Design, Synthesis and Antimycobacterial Evaluation of a Novel Class of Chemotherapeutic Agents

by

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Abstract

Mycobacterium tuberculosis (*Mtb*), one of the deadliest known human pathogens, infects ~ 2 billion people (33% of the world's population) and remains the leading infectious cause of death. According to the World Health Organization's (WHO) report, 225 million new infections will occur and ~79 million people worldwide will die of tuberculosis (TB) between the years 1998-2030. Worldwide, TB accounts for 9.3 million new cases and 2 million deaths every year. HIV infection has contributed to a significant increase in the incidence of TB globally. Further, emergence of multi-drug resistant (MDR), extensively-drug resistant (XDR) and totally-drug resistant (TDR) strains of mycobacteria has made this disease almost incurable. Therefore, there is an urgent need to discover novel antimycobacterial agents and effective therapeutic regimens.

The genome of *Mtb* encodes several enzymes involved in nucleic acid synthesis, and pyrimidine and purine biosynthesis, which differ significantly in selectivity towards their substrates and/or potential inhibitors from those in mammals. Therefore, modified nucleosides could act as selective inhibitors of mycobacterial RNA and DNA synthesis and replication. In this study, I have designed and synthesized nineteen novel deoxy, dideoxy, ribo and arabino pyrimidine nucleoside analogs containing 5-ethynyl, 5-(2-propynyloxy) and 5-hydroxymethyl uracil bases. These compounds were evaluated for their activity against several mycobacterial species (*Mtb, M. bovis* and *M. avium*). Combination therapy has been the most successful approach in the treatment of mycobacterial infections, and therefore selected compounds were investigated in combination with isoniazid and rifampicin in 2 and/or 3 drug combinations. Inhibition of intracellular mycobacterial growth within macrophages and *in vivo* activity of the novel agents in an infection model of *Mtb* was also carried out. Although, several compounds exhibited modest inhibitory activity at higher concentrations against *Mtb* and *M. bovis* they displayed unexpected synergistic interactions at lower concentrations with isoniazid and rifampicin. Interestingly, the active analogs were also found to inhibit intracellular mycobacterial replication in a human monocytic cell line infected with H37Ra. From this work, 5-hydroxymethyl-3-*N*-(2-propynyl)-3'-azido-2',3'-dideoxyuridine and 5-hydroxymethyl-3-*N*-(2-propynyl)-2',3'-dideoxyuridine emerged as the most potent compounds. Oral treatment of mice infected with *Mtb* (H37Ra) with these compounds demonstrated promising antimycobacterial effect.

I have also designed and investigated novel drug conjugates by integrating a pyrimidine nucleoside effective against mycobacteria with an existing TB drug working by a different mechanism as a new class antimycobacterial agent. To test this unique approach, 5'-mono-pyrazinoate and 3', 5'-di-pyrazinoates of 5-fluoro-

2'-deoxyuridine were synthesized. Both mono- and di-pyrazinoated conjugates were evaluated for their antimycobacterial activity alone and in combination with isoniazid and rifampicin in *in vitro* and *in vivo* experiments. In a mouse model of *Mtb* infection, a di-pyrazinoated nucleoside compound significantly reduced mycobacterial loads upon oral administration alone and when combined with a low dose of isoniazid or rifampicin, when compared to parent drugs alone, and their individual 2- or 3-drug combinations with isoniazid and rifampicin. The mono-pyrazinoated compound was less effective than di-pyrazinoated compound in mice infected with *Mtb*.

In these studies, I also investigated a new strategy for TB and/or TB-HIV co-infection by designing and synthesizing a novel co-drug incorporating an anti-HIV nucleoside analog 3'-azidothymidine (AZT) with an anti-TB drug p-aminosalicylic acid (PAS). This novel synthesized 5'-para-aminosalicylate-AZT co-drug was evaluated *in vitro* and *in vivo* for its antimycobacterial effects alone and in combinations with existing drugs, isoniazid and rifampicin. Strikingly, the results demonstrated that oral treatment with the designed co-drug provided significant inhibition of *Mtb* in the mouse model in lungs, liver and spleen at $1/20^{th}$ of the dose of parent drug p-aminosalicylic acid. Intriguingly, the co-drug when co-administered with isoniazid or rifampicin, also furnish enhanced effects over isoniazid or rifampicin alone or their combinations as expected.

In vitro cytotoxicity of the identified compounds, conjugates and co-drug was not observed up to the highest concentration tested. In the *in vivo* studies, none of the mice became sick, lost weight or died in all of the treatment groups with the new class of compounds.

From this project, new classes of effective antimycobacterial agents have emerged that have potential to be developed as a new generation of therapeutic regimens for the treatment of TB and TB-HIV co-infection. The importance of my initial finding is that they open new avenues of research to augment current therapy, shorten the duration of treatment, and avoid drug-resistance problems.

Preface

This thesis is an original work by Saurabh Garg. The animal experiments/ procedures (Protocol# AUP 279, till July 15, 2017) were approved and conducted in accordance with the University of Alberta Animal Care and Use Committee (ACUC) for Health Sciences.

Chapter 2 of this thesis has been published to the journal " Bioorganic & Medicinal Chemistry", as S. Garg, N. Shakya, N.C. Srivastav, D.Y. Kunimoto, B. Agrawal and R. Kumar, "Investigation of C-5 alkynyl (alkynyloxy or hydroxymethyl) and/or N-3 propynyl substituted pyrimidine nucleoside analogs as a new class of antimicrobial agents". I was responsible for the design and synthesis of chemical compounds, *in vitro* and *in vivo* biological activity evaluation, experimental design and execution, data collection and analysis, and manuscript writing. Dr. Shakya and Dr. Srivastav provided starting materials used in the synthesis of target compounds. Dr. B. Agrawal and Dr. Kunimoto contributed to data analysis and manuscript edits. Dr. R. Kumar was the supervisory author and was involved with concept formation, data analysis and manuscript composition. The design and synthesize of conjugates and co-drug, *in vitro* and *in vivo* biological activity evaluation, data collection and analysis in chapters 3 and 4 are my original work.

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List of Abbreviations

Ado	:Adenosine
AIDS	:Acquired immuno deficiency syndrome
AMTD	:Amplified mycobacteria direct
ARTs	:Anti-retroviarls
ATCC	:American Type Culture Collection
ATP	:Adenosine triphosphate
ATPase	:Adenosine triphosphatase
AZT	:Azidothymidine
AZT-DP	:Azidothymidine diphosphate
AZT-MP	:Azidothymidine monophosphate
AZT-TP	:Azidothymidine triphosphate
BC	:Before Christ
BCG	:Bacillus Calmette–Guérin
CC_{50}	:Cytotoxic concentration
CD_3OD	:Methanol deuterated
CFU	:Colony forming unit
CH_2Cl_2	:Dichloromethane
CH_2F	:Fluoromethyl
CH_2N_3	:Azidomethyl
CH_2NH_2	:Aminomethyl
$CHCl_3$	:Chloroform
^{13}C NMR	:Carbon 13- nuclear magnetic resonance
CNS	:Central nervous system
Combn	: Combination
Compd	: Compound
Conc	: Concentration
CRI	:Colorimetric redox indicator

d	:Doublet
D_2O	:Deuterium oxide
DC	:Dendritic cells
DCC	:Dicyclohexyl carbodiimide
dd	:Doublet of doublets
DDI	:Dideoxyinosine
Ddn	:Deazaflavin dependent nitroreductase
DEAD	:Diethyl azo dicarboxylate
DHFR	:Dihyhdrofolate reductase
DHFS	:Dihydrofolate synthase
DHPS	:Dihydropteorate synthase
dm	: Doublet of multiplet
DMAP	:4-Dimethylaminopyridine
DMF	:Dimethylformamide
DMSO	:Dimethylsulfoxide
DNA	:Deoxyribonucleic acid
DOTS	:Directly observed standard therapy
dt	: Doublet of triplets
dUMP	:Deoxyuridine monophophate
E. coli	*:Escherichia coli*
E. faecalis	*:Enterococcus faecalis*
EDTA	:Ethylene diaminetetraacetic acid
ELISA	:Enzyme-linked immunosorbent assay
EMA	:European medicine agency
EMB	:Ethambutol
ETH	:Ethionamide
FDA	:Food and Drug Administration
FIC_s	:Fractional inhibitory concentration indices
FUDR	:5-Fluoro-2'-deoxyuridine
FUDR-DP	:5-Fluoro-2'-deoxyuridine diphosphate
FUDR-MP	:5-Fluoro-2'-deoxyuridine monophosphate

FUDR-TP	:5-Fluoro-2'-deoxyuridine triphosphate
5-FdUMP	:5-Fluoro-2'-deoxyuridine monophosphate
GI	:Gastrointestinal
GTPase	:Guanosine triphosphatase
HIV	:Human immunodeficiency virus
^{1}H NMR	:Proton nuclear magnetic resonance
INH	:Isoniazid
Kg	:Kilogram
Ki	:Inhibitory constant
m	:Multiplet
M. africanum	*:Mycobacterium africanum*
M. avium	:*Mycobacterium avium*
M. bovis	:*Mycobacterium bovis*
M. caprae	*:Mycobacterium caprae*
M. intracellulare	*:Mycobacterium intracellulare*
M. microti	*:Mycobacterium microti*
M. pinnipedii	*:Mycobacterium pinnipedii*
M. smegmatis	*:Mycobacterium smegmatis*
M. tuberculosis	:*Mycobacterium tuberculosis*
M.p.	:Melting point
MABA	:Microplate alamar blue assay
MAC	:*Mycobacterium avium* complex
MDR-TB	:Multidrug-resistance tuberculosis
Me_2SO-d_6	:Dimethylsulfoxide deuterated
MeOH	:Methanol
Mg	:Miligram
MIC	:Minimum inhibitory concentration
MODS	:Microscopic observation of drug susceptibility
Mtb	*: Mycobacterium tuberculosis*
MTBC	:*Mycobacterium tuberculosis* complex
MTBDR	:Mycobacteria Direct

NAATs	:Nucleic acid amplification tests
NADH	:Nicotinamide adenine dinucleotide
NaOH	:Sodium hydroxide
NNRTIs	:Non-nucleoside reverse transcriptase inhibitors
NRA	:Nitrate reductase assay
OD	:Optical density
P. aeruginosa	*:Pseudomonas aeruginosa*
PABA	:Para-amino benzoic acid
PAS	:Para-amino salicylic acid
PCR	:Polymerase chain reaction
PMA	:Phorbol myristate acetate
PTH	:Protionamide
PZ	:Pyrazinamide
PZA	:Pyrazinoic acid
PZase	:Pyrazinamidase
q	:Quartet
REMA	:Resazurinmicrotiter assay
RIF	:Rifampicin
RNA	:Ribonucleic acid
RPMI	:Roswell Park Memorial Institute Medium
rRNA	:ribosomal Ribonucleic acid
RT	:Reverse transcriptase
s	:Singlet
S. aureus	:*Staphylococcus aureus*
S. enterica	*:Salmonella enterica*
S. typhimurium	*:Salmonella typhimurium*
SDA	:Strand displacement amplification
SMX	:Sulphamethoxazole
t	:Triplet
TB	:Tuberculosis
TDM	:Therapeutic drug monitoring

TDR-TB	:Totally drug resistant tuberculosis
TLC	:Thin layer chromatography
TMA	:Transcription mediated amplification
TMP	:Trimethoprim
TMPK	:Thymidine monophosphate kinase
TMPKmt	:Thymidine monophosphate kinase *Mycobacterium tuberculosis*
TMS	:Tetramethylsilane
TS	:Thymidylate synthetase
tRNA	:transfer Ribonucleic acid
USFDA	:United States Food and Drug Administration
UV	:Ultra violet
WHO	:World Health Organization
XDR-TB	:Extensively drug-resistant tuberculosis
µg	:Microgram
µl	:Microliter

Chapter 1

Introduction

1.1. Introduction

Tuberculosis (TB) is one of the most serious infectious diseases worldwide and is ranked among the top 10 causes of death globally (1, 2). Ninety-five percent of the deaths due to TB are reported from low or middle-income countries but this disease is also prevalent in developed countries including the United States, Canada, Japan, Europe and Australia (1-3). In Canada, the incidence of TB remains highest in ethnic minorities, aboriginals and foreign-born people (3, 4). TB prevalence is particularly severe in African and Southeast Asian countries accounting for 59% of the total global cases and high mortality (1-6).

At the beginning of the 1900's, TB was the most common cause of death due to an infectious disease in the US and Canada (1, 2). There was a gradual decline in TB cases in North America from the early 1950s but there has been resurgence since about 1984 (3-6). In 1993, the World Health Organization (WHO) declared TB a global emergency (7). The possible reason for the re-emergence of TB has been due to synergism with the HIV/AIDS epidemic, increased mobility and immigration, increased poverty and homelessness, premature dismantling of the health infrastructure for TB treatment, poor compliance of treatment regimens and emergence of multi-drug resistant variants (1-12).

Besides HIV infection, increasing numbers of other immunocompromised individuals, including those undergoing organ transplantation, cancer patients, diabetic and elderly people, are highly susceptible to primary TB infection and reactivation disease (13, 14). Since 1993, important strides to control global TB have been made. Despite these advances, in 2014 alone around 9.6 million people developed TB and 1.5 million died worldwide (15). TB has now surpassed malaria and HIV as the most lethal infectious disease worldwide (1-15).

1.2. History

Literary descriptions suggest that tuberculosis dates back millennia and is found in many ancient medical texts such as in the Old Testament of the bible (~ 1300-400 BC) (16), the writing of Hippocrates (460 BC) (17), the Vedic writing of India (~ 1500 BC) (18) and Chinese medical texts (~ 2700 BC) (19). In the seventh century B.C. the disease was described as coughing blood and in the fifth century B.C., Hippocrates described it as phthisis with coughing and chest pain (20). The ancient link of tuberculosis disease is supported by archaeological data including molecular techniques. The most well-known case was based on the Egyptian mummified remains of Nesparehan, (2400 BC: tubercular decay in the spine). Further studies through IS6110 typing depicted the presence of *Mycobacterium tuberculosis* (*Mtb*) Complex during the Egyptian pre-dynastic period (~3400 BC) (21, 22). Other reports suggested that tuberculosis was geographically widespread with fossil evidence from the fourth millennium BC to the first millennium BC. The oldest fossil evidence of tuberculosis was recovered from a submerged site in the Eastern Mediterranean from the 9000-year-old remains of a woman and infant (23). Some key events of TB diagnosis, mycobacterial identification and culture, vaccine and drug discovery are listed as a historical timeline in figure 1.1.

