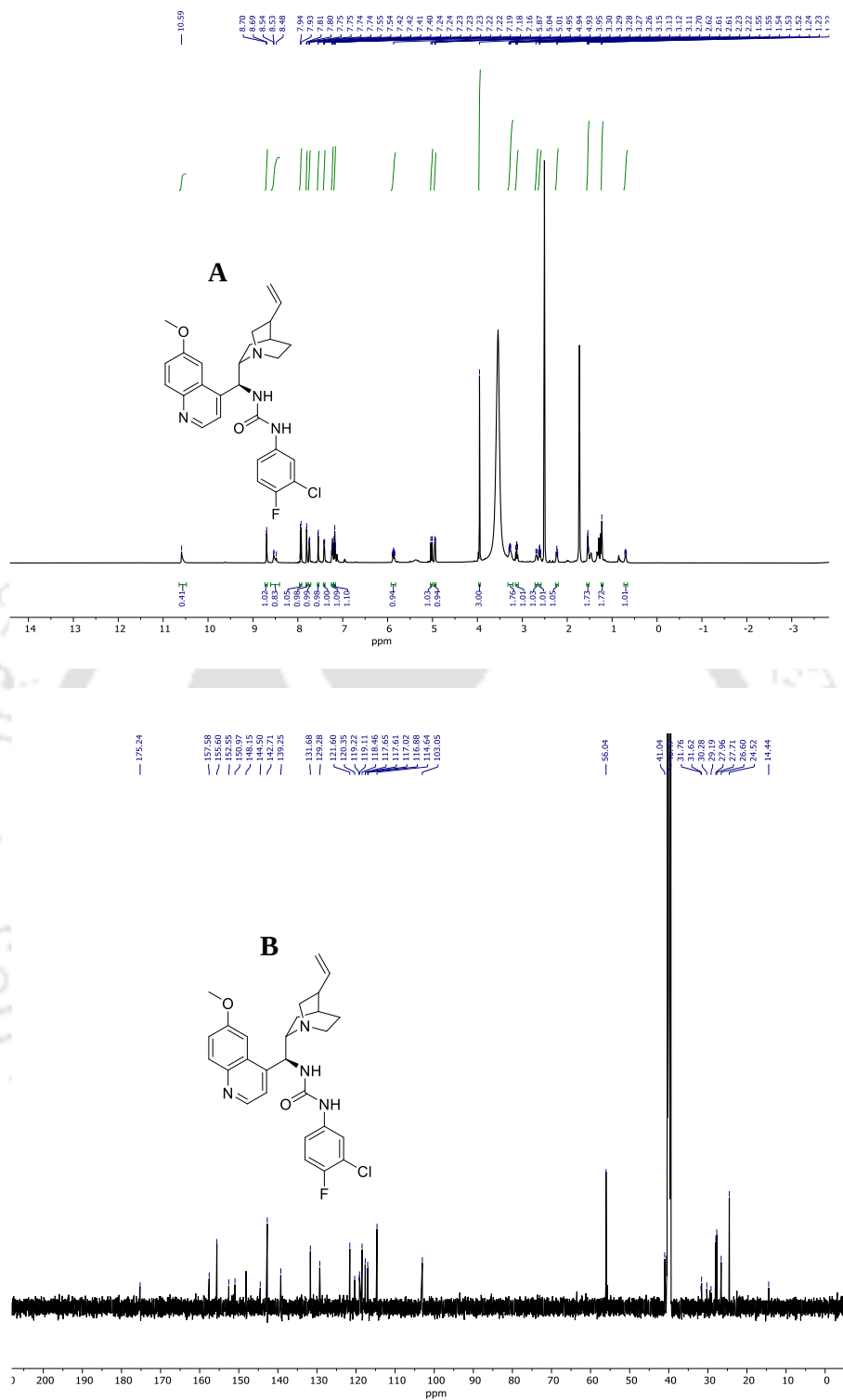


Figure 4.29 ^1H NMR (A) and ^{13}C NMR (B) spectra of compound **4.4d**.



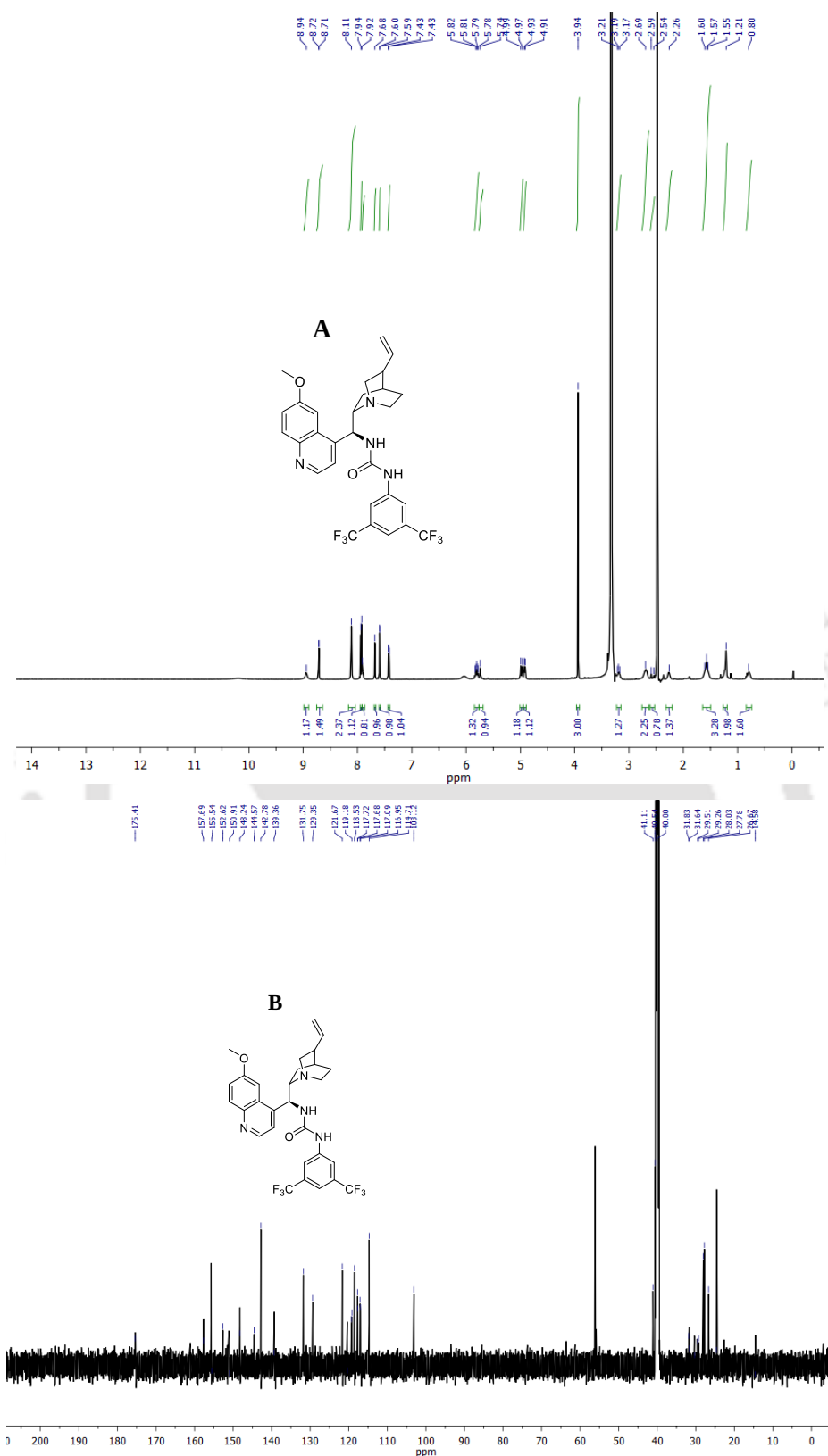


Figure 4.31 ^1H NMR (A) and ^{13}C NMR (B) spectra of compound **4.4f**.

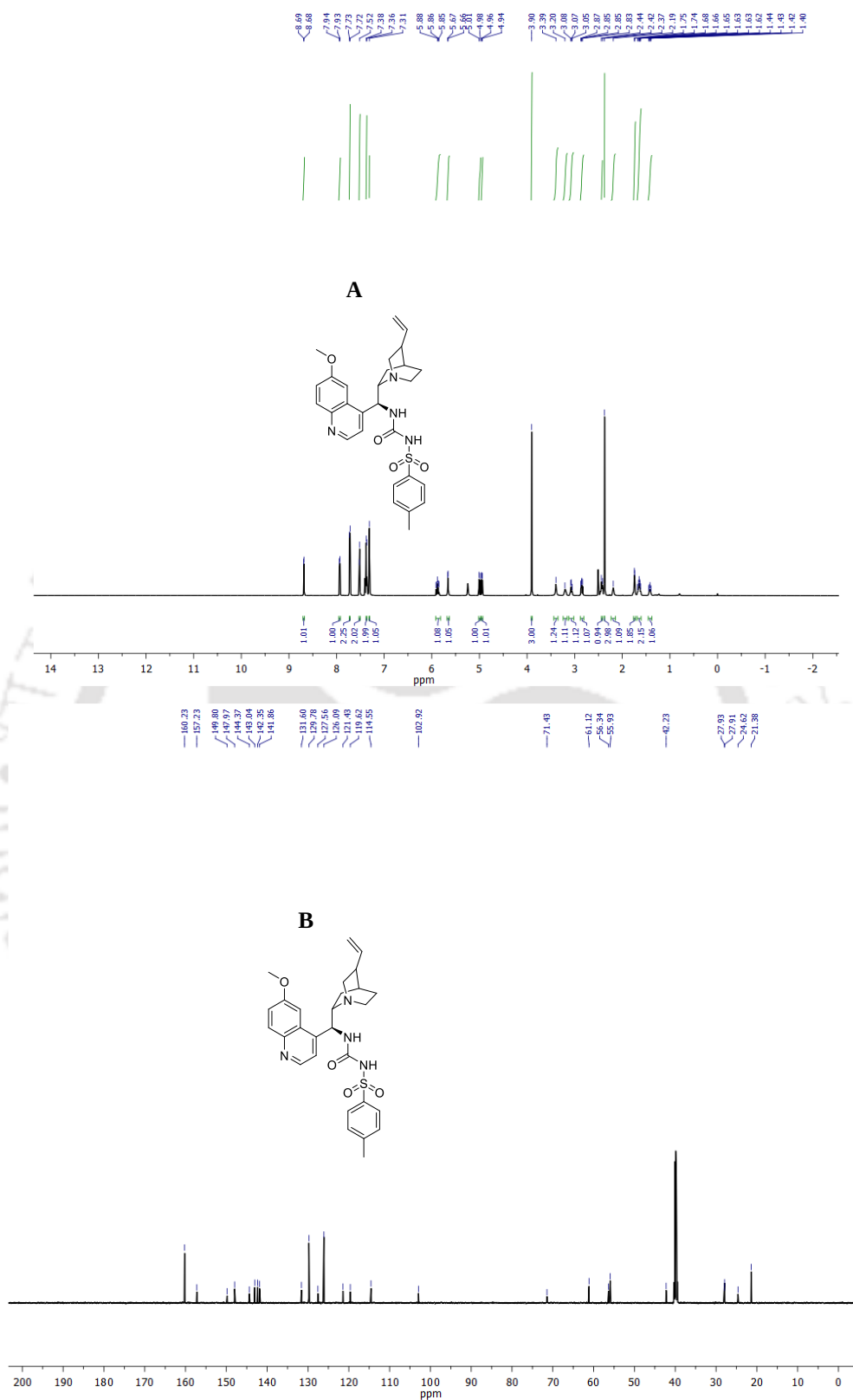


Figure 4.32 ^1H NMR (A) and ^{13}C NMR (B) spectra of compound **4.4g**.

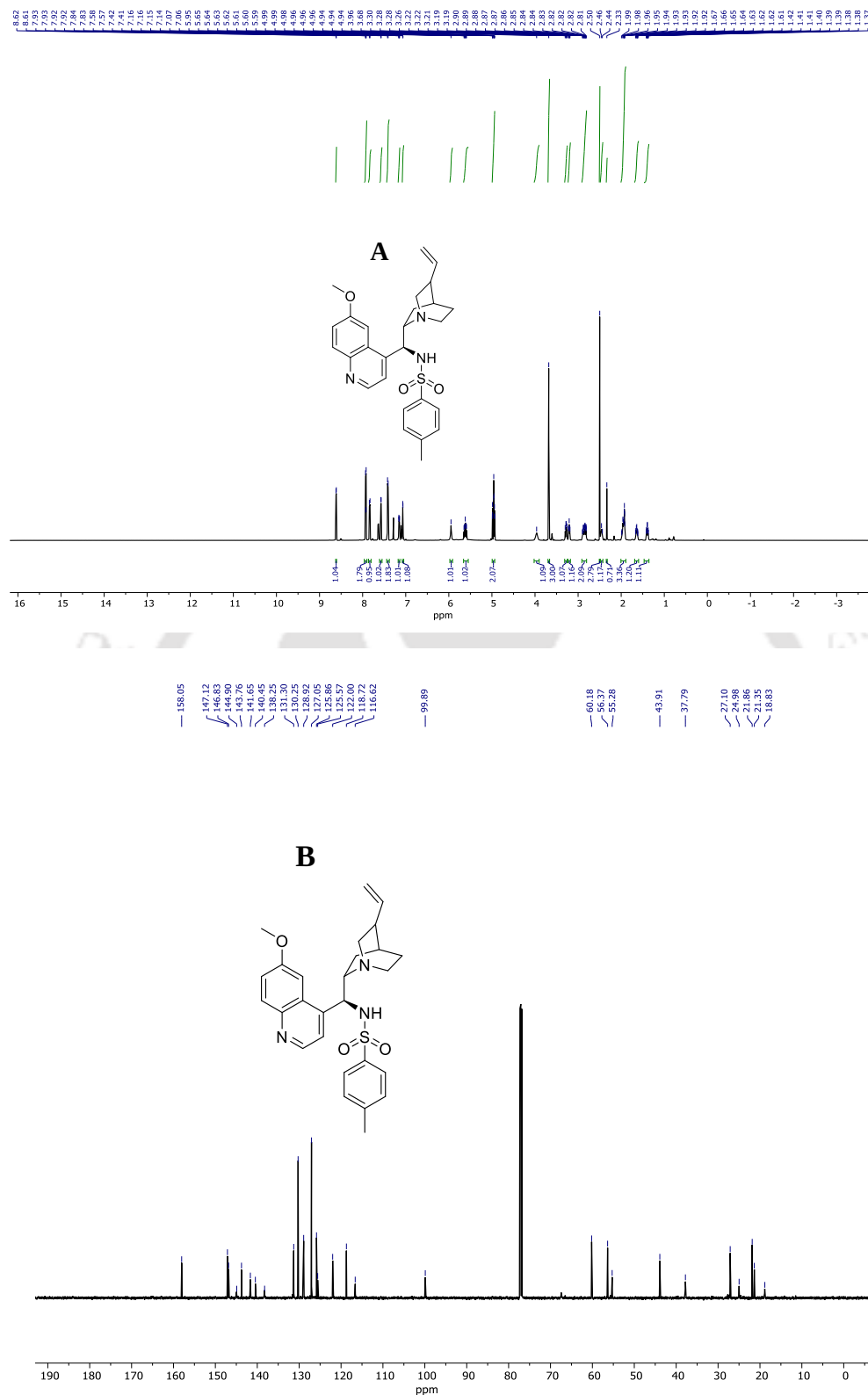


Figure 4.33 ^1H NMR (A) and ^{13}C NMR (B) spectra of compound **4.5a**.

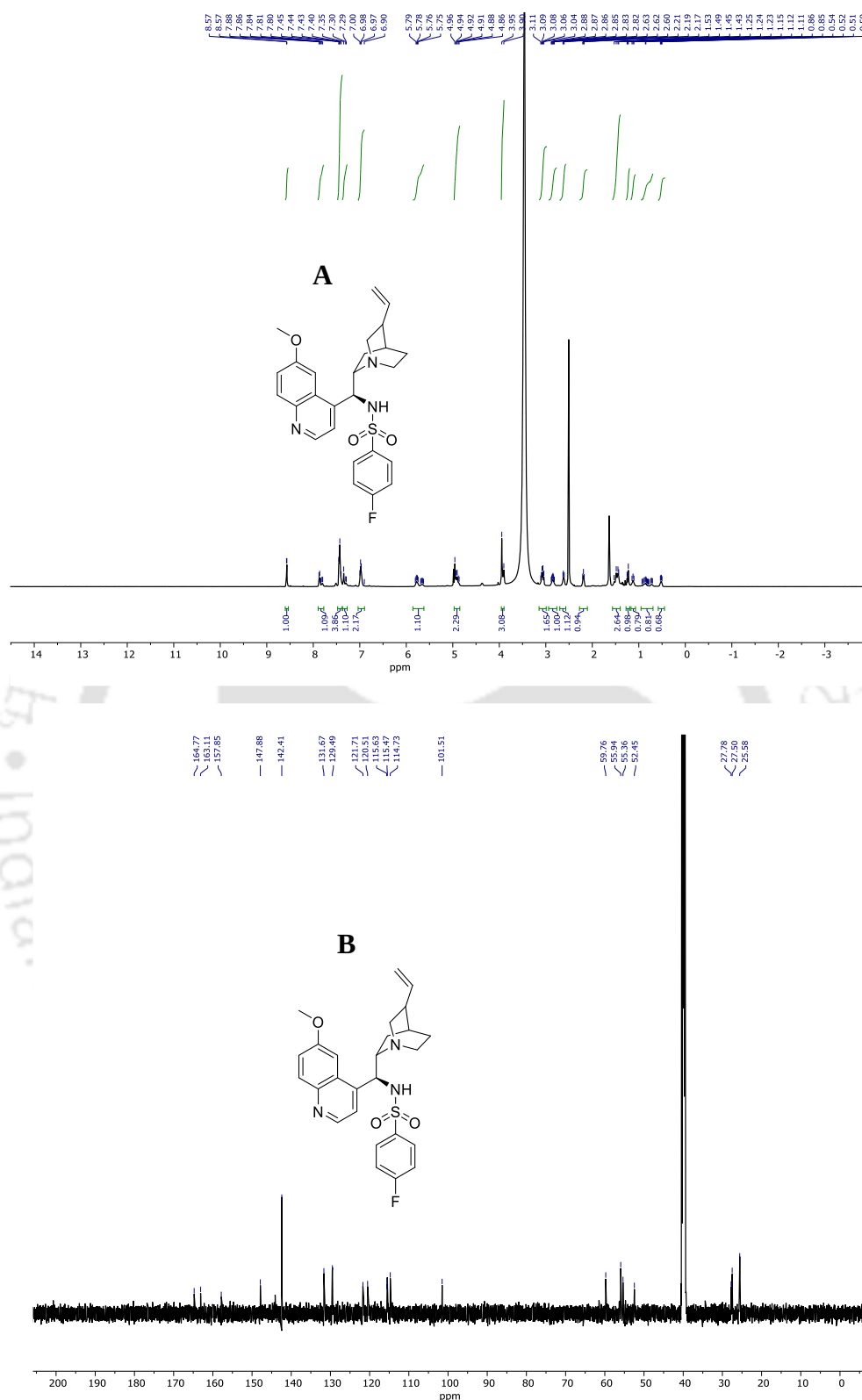


Figure 4.34 ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 4.5b.



Chapter 5

*Glutathione-responsible tumor-homing peptide
amphiphile for the co-delivery of doxorubicin and
immunosuppressive IDO1 enzyme inhibitor
promoted as chemo-immunotherapy*



5.1. Background and objective of present work

Recent studies demonstrated that the chemotherapeutic drug (like doxorubicin and oxaliplatin) mediated apoptosis and necrosis of the tumor cells also promote the host immune system via ICD through the expression of CRT on dying tumor cell surface.¹ The ICD facilitates the action of antigen-presenting DCs against cancerous cells and boosts antitumor immune response.² However, the use of only a chemotherapeutic drug for cancer treatment is often inadequate for the treatment of the patients. Serious side effects related to non-tumor-specific delivery of the chemotherapeutic drugs suppress its beneficial cancer immunogenicity and immune responses. The low penetration ability of chemotherapeutic drugs to tumor cells and detrimental tumor micro-metastases often leads to tumor recurrence. Tumors also generate various immunosuppressive mechanisms such as overexpression of immunosuppressive elements or immunocheckpoint proteins to offset the antitumor ability of the immunogenic chemotherapeutics.^{3, 4} Hence, inhibition of negative immune regulation is considered as one of the potential strategies to rejuvenate the antitumor immune response. IDO1 activity is directly associated with the generation of the immunosuppressive tumor microenvironment.⁴ Meanwhile, various chemotherapeutic drugs also showed upregulation of the IDO activity in the tumor microenvironment.^{4, 5} Therefore, the use of IDO inhibitors in combination with the chemotherapeutic agent could provide a beneficial synergistic antitumor activity. However, lipophilicity and poor pharmacokinetics behaviors of most of the chemotherapeutic drugs limit their clinical application. In this regard, various advanced nano-drug delivery systems were utilized to control the limitations of the immunomodulators and chemotherapeutic drugs to augment the effectiveness of chemoimmunotherapy^{2, 4, 6-8}

Mesoporous silica nanoparticles mediated co-delivery of oxaliplatin with IDO1 inhibitor (indoximod) in PDAC model showed improved antitumor immunity.⁶ The liposome-encapsulated co-delivery of Dox and phospholipid-conjugated indoximod prodrug showed synchronized ICD initiation and immunological modulation in metastatic breast cancer cells.² The PEG-mediated co-delivery of IDO1 inhibitor, NLG919, and paclitaxel showed enhanced antitumor efficacy in several tumor models.⁸ The CLANs mediated delivery of siIDO1 with oxaliplatin to the colorectal pancreatic cancer models revealed an improved antitumor efficacy.⁴ Folic acid-

conjugated pH-degradable nano-gel mediated delivery of NLG919 and docetaxel showed enhanced anticancer activities on the 4T1-breast cancer metastasis mouse model due to the activation of ICD and reversal of IDO1-associated immunosuppressive consequence.⁷ In the meantime, pH-sensitive hydrophilic peptide, RGD was successfully used for target-specific drug delivery to the cancer cells. The RGD-conjugated NLG919 prodrug showed enhanced IDO1 inhibitory activity and reduced systematic toxicity than the NLG919 itself. The prodrug nanovector showed enhanced antitumor immunity of PD-L1 checkpoint inhibitor.⁹ Recently developed apo-IDO1 inhibitors showed very strong IDO1 inhibition potency, but their selective delivery to tumor tissues and co-delivery with chemotherapeutic drugs in achieving combined chemotherapy with blockage of the IDO pathway to fight cancer was not investigated.^{10, 11}

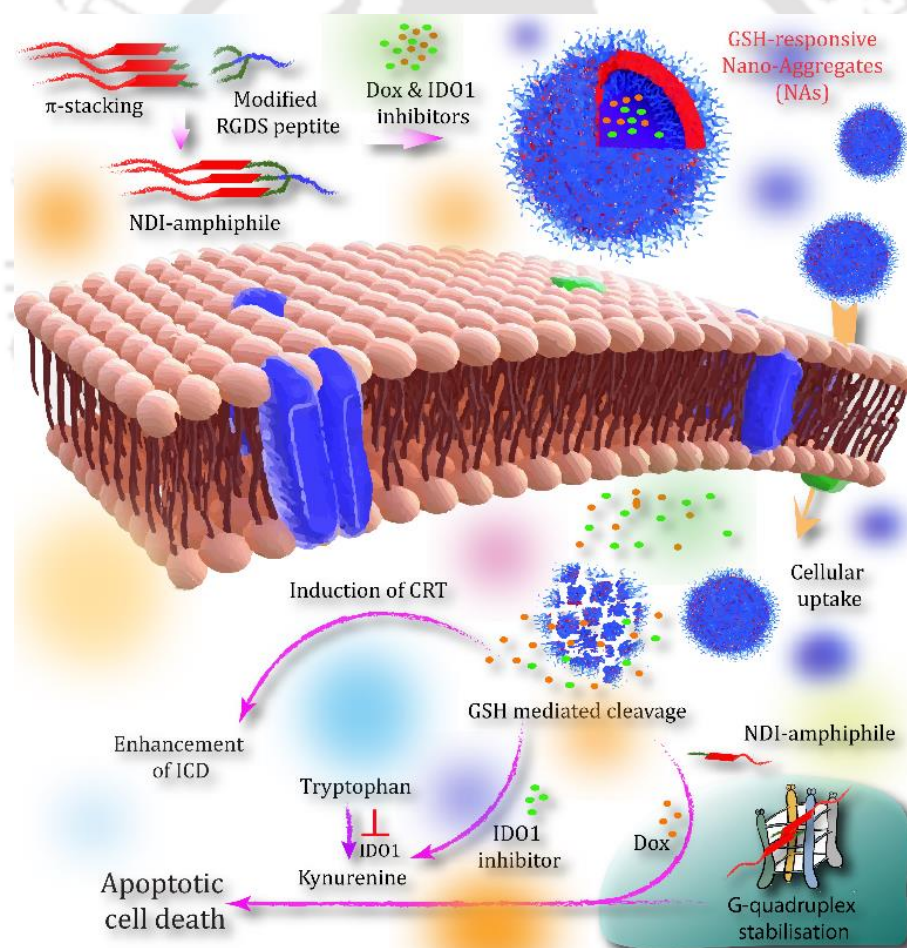
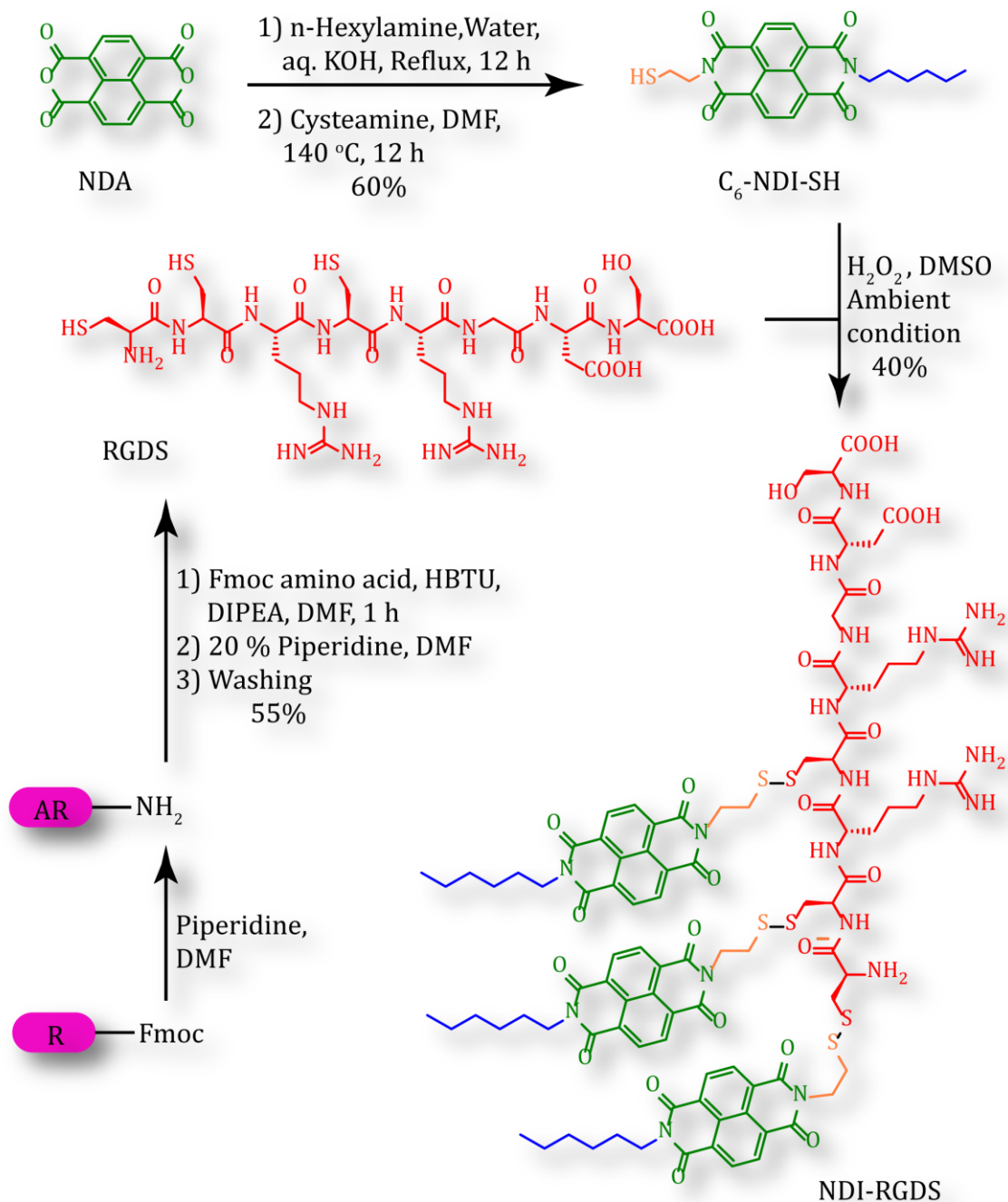


Figure 5.1 Schematic representation of the synthesis of NDI-conjugated GSH-responsive RGDS nanovehicle formulation and successful co-delivery of the chemotherapeutic agent (Dox) and immunosuppressive enzyme inhibitors to the targeted cancer cells.



Scheme 5.1 Synthetic route to **NDI-RGDS** amphiphile.

The development of stimuli-responsive nanocarriers is considered a promising strategy for target-specific delivery of antineoplastic drugs. Various cancer cell markers, including pH, redox potential, selective protein/enzyme in the tumor microenvironment, were used as stimuli. The overproduction of antioxidants such as GSH within the cancer cells minimizes the effect of ROS and reduce oxidative stress-mediated apoptosis.^{2, 7, 9, 12-14} In this regard, GSH-responsive nanocarriers were developed to deliver chemotherapeutic drugs selectively to the cancer cells.

Meanwhile, the RGD peptides have been demonstrated to significantly augment the tumor-homing competence of the drug delivery systems due to their binding to the $\alpha_v\beta_3$ integrin receptor, a cancer cell biomarker.⁹ Recent studies revealed that NDI derivatives stabilize the telomeric G-quadruplex, which aids in apoptotic cell death. The NDI chromophore was incorporated in several amphiphiles as it assists the formation of stable aggregation through π -stacking.^{15, 16} Hence, the development of NDI conjugated RGD peptide-based GSH-responsive nanocarrier may improve selective co-delivery of the drug molecules to malignant tumor cells and accomplish triggered release of drugs in the reductive tumor microenvironment. In addition, the GSH-mediated generation of NDI derivatives could also induce apoptotic cell death via stabilization of the G-quadruplex of the cancer cells (Figure 5.1). We hypothesize that potential inhibition of IDO1 activity in tumor tissues would significantly augment the immunogenic effect of Dox and other chemotherapeutic drugs, and the use of stimuli-responsive target-specific nanocarrier could enrich the chemo and immunotherapeutic efficacy of both Dox and IDO1 inhibitor to tumor tissues. Therefore, the amphiphile with triple-functions – a) RGD-mediated targeted delivery, b) GSH-responsive endogenous cleavage to release encapsulated drug molecules, and c) stabilization of G-quadruplexes by the in situ generated NDI ligands could act as a potential nanocarrier for chemoimmunotherapeutic treatments.

5.2. Results and discussions

5.2.1. Synthesis and characterization of NDI-RGDS amphiphile

The GSH-responsive modular amphiphilic **NDI-RGDS** was synthesized in two steps, as shown in Scheme 5.1. The CCRCRGDS peptide was synthesized by solid-phase peptide synthesis method using Fmoc protected amino acids. Three cysteine residues were included to prepare cleavable amphiphile with tri-alkyl chains. The trichain is known to provide better stability and drug or gene encapsulation efficiency to the amphiphiles.¹⁷ The lipophilic alkyl chain containing naphthalene diimide and thiol moieties (**C₆-NDI-SH**) was synthesized according to the reported protocol, where naphthalene-1,4,5,8-tetracarboxylic dianhydride was coupled with hexylamine and cysteamine.¹⁸ Finally, the lipophilic **C₆-NDI-SH** was then linked with the CCRCRGDS peptide via disulfide linkage in the presence of H₂O₂ in DMSO solvent. The successful synthesis of **NDI-RGDS** was confirmed by ¹H NMR, HPLC, and ESI-MS.