Figure 1.1: Timeline of Tuberculosis

1.3. Prevalence

Globally, more than 2 billion people (33% of the world's population) harbor *Mtb* in latent form. *Mtb* remains the leading infectious cause of death among adults (15, 24). Among a total of 9.6 million people who fell ill with TB, in 2014, 5.4 million were men, 3.2 million were women, and 1 million were children. More than two thirds of the global TB burden was reported in Africa and Asia and India, China, Indonesia, Ethiopia, Uganda, Russia, Ukraine, Pakistan, Brazil and South Africa account for the highest number of TB cases. Currently, there are ~10-15 million people infected with *Mtb* in the USA (1-12, 15). The overall incidence of TB in Canada is currently 4.8 cases per 100,000 (25). European countries have a TB incidence of >50 cases per 100,000 population. Cases of drug-resistant TB are prevalent worldwide in Asia, Africa, Europe, America and the Eastern Mediterranean Region (Figure 1.2) with a global estimate in 2014 of 480,000 cases of multidrug-resistance tuberculosis (MDR-TB) causing 200,000 deaths. Around 9.7% of MDR-TB cases were caused by extensively drug-resistance *Mtb* (XDR-TB, 15).

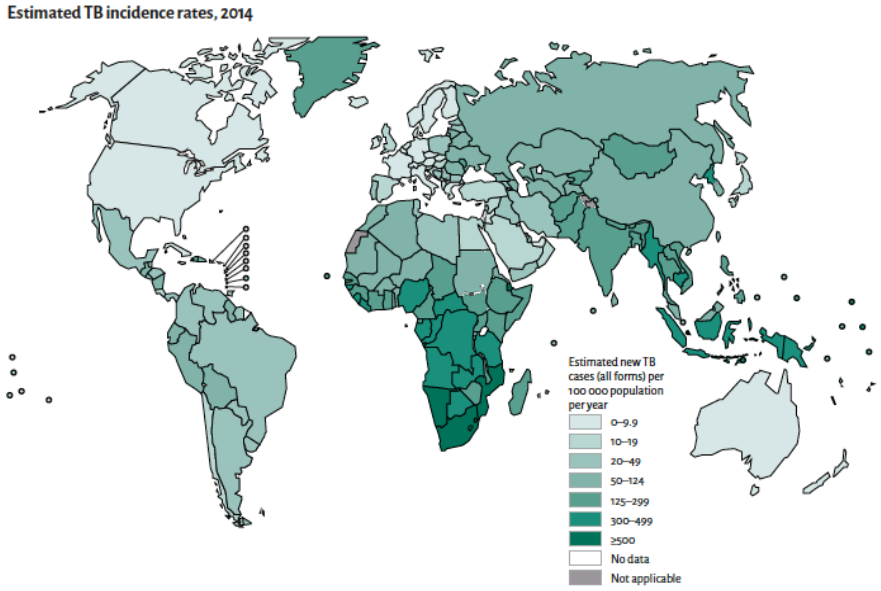

Figure 1.2: Global distribution of tuberculosis

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1.4. The causative agents

TB is caused by any one of the groups of mycobacteria known as the *Mycobacterium tuberculosis* Complex (MTBC) and *M. avium* Complex (MAC). MTBC includes *M. tuberculosis* (*Mtb*), *M. bovis, M. africanum, M. caprae, M. microti* and *M. pinnipedii*. MAC comprises *M. avium*, *M. avium* subspecies (paratuberculosis, silvaticum) and *M. intracellulare* (20, 26). Members of MTBC are highly pathogenic to humans and animals, while MAC infections cause disseminated disease in patients who are immunocompromised by HIV/AIDS and other underlying diseases (27).

1.5. *Mycobacterium tuberculosis* (*Mtb*)

Mtb is a non-motile, rod-shaped bacterium (2-4 μm in length and 0.2-0.5 μm in width). It is an obligate aerobic bacterium, found in the well-aerated upper alveoli of the lungs in TB infected patients. It has a notably slow generation time of 15-20 hours (28).

Mtb has a unique lipid rich cell wall. The cell wall of *Mtb* largely consists of long chain fatty acids termed mycolic acid linked to arabinogalactan, which is attached to the peptidoglycan and forms the mycolyl-arabinogalactan-peptidoglycan complex (Figure 1.3). This unique cell wall makes the bacterium highly impermeable to Gram stain and to water-soluble antibiotics (29). Mycobacteria are specially stained with the Ziehl-Neelsen method using carbolfuchsin, which resists decolorization with acids and alcohols and mycobacteria are thus termed “acid-fast bacilli”. After staining, organisms appear as slightly curved or straight, thin red or pink rods by light microscopy (30).

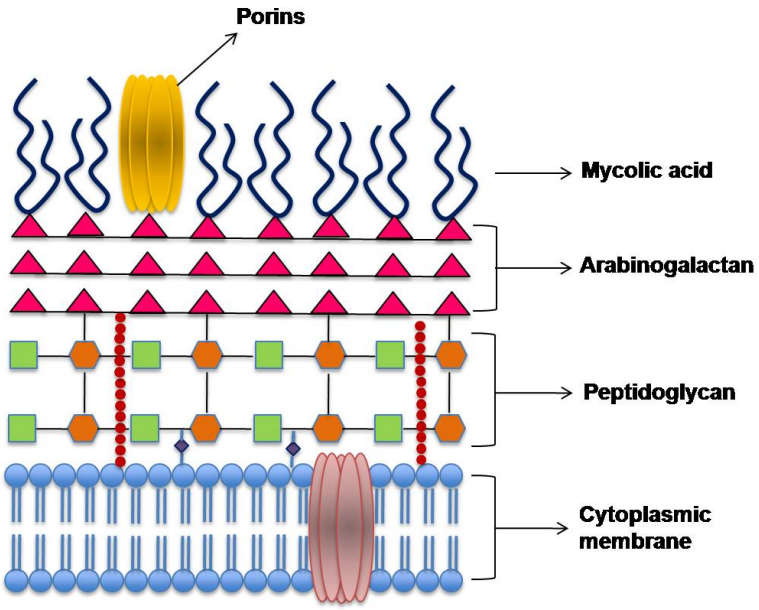

Figure 1.3: **Schematic representation of mycobacterial cell wall**

"Modified from Wu Y, Zhou A. In situ, real-time tracking of cell wall topography and nanomechanics of antimycobacterial drugs treated Mycobacterium JLS using atomic force microscopy. Chem Commun (Camb). 2009 Dec 7;(45):7021-3., © (2009), with permission of the Royal Society of Chemistry. Licence number-3945010215272"

1.6. Transmission and pathogenesis

Tuberculosis is spread through the air in the form of aerosols when a person with active pulmonary tuberculosis sneezes, coughs, spits, laughs or talks (31). Transmission of mycobacteria occurs when a person inhales aerosolized particles containing tubercle bacilli, which are transported through the upper respiratory tract to reach the alveoli of the lungs (31). In the alveoli *Mtb* is taken up by phagocytes in the lung, in particular by alveolar macrophages, and resides within intracellular phagosomes (32, 33). The bacteria survive in the phagosomes by interfering with phagosomal acidification by blocking the accumulation of ATPases and GTPases in the phagosomal vacuolar compartments (34).

The successful establishment of mycobacterial infection depends on the pathogen's ability to disrupt the host cell's defense responses and successfully survive, multiply and

persist within the host. To evade host defense systems, bacterial pathogens produce a variety of virulence factors such as mycolic acid, lipoarabinomannan, mycobactin, trehalose dimycolate, culture filterate proteins etc. that potentiate bacterial adherence and invasion and usurp host cell signaling cascades that regulate intracellular microbial survival and trafficking (35).

The factors that regulate the course and outcome of *Mtb* infection are multifaceted and involve complex interactions between the immune system of the host and survival mechanisms used by the bacilli. The initial stage involves the innate immune response that recruits dendritic cells (DCs) and other inflammatory cells at the site of infection, i.e., lungs. When DCs phagocytize and transport the pathogen or its cellular components to the lymph nodes, adaptive immunity comes into play. In the lymph nodes, dendritic cells present antigens to naive T cells, leading to their proliferation and differentiation into antigen-specific effector T cells. Under the influence of chemokines the effector T cells ($CD4^+$ and $CD8^+$) migrate to the infected lung and initiate the formation of granulomas in combination with other immune cells. Granulomas are organized structures, where infected macrophages are surrounded by epithelial cells, foamy macrophages and lymphocytes ($CD4^+$ T cells, $CD8^+$ T cells and B cells) on the periphery (34-36). Ideally, granulomas form to restrict the dispersal of *Mtb* to the rest of the lung and other organs. Most otherwise healthy people exposed to *Mtb* prevent spread of the organism from the lung but harbor living bacilli within solid granulomas, a condition known as latent TB. In a small percentage (5-10%) of individuals, bacteria are released from the granulomas that liquify and lead to active TB that has the potential to disseminate throughout the body (37). Depending on the host immune status, possible outcomes of *Mtb* infection include:

a) In the immunocompetent host, the bacilli may remain forever within the granuloma in a dormant stage as latent TB.

b) In the immunocompromised host, active disease can develop directly after infection (primary TB disease).

c) In most cases, mycobacteria remain in a quiescent stage, but upon onset of immunocompromised conditions, such as HIV infection, cancer, aging or malnutrition, active TB disease develops as a result of reactivation (secondary TB disease) (38).

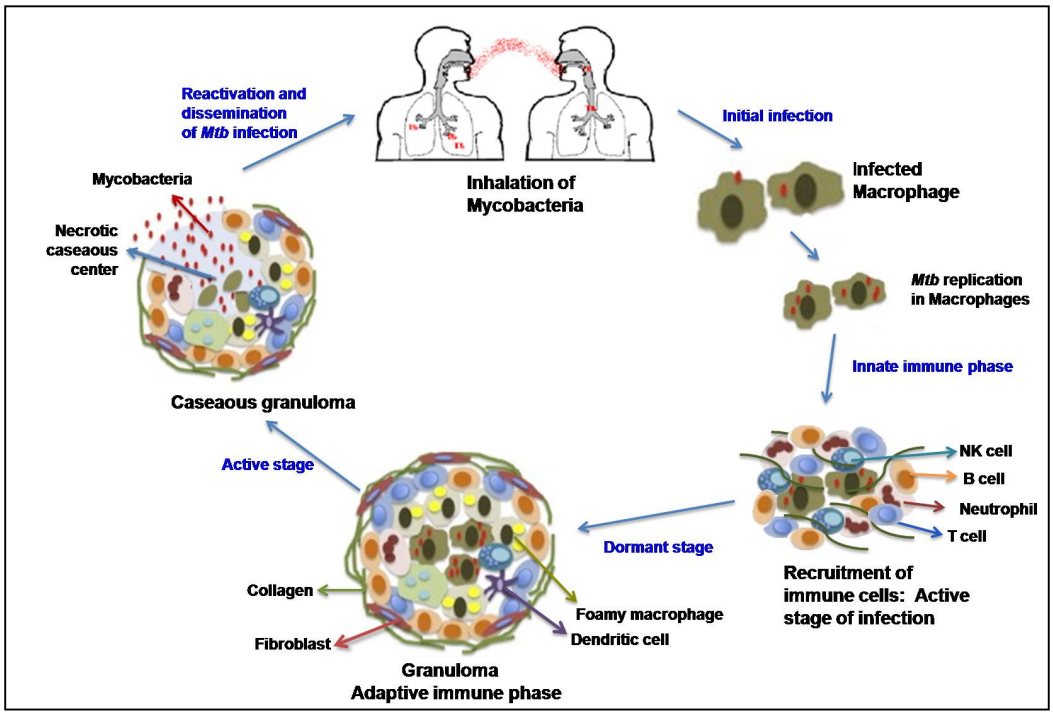

Figure 1.4: TB transmission and pathogenesis

"Modified from Rayasam GV, Balganesh TS. Exploring the potential of adjunct therapy in tuberculosis. Trends Pharmacol Sci. 2015 Aug;36(8):506-13., © (2015), with permission from Elsevier. License number-3944990853906"

1.6.1. Active vs latent TB

Active TB: Active TB is characterized by uncontrolled proliferation and dissemination of the bacilli to various sites of the body. It is a clinically symptomatic and contagious form of the disease (39).

Latent TB: In latent TB, bacilli remain in a non-replicating or dormant stage for years or even decades. Latent TB is clinically asymptomatic and a non-contagious form of the disease (39).

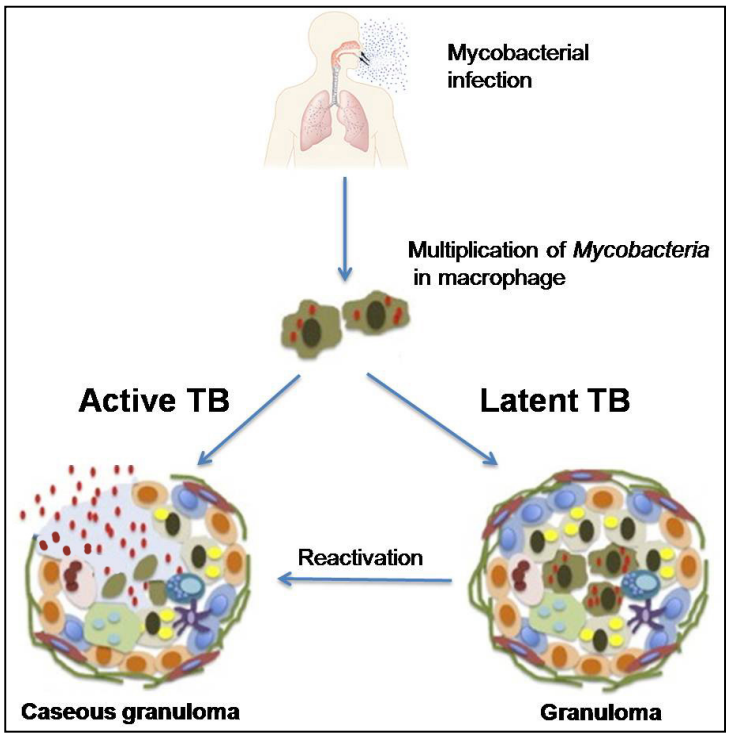

Figure 1.5: Diagrammatic representation of active and latent TB

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1.7. Symptoms

For pulmonary TB, the symptoms include a bad cough lasting three weeks or more, chest pain, weight loss, weakness or fatigue, loss of appetite, bloody sputum, chills, fever, night sweats and shortness of breath (40). Spread of TB to other organs such as lymph nodes can cause swelling under the arms or at the side of the neck. In disseminated disease, liver,

spleen and peritoneal cavity can also be affected. When TB spreads to the joints and bones, it can cause swelling and pain in the joints (40).