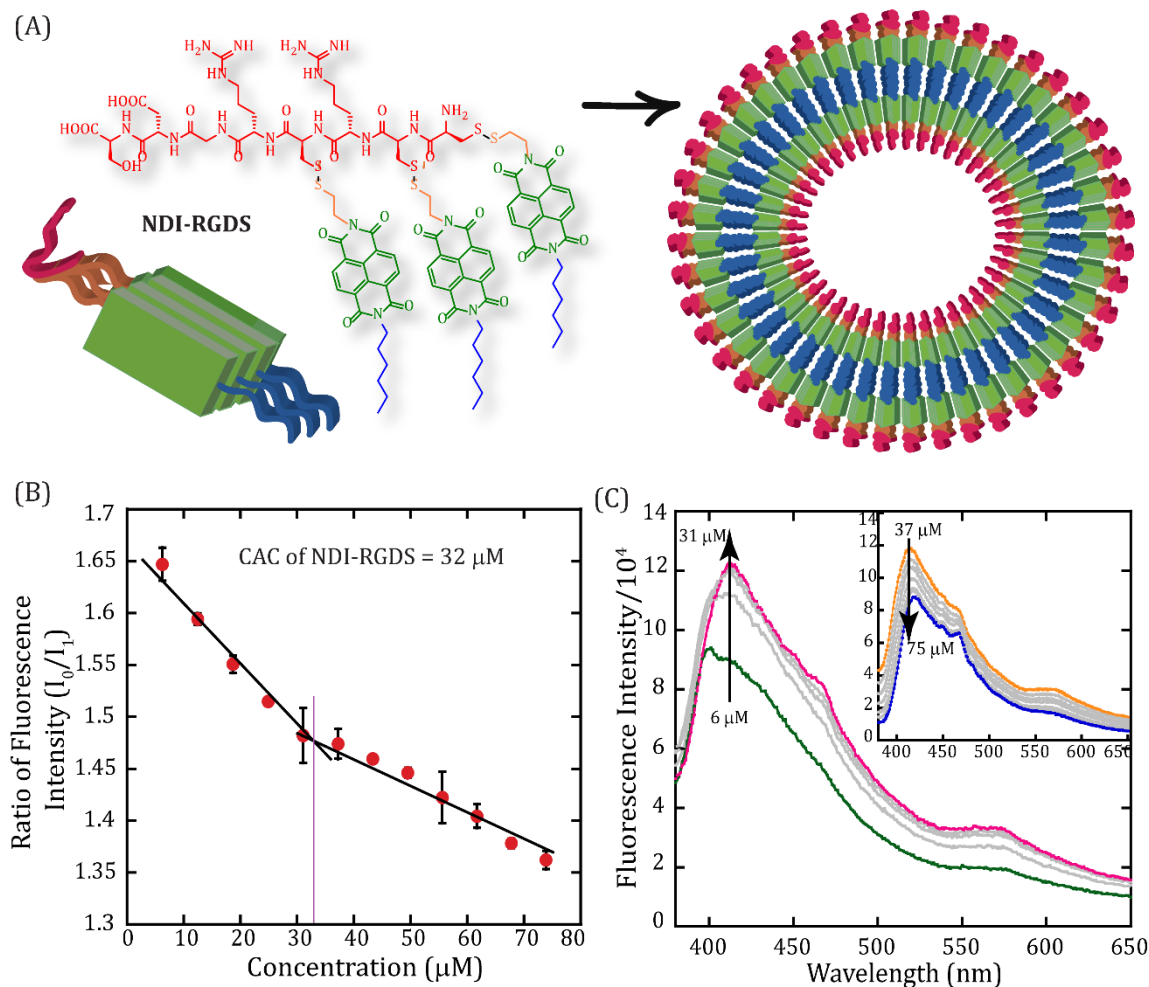


Figure 5.2 Cartoon representation for the probable formation of NAs in the aqueous medium (A). Concentration-dependent fluorescence measurements to calculate the CAC values of the amphiphile (B) and concentration-dependent fluorescence spectra of **NDI-RGDS** (C).

5.2.2. Physicochemical properties of NDI-RGDS amphiphile

The hydration of amphiphilic **NDI-RGDS** led to the formation of colloidal aggregates. The concentration-dependent investigation of NDI fluorescence properties ($\lambda_{\text{ex}} = 365 \text{ nm}$ and $\lambda_{\text{em}} = 419 \text{ nm}$ (I_0), 468 nm (I_1)) revealed a continuous increase in fluorescence intensity up to $31 \mu\text{M}$ and then decreased gradually (up to $70 \mu\text{M}$). The variation of I_0/I_1 against concentration revealed the CAC value of $32 \mu\text{M}$ (Figure 5.2). The π -stacking interactions between the NDI moieties and hydrophobic interactions could be the driving force for their self-assembly in the aqueous medium. To identify the morphology of the **NDI-RGDS** in the aqueous environment,

we performed FESEM and FETEM analyses. The FESEM (Figure 5.3A) and FETEM (Figure 5.3B) analyses

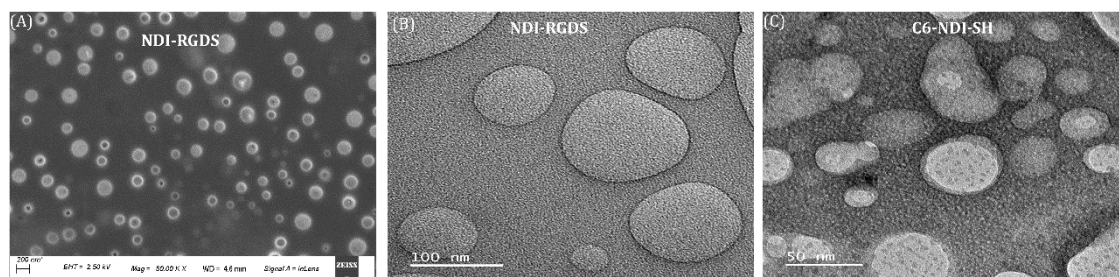


Figure 5.3 Representative FESEM (A) and FETEM (B) images of the amphiphile. Representative FETEM image of **C₆-NDI-SH** (C).

confirmed the formation of spherical NAs with a diameter of 200-300 nm. The FETEM analyses of **C₆-NDI-SH** also showed the formation of spherical aggregates, suggesting π -stacking interactions interaction of NDI moieties (Figure 5.3C). Spherical aggregation of such smaller sizes might be advantageous for the delivery of drugs due to the EPR effect in blood vessels and easy uptake behaviour within tissues. The pH-dependent (3-8.5) DLS measurements revealed its d_H are within the range of 180-200 nm, suggesting its stability in different environments (Figure 5.4A). The DLS measurements in the presence of human serum albumin (0-72 h) revealed the d_H values were within 210-240 nm (Figure 5.4B), suggesting its low protein corona effect in blood. The pH-dependent zeta potential measurements of NAs revealed a surface charge of +5.0 mV at physiological pH (the PI was 7.5), which could be due to the presence of arginine (PI = 10.76), aspartic acid (PI = 2.77), and serine (PI = 5.68) residues within the NAs. The concomitant decrease of surface potential with the increase in pH could be due to the increase in the hydration number of the NAs in the aqueous medium (Figure 5.4C). The moderate positively charged NAs and the presence of surface-exposed RGDS peptide sequence could be useful for selective targeting of the cancer cells via its interaction with the $\alpha_v\beta_3$ integrin receptor. The pH stability of the NAs instigated us to explore their thermal stability. The higher phase transition temperature (T_m) validates the thermal stability of the NAs. The temperature-dependent fluorescence anisotropy measurements of DPH encapsulated NAs demonstrated the T_m value of 54 °C (Figure 5.4D), which is much

higher than the DPPC ($T_m = 41\text{ }^{\circ}\text{C}$). The π -stacking interactions between the NDI moieties present within the hydrophobic core of the NAs might be responsible for its higher T_m value than that of DPPC. The higher T_m value suggests its better drug retention ability at normal body temperature.

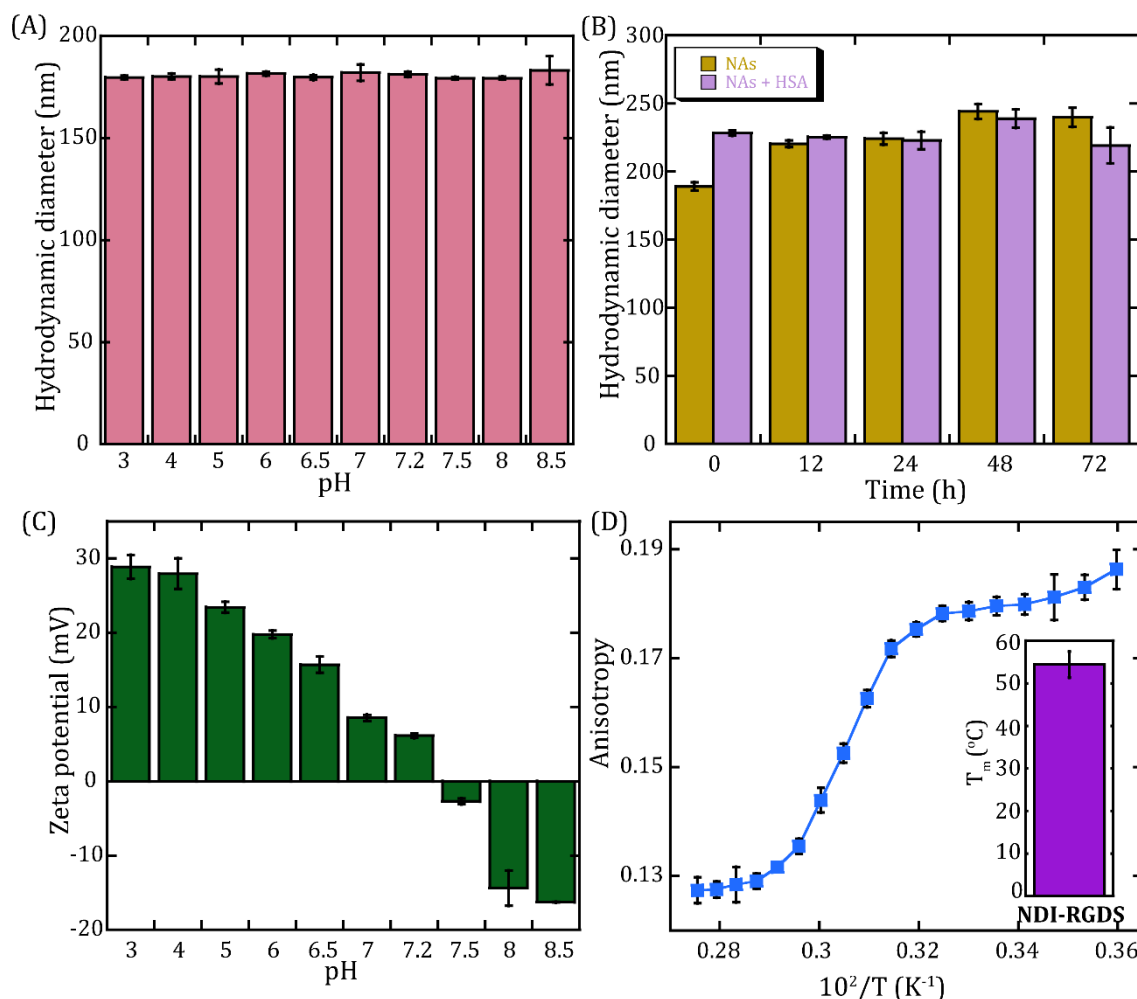


Figure 5.4 Variation of hydrodynamic diameter at different pH (A) and NAs in the absence/presence of HSA protein at different time intervals (B). Zeta potential (C) of the amphiphile at different pH. The phase transition temperature of the amphiphile (D).

5.2.3. GSH-mediated degradation of the NDI-RGDS-based nanocarrier

The pH and thermal stability motivated us to scrutinize whether the NAs can be deformed in a controlled mode in the presence of stimuli (GSH). The high level of GSH is a recognized biomarker of cancer cells. The presence of disulfide bonds in the core structure of the NAs might be susceptible to the elevated level of GSH selectively. To

confirm the GSH-mediated deformation, the NAs were incubated at 37 °C for 12 hours in the presence of a high level of GSH (10 mM). The RAMAN spectroscopic analysis revealed the cleavage of S-S bond linkage (at 510 nm) under the redox environment, which could be the primary reason for its morphological changes of the NAs (Figure 5.5A).¹⁹ The FETEM studies revealed a significant difference in the morphology of the NAs, suggesting its instability in the presence of a high concentration of GSH (Figure 5.5B). Hence, pH and thermal stability and GSH-responsive deformation of the NAs could be beneficial for cancer cell-specific drug-release application.

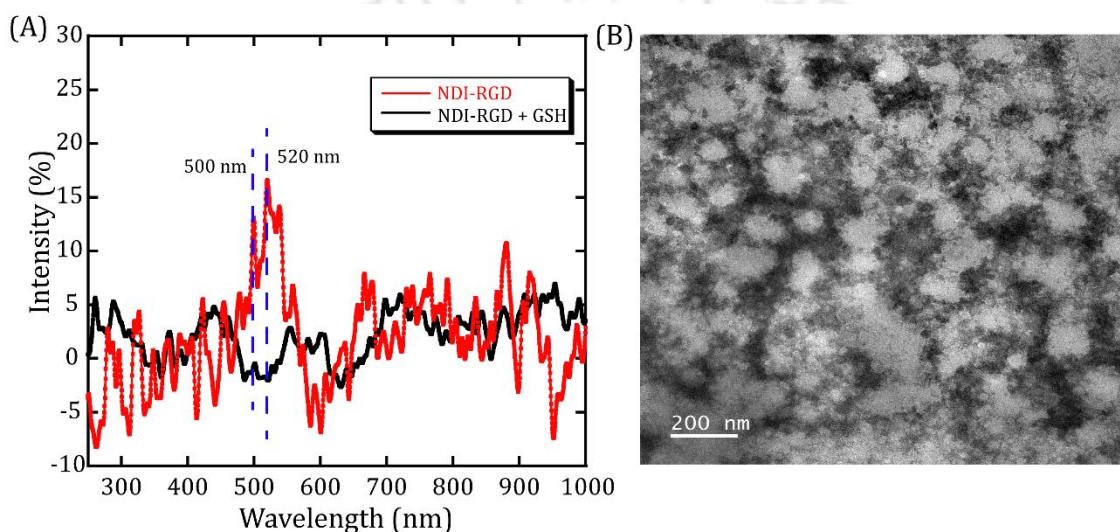


Figure 5.5 RAMAN spectroscopic analysis of **NDI-RGDS** in the absence and presence of GSH (A). Representative FETEM analysis of **NDI-RGDS** in the presence of GSH (B).

5.2.4. Binding and stabilization of G-quadruplex

The NDI derivatives are known to stabilize the telomeric G-quadruplex DNA, which supports the apoptosis of cancerous cells.¹⁸ In this regard, the stabilization of the human telomeric G-quadruplex (5'-AGGGTTAGGGTTAGGGTTAGGG-3') was evaluated in the presence of **C₆-NDI-SH**, which gets generated after the GSH-mediated degradation of the nanocarrier. We performed the fluorescence-based **C₆-NDI-SH** and G-quadruplex binding analysis. The inherent fluorescence property of the NDI scaffold was monitored after subsequent addition of G-quadruplex to **C₆-NDI-SH** solution. The fluorescence emission intensity of the **C₆-NDI-SH** ($\lambda_{\text{ex}} = 365$; $\lambda_{\text{em}} = 419$ (I_0) and 468 (I_1) nm were found to be enhanced upon incremental addition of G-quadruplex. Furthermore, the concomitant decrease of I_0/I_1 emission intensity

also suggested the intercalated binding mode of **C₆-NDI-SH** within the G-quadruplex (Figure 5.6A and 5.6B). In addition, we performed the CD spectroscopic-based G-quadruplex melting assay to investigate the stabilization of G-quadruplex. Upon adding **C₆-NDI-SH** to the G-

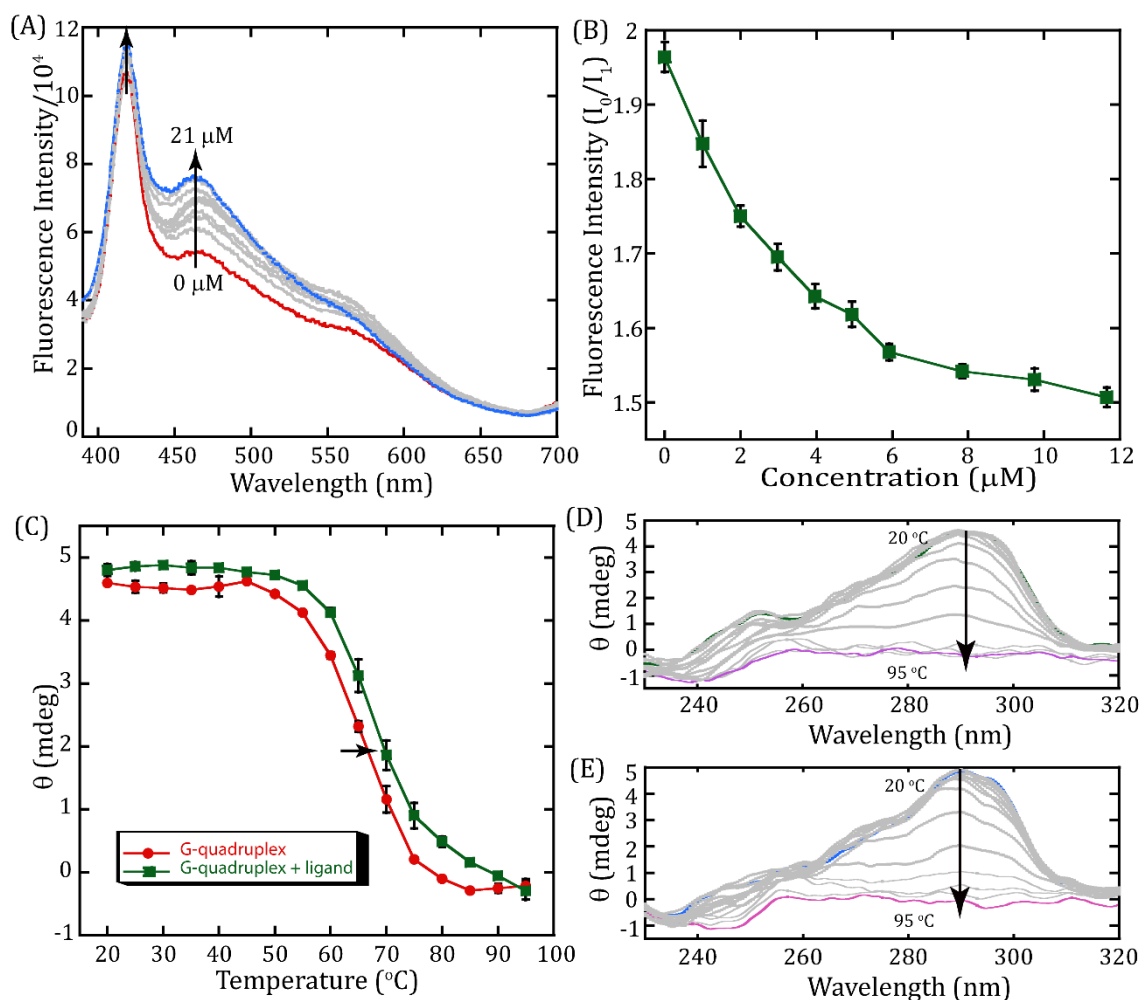


Figure 5.6 Fluorescence-based binding analysis of **C₆-NDI-SH** with G-quadruplex. Emission spectra of **C₆-NDI-SH** (5 μ M) (A) and intensity ratio (B) with increasing concentration of G-quadruplex (0 - 21 μ M). Plot (C) and spectra of Circular dichroism-based G-quadruplex melting assay in the absence (D) and presence (E) of **C₆-NDI-SH**.

quadruplex, there was a slight change in the CD spectra, which suggested the integrity of the G-quadruplex topology. Further, the temperature-dependent difference in the secondary structure of G-quadruplex at 290 nm was analyzed in the presence and absence of **C₆-NDI-SH**. The T_m of G-quadruplex enhanced from 65 $^{\circ}$ to 73 $^{\circ}$ C in the presence of **C₆-NDI-SH**, which suggested the stabilization of G-

quadruplex by **C₆-NDI-SH** (Figure 5.6C, 5.6D and 5.6E). Hence, the fluorescence and CD analyses revealed that **C₆-NDI-SH** could be a suitable ligand of G-quadruplex DNA. Therefore, the GSH mediated disruption of **NDI-RGDS** NAs might be advantageous for inducing apoptosis in cancer cells through a significant stabilization of human telomeric G-quadruplex.

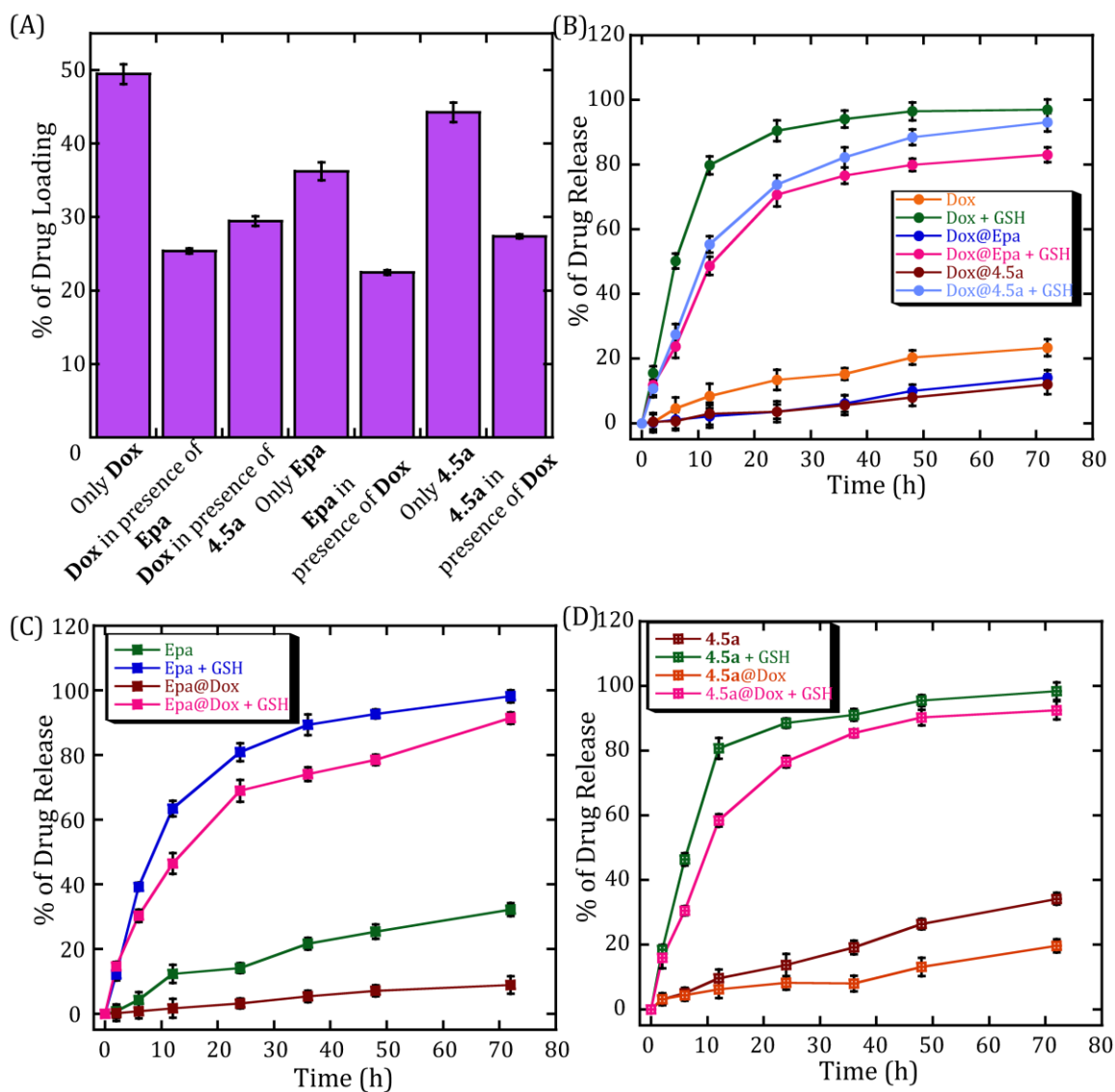


Figure 5.7 HPLC-based loading (A) and release (B, C, and D) profiles of Dox, Epacadostat (Epa), and compound **4.5a**. The release profiles were investigated both in the absence and presence of GSH.

5.2.5. Drug loading and release efficiency of NDI-RGDS-based nanocarrier

The GSH-responsive deformation of NAs encouraged us to explore its encapsulation and release efficiencies of chemo and immunotherapeutic drugs. The dual chemo and immunotherapeutic drug delivery systems were more advantageous for their selectivity and co-action towards cancer treatment.^{2, 9, 20} In this regard, the chemotherapeutic drug, Dox along with IDO1 inhibitors, Epacadostat (developed by Incyte Corporation) or **4.5a** (quinine derivative developed by our group) were encapsulated into the NAs.^{10, 21} The HPLC-based analyses revealed the encapsulation efficiency of Dox (Dox@NA), Epacadostat (Epacadostat@NA), and **4.5a**(**Q5a**@NA) were found 50%, 36%, and 44%, respectively. Whereas the co-encapsulation (dual drug) of Dox;Epacadostat@NA, and Dox;**4.5a**@NA was 25;22 and 29;27%, respectively (Figure 5.7). This moderate co-encapsulation efficiency and positive surface potential of the spherical NAs (at physiological pH) could be beneficial for the co-delivery of chemo and immunotherapeutic drug molecules to the cancer cells. The drug release efficacy of the NAs was also analysed with the HPLC-based assay (Figure 5.7). The studies showed 23 and 97% of Dox were leached after 72 h in the absence and presence of GSH, respectively, from Dox@NA. The NAs loaded with Epacadostat, and **4.5a** showed 32 and 34% release efficiency after 72 hours, respectively, in the absence of GSH. Nevertheless, GSH improved the drug release efficacy of Epacadostat@NA and **4.5a**@NA up to 97 and 98%, respectively. The release of drugs from Dox;Epacadostat@NA (14;9%) and Dox;**4.5a**@NA (12;19%) were also very slow in the absence of GSH. However, upon GSH treatments, the Dox;Epacadostat@NA (83;91%) and Dox;**4.5a**@NA (98;95%) displayed a sustained release of drugs. Such stimuli-responsive drug release profiles of the RGDS-decorated NAs could restrict the premature release of drugs in the blood circulation system and allow the controlled release of drugs to the targeted cancer cells in the presence of a high concentration of GSH. The DLS measurements also demonstrated that the size of the co-encapsulated drug-containing NAs was within 170-250 nm. The zeta potential analysis showed that the surface potential of Dox;Epacadostat@NAs and Dox;**4.5a**@NA were +6.86 and +7.11 mV, respectively (Figure 5.8).

5.2.6. GSH-mediated selective release of encapsulated drugs within the cancer cells

In order to demonstrate the cellular activities of the NAs and drug@NA, initially, the viability of MDA-MB-231 (triple-negative breast cancer) and PBMC (healthy human) cells was determined by MTT assay. The IC_{50} of free NAs in MDA-MD-231 and PBMC cells were 10.13 and 90.21 μ M, respectively (Figure 5.13A). The anthracycline-based anticancer drug Dox showed significant toxicity against MDA-MB-231 cells under

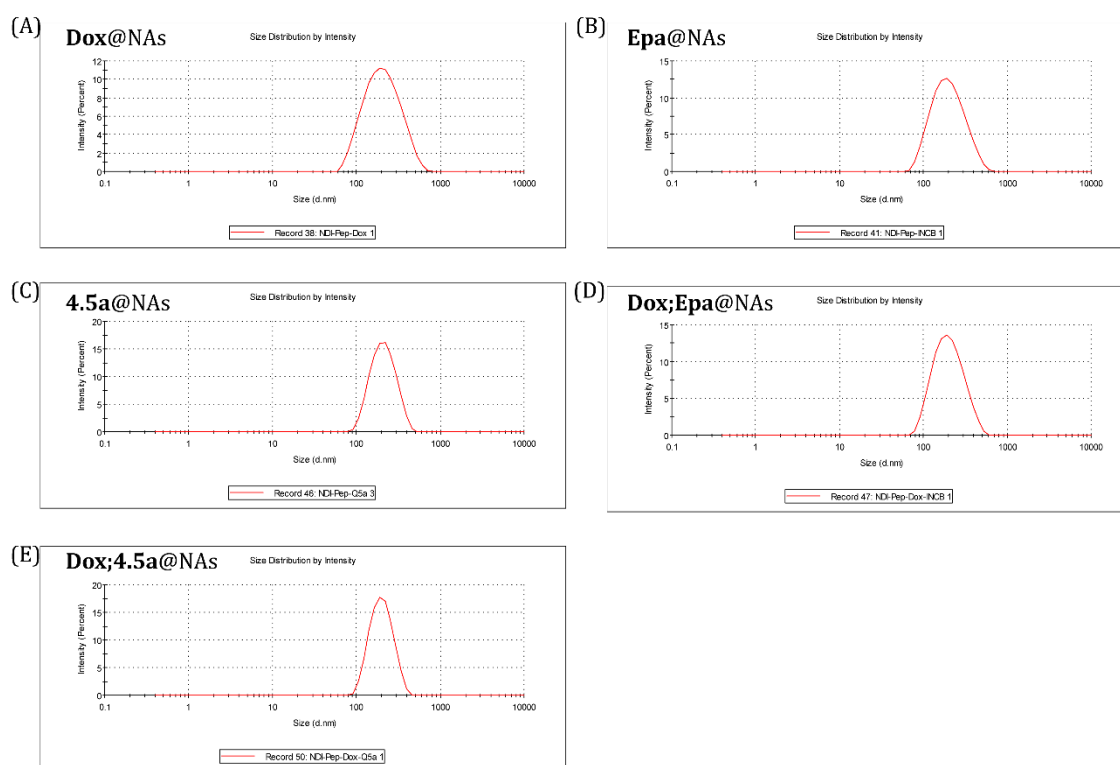


Figure 5.8 Intensity weighted distribution of drug loaded NAs (Dox@NAs (A), Epa@NAs (B), **4.5a**@NAs(C), Dox;Epa@NA (D), Dox;**4.5a**@NA (E)) from DLS measurements of the amphiphile in aqueous solutions.

similar conditions with IC_{50} values of 0.96 μ M, which is in accordance with the reported literature.²² Meanwhile, the Epacadostat, and **4.5a** treated (0-50 μ M) cells showed no or negligible toxicity even after 72 hours (Figure 5.9A). The nontoxicity of IDO1 inhibitors Epacadostat and **4.5a** are in accordance with the reported literature.¹⁰ The IDO1 inhibitors are well-known non-toxic agents that boost our immune system to fight against cancer and other diseases.²³ The IC_{50} of Dox@NA, Epacadostat@NA, **4.5a**@NA, Dox;Epacadostat@NA and Dox;**4.5a**@NA were 3.10, 8.62, 7.34, 4.36, and 4.70 μ M (with respect to the vehicle concentration), respectively

(Figure 5.9B). The superior cytotoxicity of Dox@NA, Dox;Epacadostat@NA and Dox;4.5a@NA could be due to both the improved drug uptake efficiency and cytotoxicity of the released Dox molecules. Though the effective drug concentrations were lower in the drugs loaded nano-vehicles, it was able to show significant toxicity than only nano-vehicle and very similar toxicity with that of free Dox molecules. The outcome of these MTT studies revealed that the **NDI-RGDS**-based nanocarrier could be a potential drug delivery vehicle. It also suggests that the cytotoxicity of Dox@NA, Dox;Epacadostat@NA and Dox;4.5a@NA could be due to the cleavage of NAs and release of Dox molecules within the cellular environments.

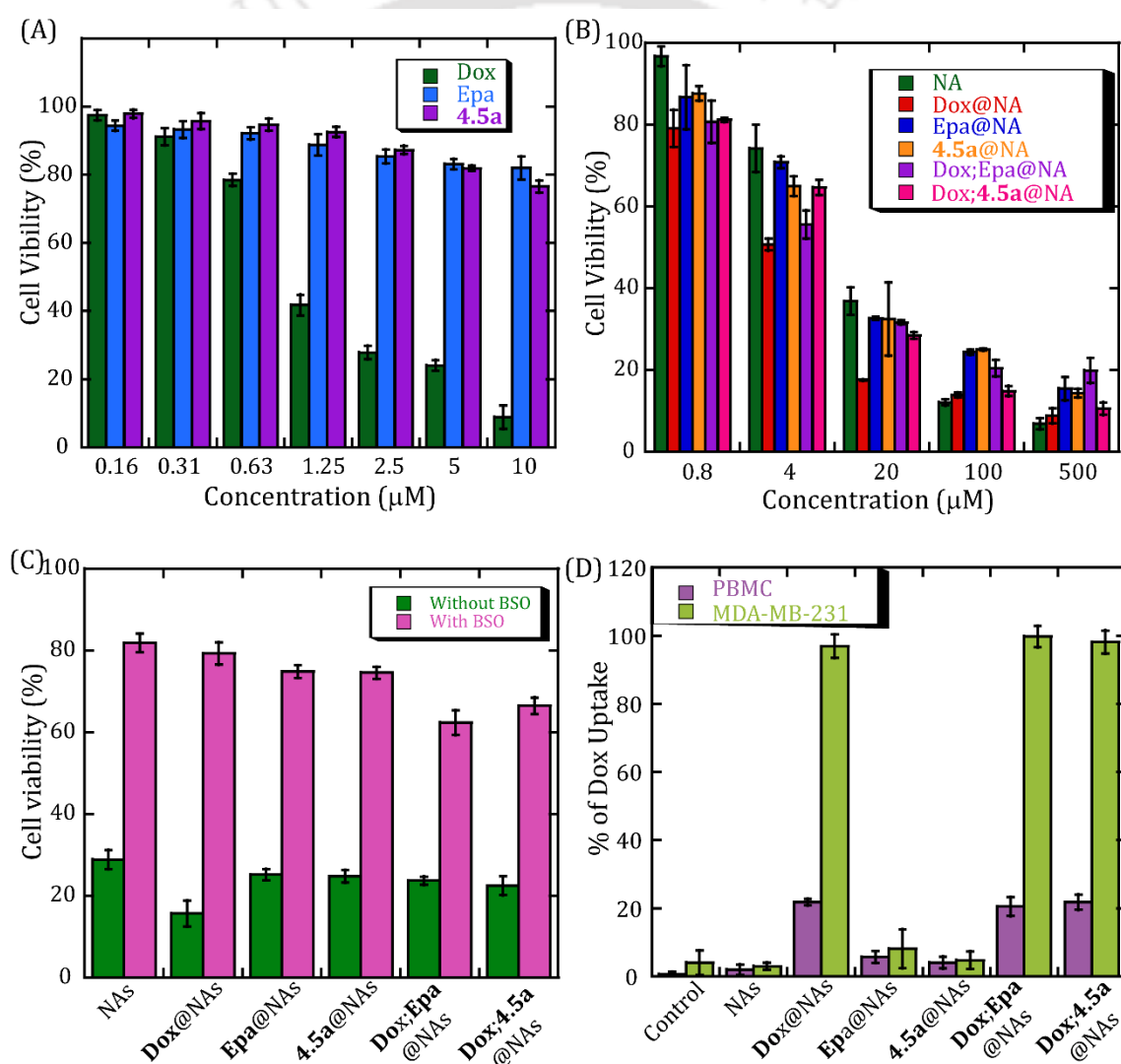


Figure 5.9 MTT-based cell viability assay. Viability of MDA-MB-231 cells after 72 hours of the treatment with drug molecules, Dox, Epacadostat (Epa), and compound 4.5a (A). Viability of MDA-MB-231 cells 72 hours after different treatments (B).