1.8. Diagnosis

According to WHO standards for diagnosis of TB, a person with a chronic cough lasting for two-three weeks duration or more should be examined for TB (41) by chest radiography, sputum smear microscopy, mycobacterial culture, and nucleic acid amplification tests (NAATs) (42).

1.8.1. Chest radiography

A posterior-anterior and lateral view of chest radiography is usually the first step to diagnose active TB infection. Chest x-rays of an adult patient with active TB show infiltrates or consolidation and cavities in the posterior apical parts of the upper lobes, the apical segment of the lower lobes and the anterior segments of the upper lobes. However, chest radiography cannot provide a definitive diagnosis of TB on its own and it should be followed by microbiological, immunological and molecular tests for TB infection (42).

1.8.2. Sputum smear microscopy (acid-fast staining)

Sputum smear microscopy is the most widely used test for laboratory diagnosis of TB, where two dyes, carbolfuchsin and auramine-rhodamine, are used. Briefly, the patient's sputum sample, collected early in the morning, is spread on a microscopic glass slide. A phenol-carbolfuchsin stain is added to the sample followed by heating to enable the dye to penetrate the mycolic acid layer of the mycobacterial cell wall. The stain, after binding to the mycolic acid layer, becomes resistant to decolorizing by acid-alcohol. The bacilli then appear

as red or pink rods when examined by light microscopy (43). The major drawback of acid-fast staining is that it cannot distinguish between tuberculous and non-tuberculous mycobacteria (44).

1.8.3. Culture of mycobacteria

Solid and liquid media are used to detect TB bacilli in sputum specimens. For solid medium culture, the sputum specimen is placed on Lowenstein-Jensen solid agar or 7H11 solid medium and allowed to grow at room temperature. After 3 to 4 weeks, the growth of TB bacilli can be detected on the solid medium as buff-colored colonies (44). For liquid culture, 7H9 medium is used for the detection of *Mtb* growth from sputum samples. Liquid culture in an automated system (e.g. BACTEC) is more sensitive and rapid for detection of *Mtb* where mycobacteria can be detected in 10-15 days (45).

1.8.4. Nucleic acid amplification tests (NAAT)

NAAT methods are widely used for the detection of the mutations in the rpoB, katG and gyrA genes of the mycobacteria. Health Canada has approved the following assays which are commercially available: Roche (COBAS Taqman MTB; real-time PCR), Becton Dickson (BD ProbeTec, strand displacement amplification (SDA)), Line probe assays such as HainLifescience (Genotype Mycobacteria Direct, PCR (MTBDR)), Gen-Probe (Amplified *Mtb* Direct (AMTD), transcription mediated amplification (TMA), and Cepheid (XpertMTB/RIF, automated cartridge-based nested PCR). These techniques allow a fast and accurate detection of the mycobacterial species and resistance genes (46).

1.8.4.1. Line probe assays

Line probe assays are NAAT used to detect *Mtb* as well as genetic mutations responsible for antibiotic resistance in *Mtb*. These are commercially available as INNO-LiPA Rif TB kit (Innogenetics, Gent, Belgium) and Genotype MTBDR plus assay (Hain Lifescience, Nehren, Germany). The test involves DNA extraction, multiplex PCR and solid phase reverse hybridization on test strip. Mutations in the target region will restrict the hybridization and subsequent color development. This assay can detect MDR strains of mycobacteria within a few hours (46-48).

1.8.4.2. GeneXpert MTB/RIF assay

WHO recommends the GeneXpert MTB/RIF assay for the diagnosis of MDR-TB (49). It is an automated PCR test that can detect mycobacterial DNA and rifampicin-resistant strains of mycobacteria in sputum specimens within two hours. In this assay, sample processing, DNA extraction and PCR amplification are integrated into a single use GeneXpert cartridge. This test provides a highly accurate diagnosis in a single test (49, 50).

1.9. Antimicrobial susceptibility testing

Inexpensive non-commercial techniques have been developed to test drug susceptibility in non-reference laboratories. The methods, endorsed by WHO, are microscopic observation of drug susceptibility (MODS), colorimetric redox indicator (CRI), and nitrate reductase assay (NRA) (49).

1.9.1. Microscopic observation of drug susceptibility (MODS)

This is an inexpensive, rapid, reliable and simple method for the detection of drug-susceptible and drug-resistant TB. This microscopic method examines the growth of the bacteria growing in liquid medium in absence or presence of the drug. The MODS test is only used for isoniazid and rifampicin susceptible testing (50-52).

1.9.2. Colorimetric redox indicator (CRI) methods

These methods are based on the reduction of a color indicator added to liquid culture medium (7H11 agar) in 96-well microplates containing bacterial sample and anti-TB drugs (51-52).

1.9.2.1. Microplate Alamar Blue assay (MABA)

This is a colorimetric drug-susceptibility method that uses a redox indicator, Alamar Blue dye. The dye changes color from blue to pink to indicate bacterial growth and is measured with a fluorimeter at an excitation of 530 nm and emission of 590 nm. In brief, the susceptibility test is performed using Middlebrook 7H9GC medium in 96-well plates. The inocula are prepared from actively growing bacteria collected from Lowenstein-Jensen slants. Mycobacterial strains are diluted with medium and added to 96 well plates along with anti-TB drugs at various concentrations. Plates are incubated for 6 days and Alamar Blue dye is added to the plates. After incubation for 24h, plates are observed visually for color changes and also read by spectrophotometer. Percent inhibition is calculated using O.D. readings using the formula [% inhibition = 100 - (OD in the presence of test drug − background OD/OD in the absence of drug − background OD) x 100]. The minimum inhibitory

concentration (MIC) is defined as the lowest concentration of drug that prevents color change from blue to pink or provides 95-100% inhibition in mycobacterial growth (51, 52).

1.9.2.2. Resazurin microtiter assay (REMA)

REMA is also used for mycobacterial susceptibility testing. It is based on the same principle as described for the MABA assay. Resazurin is less expensive than Alamar Blue (51).

1.9.2.3. Nitrate reductase assay (NRA)

The NRA method is based on the ability of *Mtb* to reduce nitrate to nitrite by incorporating potassium nitrate in Lowenstein-Jensen medium. The reduction of nitrite is detected by using Griess reagent (concentrated hydrochloric acid, sulphanilamide and *n*-1-naphthylethylenediamine dihydrochloride). In brief, the diluted bacterial suspension is inoculated into Lowenstein-Jensen medium with and without antibiotics and incubated at 37^0C for 7 days. After 7 days' incubation, 500 µl Griess reagent is added and plates are observed visually for color changes. In the presence of drugs, the color changes to pink representing resistance to the antibiotic. WHO data showed that NRA is highly sensitive and specific for the detection of rifampicin and isoniazid resistance (51-53).

1.9.3. BACTEC MGIT assay

The BACTEC MGIT 960 system (Mycobacteria Growth Indicator Tube, Becton Dickinson Co.) is a fully automated, non-radiometric instrument used for the detection of the growth of mycobacteria and drug susceptibility testing. This method is used to determine drug susceptibility for the resistant strains. This method is more sensitive and specific than

the earlier BACTEC 460 method (54). It contains a non-radioactive 7H9 broth medium and an oxygen-labile fluorescent indicator at the bottom of each tube. When mycobacteria use oxygen, the fluorescent indicator gets excited, the resulting fluorescence is examined by a UV source. This method takes an average of 7 days to determine drug susceptibility for resistant strains (54).

1.10. Prevention of TB

Transmission of TB bacilli can be prevented through adequate ventilation, limited contact with patients and proper vaccination. Bacillus Calmette–Guerin (BCG) vaccine is an attenuated strain of *M. bovis* that is available for immunization against TB (55). This vaccine is administered intradermally in the deltoid region of the upper arm. BCG vaccine has shown protection against the development of TB infection in children but it has low to modest efficacy against adult pulmonary TB ranging from 0–80% (55). It has been shown that BCG effectively boosts the immune response against primary infection but has limited effects on the subsequent course of dormancy and reactivation (56). Several investigators have been working on developing a modified BCG as a next generation TB vaccine, but so far clinical trial results have not been promising (56).

1.11. Introduction to anti-tuberculosis drugs

In the absence of effective preventive measures, drug therapy remains the major tool in controlling TB disease. Treating tuberculosis is challenging since it requires early diagnosis, drug-susceptibility screening and the administration of effective combined treatment regimens.

The first antituberculosis agent, streptomycin, was developed as an antibiotic in 1943

at Rutgers University in the laboratory of Selman Waksman (57). In 1944, a patient with tuberculosis infection was successfully treated with streptomycin (57-59). In 1948, the British Medical Research Council conducted the first large-scale clinical trial of streptomycin in TB patients (57-59). Although it was quite effective, organisms developed resistance resulting in a high relapse rate (59).

After streptomycin, two new antimycobacterial agents, thiacetazone and para-aminosalicylic acid were introduced in the clinic. Combination therapy of either of these drugs with streptomycin improved the clinical outcomes in streptomycin-resistant cases. Introduction of isoniazid around 1950 resulted in dramatic improvement in TB treatment and soon isoniazid became the drug of choice. After isoniazid, many other drugs were also introduced including pyrazinamide (1952), cycloserine (1952), ethionamide (1956), rifampin (1957), and ethambutol (1962) (60).

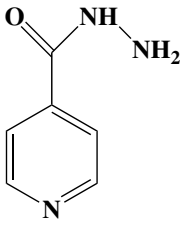
1.11.1. Treatment of tuberculosis

There are currently 20 drugs approved by the U.S. Food and Drug Administration (FDA) for treating TB. The first-line anti-TB agents that form the core of treatment regimens include isoniazid, rifampicin, pyrazinamide and ethambutol (61). Because *Mtb* is a slow growing bacterium and difficult to kill, TB treatment requires a minimum of six months therapy under the DOTS (Directly Observed Standard Therapy) program in two phases: the first two months with four first-line drugs isoniazid, rifampicin, pyrazinamide and ethambutol followed by 4 month's treatment with isoniazid plus rifampicin (61). The second line drugs have lower efficacy, more serious side effects and are used for drug-resistant TB treatment (62).

1.11.2. Anti-tuberculosis drugs and their mechanisms of action

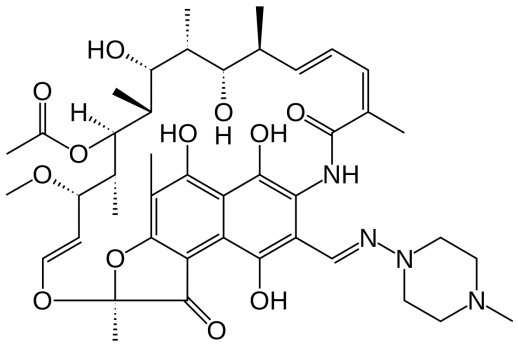
1.11.2.1. First-line anti-tuberculosis drugs

1.11.2.1.1. Isoniazid (INH)



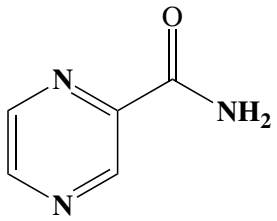
INH was first discovered in 1952 (63). It acts as a bactericidal agent (63). It has an MIC of 0.01-0.2μg/mL for fast replicating mycobacteria (i.e., other than *Mycobacterium tuberculosis*) (63). It is a prodrug (an inactive form of the drug) that is activated by the mycobacterial enzyme, catalase-peroxidase (KatG). After the formation of the isonicotinic acyl-NADH adduct from isonicotinic acid and NADH, it binds to the enoyl-acyl carrier protein reductase known as InhA and blocks the natural substrate enoyl-AcpM and fatty acid synthase. This results in inhibition of mycolic acid synthesis, compromising the formation of the mycobacterial cell wall. INH is bactericidal against dividing bacteria and bacteriostatic against slow growing mycobacteria (62, 63). The recommended daily dose of INH in adults is 5 mg/kg/day (max 300 mg daily) whereas the recommended dose for children is 8 to 12 mg/kg/day (64, 65). INH is metabolized in the liver and its metabolites are excreted in the urine (66). INH may show toxicity to the liver, circulatory system and peripheral nervous system, resulting in acute hepatitis, peripheral neuropathy and haemolytic anemia (67).

1.11.2.1.2. Rifampicin (RIF)



RIF was discovered in 1966 and introduced in 1972 as an antituberculosis drug (63). It has very potent *in vitro* activity against *Mtb* (MIC = 0.05-0.5 μg/mL) (63). It is a semi-synthetic, bactericidal agent derived from the gram-positive bacterium, *Amycolatopsis rifamycinica.* It inhibits DNA-dependent RNA polymerase in bacterial cells by binding to its β-subunit, thereby inhibiting bacterial DNA transcription to RNA and subsequent translation to proteins (62, 63). RIF at a daily dose of 10 mg/kg (up to 600 mg/day) or an intermittent regimen of 10 mg/kg orally, is effective in humans (64). However, *Mtb* quickly develops resistance to rifampicin (68). The side effects of RIF include hepatitis with elevation of bile and bilirubin, anemia, leucopenia, thrombocytopenia, bleeding, fever, eosinophilia, purpura, hemolysis and nephrotoxicity (67, 68).

1.11.2.1.3. Pyrazinamide (PZ)

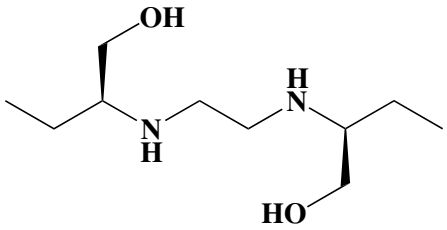


PZ was discovered in 1952. It mainly acts bacteriostatically but can be bactericidal for replicating *Mtb* (63). PZ has MICs in the range of 20-100 μg/mL against *Mtb* (63). This

drug targets a crucial enzyme involved in fatty acid synthesis of the mycobacteria. PZ is a prodrug and in acidic conditions gets converted into the active drug pyrazinoic acid by *Mtb* pyrazinamidase. The pyrazinoic acid then inhibits the enzyme fatty acid synthase I, required by the mycobacterium to synthesize components of mycolic acid (69, 70).