Viability of MDA-MB-231 cells in the absence and presence of BSO (50 μ M), a GSH inhibitor (after 72 hours of treatment) (C). The concentration of NAs and drug encapsulated NAs were 50 μ M. FACS-based Dox uptake in both PBMC and MDA-MB-231 cells after 8 hours of the treatment of different Dox loaded NAs (D).

The endogenous stimulus like GSH is present in higher concentration in cancerous cells (including MDA-MB-231 cells) than the normal cells.¹⁰ To investigate whether the GSH mediated degradation of the nano-vehicles is the primary mechanism of drug release to the cancer cells, we carried out MTT-based cytotoxicity assay of drug-loaded nanocarriers in the presence of GSH inhibitor, BSO in MDA-MB-231 cells (Figure 5.9C). BSO specifically blocks the rate-limiting step of GSH biosynthesis via γ -glutamyl-cysteine synthase, which also restores the sensitivity of resistant tumors to drugs.²⁴ The increase in cell viability for drug-loaded nanocarriers in the presence of BSO confirmed the GSH mediated degradation of the nanocarriers in MDA-MB-231 cells.

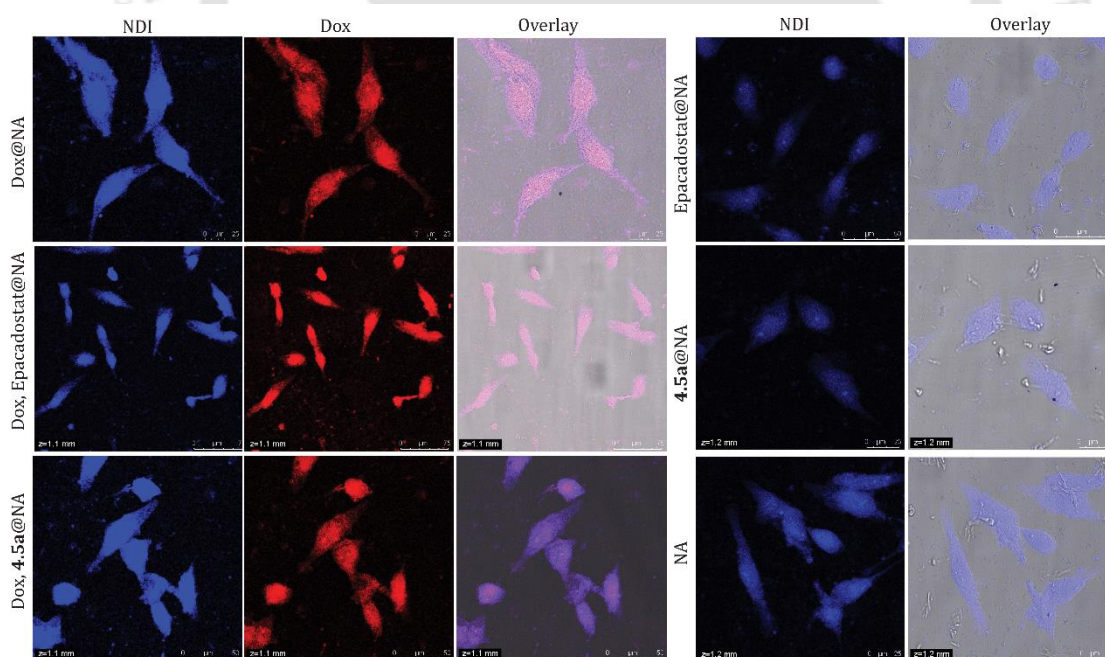


Figure 5.10 Representative CLSM images of the MDA-MB-231 cells incubated with 50 μ M of drug-loaded NAs for 8 h. The blue channel (NDI), red channel (Dox), overlay of the blue and red channels illustrate the Dox uptake efficacy by MDA-MB-231 cells.

To investigate the target-specific cellular uptake efficacy of **NDI-RGDS**-based nanocarrier via integrin $\alpha_v\beta_3$ receptor of the cancer cell, we performed the FACS

analysis of drug-loaded vehicles against MDA-MB-231 and PBMC (healthy human) cells. The FL2 fluorescence channels were monitored for the uptake of vehicles and Dox, respectively, after 8 hours of treatment of drug-loaded nano-vehicles. The outcome of the FACS analysis revealed the elevation of fluorescence intensity for Dox molecules in cancerous MDA-MB-231 cells compared to that of healthy PMBC cells (Figure 5.9D) suggesting the selective uptake and delivery of drug-loaded NAs in cancerous cells. In addition, CLSM-based uptake assay was performed in MDA-MB-231 cells (Figure 5.10). As expected, the microscopic images of the cells revealed the internalization of both vehicles and Dox after 8 hours of treatment of drug encapsulated NAs. Hence, both FACS and CLSM analyses revealed the successful drug delivery by the **NDI-RGDS**-based NAs.



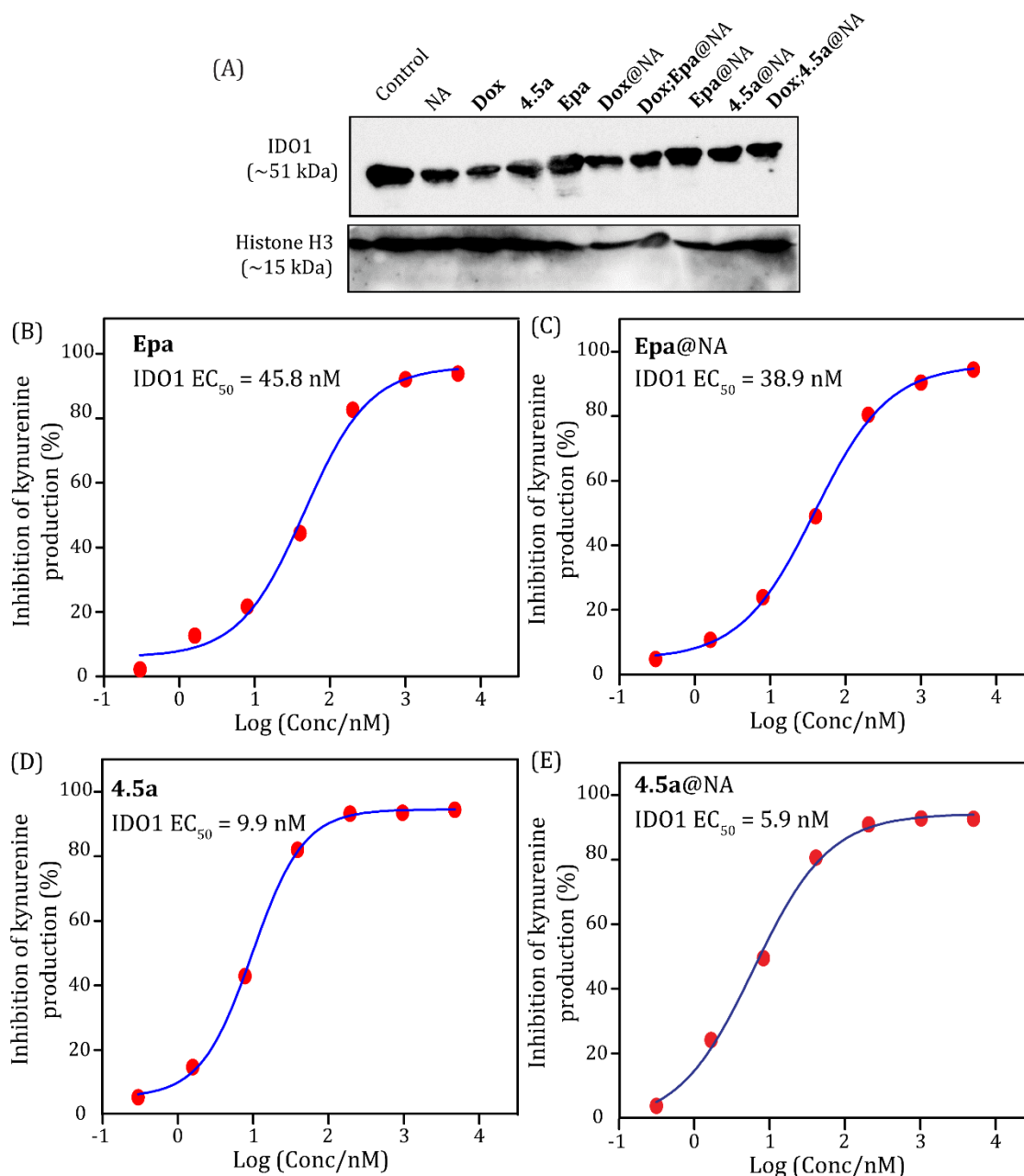


Figure 5.11 The expression levels of IDO1 protein in MDA-MB-231 cells across different treatments (A). Antibodies against the IDO1 enzyme was used to develop the blot. The concentration of IFN- γ was 50 ng/ mL. HPLC-based cellular (MDA-MB-231) IDO1 inhibitory assay. Plots of concentration of kynurenine in the presence of different concentrations of Epa (B), Epa@NA (C), 4.5a (D) and 4.5a@NA (E).

5.2.7. Cellular IDO1 inhibition by drug-loaded NDI-RGD nano-aggregates

Accumulating evidence has suggested that the inhibition of IDO1 activity promotes anticancer immune responses that contribute to the conventional chemotherapeutic effects.^{10, 21} In this regard, IDO1 inhibitory activities were

analysed with only NAs, and IDO1 inhibitor (Epacodastat or **4.5a**) encapsulated NAs in the MDA-MB-231 cell line (Figure S13). The IFN- γ was used to induce the expression of the IDO1 enzyme in MDA-MB-231 cells. Initially, the expression level of the IDO1 enzyme in the MDA-MB-231 cell line across different treatments was analysed by the western-blot analysis using IDO1-specific antibody. The outcome of the western-blot analysis revealed that there were no significant changes in the expression level of the IDO1 enzyme after different treatments (Figure 5.11A). In the meantime, kynurenine production inhibition in cultured media of different treatments was quantified with HPLC. The results revealed the reduction of kynurenine level with the increase in the concentration of the treatments. The calculated EC₅₀ of free Epacodastat and **4.5a** were 45.8 and 9.9 nM, respectively. The IDO1 inhibition efficacy improved with epacodastat@NA (EC₅₀ of 38.9 nM) and **4.5a**@NA (EC₅₀ of 5.9 nM), which could be due to the higher cellular uptake efficiency of IDO1 inhibitors via RGDS-mediated endocytosis of the NAs. Hence, drug encapsulated NAs might be advantageous in inhibiting IDO1 mediated immunosuppression and boost the prospect chemotherapeutic effects of Dox (Figure 5.11).

5.2.8. Generation of ICD signal in cancer cell by drug-loaded NDI-RGDS nano-aggregates

The preclinical and clinical studies revealed that Dox, the front-line chemotherapeutic drug for breast cancer, induces apoptosis and promotes ICD to show antitumor activity.¹ Meanwhile, the G-quadruplex binding ligands are also known to stimulate ICD.²⁵ The induction of ICD signal stimulates the expression of immunogenic markers, including CRT (cellular stress), on the surface of dying cancer cells, which provides an eat-me signal for APC. In this regard, we performed western-blot analysis using CRT antibody and monitored the CRT expression levels on the surfaces of MDA-MB-231 cells before and after (72 h) the treatment of drug-loaded NAs. The western-blot analysis revealed a significant level of increase in CRT expression after the treatments with NA, free Dox, Epa, **4.5a**, Dox@NA, Epa@NA, **4.5a**@NA, Dox;Epa@NA and Dox;**4.5a**@NA, suggesting an improvement of ICD signal generation efficacy after the treatments (Figure 5.12). This enhancement of ICD through CRT expression could be due to the inhibition of the IDO1 activity and

stabilization of G-quadruplex within the tumor immune milieu. Overall, the amplification of CRT expression due to the selective delivery of chemo and immune therapeutic drugs could be productive for anticancer treatment.^{25, 26}

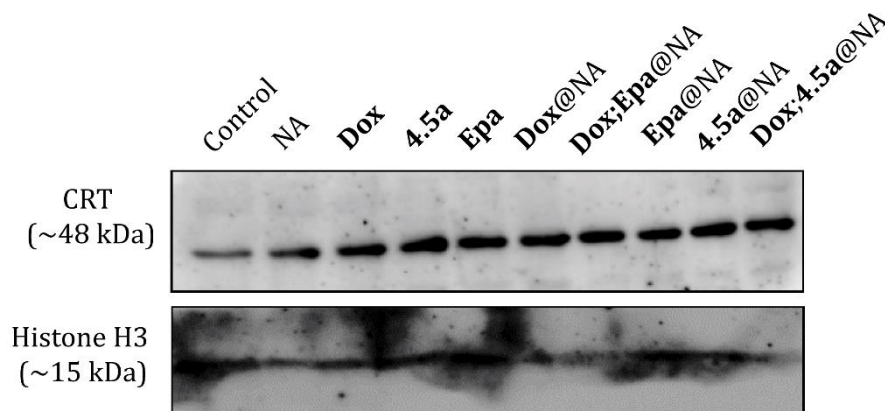


Figure 5.12 The expression levels of CRT protein in MDA-MB-231 cells across different treatments (A). Antibodies against the CRT protein was used to develop the blot.

5.2.9. Cellular G-quadruplex stabilization and induction of cancer cell apoptosis

The stabilization of cellular G-quadruplex is directly associated with the induction of apoptosis of the cancer cells.^{18, 25, 27} The presence of NDI moiety and the outcome of the fluorometric and CD studies of NAs with the human G-quadruplex prompted us investigated its role in cancer cell apoptosis. The MTT-based cell viability assay showed much higher toxicity of the NAs in MDA-MB-231 ($IC_{50} = 10.13 \mu M$) in comparison to that of healthy PBMC cells ($IC_{50} = 90.21 \mu M$) (Figure 5.13A). The cytotoxicity of NAs against MDA-MB-231 cells is presumed to be due to the RGDS targeted delivery and the involvement of G-quadruplex stabilization mediated anti-cancerous activity of the NAs. The cleavage of NAs in the presence of the GSH would generate **C₆-NDI-SH** inside the cancer cells. Hence, the effect of NAs on the stabilization of cellular G-quadruplex was investigated by monitoring the enhancement of G-quadruplex structures in MDA-MB-231 cells using western-blot and FACS analysis. The western-blot analysis of NAs in MDA-MB-231 cells was performed using c-MYC antibody. The outcome of this western-blot analysis revealed

a concentration dependent suppression of MYC protein after the treatment with only NAs (Figure 5.13F). For FACS analysis the cells were stained with propidium iodide, and the dot plot of PI-A against PI-H was used to analyze the G-quadruplex structures (Figure 5.13B). The elevation of G-quadruplex structures by 3% was observed in the presence of NAs compared with the basal condition.²⁵ This observation suggested that cellular G-quadruplex stabilization could be one of the probable reasons for apoptotic MDA-MB-231 cell death by the NAs.

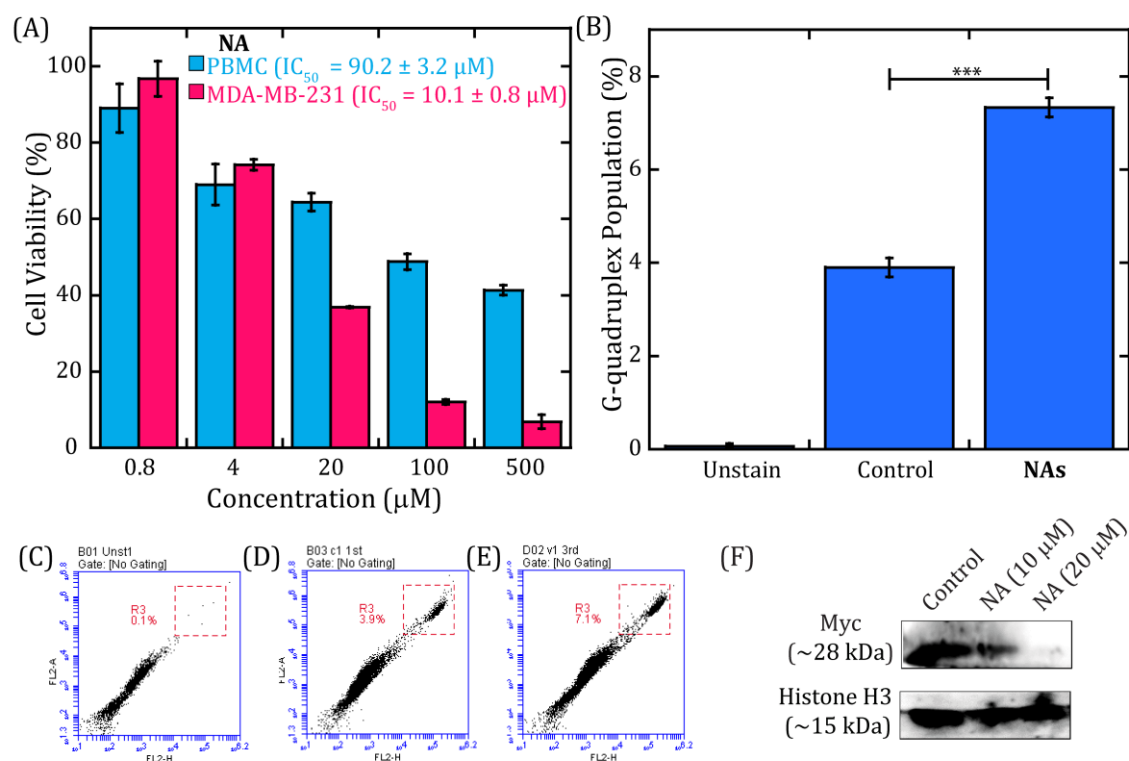


Figure 5.13 MTT-based cell viability assay. Viability of PBMC and MDA-MB-231 cells after 72 hours of the treatment with NA (A). Plot of FACS-based quantification of cellular (MD-MB-231) G-quadruplex after the treatment of NA (B). FACS data of cellular (MDA-MB-231) G-quadruplex after untreated PI (C), treated PI (D) and NA treated PI stained cells (E). The expression levels of c-Myc protein in MDA-MB-231 cells across different treatments (A). Antibodies against the Myc protein was used to develop the blot.

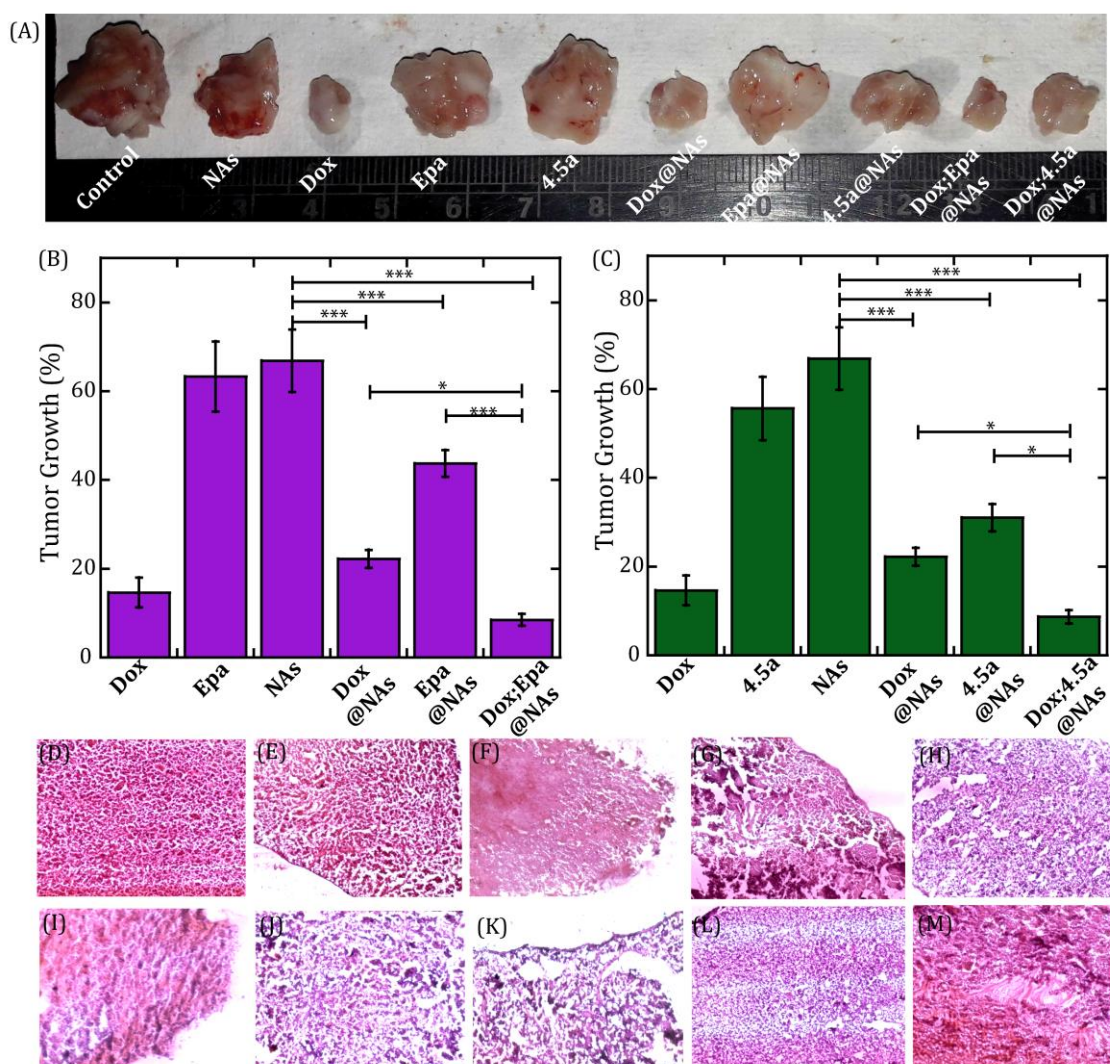


Figure 5.14 Size of solid tumor in the female Swiss albino mice after 15th day of the treatments (A). The Ehrlich ascites carcinoma (EAC) cells were injected into the thigh muscle of the Swiss albino mice to grow the tumor. Level (%) of tumor growth after the 15th day of the treatments (B and C). Immunotoxicity assessment of spleen by hematoxylin and eosin staining after treatments with control (D), NA (E), Dox (F), Epa (epacadostat) (G), **4.5a** (H), Dox@NA (I), epacadostat@NA (J), **4.5a**@NA (K), Dox;epacadostat@NA (L) and Dox;**4.5a**@NA (M).

5.2.10. *In vivo* antitumor activity

The NA-mediated selective delivery of chemo-immunotherapeutic drugs to cancerous cells and an increase in ICD signals prompted us to investigate its *in vivo* antitumor efficacy murine Ehrlich ascites carcinoma (EAC) solid tumor model. The mice were treated with only NAs, free Dox, free Epacadostat, free **4.5a**, Dox@NA,

epacadostat@NA, **4.5a**@NA, Dox;epacadostat@NA, and Dox;**4.5a**@NA, respectively. The vehicle concentration was fixed to 20 mg/kg per dose in each treatment, and free drugs were treated according to their effective loading concentration in the vehicle. The inhibition of relative tumor growth was analyzed on the 15th day of treatments (Figure 5.14A). Only vehicle treatment showed 34% regression of tumor growth which might be due to the stabilization of G4. There was no significant difference in tumor growth inhibition (TGI) for both free Dox and Dox-loaded vehicles. Both the treatments showed a TGI of 86% and 80%, respectively. In contrast, an increase of TGI was observed for epacadostat@NA (TGI of 56%) than only NA (TGI of 34%) and free epacadostat (TGI of 37%) (Figure 5.14B) treated mice. A remarkable regression of the tumor growth was observed for Dox;epacadostat@NA (TGI of 91%) treated mice, which was comparable to that of free NA, Dox@NA and epacadostat@NA treated mice (Figure 6B). Similarly, the enhancement of TGI was also identified for **4.5a**@NA (TGI of 69%) than only NA (TGI of 34%) and free **4.5a** (TGI of 44%) treated mice (Figure 5.14C). A notable suppression of the tumor growth was observed for Dox;**4.5a**@NA (TGI of 91%) treated mice, comparable to that of only NA, Dox@NA, and **4.5a**@NA treated mice (Figure 5.14C). However, the effective dual drug concentrations were lower compared to mono encapsulated drugs. The observed robust tumor regression could be due to the selective drug delivery ability of the NAs. The outcomes of these studies suggest a comparable efficacy of the chemotherapeutic drug molecule in the absence and presence of immunotherapeutic drug molecules for anticancer treatments.

The tissue architectures after the treatments were further investigated by the hematoxylin and eosin (H&E) staining method. The tumor-bearing mice showed substantial splenic inflammation and disruption of the systematic arrangement of the splenocytes (Figure 5.14D-M). The free Dox treatment showed a significant splenic inflammation. However, Dox@NA, Dox;epacadostat@NA, and Dox;**4.5a**@NA showed reduced splenic inflammation. Whereas free form of epacadostat, **4.5a**, and epacadostat@NA, **4.5a**@NA treated spleen groups showed no significant splenic inflammation compared to the control one. The inflammation of free Dox compared to Dox@NA, Dox;epacadostat@NA and Dox;**4.5a**@NA revealed a nonspecific action of Dox, which might freeze out robust regression of tumor growth in Dox treated mice. Therefore, H&E staining studies revealed a significant reduction of splenic

inflammation and disruption of the systematic arrangement of the splenocytes by drug-loaded NA. Thus, the biocompatible NDI-RGDS-based NA is a potent chemo-immunotherapeutic drug delivery nano-vehicle.

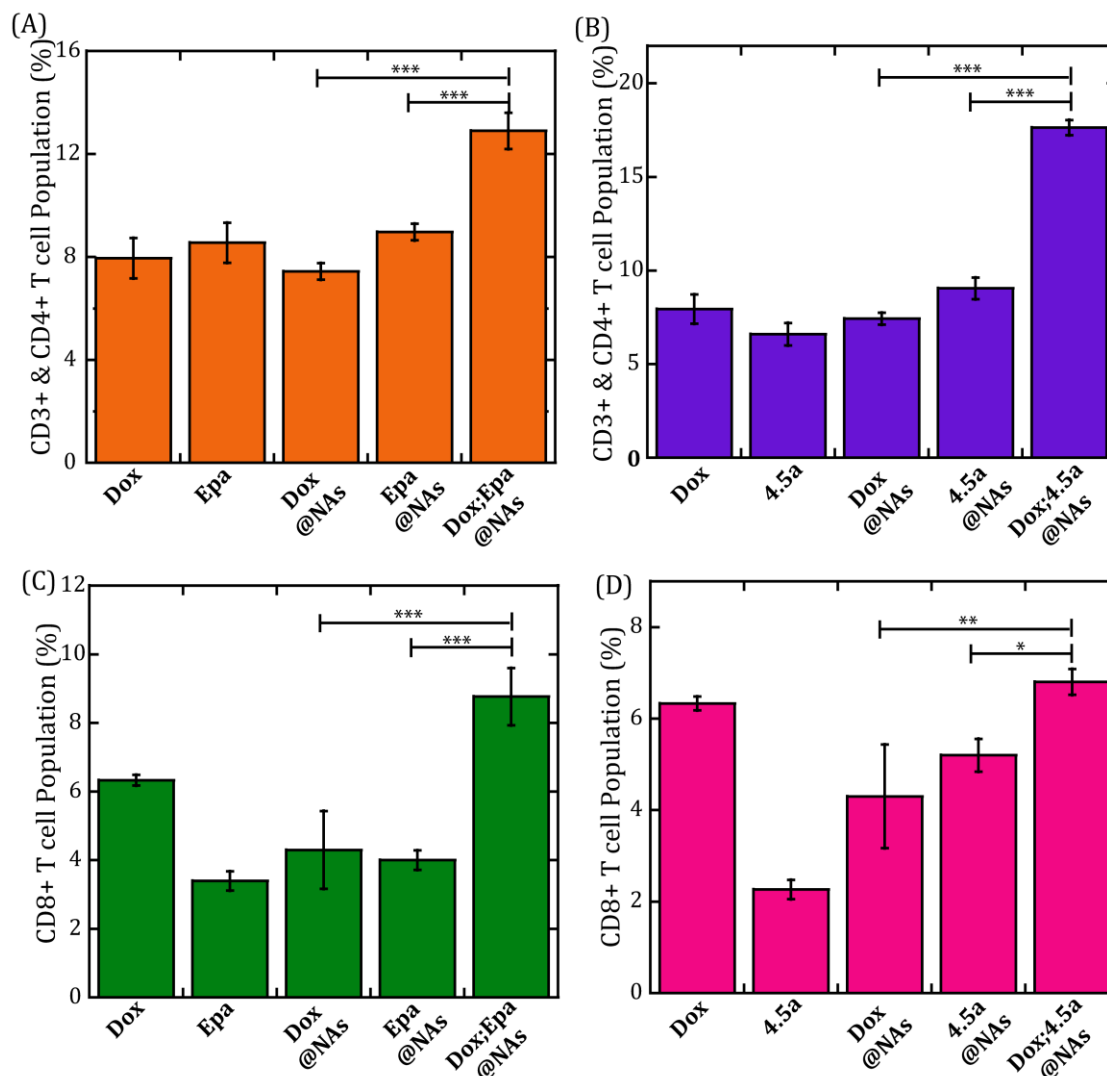


Figure 5.15 Immunophenotyping of CD3+CD4+ T cells in drug treatment groups (A and B). Modulation of CD8+ T cell number post-co-delivery regimen in drug treatment groups (C and D).

To further confirm the involvement of the immune signal in the antitumor activity of drug-loaded nanocarrier, the CD3+, CD4+, and CD8+ T cell populations in the spleen were evaluated by FACS analysis (Figure 5.15). The FACS results showed a significant increase in CD3+, CD4+, and CD8+ T cells population after different drug-loaded NAs treatments. The population of CD3+ with CD4+ T cell with Dox;Epaacadostat@NA showed (13%) an incremental effect compare to Dox@NA

(7.4%) and Epacadostat@NA (9%) in treated mice (Figure 5.14A). Similarly, the enhancement of CD8⁺ T cells population was also observed for Dox;Epacadostat@NA (8.8%), compared with Dox@NA (4.3%) and Epacadostat@NA (4%) treated mice (Figure 5.15C). A similar upregulation of CD3⁺ with CD4⁺ T cell (17.4%) and CD8⁺ T cell (6.8%) population were also identified for Dox;**4.5a**@NA in comparison to Dox@NA (7.4% and 4.3% respectively) and **4.5a**@NA (9% and 5.2% respectively) treated mice (Figure 5.15B and 5.15D). This trivial increase in the T cell population of dual-drug-loaded nanocarriers could be due to the lower immunotherapeutic drug loading efficiency than the corresponding Dox@NA, epcadostat@NA, and **4.5a**@NA formulations. Total CD4⁺ T cells in mice spleen showed an upregulated proliferation than the cocktail treatment groups. As CD4⁺ T cells represent a large domain of helper and regulatory CD4 T cells hence a detailed study will further confirm the complex immune modulation procedure, but these *in vivo* findings confirm the co-delivery regimen significantly modulate the immune profile in the lymphoid organ. In addition, the population of CD3⁺ T cells with low CD4 expression, i.e., CD8⁺ T cells (cytotoxic T cells) was also analysed. In this studies, we showed a significant alteration of cytotoxic T cell frequency in the cocktail drug treatment groups. Again, further study of cytokine release and differentiation phenotype alteration will confirm the detailed cytotoxic T cell modulation in murine spleen post drug treatment. Overall, this single or dual-drug encapsulated nanocarrier selectively delivered the drug molecules to the cancerous cells and improved the T cells population, which is advantageous for antitumor activity.

5.3. Summary

In summary, we successfully engineered a peptide amphiphile-based target-specific chemoimmunotherapeutic drug delivery system to augment therapeutic efficiency. The GSH-sensitive NDI-conjugated RGDS amphiphile self-assembled in aqueous solution to form nano-aggregates. This nano-vehicle synchronously delivered and released hydrophobic chemotherapeutic drug, Dox and IDO1 inhibitor, epacadostat, or quinine derivative (**4.5a**) to the targeted cancer cells. The delivery of encapsulated Dox and IDO1 inhibitor showed an anticancer effect and promoted the production of CRT. The anticancer activity of the NAs could be due to the stabilization of G-quadruplex by the NDI ligand. We believe that this GSH-responsive RGDS

peptide amphiphile will benefit the development of advanced cancer cell targeting nano-vehicle to accomplish proficient delivery of hydrophobic drugs and rejuvenate the immune response.