The recommended dose of PZ is 20-25 mg/kg daily or 30-40 mg/kg three times a week (71). PZ is metabolized in the liver and its metabolic products are excreted by the kidneys (70). Common side effects of PZ include skin rash, nausea, vomiting, hepatotoxicity, anorexia, hyperuricemia, sideroblastic anemia, dysuria, joint pains (arthralgia), urticaria, pruritus, malaise, interstitial nephritis, porphyria and fever (71).

1.11.2.1.4. Ethambutol (EMB)

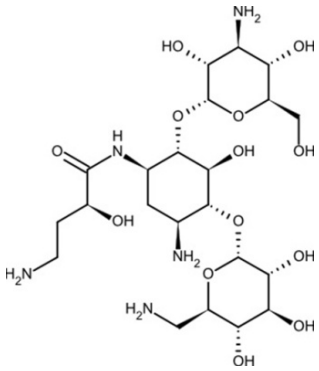


EMB is a bacteriostatic drug developed in 1961 (63). This drug interferes with the synthesis of mycobacterial cell wall component arabinogalactan, by inhibiting the enzyme arabinosyl transferase (62, 63). EMB is well absorbed in the gastrointestinal tract, and is efficiently distributed in body tissues. However, fifty percent of a given dose is excreted unchanged in urine (72). Ethambutol is used at 15-25 mg/kg once daily doses for 6-8 weeks concurrent with isoniazid therapy depending on the clinical situation (73). The major side effects of EMB are peripheral neuropathy, red-green color blindness, arthralgia, hyperuricemia and optic neuritis (74).

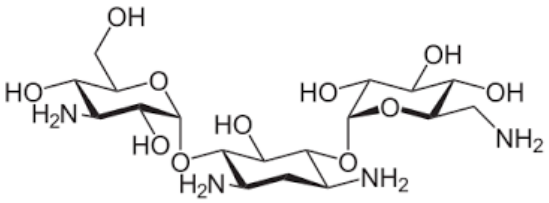
1.11.2.2. Second-line anti-tuberculosis drugs

Second-line anti-mycobacterial drugs include aminoglycosides (amikacin and kanamycin), polypeptides (capreomycin), thioamides (ethionamide and prothionamide), oxazolidinone (linezolid), para-aminosalicylic acid, cycloserine and fluoroquinolones (ciprofloxacin, ofloxacin, levofloxacin and moxifloxacin) (75). In comparison to first-line drugs, second-line drugs possess lower efficacy, unfavorable pharmacokinetic profiles and more serious adverse events. Therefore, second-line drugs are used in combination with first-line drugs for the treatment of drug-resistant MDR and XDR tuberculosis and for therapy of mycobacterium other than *Mtb* (e.g. MAC) (76). Multidrug resistant tuberculosis (MDR-TB) is defined as resistance to the first-line drugs INH and RIF (76). Extensively drug resistant tuberculosis (XDR-TB) is defined as resistance to INH and RIF plus any fluoroquinolone and with 1 or 2 injectable anti-TB drugs such as kanamycin, amikacin and capreomycin (76).

1.11.2.2.1. Aminoglycosides



Amikacin

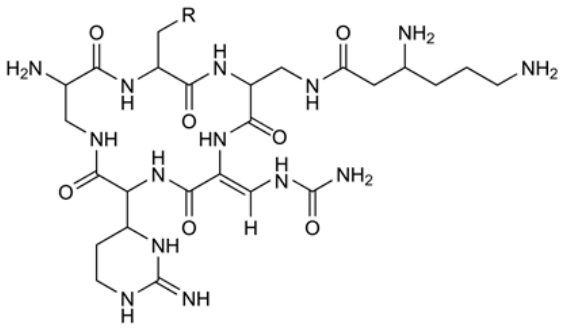


Kanamycin

Both amikacin and kanamycin are aminoglycosides. These are injectable drugs and used in the treatment of MDR-TB (77). They inhibit protein synthesis by binding to the 30S subunit of the bacterial ribosome. These aminoglycosides have a narrow therapeutic window (78). Their optimal dosage regimens are not very clear, however, they are recommended to be

used in the range of 15-30 mg/kg/day dose (78). Therapeutic drug monitoring (TDM) has helped to reduce the side effects associated with this class of agents. Main adverse events seen with them include auditory, vestibular and renal toxicity as well as dizziness (78).

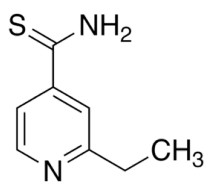
1.11.2.2.2. Polypeptides



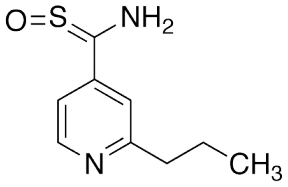
Capreomycin

Capreomycin is a peptide antibiotic that is currently used to treat MDR-TB (79). It inhibits mycobacterial growth by blocking protein synthesis at the ribosomal level by interfering with the formation of the 30S subunit initiation complex and blocks tRNA translocation from A to P site (79). For this drug, a daily intramuscular dose of 15-30 mg/kg is administered to the patients (80). However, capreomycin use is limited due to its vestibular, liver and renal toxicities (79).

1.11.2.2.3. Thioamides



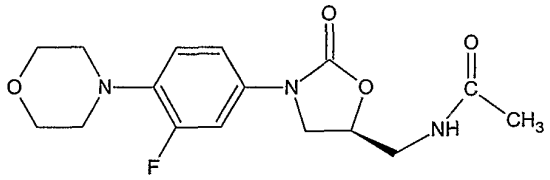
Ethionamide



Prothionamide

Thioamide drugs, ethionamide (ETH) and prothionamide (PTH) are the most frequently used anti-mycobacterial drugs for the treatment of drug-resistant TB (81). ETH is a derivative of isonicotinic acid and has been used since 1956 as an antituberculosis agent. Both ETH and PTH are prodrugs. Their activation occurs by EtaA/EthA (flavin-dependent monooxygenase). Both drugs, like INH, target InhA to inhibit mycolic acid biosynthesis (81). Patient tolerance with these drugs can be challenging because of abdominal pain, vomiting, nausea, anorexia and gastrointestinal toxicity (80). Their daily recommended dose is 15-20 mg/kg (80).

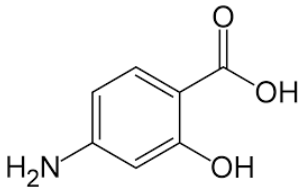
1.11.2.2.4. Oxazolidinones



Linezolid

Linezolid is a first-generation of the oxazolidinone class of drug that inhibits protein synthesis by binding the 23S rRNA in the 50S ribosomal subunit of bacteria (82). It is effective against MDR-TB (82). Linezolid is given at a dose of 600 mg/day (80, 83). Significant adverse effects of linezolid are thrombocytopenia, peripheral neuropathy, myelo suppression and optic neuritis (78). Second-generation oxazolidinone are under development with fewer side effects.

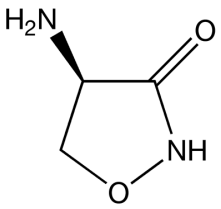
1.11.2.2.5. Aminosalicylic acid



para-Aminosalicylic acid

Para-Aminosalicylic acid (PAS) is a potent antituberculosis agent. It gets incorporated into the folate pathway by dihydropteorate synthase (DHPS) and dihydrofolate synthase (DHFS) to generate a hydroxyl dihydrofolate antimetabolite. This antimetabolite further inhibits the bacterial dihyhdrofolate reductase (DHFR) enzyme (84, 85). PAS is mostly used to treat XDR-TB in combination with INH and streptomycin (84). An oral dose of PAS at 150mg/kg/day is effective (80). PAS is extremely toxic. Gastrointestinal disturbances are the most common adverse events of PAS (85).

1.11.2.2.6. Cycloserine

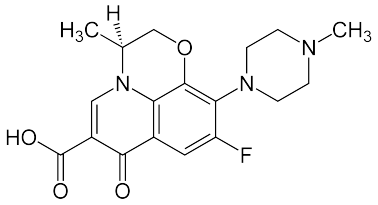


Cycloserine

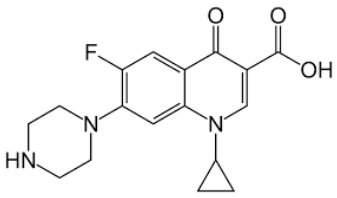
Cyloserine is a cyclic peptide anti-tuberculosis drug. It is effective in the treatment of MDR and XDR tuberculosis (76, 77, 86). The exact mechanism of cycloserine is not known, but it has been suggested that it inhibits peptidoglycan synthesis, which is required to form the mycobacterial cell wall (77). A daily-recommended dose of cyloserine is 15-20 mg/kg (80). Despite its utility against MDR and XDR strains, cycloserine use is limited due to its

CNS toxicity and other side effects such as psychosis, rashes, convulsions and depression (86).

1.11.2.2.7. Fluoroquinolones



Levofloxacin



Ciprofloxacin

Levofloxacin and ciprofloxacin are the most studied agents of the fluoroquinolone class (87, 88). These drugs target DNA gyrase, a type II topoisomerase consisting of two A and two B subunits encoded by the *gyrA* and *gyrB* genes, respectively. The DNA gyrase is a necessary enzyme for uncoiling of the circular chromosome during replication. Due to efficacy and favorable side effect profile, these fluoroquinolones have been used to treat MDR-TB for decades (87). A single dose of ciprofloxacin at 750-1500 mg/day and levofloxacin at 500 mg/day are used clinically (80). Some common adverse effects of fluoroquinolones are dizziness, gastrointestinal intolerance, rashes, hypersensitivities and headache (88).

1.12. Mycobacterial cell wall permeability barrier

The mycobacterial cell wall is structurally different from that of Gram-positive and Gram-negative bacteria (89-91). The mycobacterial cell wall is composed of mycolic acids, which are covalently linked to the arabinogalactan-peptidoglycan complex. The cell wall is also composed of phosphotidyl-myo-inositol derived glycolipids such as lipoarabinomannan

and lipomannan (89-91). The mycolyl-arabinogalactan-peptidogalactan complex creates a permeability barrier to the entry of both hydrophobic and hydrophilic molecules (92). However, hydrophobic drugs can penetrate lipophilic cell wall by passive diffusion, whereas the hydrophilic molecules can enter through porins (93, 94).

1.12.1. Passive diffusion

Anti-tuberculosis drugs INH, RIF, PZA and ETH mainly enter into mycobacterial cells by passive diffusion through the lipid-rich environment (94, 95). The passive transport is facilitated due to the interactions between structural lipids and small molecules (94, 96).

1.12.2. Porins

Water-filled open channels may allow the penetration of hydrophilic molecules into mycobacteria (94-98). Two classes of porins, Omp-A-like and MspA-like, have been identified in mycobacteria (94, 97). Omp-A-like porins are present mainly in *Mtb.* MspA-like porins have been characterized in *M. smegmatis, M. bovis* as well as in *Mtb* (97).

Mycobacteria reside in the cellular as well as necrotic regions of granulomas, therefore effective penetration of drugs through cellular layers and bacterial membranes are essential to eradicate the mycobacterial infection (94-98). Physicochemical studies conducted with a large number of anti-mycobacterial agents suggest that molecules with a small number of aromatic rings and low molecular weights can diffuse efficiently through host cellular areas and through the mycobacterial cell wall and are more likely to exhibit potent effects (99). Poor drug penetration can lead to the emergence of resistant variants due to their sub-inhibitory concentrations at the site of infection (94, 99).

1.13. Antimycobacterial drug-resistance

Combination therapy has been highly effective in treating TB and preventing drug-resistance and treatment failure. Multiple drugs are used to treat TB (100) **i**. to reduce the probability of spontaneous mutations that confer resistance to individual drugs, **ii**. to reduce the frequency of resistance development against a drug and **iii**. to inhibit mycobacteria optimally by using agents that act on different targets (101). The spontaneous mutation rates for RIF, INH, PZA, streptomycin, and ETH per bacterium are $2.25x10^{-10}$, $2.56x10^{-8}$, $1x10^{-3}$, $2.95x10^{-8}$, $1.0x10^{-7}$, respectively (101, 102). Therefore, the greater the bacterial burden, the more likely it contains genetically mono-resistant mutants.

By combining different drugs, several important mechanisms in mycobacteria can be simultaneously targeted allowing a more complete inhibition of mycobacterial replication. This practice makes it harder for the organism to develop resistance against individual drugs.

1.14. Mechanism of resistance in mycobacteria

Mycobacteria use several strategies to develop resistance against antimycobacterial agents. Resistance is not developed in *Mtb* through transfer of plasmids, but rather through mutations in the target gene. Therefore, reversal in resistance to a drug is never observed. This means that development of resistance to a drug or combination of drugs in the host has good probability of spreading the drug resistance in the community (103). Genetic studies have shown that resistance of *Mtb* to drugs is due to the spontaneous mutations in genes that encode either the target of the drug or enzymes that are involved in drug activation (104-106).

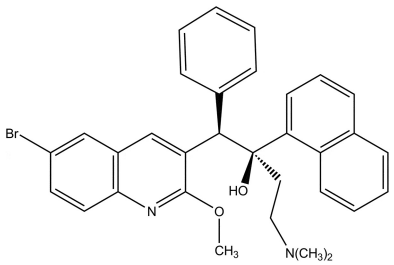
INH resistant strains carry mutations at codon 315 of the *katG* gene. The *katG* S315T mutation is the most common mutation in INH-resistant strains, accounting for 50-95% of INH-resistance in clinical isolates. Resistance to INH also occurs by mutations in *inhA* active

sites and in its promoter region, which lower the *inhA* affinity to the INH-NAD adduct (107). Resistance to INH can also occur due to mutation in the promoter region of mabA (fabG1)/inhA. Strains resistant to the other antituberculosis drug PZ, that kills non-growing persister mycobacteria, have a mutation in the *pncA* gene encoding the enzyme pyrazinamidase. This is the main mechanism of resistance in *Mtb* (108). RIF-resistant strains possess mutations at base pair 81 of the central region of the *rpoB* gene that encodes the β-subunit of RNA polymerase (109). Ethambutol resistance occurs due to mutation in the gene *embB* at codon 306 encoding target enzyme arabinosyltransferase (110).

1.15. New drugs for TB

Recently two new drugs have been developed for the treatment of drug-resistant tuberculosis. In view of the urgent need, the United States Food and Drug Administration (USFDA) granted accelerated approval of bedaquiline and the European Medicine Agency (EMA) approved delaminid (111). These and certain other anti-TB drugs currently at various stages of clinical and preclinical development, are described below (111-114):

1.15.1. Diarylquinolines

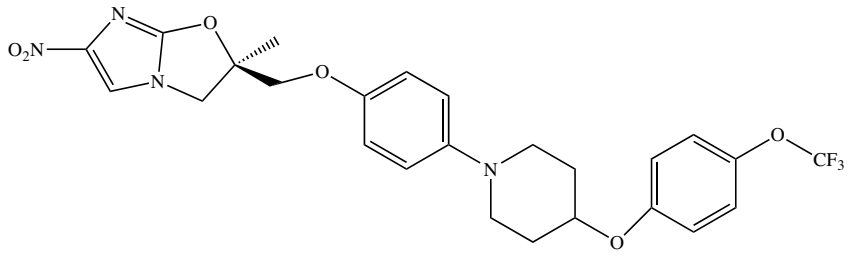


Bedaquiline

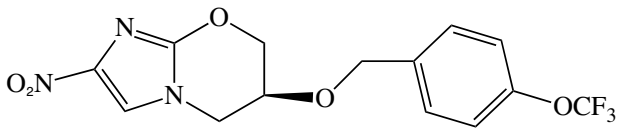
The recently approved drug bedaquiline emerged as a result of high throughput screening of compounds against *M. smegmatis* (111). It is a bactericidal agent (111).

Bedaquiline is also known as TMC 207. This drug belongs to the diarylquinoline class of compounds. Bedaquiline has been approved for the treatment of MDR pulmonary TB and inhibits the mycobacterial ATP-synthase pathway. After processing in the liver by the cytochrome P450 isoenzyme, bedaquiline gets converted to a less active metabolite. However, this drug is minimally excreted by the kidneys (111, 112). Bedaquiline use is highly restricted due to its severe liver and kidney toxicities with potential fatal outcome. Therefore, it is administered with vigilance in TB patients (112). Further, it has been observed that *in vitro* susceptibility does not appear to correlate with efficacy (113).

1.15.2. Nitroimidazoles



Delamanid



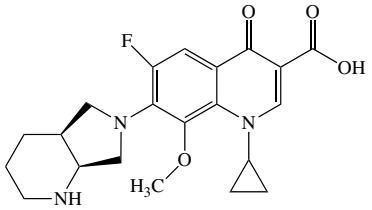
Pretomanid

Delamanid is other newly approved drug for the treatment of drug-resistant TB. It belongs to the nitroimidazole class of compounds. Nitroimidazoles are structurally related to metronidazole, which is currently used to treat protozoal and anaerobic bacterial infections (114). Nitroimidazoles emerged in the 1990s to treat non-replicating *Mtb.* Delamanid, also

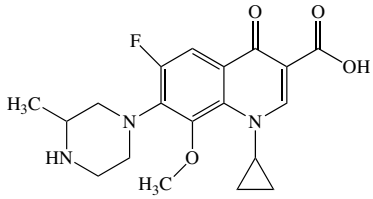
known as OPC-67683, is a dihydro-nitroimidazole prodrug that gets activated by the deazaflavin dependent nitroreductase (Ddn) (115). It inhibits the synthesis of mycobacterial cell wall components, methoxymycolic and ketomycolic acids and is administered orally, exhibiting bactericidal activity (115). Delamanid has a plasma half-life of 30-38 hours (116).

Pretomanid (PA-824) is another nitroimidazole that is being developed. The pretomanid prodrug is activated by the mycobacterial enzyme F_{420}-deazaflavin-dependent nitroreductase (114-117). Both delamanid and pretomanid produce lethal reactive nitrogen species that interfere with the anaerobic pathway of mycobacteria respiration. Pretomanid is currently in phase 3 trials (114-117).

1.15.3. Fluoroquinolones



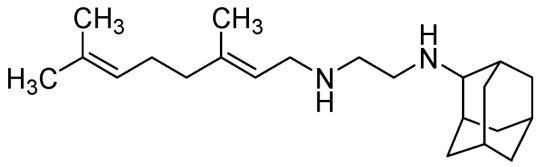
Moxifloxacin



Gatifloxacin

Fluoroquinolones are known as broad-spectrum antibiotics that target DNA gyrase and DNA topoisomerase in many bacteria (115). They are highly active against MDR-TB. Moxifloxacin and gatifloxacin are two of the new generation of fluoroquinolones that are currently being evaluated in phase III clinical trials for MDR-TB therapy (115). In clinical trials, gatifloxacin is being evaluated as a substitute for ethambutol where as moxifloxacin is being tested in place of ethambutol or isoniazid with a goal of shortening the duration of TB treatment (115, 117).

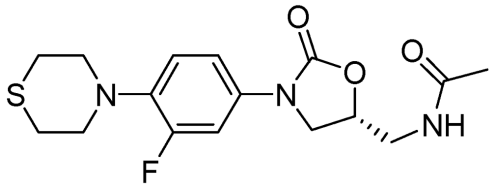
1.15.4. Ethylenediamines



SQ109

SQ109 is a novel 1,2-ethylenediamine compound being developed for the treatment of TB infections (118). SQ109 is an analog of ethambutol (118). It targets MmpL3, a membrane protein that transports trehalose monomycolate into the cell envelope which is essential for mycolic acid biogenesis (119). SQ109 is active against both drug-susceptible and drug-resistant TB. Currently, it is in phase II clinical studies (114, 115, 131).

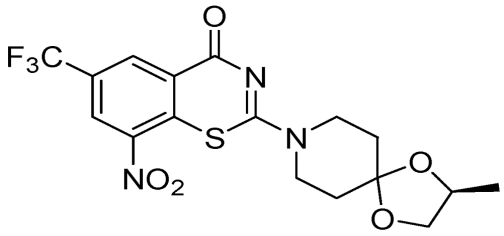
1.15.5. Oxazolidinones



Sutezolid

Sutezolid, also known as PNU-100480, is being investigated for MDR and XDR-TB (120). Sutezolid inhibits protein synthesis by blocking part of the ribosome (121). Sutezolid is a linezolid derivative and is more active (MIC = 0.0625–0.5 μg/mL) than the parent compound linezolid (122). Sutezolid possesses similar *in vitro* efficacy as INH and RIF (122). It has lower MICs then linezolid towards clinical isolates of MDR-TB. Currently it is in Phase II clinical trials in patients with MDR-/XDR-TB (123).

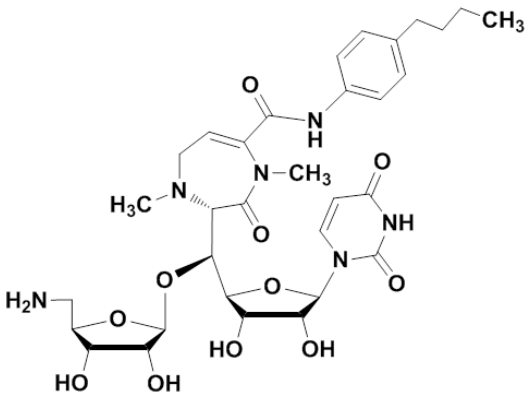
1.15.6. Benzothiazinones



BTZ-043

BTZ-043 is a member of the benzothiazinone class of compounds. It is highly active against *Mtb* (MIC = 1–10 ng/mL) (124). BTZ-043 is also active against MDR and XDR strains of mycobacteria. Activity of BTZ-043 against other actinobacterial species has also been reported (125). It inhibits cell wall biosynthesis, and targets the DprE1 (Rv3790) subunit of the enzyme decaprenylphosphoryl-beta-D-ribose 2'-epimerase (126). A positive feature of BTZ-043 is that it has good oral bioavailability (127).

1.15.7. CPZEN



CPZEN-45

CPZEN-45 (caprazamycin B), a nucleoside antibiotic, is produced by *Streptomyces* species. CPZEN contains a ribose sugar and a diazepinone ring system base (128). It was found to be active against both replicating and non-replicating mycobacteria with an MIC of

1.56 μg/mL against wild type *Mtb* (H37Rv) and an MIC of 6.25 μg/mL against an MDR strain of *Mtb* (128-130). It was also effective in a pulmonary tuberculosis mouse model without causing significant toxicity (129-131).

1.16. Purine nucleoside analogs

Purine nucleoside analogs have also been investigated that interfere with siderophore biosynthesis in *Mtb* under iron-limiting conditions. Their activity was presumed to be due to the inhibition of the adenylate-forming enzyme *MbtA*, which is involved in biosynthesis of the mycobactins (132). Among purine nucleosides, triazole derivatives of 5′-*O*-[*N*-(salicyl)sulfamoyl]adenosine were found to be inhibitors of aryl acid adenylating enzymes (AAAE) involved in siderophore biosynthesis of *Mtb* (H37Rv) (133).

Adenosine (Ado) kinase is a purine salvage enzyme that phosphorylates adenosine to adenosine-monophosphate (134). A number of adenine nucleosides were evaluated as substrates and inhibitors of Ado kinase from *Mtb*. 2-Aza-adenosine, 8-aza-9-deaza-adenosine and 2-fluoroadenosine, were noted to be the best substrates while *N*-1-benzyladenosine, 2-fluoroadenosine, 6-cyclopentyloxy purine riboside and 7-iodo-7-deaza adenosine emerged as the most potent compounds with promising MICs against *Mtb* (134).

1.17. Pyrimidine nucleoside analogs

Mycobacterial thymidine monophosphate kinase (TMPKmt) has been investigated as one of the potential targets for the design of a new class of antimycobacterial drugs (135). TMPKmt is an essential enzyme for DNA synthesis in mycobacteria and other bacteria (135). It has only 22% sequence identity with the human isoenzyme (135). Munier-Lehmann *et al*. and Vanheusden *et al*. (135, 136) reported that monophosphates of 2’-chloro-2’-

deoxythymidine and azidothymidine (AZT) are potent inhibitors of TMPKmt with K_i values of 19 and 10 μM, respectively. These authors also described a series of 2'-deoxy and 2'-ribofluoro nucleotides containing a 3'-C-branched chain of CH_2NH_2, CH_2N_3 and CH_2F substituents with K_i values of 10.5, 12, and 15 μM, respectively. Among a large number of 5-substituted pyrimidine nucleoside they found that 5-Br-dUMP had the highest inhibition of TMPKmt with a K_i value of 33 μM. Their continued efforts also lead to the identification of bicyclic analogues of thymidine as inhibitors for TMPKmt with Ki values in the range of 4.5-3.5 μM. However, when they evaluated their TMPKmt inhibitors in mycobacterial culture, none of them showed any inhibition of *Mtb* replication in concentrations up to 100 μg/mL.

In the search of new classes of antituberculosis agents, our research group (Kumar et al.) designed, synthesized and investigated a variety of pyrimidine nucleosides substituted at 2-, 4-, 5- and/or 6- positions of the pyrimidine base with various deoxy, ribo, arabino, dideoxy and acyclic sugar moieties. We discovered that 5-alkynated pyrimidine nucleosides exhibit potent activities against mycobacteria in cell-based assays at low concentrations (137, 138). Later, our lab (139) reported that pyrimidine nucleoside analogs with a long alkynyl chain at the C-5 position of the base, inhibit *Mtb* (H37Ra) at a concentration of 1-5 μg/mL. These nucleoside analogs also showed sensitivity against a RIF-resistant strain of *Mtb* (H37Rv). However, they had poor water solubility due to their high lipophilicity. In further studies, it was described that nucleoside analogs with a 5-arylalkyl substituents not only displayed potent *in vitro* activities against *Mtb* (H37Ra) and *M. bovis* (MIC_{50} at 0.5-5 μg/mL) but also had potential to inhibit mycobacterial replication *in vivo* in a mouse model of *Mtb* infection (139). This study provided the first evidence of antimycobacterial activity for 5-substituted pyrimidine nucleosides in an animal model as a potential for a new class of antituberculosis agents (139).

Initial findings by our laboratory were followed by the research group of Kogler et al.,

who investigated a series of 5-alkynyl substituted 2'-deoxyuridine monophosphates as selective and potent inhibitors of mycobacterial flavin-dependent thymidylate synthase (140).

1.18. Rational and Hypothesis

Nucleoside classes of compounds or drugs are mainly explored for their antiviral and anticancer properties (141). Nucleoside analogs containing a pyrimidine or purine base can compete with natural nucleosides and/or nucleotides and interfere in DNA and/or RNA synthesis pathways (142). Nucleoside analogs possessing a ribose, deoxy or dideoxy ribose sugar moiety can selectively inhibit enzymes involved in viral and bacterial replication and thereby terminate their DNA and/or RNA synthesis. As a result, nucleoside derivatives can exhibit a wide variety of antibacterial and antiviral properties (142).

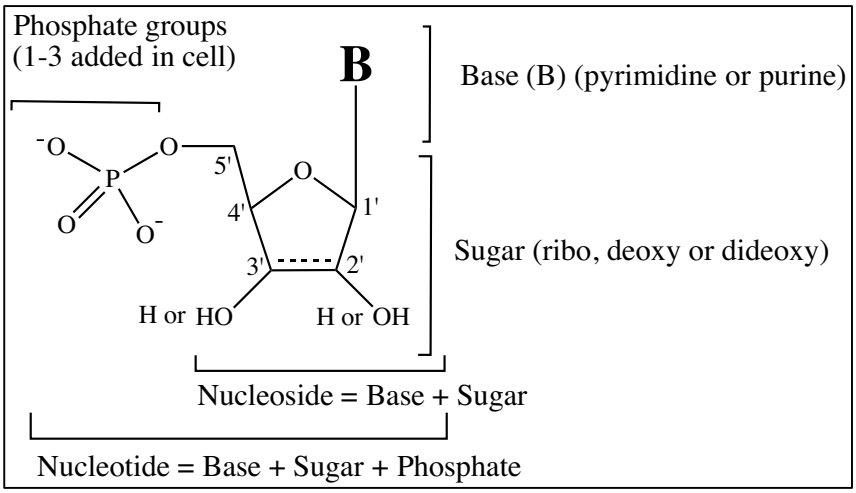


Figure 1.6: Structure of a nucleoside and a nucleotide

The complete genome sequence of *Mtb* has identified many genes that encode several enzymes involved in nucleic acid synthesis, and pyrimidine and purine biosynthesis (143). This suggests that modified nucleosides could target key enzymes involved in mycobacterial RNA and DNA processes. Besides mycobacterial TMPKmt and thymidylate synthase there are a number of other enzymes such as ribonucleotide reductase, dihydrofolate reductase, and

DNA ligase that are also involved in purine and pyrimidine metabolism (143). These bacterial enzymes differ significantly from those in mammals in regards to selectivity towards their substrates and/or potential inhibitors. Thus, enzymes involved in nucleic acid synthesis and metabolism are attractive targets for drug design against bacterial as well as mycobacterial infections. Investigation of novel pyrimidine nucleosides capable of interfering with nucleic acid biosynthetic pathways of bacteria is one of several rational and yet unexplored approaches for investigation of new classes of anti-mycobacterial agents.