5.4. Experimental section

5.4.1. General information

All reagents were purchased from commercial sources and used directly without further purification. The ^1H NMR and ^{13}C NMR were recorded at 600 and 151 MHz with Bruker spectrometer, respectively. The chemical shifts were reported in parts per million (δ) using TMS as an internal standard with $\text{DMSO}-d_6$ solvent. The J values and chemical shifts (δ_{ppm}) were reported in Hertz (Hz) and ppm, respectively. Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), m (multiplet), and br (broadened). Chromatographic purification was performed on the Ultimate 3000 (Dionex) Thermo Scientific system. ESI-MS was recorded at Agilent Q-TOF mass spectrometer with Z-spray source using built-in software for analysis of the recorded data. DLS and zeta potentials were measured using Zetasizer Nano-ZS90 (Malvern). Fluorescence measurements were performed with a Fluoromax-4 spectrofluorometer (HORIBA). CD experiments were performed on a Jasco J-1500 spectropolarimeter. The FESEM and FETEM images were recorded using the Zeiss Gemini 300 field emission scanning electron microscope and JEOL JEM 2100 transmission electron microscope, respectively. RAMAN spectra were measured in Horiba Jobin Vyon, LabRam HR laser micro Raman system. Confocal microscopic images were collected in Leica/TCS SP8 microscope. Analytical HPLC was performed in 1260 Infinity II, Agilent instrument. Multiscan Go, Thermo Scientific was used for UV analysis of 96-well plates. FACS analysis was conducted in the BD Accuri C6 system. Ultrapure water (Milli-Q system, Millipore, Billerica, MA) was used for the preparation of buffers. The stock solutions of compounds were prepared in DMSO. The cells were cultured in DMEM (GIBCO) supplemented with 10% FBS (GIBCO) and penicillin-streptomycin solution (GIBCO; 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin). Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 $^\circ\text{C}$. Animals were housed in an animal facility room, Department of Zoology, the University of Calcutta at 25 ± 2 $^\circ\text{C}$, with a relative humidity of nearly 45 ± 5 % with alternative 12 hours day/ night cycle with access to food and water. All the

experiments were carried out as per guideline of the Committee for the CPCSEA, Government of India (Registration No: 885/GO/Re/S/05/CPCSEA), and approved by the Institutional animal ethical committee (IAEC), University of Calcutta.

5.4.2. Synthesis and characterisation of the compounds

5.4.2.1. Synthesis of the compounds

5.4.2.1.1. Synthesis of C₆-NDI-SH

To a stirring aqueous solution of 1,4,5,8- naphthalenetetracarboxylic acid dianhydride (2.0 g, 7.46 mmol), 35 mL of aqueous KOH (1 M) solution was added.²⁸ The mixture was heated until the compound completely gets dissolved. The pH of the solution was adjusted with H₃PO₄ solution (1 M) to pH 6.4. Then n-hexyl amine (0.98 ml, 7.46 mmol) was then added to the solution. The pH of the solution was re-adjusted to pH 6.4 with H₃PO₄ solution (1M). The resulting mixture was refluxed overnight and then allowed to cool to room temperature. After that, acetic acid (5 mL) was added to the solution to get a solid precipitate, which was then filtered and washed with water. The product was dried to get off-white solid and used for the next step without further purification.

To a stirring solution of the above crude product (500 mg, 1.42 mmol) in DMF, cystamine (143 mg, 1.85 mmol) was added. The mixture was refluxed at 140 °C for a day. The reaction mixture was cooled to room temperature. Water (50 mL) and CH₂Cl₂ (50 mL) were added to the solution. The organic portion was separated and concentrated under reduced pressure. The target compound was further purified using HPLC with a C18 column (Luna 5 µm) and acetonitrile-water as a mobile phase.

5.4.2.1.2. Synthesis of RGDS peptide

The designed peptide was prepared by the solid-phase peptide synthesis method.²⁹ Rink amide MBHA resin and Fmoc- protected amino acids were used for synthesis. Initially, 20% piperidine/DMF solution was used for resin activation. After that, Fmoc-protected Ser (4 equivalents with respect to resin) was coupled with resin in the presence of HBTU (4 equivalents with respect to resin), DIPEA (8 equivalents with respect to resin) in DMF solvent. The reaction mixture was stirred for one hour, and resin was washed with DMF several times. Then, Fmoc deprotection was performed with 20% piperidine/DMF (V/V) for 30 minutes, followed by washing

with DMF. The coupling and deprotection reactions were repeated until the desired sequence was synthesized. Finally, deprotection of resin and peptide was performed with TFA containing TES for 2-3 hours shaking. The resin was washed with DCM, and the solution was concentrated in a rotary evaporator. The desired peptide was precipitated in cold diethyl ether and lyophilized. The crude peptide was taken for HPLC purification with a C18 column (Luna 5 μ m) and acetonitrile-water as mobile phase and lyophilized.

5.4.2.1.3. Synthesis of NDI-RGDS peptide

To a stirring solution of peptide (50 mg, 0.06 mmol) and **C₆-NDI-SH** (68 mg, 0.18 mmol) in DMSO, 30 % H₂O₂ (1 % V/V) was added.²⁹ The solution was stirred for 24 hours under ambient conditions. Maximum DMSO was removed under reduced pressure at 70 °C, and the crude was washed with water. The precipitate was then dissolved in acetonitrile and purified using HPLC with a C18 column (Luna 5 μ m) and acetonitrile-water as mobile phase.

5.4.2.2. Characterization of synthesized compounds

C₆-NDI-SH: As brown solid; ¹H NMR (600 MHz, DMSO-*d*₆) δ_{ppm} 8.67 (4H, s), 4.13 – 4.00 (4H, m), 1.66 – 1.63 (4H, m), 1.36 – 1.33 (4H, m), 1.30 – 1.29 (2H, m), 1.23 (1H, s), 0.86 (3H, *J* = 12 Hz, t). ¹³C NMR (150 MHz, DMSO-*d*₆) δ_{ppm} 163.1, 130.9, 126.7, 126.6, 40.4, 31.4, 27.8, 26.6, 22.4, 14.4. ESI-MS calcd. for C₂₂H₂₁N₂O₄S (M-H)⁻: 409.1228, found: 409.9826.

RGDS peptide: As white powder (55%); ¹H NMR (600 MHz, DMSO-*d*₆) δ_{ppm} 8.76 – 8.75 (1H, m), 8.42 – 8.40 (1H, m), 8.27 – 8.19 (6H, m), 8.09 – 8.07 (2H, m), 8.02 – 7.99 (1H, m), 7.63 – 7.62 (2H, m), 7.43 (6H, brs), 6.97 (3H, brs), 4.94 (1H, brs), 4.67 – 4.65 (1H, m), 4.50 – 4.48 (2H, m), 4.44 – 4.42 (2H, m), 4.29 – 4.24 (1H, m), 3.79 – 3.72 (3H, m), 3.71 – 3.69 (1H, m), 3.10 – 3.09 (4H, m), 3.01 – 2.88 (1H, m), 2.87 – 2.85 (2H, m), 2.84 – 2.81 (1H, m), 2.79 – 2.75 (1H, m), 2.75 – 2.73 (1H, m), 2.71 – 2.68 (1H, m), 2.39 – 2.36 (1H, m), 1.55 – 1.53 (2H, m), 1.52 – 1.50 (5H, m). ESI-MS calcd. for C₃₀H₅₅N₁₄O₁₂S₃ (M+H)⁺: 899.3281, found: 899.3348.

NDI-RGDS peptide: As brown solid (40%); ^1H NMR (600 MHz, $\text{DMSO-}d_6$) δ_{ppm} 8.74 – 8.73 (1H, m), 8.68 (12H, s), 8.41 – 8.40 (1H, m), 8.24 – 8.19 (6H, m), 8.08 – 8.06 (2H, m), 8.03 – 8.01 (1H, m), 7.52 – 7.51 (2H, m), 7.33 (6H, brs), 6.81 (3H, brs), 4.99 (1H, brs), 4.69 – 4.65 (1H, m), 4.50 – 4.48 (2H, m), 4.44 – 4.42 (2H, m), 4.28 – 4.24 (1H, m), 4.09 – 4.05 (12H, m), 3.78 – 3.72 (3H, m), 3.64 – 3.62 (1H, m), 3.10 – 3.09 (4H, m), 3.00 – 2.97 (1H, m), 2.88 – 2.85 (1H, m), 2.84 – 2.81 (2H, m), 2.78 – 2.74 (1H, m), 2.73 – 2.71 (1H, m), 2.65 – 2.62 (1H, m), 2.39 – 2.37 (1H, m), 1.68 – 1.63 (8H, m), 1.55 – 1.51 (5H, m), 1.38 – 1.31 (24H, m), 0.87 (9H, $J = 12$ Hz, t). ESI-MS calcd. for $\text{C}_{96}\text{H}_{117}\text{N}_{20}\text{O}_{24}\text{S}_6$ ($\text{M}+3\text{H}$) $^{3+}$: 708.8964, found: 708.5202.

5.4.3. Critical aggregation concentration measurement

The fluorescence property of the NDI-core was used to determine the CAC of the amphiphile. The aqueous solution of **NDI-RGDS** amphiphile was prepared by directly dispersing lyophilized amphiphile in 10 mM PBS buffer, pH 7.2 with varying concentrations of 0-75 μM . The fluorescence spectra were recorded in the emission range of 380 nm to 680 nm with $\lambda_{\text{ex}} = 365$ nm at 5 nm slit. The plot of intensity ratios of I_0 (419 nm) and I_1 (468 nm) with the concentration of the NAs was used to calculate the CAC values.²²

5.4.4. Morphological analysis

The NAs were prepared by the thin-film hydration method in HEPES buffer (20 mM HEPES, 100 mM KCl, pH 7.2) according to the reported procedure.¹² The methanol solution of the amphiphile was dried under reduced pressure for 3 hours to prepare the thin film. Then, the mentioned buffer was added and heated to 60-70 $^{\circ}\text{C}$ for 10 to 15 minutes. After that, the solution was vortexed for 15 minutes and sonicated 10 to 15 times with cooling and sonication cycles. The FESEM analysis was performed with 100 μM of the above-prepared solution using a drop-casting method on an aluminum foil-covered glass slide. The dried sample was analyzed using the voltage of 2-5 kV in the FESEM instrument. The FETEM analysis of the NAs was performed according to the reported procedure with the above-prepared solution.^{12, 22} Briefly, the suspended solution (10 μL of 100 μM) was allowed to absorb on a carbon-coated copper grid for one minute. The grid was then carefully blotted with filter paper and allowed to dry for 10 to 15 minutes at 37 $^{\circ}\text{C}$. Then uranyl acetate (5-

10 μ L of 2% solution in water) was added to the grid to stain for another one minute. Then the solution was removed, and the grid was dried overnight at 37 °C. The images were collected at a maximum accelerating voltage of 200 kV.

5.4.5. Dynamic light scattering and zeta potential measurements

The NAs were prepared according to the mentioned method as described in the earlier section. Different buffer solutions were freshly prepared using an isosmotic 10 mM buffering agent and 10 mM salt with a varying pH range of 3 to 8.5. Citric acid and trisodium-citrate were used for pH 3.0–6.5 and (MOPS) for pH 7.0. Tris-HCl were used for pH 7.2–8.5. Both the hydrodynamic diameter and zeta potential of the NAs (100 μ M) were measured at 25 °C. Similarly, the hydrodynamic diameter of the NAs in the absence and presence of HSA (10 μ M) was monitored for 72 hours.¹²

5.4.6. Phase transition temperature measurements

Temperature-dependent steady-state anisotropy measurements were performed to determine the T_m of the NAs as reported earlier.¹² The fluorescent probe DPH was used for this experiment. The NAs were prepared according to the described method in the previous section. Then, DPH solution (10 μ M) was incorporated into the NAs (100 μ M) under tumbling conditions (kept for overnight at room temperature). After that, the degree of anisotropy (r) of DPH (λ_{ex} = 350 nm; λ_{em} = 445 nm) was measured with varying temperatures from 5 - 90 °C. The T_m value was calculated from the plots of the degree of anisotropy (r) of the DPH probe as a function of temperature.

5.4.7. RAMAN experiment

The amorphous samples of both GSH untreated and treated NAs were used for RAMAN spectral analysis.¹⁹ Briefly, 1 mM of nano-vehicle was treated with GSH (10 mM in PBS buffer) at pH 7.2 for 72 hours at room temperature. The suspended solution was then centrifuged at 14000 rpm for 15 minutes. The precipitate was washed with PBS buffer and lyophilized. The lyophilized powder was then used for RAMAN spectral analysis with 488 nm laser excitation.

5.4.8. Preparation of G-quadruplex DNA

The oligonucleotide, human telomeric G-quadruplex DNA sequence, 5'-AGGGTTAGGGTTAGGGTTAGGG-3' was dissolved in 10 mM Tris-HCl buffer containing 100 mM KCl at pH 7.2. Then the solution was heated at 95 °C for 10 minutes and allow for gradual cooling at room temperature for overnight. The formation of G-quadruplex was confirmed by CD.³⁰

5.4.9. Fluorescence-based G-quadruplex binding analysis

The change of fluorescence emission intensity for the NAs in the absence and presence of G-quadruplex was carried out to measure binding affinity. All measurements were carried out with a 10 mm path-length quartz cell. Fluorescence emission spectra of NAs (5 μ M) was recorded in the emission range of 380 nm to 680 nm with $\lambda_{\text{ex}} = 365$ nm at 7 nm slit in the presence of G-quadruplex (0 μ M to 12 μ M). The plot of intensity ratios of I_0 (419 nm) and I_1 (at 468 nm) in the emission spectra against concentrations of G-quadruplex was used to identify the binding properties.³⁰

5.4.10. CD-based G-quadruplex melting measurement

The G-quadruplex melting experiment in the absence and presence of NAs was performed according to the reported protocol.³⁰ Briefly, G-quadruplex (10 μ M) in buffer (10 mM Tris-HCl buffer containing 100 mM KCl at pH 7.2) without or with NAs (20 μ M) were taken in a quartz cuvette with a 0.2 cm path length for recording the CD spectra. The spectra were collected over a range of 20–95 °C (at 5 °C intervals) from 230 to 320 nm (0.1 nm intervals) at a fixed slit width of 1 nm. Finally, the molar ellipticity at 290 nm against temperature was analyzed to calculate the melting temperature of G-quadruplex.

5.4.11. HPLC-based drug loading and release experiment

The nano-vehicle (5 mM) was prepared as mentioned earlier in the presence of different drugs (Vehicle: drug; 200:1 w/w). The un-encapsulated drugs were removed by centrifuge (14000 rpm for 30 minutes at room temperature). Then the precipitate was washed with the same buffer (HEPES buffer, pH 7.2), and the concentration was adjusted to 1 mM. After that, the suspended solution was divided into 100 μ L solution and incubated in the absence and presence of GSH (50 mM) of

their respective time up to 72 h. For the experiment in the presence of GSH, 100 μL of acetonitrile was added to the suspension solution, and the precipitate was removed by centrifugation. For the experiment in the absence of GSH, the suspension was centrifuged, and 20 μL of the clear solution was used for HPLC analysis. Acetonitrile and water were used as a mobile phase through a multiple gradient system with a flow rate of 0.5 mL/minutes, and the chromatogram was recorded at 254 nm wavelength. Finally, the vesicles were lysed with GSH to release the loaded drugs. The area of the respective drug was proportional to the concentration of the released drugs. The loading of the drug was calculated from both the concentration of released drugs and incubated concentration. The release % was calculated with respect to the drug released after cleavage of vehicle.^{10,12}

5.4.12. Cellular cytotoxicity assay

The MDA-MB-231 cells (human breast adenocarcinoma) and PBMC (human healthy) were used for cell viability study according to the reported procedure in section 2.4.7.¹⁰

5.4.13. Cellular uptake assay

The cellular uptake efficiencies of the drugs or drug@nano-carrier were measured by CLSM and FACS analysis, as mentioned earlier.¹² For microscopy analysis, MDA-MB-231 cells were seeded on 22 mm square coverslip into 6-well plates at a density of 1.5×10^5 cells per well and incubated overnight in complete media into the CO₂ incubator. Cells were then treated with different compounds at their specific concentration and incubated for another 8 hours in 1% FBS containing media. Cells were washed with PBS buffer, pH 7.2, and fixed with 4% formaldehyde at 37 °C. Coverslips were then washed with PBS and mounted over a drop of glycerol over a glass slide. Finally, the dried slide was used for microscopic analysis in a confocal laser scanning microscope by giving $\lambda_{\text{ex}} = 405 \text{ nm}$, $\lambda_{\text{em}} = 410\text{--}480 \text{ nm}$ for the nano-vehicle (NDI) and $\lambda_{\text{ex}} = 488 \text{ nm}$, $\lambda_{\text{em}} = 560\text{--}630 \text{ nm}$ for Dox. For FACS analysis, MDA-MB-231 cells were cultured similar to that mentioned for the CLSM experiment. The treated cells were washed with PBS buffer, pH 7.2, and trypsinized (0.05%; GIBCO) for 2 minutes at 37 °C. Cells were collected by centrifugation at 800 rpm at 4°C for 5 minutes and then re-suspended in 300 μL of PBS buffer at pH 7.2

supplemented with 2% FBS. Finally, both the vehicle and drug uptake were analysed by flow cytometry.