Peer-reviewed studies reveal that the C-5 position of the base of pyrimidine nucleosides is a key position for molecular modifications, as this site lies in the major groove of DNA and has steric freedom (144). 5-Alkynyl modifications at the C-5 position can stabilize the DNA duplex structure (145). Additionally, 5-alkynated groups are more hydrophobic than the methyl group present in the natural nucleoside thymidine, allowing for their incorporation into DNA and/or RNA (145). The increased hydrophobicity may also facilitate their entry into bacterial cells (146). It is hypothesized that novel 5-substituted alkynylated pyrimidine nucleoside analogs could interfere with mycobacterial nucleic acid metabolism pathways by acting as inhibitors or competitive substrates, thus altering DNA and RNA synthesis.

An expectation of new TB drugs is that they should have shorter, more effective and more easily tolerated therapeutic regimens. In this regard, modifying existing drugs is an attractive and promising strategy to find new treatment regimens for controlling TB infections.

Pyrazinamide (PZ) and para-aminosalicylic acid (PAS) are two unique anti-TB drugs since they have efficacy against MDR and XDR-TB (147-149). In addition, PZ is also effective against latent tubercle bacilli (148). However, PZ and PAS require high therapeutic doses and suffer from associated severe gastrointestinal and liver toxicities as well as resistance problems

(150, 151). To overcome these limitations, various ester prodrugs of PZ and PAS were investigated (152, 153). However, despite improved *in vitro* antimycobacterial activity of their esters, they failed to provide efficacy in mice. To improve compliance and prevent onset of resistance, it was postulated that conjugation of pyrazinoic acid (PZA, an active form of PZ) and PAS with nucleosides possessing anti-mycobacterial activity, could provide a beneficial approach. The novel conjugates would reduce dosing and improve the toxicity profile, while also providing improved efficacy due to slow release of the two drugs acting on two different targets. To the best of our knowledge such conjugates of PZA and PAS with nucleosides have never been explored.

For a candidate drug to be effective, it must possess desirable pharmacokinetic properties that are determined by absorption, distribution, metabolism and excretion (ADME) of a drug. Nucleoside analogs suffer from poor oral bioavailability due to low intestinal permeability and high polarity (154). Drug bioavailability is reliant on an enterocyte P-glycoprotein (P-gp) an ATP dependent drug efflux pump, which can actively pump back drugs into the duodenum, and on CYP450 cytochrome enzymes, metabolizing the drug before it reaches the systemic circulation. Inhibition or induction of P-gp and CYP450 can thus impact drug bioavailability (155). It is not well understood whether nucleoside analogs have CYP450 inhibitory activity, if that were the case, then drug concentration may be increased in plasma, thus increase their activity. If these nucleoside analogs act as inducers of CYP450 enzyme then the effective plasma concentration may be decreased and thus not only decrease the activity but may also affect other drugs used in combination. In animal models inhibition or induction might then be expected to affect the inherent activity of the nucleoside analog or combination.

Based on the above rationales and hypotheses the specific aims of my research are:

1. To design, synthesize and investigate *in vitro* and *in vivo* antimycobacterial properties of novel 5-substituted pyrimidine nucleoside analogs possessing various sugar moieties.

2. To design and synthesize a novel drug conjugate of PZA and 5-fluoro-2'-deoxyuridine (FUDR) to investigate its antimycobacterial effect *in vitro* and *in vivo* alone and in combination with INH or RIF.

3. To design and synthesize a novel co-drug of PAS and 3'-azido-2',3'-dideoxythymidine (AZT) to investigate its antimycobacterial potential *in vitro* and *in vivo* alone and in combination with INH or RIF.

The overall goals of this research are to design, synthesize and investigate the *in vitro* and *in vivo* antimycobacterial properties of novel pyrimidine nucleosides and prodrugs as new classes of antituberculosis agents.

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Chapter 2

Investigation of C-5 alkynyl (alkynyloxy or hydroxymethyl) and/or N-3 propynyl substituted pyrimidine nucleoside analogs as a new class of antimycobacterial agents*

*A version of this chapter has been published: ***Garg S et al. 2016. Bioorg Med Chem. 24:5521-5533.*** I designed and performed all of the experiments and wrote the manuscript arising from this chapter. Drs. R. Kumar (supervisor), D.Y. Kunimoto and B. Agrawal contributed to the concept for these studies, data analyses and manuscript composition.

2.1. Introduction

Tuberculosis (TB), one of the earliest known human airborne infectious diseases, is caused by the bacterium, *Mycobacterium tuberculosis* (*Mtb)* (1, 2). One-third of the world population is latently infected with *Mtb* and at a risk of developing active TB. Despite the availability of more than 20 approved drugs for the treatment of TB, the current multifaceted TB epidemic continues to grow at an alarming rate. The toxic side effects, the lengthy treatment regimens, poor patients compliance with multiple chemotherapeutic agents, the prevalence of co-infection with HIV and the increasing number of cases of multi/extensively/totally drug resistant tuberculosis (MDR/XDR/TDT-TB), have seriously compromised the current treatments and made the control of mycobacterial infections highly challenging (3-5).

Bacille Calmette–Guerin (BCG) vaccine has been used for the prevention of TB, however it has modest efficacy at best and does not prevent the reactivation or establishment of latent TB (6, 7). Recently, MVA85A vaccine was designed to increase the efficacy of the BCG vaccine but it failed in clinical trials (8, 9). In the absence of effective vaccine against mycobacterial infections, new classes of antimycobacterial agents and effective regimens are immediately required for the management and treatment of wild-type and drug-resistant infections.

Pyrimidine nucleosides are building blocks of DNA and RNA (10). Their analogs have been explored widely as antiviral and anticancer drugs (10, 11). For example, anti-HIV drug 3'-azidothymidine (AZT), anti-cancer drug 5-fluoro-2'-deoxyuridine (FUDR) and 2',2'-difluoro-2'-deoxycytidine (gemcitabine) comprise a major group of chemotherapeutic drugs (12, 13). However, modified pyrimidine nucleosides have not been extensively investigated as antimicrobial agents (11, 14). Piskur *et al.* reported that gram-negative bacteria were

susceptible to AZT (12). Jordheim *et al.* demonstrated antibacterial activity of gemcitabine against MRSA (15, 16) and Beck *et al.* (17, 18) showed 5-FUDR to be active against some gram-positive (*S. aureus* and *E. faecalis*) and gram-negative (*S. typhimurium*, *E. coli* and *P. aeruginosa*) bacteria.

Enzymes involved in nucleic acid synthesis and metabolism are attractive targets for drug design against mycobacterial infections, since a number of enzymes involved in nucleic acid metabolism are significantly different between mycobacteria and their human hosts in terms of selectivity towards their substrates and/or potential inhibitors (19). For example, thymidine monophosphate kinase (TMPK) is essential for DNA synthesis in mycobacteria (19). It has only 22% sequence identity with the human TMPK isoenzyme. Similarly, dihydrofolate reductase (DHFR) of *Mtb* shows only 26% identity with the human DHFR (20).

The C-5 position at the base of pyrimidine nucleosides is the key position for molecular modifications, as this site lies in the major groove of DNA and has steric freedom (21, 22). Kottysch *et al.* (23) suggested that alkynyl and propynyl modifications at the C-5 position of 2'-deoxythymidine stabilize the DNA duplex structure (Figure 2.1). Additionally, these groups are more hydrophobic than the methyl group, allowing their incorporation into DNA and/or RNA (24). Our laboratory previously found that pyrimidine nucleosides with 5-alkynyl substituents exhibit potent and selective anti-mycobacterial activity (25-33).

Incorporation of a fluorine atom at the 2'-position of pyrimidine nucleosides has provided compounds, which are excellent substrates for phosphorylation by kinases. Further, it has been observed that the presence of the 2'-ribofluoro group and an arabino hydroxyl group at the C-2' position leads to stabilization of glycosidic bond against phosphorolysis while retaining biological activities of the parent nucleosides (32, 33). Mizrahi *et al.* (25)

demonstrated that dideoxy nucleoside could serve as good substrates for *Mtb* DNA polymerase.

In this study, a series of 5-ethynyl, 5-(2-propynyloxy) and 5-hydroxymethyl derivatives of 2'- and 3'-substituted deoxy and dideoxy uridines were synthesized to investigate their antibacterial effects against various mycobacteria (*Mtb, M. bovis* and *M. avium*). In this work, several newly synthesized compounds inhibited replication of *Mtb* and *M. bovis* in *in vitro* assays. Interestingly, in these studies, we observed that combining moderately effective nucleosides with known antituberculosis agents provided unpredicted synergistic effects against *Mtb*. We also noted a significant inhibition of *Mtb* growth in mice infected with *Mtb* despite their weak *in vitro* activity.

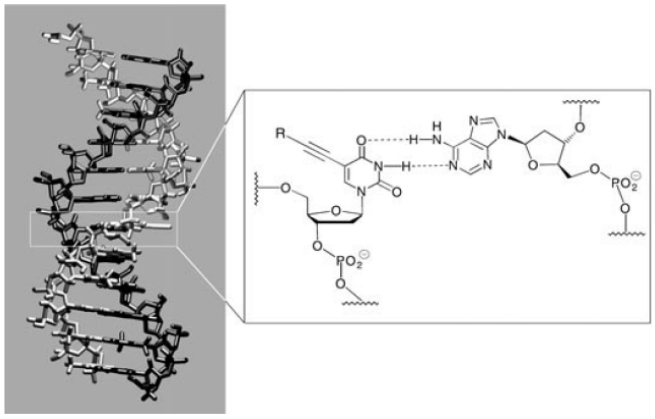


Figure 2.1.

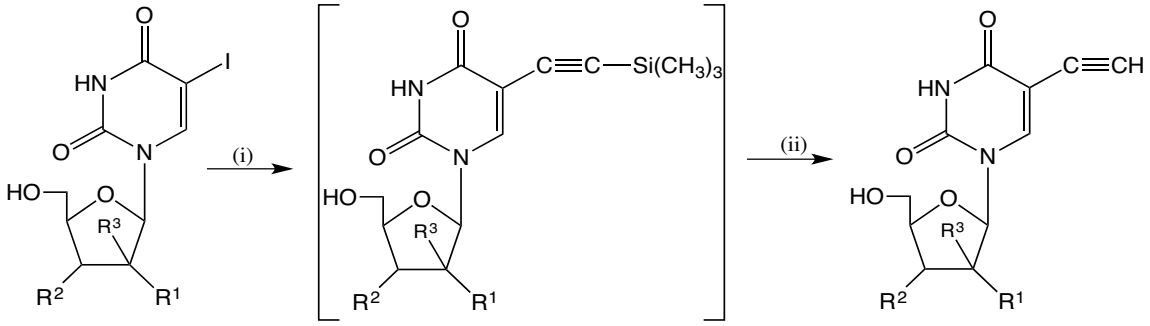
The figure has been reprinted from reference (23) with permission.

2.2. Material and Methods

2.2.1. Chemistry

The synthesis of target 5-alkynyl pyrimidine nucleosides, 5-ethynyluridine (**6**), 5-ethynyl-2'-

arabinouridine (**7**), 5-ethynyl-2',3'-dideoxy-3'-fluorouridine (**8**), 5-ethynyl-2',3'-dideoxy-3'-azidouridine (**9**) and 5-ethynyl-2',3'-dideoxyuridine (**10**) was carried out by a coupling reaction of respective 5-iodo derivatives with trimethylsilyl acetylene. Thus, 5-iodouridine (**1**), 5-iodo-2'-arabinouridine (**2**), 5-iodo-3'-fluoro-2',3'-dideoxyuridine (**3**), 5-iodo-3'-azido-2',3'dideoxyuridine (**4**) and 5-iodo-2',3'-didehydro-2'-3'-dideoxyuridine (**5**) were reacted with trimethylsilylacetylene in diisopropylethylamine in the presence of a catalytic amount of tetrakis(triphenylphosphine)palladium(0) chloride and copper(I)iodide using the Sonogashira reaction (34). The resulting products were deprotected with methanolic sodium methoxide at room temperature using the method of Robins *at el.* (35) to yield the desired compounds (**6-10**) in 51-93% yields (Scheme 2.1).



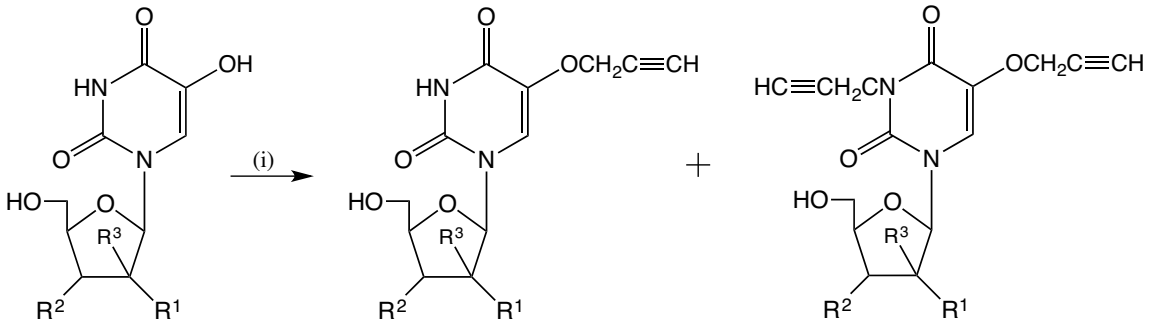
1-5 **6-10**

1, R^1 = OH, R^2 = OH, R^3 = H
2, R^1 = H, R^2 = OH, R^3 = OH
3, R^1 = H, R^2 = F, R^3 = H
4, R^1 = H, R^2 = N_3, R^3 = H
5, R^1 = H, R^2 = H, R^3 = H

6, R^1 = OH, R^2 = OH, R^3 = H
7, R^1 = H, R^2 = OH, R^3 = OH
8, R^1 = H, R^2 = F, R^3 = H
9, R^1 = H, R^2 = N_3, R^3 = H
10, R^1 = H, R^2 = H, R^3 = H

Scheme 2.1. Reagents and conditions: (i) trimethylsilylacetylene, disopropylethylamine, copper(I)iodide, tetrakis(triphenylphosphine)palladium(0), anhydrous dimethylformamide, 25°C; (ii) sodium methoxide, methanol, 25°C.

Alkylation of 5-hydroxy pyrimidine nucleosides (**11-15**) with propargyl bromide in presence of sodium hydroxide in aqueous methanol under nitrogen atmosphere, provided a mixture of the desired mono-alkylated 5-(2-propynyloxy)- (**16, 18, 20, 22** and **24**) as well as di-alkylated 5-(2-propynyloxy)- 3-*N*-(2-propynyl) (**17, 19, 21, 23** and **25**) nucleoside analogues in 12-71% yields (Scheme 2.2) (36).

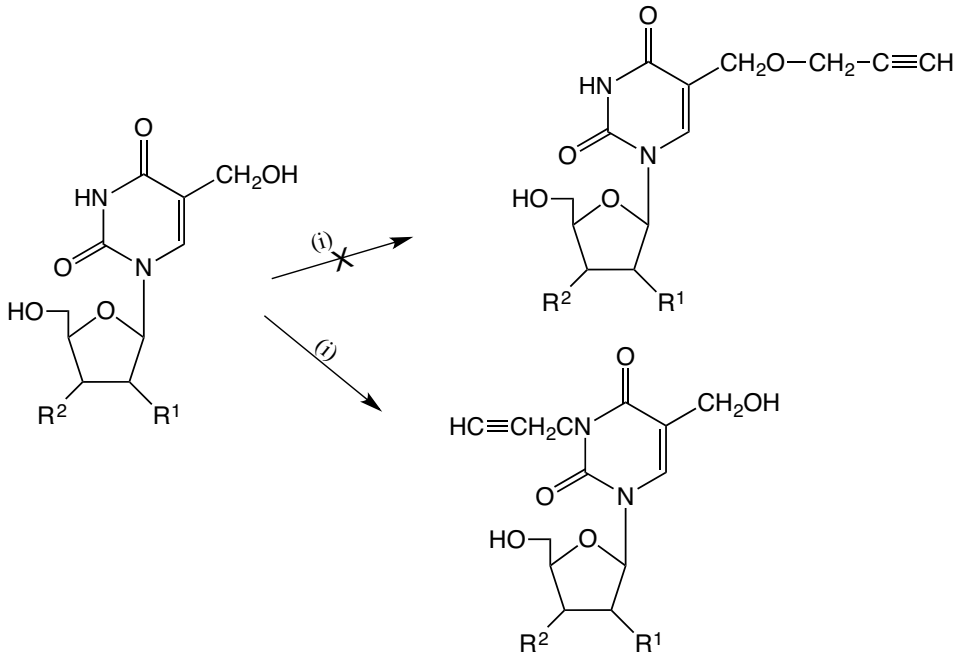


11-15 **16,18,20,22,24** **17,19,21,23,25**

11, R^1 = OH, R^2 = OH, R^3 = H **16**, R^1 = OH, R^2 = OH, R^3 = H **17,** R^1 = OH, R^2 = OH, R^3 = H
12, R^1 = OCH_3, R^2 = OH, R^3 = H **18,** R^1 = OCH_3, R^2 = OH, R^3 = H **19,** R^1 = OCH_3, R^2 = OH, R^3 = H
13, R^1 = H, R^2 = OH, R^3 = OH **20,**R^1 = H, R^2 = OH, R^3 = OH **21,** R^1 = H, R^2 = OH, R^3 = OH
14, R^1 = F, R^2 = OH, R^3 = H **22,** R^1 = F, R^2 = OH, R^3 = H **23**, R^1 = F, R^2 = OH, R^3 = H
15, R^1 = H, R^2 = F, R^3 = H **24,** R^1 = H, R^2 = F, R^3 = H **25,** R^1 = H, R^2 = F, R^3 = H

Scheme 2.2. Reagents and conditions: (i) propargyl bromide, sodium hydroxide, methanol, water, 25°C.

Similar alkylation of 5-hydroxymethyl pyrimidine nucleosides (**26-29**) with propargyl bromide, however, did not give a C-5 alkylated product and afforded N-3 alkylated nucleosides (**30-33**) only in 44-71% yields (Scheme 2.3). The ^{1}H NMR and ^{13}C NMR spectra of **17, 19, 21, 23, 25** and **30-33** provided conclusive evidence for N-3 alkylated products because the NH protons disappeared and new carbon peaks were introduced. Some of the starting materials and all of the reagents were purchased from Sigma Aldrich, Canada.



26-29 **30-33**

26, R^1 = H, R^2 = OH
27, R^1 = OH, R^2 = OCH_3
28, R^1 = H, R^2 = N_3
29, R^1 = H, R^2 = H

30, R^1 = H, R^2 = OH
31, R^1 = OH, R^2 = OCH_3
32, R^1 = H, R^2 = N_3
33, R^1 = H, R^2 = H

Scheme 2.3. Reagents and conditions: (i) propargyl bromide, sodium hydroxide, methanol, water, 25°C.

2.2.2. Experimental section

Melting points were determined with an electrothermal melting point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were determined for samples in Me_2SO-d_6 or CD_3OD on a Bruker AM 600 spectrophotometer using TMS as an internal standard. ^{13}C NMR spectra were determined, where methyl and methyne carbon resonance appear as positive peaks and methylene and quaternary carbon resonances appear as negative peaks. Chemical shifts are given in ppm relative to TMS and signals are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublets), dt (doublet of triplets) and dm (doublet of multiplet). The assignment of exchangeable proton (OH, NH) was confirmed by the addition of D_2O. All of the final compounds had >95% purity determined by microanalysis. Microanalysis results were within ±0.4% of the theoretical values for all elements listed unless otherwise indicated. Silica gel column chromatography was carried out using Merck 7734 silica gel (100-200 μM particle size). Thin-layer chromatography (TLC) was performed with Machery-Nagel Alugram Sil G/UV silica gel slides (20 μM thickness).

2.2.2.1. 5-Ethynyluridine (6)

Tetrakis(triphenylphosphine) palladium (0) (31 mg, 0.026 mmol), copper(I)iodide (10 mg, 0.052 mmol), diisopropylethylamine (69 mg, 0.53 mmol) were added to a solution of 5-iodouridine (**1**, 100 mg, 0.26 mmol) in anhydrous dimethylformamide (20 mL). The reaction mixture was stirred at room temperature for 6 hrs in a nitrogen atmosphere. The progress of the reaction was monitored by TLC in ethylacetate/hexane (50:50, v/v). After 6 h of stirring, 15 drops of 5% disodium salt of EDTA/H_2O were added to the reaction mixture and the contents were concentrated *in vacuo*. The residue obtained was purified on a silica gel column using

MeOH/dichloromethane (4:96, v/v) as an eluent. The acquired trimethylsilylacetylene intermediate compound was directly treated with 0.2 M sodium methoxide in methanol at room temperature with stirring for 8 h. The reaction mixture was neutralized with Dowex 50 (H^+) resin and filtered, and the filtrate was evaporated. The residue obtained was purified on a silica gel column using MeOH/dichloromethane (3:97, v/v) as an eluent to provide **6** (44 mg, 86%) as a solid, M.p. 188-190°C; ^{1}H NMR (DMSO-d6) δ: 3.62-3.65 (m, 1H, H-5'), 3.72-3.76 (m, 1H, H-5'), 3.91-3.93 (m, 1H, H-4'), 4.03-4.11 (m, 2H, H-2' & H-3'), 4.17 (s, 1H, CH), 5.12 (d, 1H, J = 5.4 Hz, 5'-OH), 5.30 (t, 1H, J = 4.8 Hz, 3'-OH), 5.48 (d, J = 5.4 Hz, 1H, 2'-OH), 5.79 (d, 1H, J = 4.8 Hz, H-1'), 8.44 (s, 1H, H-6), 11.70 (s, 1H, NH); ^{13}C NMR (DMSO-d6) δ: 60.65 (C-5'), 69.76 (C-2'), 74.43 (C-3'), 76.78 (5-ethynyl-C), 85.19 & 85.16 (C-4' & 5-ethynyl-C), 88.86 (C-1'), 98.08 (C-5), 145.12 (C-6), 150.13 (C-4), 162.08 (C-2). Anal. Calcd for $C_{11}H_{12}N_2O_6$: C 49.26, H 4.51, N 10.44. Found C 49.58, H 4.76, N 10.21 (Appendix 1).

2.2.2.2. 5-Ethynyl- 2'-arabinouridine (7)

This reaction was carried out with 5-iodo-2'-arabinouridine (**2,** 100 mg, 0.270 mmol) using similar reaction condition as described for **6** to afford **7** as a solid in 51% yield; M.p. 80-85°C; ^{1}H NMR (DMSO-d6) δ: 3.62-3.65 & 3.67-3.70 (dm, 2H, H-5'), 3.87 (q, 1H, J = 1.8 Hz, H-4'), 4.01 (q, 1H, J = 3.6 Hz, H-3'), 4.07 (m, 1H, CH), 5.12 (t, 1H, J = 5.4 Hz, 5'-OH), 5.58 (d, 1H, J = 4.2 Hz, 3'-OH), 5.68 (q, 1H, J = 4.8 Hz, 2'-OH), 6.08 (d, 1H, J = 4.2 Hz, H-1'), 8.43 (s, 1H, H-6), 11.72 (s, 1H, NH); ^{13}C NMR (DMSO-d6) δ: 30.73 (C-5'), 61.21 (5-ethynyl-C), 75.61 (C-3'), 76.17 (C-2'), 85.53 & 85.50 (C-4' & 3-ethynyl-C), 86.39 (C-1'), 111.19 (C-5), 148.63 (C-6), 150.10 (C-4), 162.24 (C-2). Anal. Calcd for $C_{11}H_{12}N_2O_6$: C 49.26, H 4.51, N 10.44. Found C 49.39, H 4.58, N 10.61 (Appendix 2).

2.2.2.3. 5-Ethynyl-3'-fluoro-2',3'-dideoxyuridine (8)

This reaction was carried out with 5-iodo-3'-fluoro-2',3'dideoxyuridine (**3,** 45 mg, 0.126 mmol) using similar reaction condition as described for **6** to afford **8** as a semi solid in 57% yield; ^{1}H NMR (DMSO-d6) δ: 2.25-2.30 (m, 1H, H-2'), 2.32-2.39 (m, 1H, H-2'), 3.62-3.67 (m, 2H, H-5'), 4.13 (s, 1H, 5-CH), 4.18 & 4.23 (dt, 1H, J = 3.0 Hz & J = 3.6 Hz, H-4'), 5.25 & 5.34 (dd, 1H, J = 4.8 Hz & J = 4.2 Hz, H-3'), 5.29 (t, 1H, J = 4.8 Hz, 5'-OH), 6.17 (q, 1H, J = 6 Hz, H-1'), 8.25 (s, 1H, H-6), 11.71 (s, 1H, NH); ^{13}C NMR (DMSO-d6) δ: 59.37 (C-5'), 72.32 (C-2'), 79.72 (5-ethynyl-C), 85.59 and 85.54 (C-4' and 5-ethynyl-C), 87.39 and 87.26 (d, C-1'), 98.23 and 98.17 (d, C-3'), 127.42 (C-6), 138.29 (C-5), 151.54 (C-4), 160.12 (C-2). Anal. Calcd for $C_{11}H_{11}FN_2O_4$: C 51.97, H 4.36, N 11.02. Found C 51.60 H 4.76 N 10.98 (Appendix 3).

2.2.2.4. 5-Ethynyl-3'-azido-2',3'-dideoxyuridine (9)

This reaction was carried out with 5-iodo-3'-azido-2',3'-dideoxyuridine (**4,** 100 mg, 0.265 mmol) using similar reaction condition as described for **6** to afford **9** as a solid in 74% yield; M.p. 92-95°C; ^{1}H NMR (DMSO-d6) δ: 2.30-2.34 (m, 1H, H-2'), 2.43-2.48 (m, 1H, H-2'), 3.60 (d, 1H, J = 1.2 Hz, H-5'), 3.69 (q, 1H, J = 4.8 Hz, H-5'), 3.85 (t, 1H, J = 1.8 Hz, H-4'), 4.11 (d, 1H, J = 1.2 Hz, 5-CH), 4.40 (q, 1H, J = 5.4 Hz, H-3'), 5.34 (t, 1H, J = 4.2 Hz, 5'-OH), 6.03 (t, 1H, J = 5.4 Hz, H-1'), 8.29 (d, 1H, J = 1.2 Hz, H-6), 11.67 (s, 1H, NH); ^{13}C NMR (DMSO-d6) δ: 37.41 (C-5'), 59.66 (C-3'), 60.61 (C-2'), 76.87 (5-ethynyl-C), 84.91 & 85.09 (C-4' & 5-ethynyl-C), 85.37 (C-1'), 98.03 (C-5), 144.94 (C-6), 149.85 (C-4), 162.14 (C-2). Anal. Calcd for $C_{11}H_{11}N_5O_4$: C 47.66, H 4.00, N 25.26. Found C 47.48 H 3.77 N 25.66 (Appendix 4).