5.4.14. Cellular G-quadruplex stabilization assay

According to the previously reported procedure, cellular G-quadruplex stabilization in the presence of **C6-NDI-SH** ligand was analysed in MDA-MB-231 cells.²⁵ Cells were seeded into 6-well plates at a density of 1.5×10^5 cells per well and incubated overnight in complete media into the CO₂ incubator. Cells were then treated with different concentrations of ligand and incubated for another 72 hours in complete media. Cells were then washed and trypsinized according to the earlier mentioned process and prepared for FACS analysis.²⁵ The G-quadruplex structures were examined by flow cytometry by gating the dot plot PI-A vs. PI-H.

5.4.15. Western Blot analysis

The MDA-MB-231 cells (seeded into 6-well plates at a density of 1.5×10^5 cells per well) were incubated in the presence of IFN- γ (50 ng/mL) along with tryptophan (150 μ M) and different compounds at their specific concentration for 20 h to analyze the expression of IDO1 protein.¹⁰ For C-myc expression analysis, cells were treated with **C6-NDI-SH** for 72 h according to the reported procedure.¹⁸ The culture media was cast off, and the cells were lysed by lysis buffer containing 1(M) Tris (pH 6.8), 20 % glycerol, 4 % SDS, 400 mM DTT, and 0.004 % bromophenol blue and lysed cells were stored at -20 °C for further analysis. Protein extracts were separated by 12 % SDS-PAGE and transferred into nitrocellulose blotting membrane. The skimmed milk was used for blocking. The solution was incubated for 2 hours with the specific antibody. The IDO-specific antibody, H3, and C-myc antibody were used in dilutions of 1:500, 1:500, and 1:5000, respectively. The proteins of interest were detected by HRP-conjugated goat anti- Rabbit antibody in dilution 1:5000 for 2 hours. Further, the visualization was performed using the ECL western blotting substrate.

5.4.16. Cellular IDO1 inhibition measurement

The cellular enzyme activity assay was performed according to the reported procedure as mentioned in section 4.4.6. with minor modifications.¹⁰ MDA-MB-231

cells were cultured in the absence and presence of compounds with IFN- γ (50 ng/mL) and tryptophan (150 μ M) according to the mentioned procedure for 20 h.¹⁰

5.4.17. *In vivo* antitumor efficacy measurement

EAC cells were maintained in the ascetic form in Swiss albino mice by intraperitoneal transplantation every 10th day on each mouse. The ascetic fluid was drawn on 7th day of implantation according to the previously described protocol.³¹ Isolated cells were diluted in sterile PBS. 2×10^6 cells were then implanted on the thigh muscle of all the animals selected for the experiment. Swiss albino mice were divided into ten groups ($n = 3$ each group). Post inoculation of the cells in thigh muscle on day 0, mice in their respective group were allowed to treat for 5 days on every alternate day at the treatment of 25 mg/kg (with respect to vehicle) body weight. All the mice were sacrificed on day 15.³¹

5.4.18. *In vivo* immune performance measurement

Single cells were prepared from lymphoid organs according to previously published protocol.^{31, 32} Briefly, the lymphoid organs were dissected out and minced in RPMI with 10% FBS on ice. The suspension was allowed to pass through a single cell strainer, and RBC was lysed using RBC lysis buffer (when required). After single-cell separation, cells were washed in PBS, and viability was counted before processing for flow cytometry. Immunophenotyping was performed according to previously published protocol using the following antibodies: FITC-CD3 (Miltenyi), APC-CD8 (Miltenyi). Results were recorded using BD Accuri C6 (BD biosciences).

5.4.19. Immuno-toxicity measurement

Spleen was harvested post-treatment for tissue processing and sectioning. Organ (5 μ m sections) of all the groups were nuclear stained with hematoxylin and cytosol stained with eosin. Post-processing the tissues were thoroughly examined to investigate the cellular toxicity.²²

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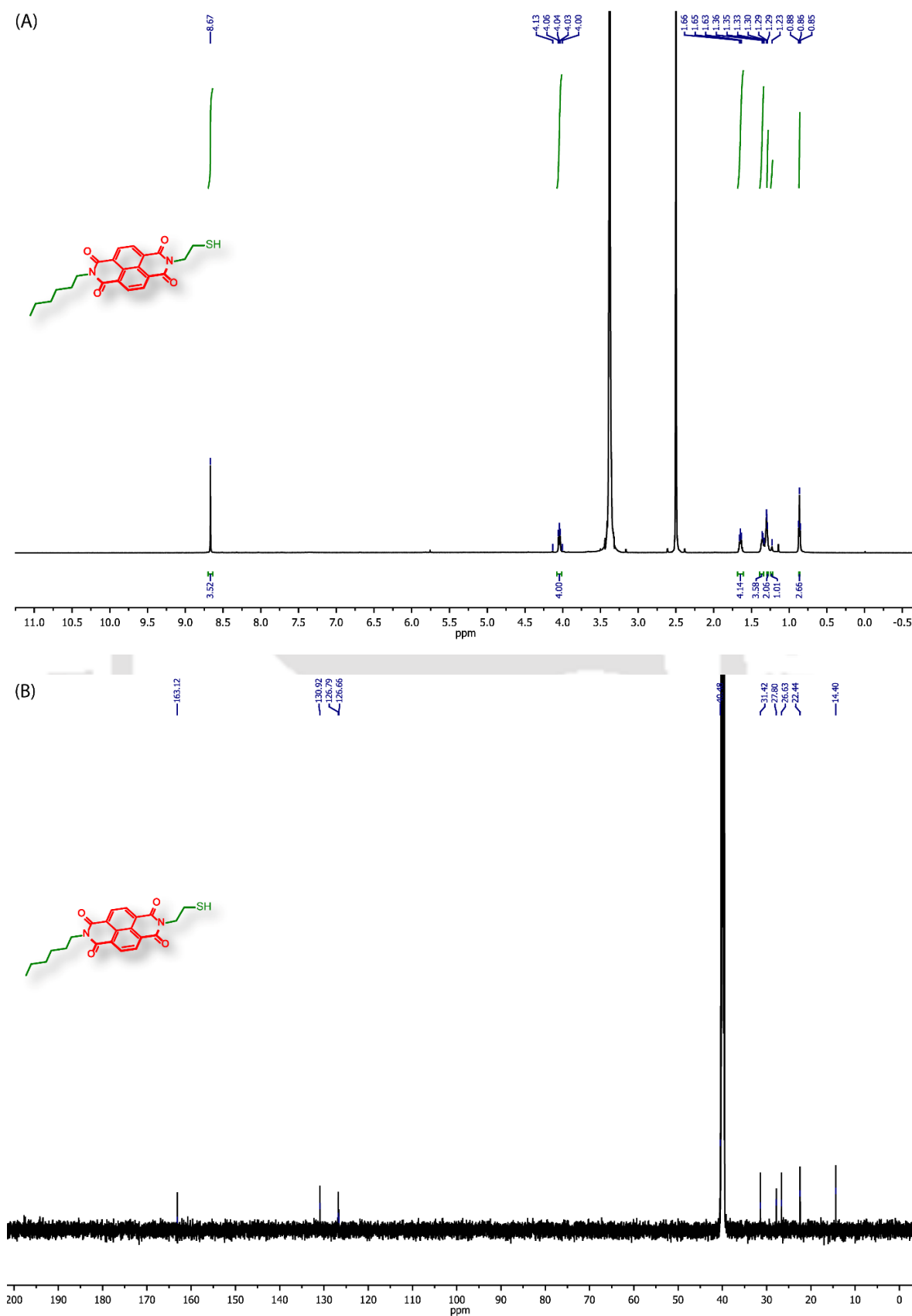
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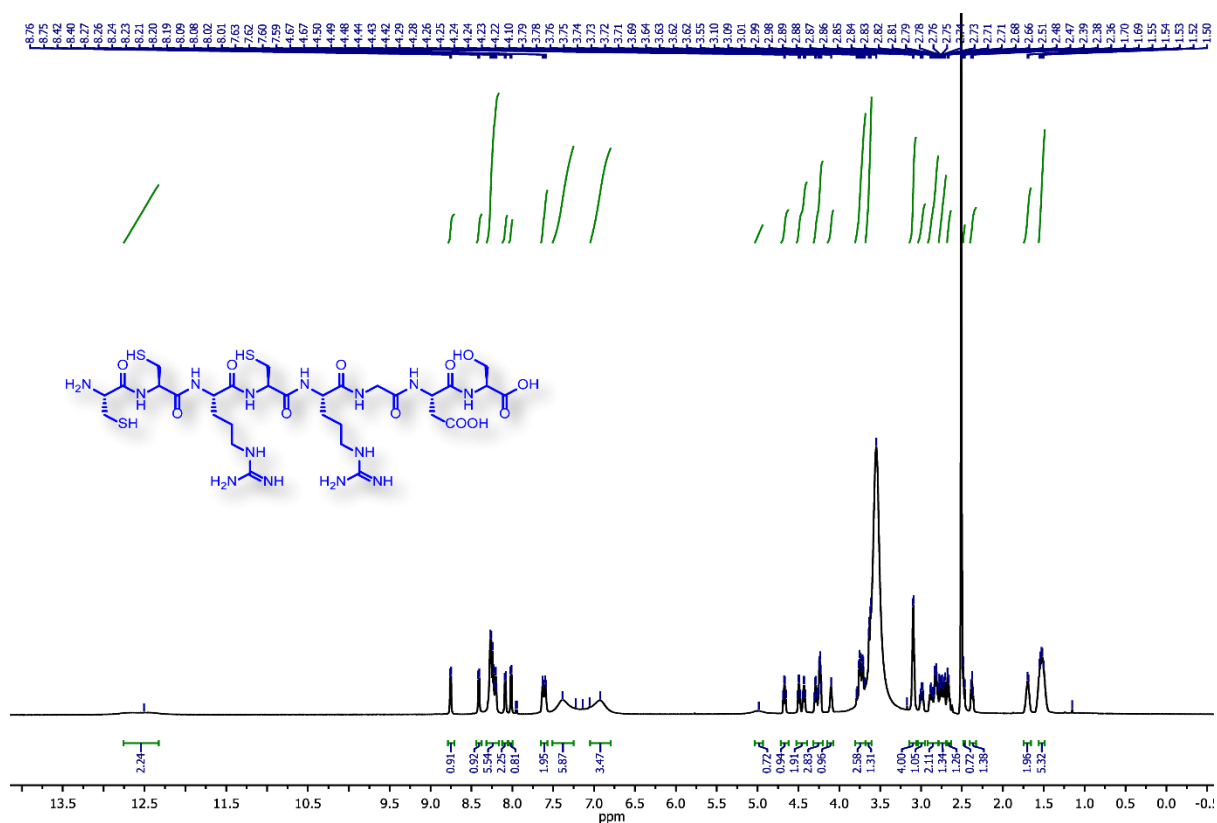
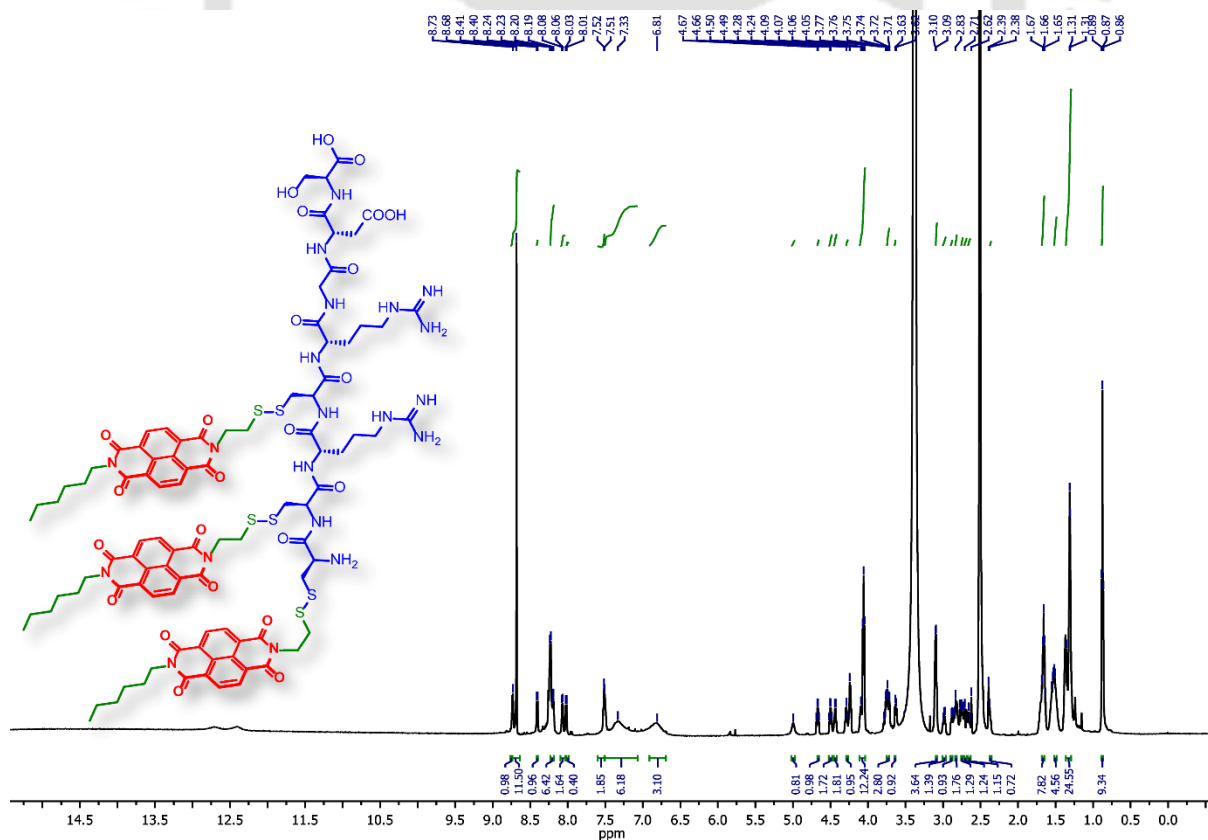
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5.6. NMR spectra of the synthesized compound



Figure 5.16 ^1H NMR spectra of compound RGDS.Figure 5.17. ^1H NMR spectra of compound NDI-RGDS.

6. Thesis Conclusion

In this thesis, we have successfully described the role of IDO1 in immunosuppression process in different life-threatening diseases especially cancer. Additionally, the importance of IDO1 inhibition has been described in **Chapter 1**. The L-Trp mimic *1H*-indazole derivatives (in **Chapter 2**) shows moderate IDO1 inhibitory activity. Though, *1H*-indazole derivatives occupies active pocket, it is unable to bind heme-iron which might be a possible reason for its moderate inhibition activity. The 4,5-disubstituted 1,2,3-triazoles (In **Chapter 3**) binds with both ferric and ferrous-heme to perturb IDO1 activity. Triazoles also have shown T cell activity which controls tumor growth. Apart from the heme binding inhibitors, dual apo and heme targeting quinine derivatives have shown a robust and selective IDO1 inhibitory activity (In **Chapter 4**). To improve the efficacy of IDO1 inhibitor along with chemotherapeutic drug in cancer cell, a novel peptide-based NAs have been designed (In **Chapter 5**). The NAs selectively deliver chemo and immuno drugs and improve ICD of the cancer cells. Overall, this thesis deals with different IDO1 inhibitors for the improvement of chem-immunotherapy.

7. Future Prospects

This thesis elucidates different potent IDO1 inhibitors and novel delivery techniques for improvement of drug efficacy into the targeted cells. Initial biophysical and cellular data implies their therapeutic potential against immunotherapeutic treatments. Best inhibitory potency has been obtained with apo-IDO1 targeting quinine derivatives (a natural product derivative, having drug likeness properties). Hence, these quinine derivatives can have profound application in therapeutic use in the near future. But several other experimental evidences remain to conclude their potential against several life-threatening diseases. Recently, delivery vehicles have been tremendously used for targeted delivery of drugs in cancer cells. We have also developed a stimuli-responsive vehicle which can deliver dual chemo and immune drug to the cancer cells. The vehicle has also shown target specific toxicity specifically in cancer cells which gives additional benefit for inducing apoptosis in cancer cells. This arena of vehicle toxicity can be explored in the future. Hope, these inhibitors and technique could be the 'spot-light' of cancer immunotherapy in due time.

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1. **Pradhan, N.†**; Chatterjee, S.†; Dey, S.; Banerjee, A.; Bhattacharyya, A.*; Manna, D.*, “Glutathione-responsible tumor-homing peptide amphiphile for the co-delivery of doxorubicin and immunosuppressive enzyme inhibitor promoted as chemo-immunotherapy” *Adv. Ther.* (Under review) (+ = Equal contribution)
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