2.2.2.5. 5-Ethynyl-2',3'-dideoxyuridine (10)

This reaction was carried out with 5-iodo-2',3'didoxyuridine (**5,** 100 mg, 0.295 mmol) using similar reaction condition as described for **6** to afford **10** as a solid in 93% yield; M.p. 178-180°C; ^{1}H NMR (DMSO-d6) δ: 1.88-1.96 (m, 2H, H-3'), 2.07-2.12 (m, 1H, H-2'), 3.59-3.61 (m, 1H, H-5'), 3.79-3.83 (m, 1H, H-5'), 4.10-4.13 (m, 2H, CH & H-4'), 5.26 (t, 1H, J = 4.8 Hz, 5'-OH), 5.96 (q, 1H, J = 3.0 Hz, H-1'), 8.53 (s, 1H, H-6), 11.64 (s, 1H, NH); ^{13}C NMR (DMSO-d6) δ: 23.34 (C-5'), 32.91 (C-3'), 61.68 (C-2'), 77.14 (5-ethynyl-C), 82.69 (C-4' & 5-ethynyl-C), 86.44 (C-1'), 97.13 (C-5), 145.21 (C-6), 149.88 (C-4), 162.35 (C-2). Anal. Calcd for $C_{11}H_{12}N_2O_4$: C 55.93, H 5.12, N 11.86. Found C 55.51 H 5.22 N 11.41 (Appendix 5).

2.2.2.6. 5-(2-Propynyloxy)uridine (16) and **5-(2-propynyloxy)-3-*N*-(2-propynyl)uridine (17)**

To a solution of 5-hydroxy uridine (100 mg, 0.38 mmol) in methanol (3 mL), water (2 mL) and 1N NaOH (1 mL) propargyl bromide (0.05 mL, 0.57 mmol) was added and the reaction mixture was stirred at room temperature for 12 h. The progress of reaction was monitored by TLC in MeOH/$CHCl_3$ (15:85, v/v). The solvent was removed *in vacuo* and the crude product thus obtained was purified on a silica gel column using MeOH/$CHCl_3$ (5:95, v/v) to yield **17** (15 mg, 12%) as a solid; M.p. 140-145°C; ^{1}H NMR (DMSO-d6) δ: 3.21-3.23 (m, 1H, 3-N-propynyl CH), 3.64-3.67(m, 1H, H-5'), 3.68 (t, J = 2.4 Hz, 1H, 5-O-propynyl CH), 3.73-3.76 (m, 1H, H-5'), 3.95 (q, J = 9.6 Hz, 1H, H-4'), 4.07 (q, J = 4.2 Hz, 1H, H-3'), 4.15 (m, 1H, H-2'), 4.60 (t, J = 2.4 Hz, 2H, 3-N-CH_2), 4.69 (d, J = 1.2 Hz, 2H, 5-O-CH_2), 5.18 (d, J = 5.4 Hz, 1H, 5'-OH), 5.30 (t, J = 4.8 Hz, 1H, 3'-OH), 5.50 (d, J = 6.0 Hz, 1H, 2'-OH), 5.91 (d, J = 4.8 Hz, 1H, H-1'), 7.97 (s, 1H, H-6); ^{13}C NMR (DMSO-d6) δ: 30.82 (C-5'), 58.46 (3-N-CH_2), 61.04 (5-O-CH_2), 70.19 (C-2'), 71.43 (3-N-propynyl CH), 73.74 (3-N-propynyl C), 74.31 (C-3'), 79.71 (5-O-propynyl

C), 85.15 (5-O-propynyl CH), 85.40 (C-4'), 89.65 (C-1'), 124.81 (C-6), 132.44 (C-5), 149.15 (C-4), 158.20 (C-2). Anal. Calcd for $C_{15}H_{16}N_2O_7$: C 53.57, H 4.80, N 8.33. Found C 53.26, H 4.95, N 8.62 (Appendix 6).

Further elution with MeOH/$CHCl_3$ (10:90, v/v) yielded **16**, (65 mg, 57%) as a solid; M.p. 170-175°C; ^{1}H NMR (DMSO-d6) δ: 3.61-3.65 (m, 1H, H-5'), 3.66 (t, J = 2.4 Hz, 1H, 5-O-propynyl CH), 3.70-3.73 (m, 1H, H-5'), 3.92 (t, J = 3.0 Hz, 1H, H-4'), 4.24 (q, J = 4.8 Hz, 1H, H-3'), 4.11 (q, J = 5.4 Hz, 1H, H-2'), 4.65 (d, J = 1.8 Hz, 2H, 5-O-CH_2), 5.14 (d, J = 5.4 Hz, 1H, 5'-OH), 5.24 (t, J = 4.8 Hz, 1H, 3'-OH), 5.43 (d, J = 5.4 Hz, 1H, 2'-OH), 5.85 (d, J = 5.4 Hz, 1H, H-1'), 7.82 (s, 1H, H-6), 11.62 (s, 1H, NH). ^{13}C NMR (CD_3OD) δ: 57.80 (C-5'), 60.83 (5-O-CH_2), 70.06 (C-2'), 74.69 (C-3'), 76.72 (5-O-propynyl C), 85.01 (C-4'& 5-O-propynyl CH), 89.20 (C-1'), 125.41 (C-6), 133.20 (C-5), 149.92 (C-4), 160.68 (C-2). Anal. Calcd for $C_{12}H_{14}N_2O_7.H_2O$: C 45.56, H 5.06, N 8.86. Found C 44.99, H 5.35, N 8.89 (Appendix 7).

2.2.2.7. 5-(2-Propynyloxy)-2'-O-methyluridine (**18**) and 5-(2-propynyloxy)-3-*N*-(2-propynyl)-2'-O-methyluridine (**19**)

To a solution of 5-hydroxy-2'-O-methyluridine (100 mg, 0.36 mmol) in methanol (3 mL), water (2 mL) and 1N NaOH (1 mL) propargyl bromide (0.1 mL, 1.08 mmol) was added and the reaction mixture was stirred at room temperature for 24 h. The progress of reaction was monitored by TLC in MeOH/$CHCl_3$ (5:95, v/v). The solvent was removed *in vacuo* and the crude product thus obtained was purified on a silica gel column using MeOH/$CHCl_3$ (5:95, v/v) as an eluent to yield **19** (28 mg, 22%) as a solid; M.p. 140-145°C; ^{1}H NMR (DMSO-d6) δ: 3.21-3.23 (m, 1H, 3-N-propynyl CH), 3.43 (s, 3H, 2'-OCH_3), 3.65-3.68 (m, 1H, H-5'), 3.70 (t, J = 2.4 Hz, 1H, 5-O-propynyl CH), 3.73-3.77 (m, 1H, H-5'), 3.91 (t, J = 4.8 Hz, 1H, H-4'), 3.97 (m, 1H, H-

2'), 4.24 (q, J = 4.8 Hz, 1H, H-3'), 4.60 (q, J = 2.4 Hz, 2H, 3-N-CH_2), 4.69 (d, J = 2.4 Hz, 2H, 5-O-CH_2), 5.26 (d, J = 6.0 Hz, 1H, 5'-OH), 5.36 (t, J = 9.0 Hz, 1H, 3'-OH), 6.0 (d, J = 4.8 Hz, 1H, H-1'), 8.01 (s, 1H, H-6); ^{13}C NMR (CD_3OD) δ: 30.12 (C-5'), 57.48 (2'-O-CH_3), 57.91 (3-N-CH_2), 60.12 (5-O-CH_2), 68.31 (C-2'), 70.15 (3-N-propynyl C), 76.69 (5-O-propynyl C), 83.98 (C-4' & 5-O-propynyl CH), 84.74 (C-3' & 3-N-propynyl CH), 88.06 (C-1'), 124.23 (C-6), 132.43 (C-5), 148.79 (C-4), 158.97 (C-2). Anal. Calcd for $C_{16}H_{18}N_2O_7$: C 54.86, H 5.18, N 8.00. Found: C 54.46, H 5.28, N 7.78 (Appendix 8).

Further elution with MeOH/$CHCl_3$ (7:93, v/v) yielded **18** (42 mg, 37%) as a solid; M.p. 170-175°C; ^{1}H NMR (DMSO-d6) δ: 3.38 (s, 3H, 2'-OCH_3), 3.63-3.66 (m, 1H, H-5'), 3.68 (t, J = 4.8 Hz, 1H, 5-O-propynyl CH), 3.70-3.74 (m, 1H, H-5'), 3.88 (t, J = 4.8 Hz, 1H, H-4'), 3.93 (q, J = 3.0 Hz, 1H, H-2'), 4.22 (q, J = 4.8 Hz, 1H, H-3'), 4.66 (d, J = 2.4 Hz, 2H, 5-OCH_2), 5.22 (d, J = 6.0 Hz, 1H, 5'-OH), 5.30 (t, J = 9.0 Hz, 1H, 3'-OH), 5.94 (d, J = 5.4 Hz, 1H, H-1'), 7.86 (s, 1H, H-6), 11.64 (s, 1H, NH); ^{13}C NMR (CD_3OD) δ: 57.41 (2'-OCH_3), 57.75 (C-5'), 60.23 (5-O-CH_2), 68.50 (C-2'), 76.69 (5-O-propynyl C), 83.81 (C-3'), 84.82 (C-4' & 5-O-propynyl CH), 87.24 (C-1'), 125.07 (C-6), 133.14 (C-5), 149.50 (C-4), 160.60 (C-2). Anal. Calcd for $C_{13}H_{16}N_2O_7$: C 50.00, H 5.16, N 8.97. Found: C 49.60, H 5.30, N 8.57 (Appendix 9).

2.2.2.8. 5-(2-Propynyloxy)-2'-arabinouridine (20) and **5-(2-propynyloxy)-3-*N*-(2-propynyl)-2'-arabinouridine (21)**

To a solution of 5-hydroxy-2'-arabinouridine (50 mg, 0.19 mmol) in methanol (3 mL), water (2 mL) and 1N NaOH (1 mL) propargyl bromide (0.03 mL, 0.29 mmol) was added and the reaction mixture was stirred at room temperature for 12 h. The progress of reaction was monitored by TLC in MeOH/$CHCl_3$ (15:85, v/v). The solvent was removed *in vacuo* and the

crude product thus obtained was purified on a silica gel column using MeOH/$CHCl_3$ (5:95 v/v) as an eluent to give **21** (15 mg, 23%) as a solid; M.p. 190-195°C; ^{1}H NMR (DMSO-d6) δ: 3.51-3.75 (m, 5H, H-5', H-4', H-3' & H-2'), 4.15 (t, J = 5.4 1H, 3-N-propynyl CH), 4.24 (q, J = 2.4 Hz, 1H, 5-O-propynyl CH), 4.62 (t, J = 2.4 Hz, 2H, 3-N-CH2), 4.64 (dd, J = 2.4 Hz, 2H, 5-O-CH2), 5.16-5.20 (m, 1H, 5'-OH), 5.61 (t, J = 4.2, 1H, 3'-OH), 5.68 (q, J = 4.8, 1H, 2'-OH), 6.19 (d, J = 5.4, H-1'),7.41 (s, 1H, H-6); ^{13}C NMR (DMSo-d6) δ: 31.58 (C-5'), 61.55 (3-N-CH_2), 71.48 (5-O-CH_2), 74.38 and 74.71 (C-3' and 3-N-propynyl CH) 76.41 (3-N-propynyl C), 76.91 (C-2'), 78.20 (5-O-propynyl C), 84.33 and 84.40 (C-4' and 5-O-propynyl CH), 87.20 (C-1'), 129.00 (C-6), 133.39 (C-5), 151.00 (C-4), 162.05 (C-2). Anal. Calcd for$C_{15}H_{16}N_2O_7$: C 53.57, H 4.80, N 8.33. Found C 53.68, H 4.98, N 8.12 (Appendix 10).

Further elution with MeOH/$CHCl_3$ (7:93, v/v) yielded **20**, (35 mg, 61%) as a solid; M.p. 150-155°C; ^{1}H NMR (DMSO-d6) δ: 3.64-3.68 (m, 2H, H-5'), 3.69-3.73 (m, 1H, H-4'), 3.79 (q, J = 4.2 Hz, 1H, H-3'), 4.00 (q, J = 3.6 Hz, 1H, H-2'), 4.09 (q, J = 4.8 Hz, 1H, 5-O-propynyl CH), 4.63 (d, J = 2.4 Hz, 2H, 5-O-CH_2), 5.20 (t, J = 5.4 Hz, 1H, 5'-OH), 5.52 (d, J = 4.8 Hz, 1H, 4'-OH), 5.63 (d, J = 5.4 Hz, 1H, 3'-OH), 6.03 (d, J = 4.8, 1H, H-1'), 7.61 (s, 1H, H-6), 11.61 (s, 1H, NH); ^{13}C NMR (DMSO-d6) δ: 58.72 (C-5'), 60.79 (5-O-CH_2), 75.30 (C-3'), 75.85 (C-2'), 79.47 (5-O-propynyl C), 84.82 & 84.79 (C-4' & 5-O-propynyl CH), 85.42 (C-1'), 128.58 (C-6), 131.82 (C-5), 149.66 (C-4), 160.04 (C-2). Anal. Calcd for $C_{12}H_{14}N_2O_7$: C 48.33, H 4.73, N 9.39. Found C 48.11, H 4.89, N 9.57 (Appendix 11).

2.2.2.9. 5-(2-Propynyloxy)-2'-ribofluorouridine (22) and **5-(2-propynyloxy)-3-*N*-(2-propynyl)-2'- ribofluorouridine (23)**

To a solution of 5-hydroxy-2'-ribofluorouridine (100 mg, 0.38 mmol) in methanol (3 mL), water

(2 mL) and 1N NaOH (1 mL) propargyl bromide (0.05 mL, 0.57 mmol) was added and the reaction mixture was stirred at room temperature for 12 h. The progress of reaction was monitored by TLC in MeOH/$CHCl_3$ (10:90, v/v). The solvent was removed *in vacuo* and the crude product thus obtained was purified on a silica gel column using MeOH/$CHCl_3$ (5:95 v/v) as an eluent to give **23** (26 mg, 20%) as a solid; M.p. 130-135°C; ^{1}H NMR (DMSO-d6) δ: 3.22 (q, J = 5.4 Hz, 1H, 3-N-propynyl CH), 3.68 (t, J = 2.4 Hz, 2H, H-5'), 3.85-3.88 (m, 1H, H-4'), 3.99 (d, J = 7.2 Hz, 1H, H-3'), 4.23-4.30 (m, 1H, 5-O-propynyl CH), 4.59 (q, J = 2.4 Hz, 2H, 3-N-CH_2), 4.66 (d, J = 1.8 Hz, 2H, 5-O-CH_2), 5.06 & 5.14 (2q, J = 1.8 Hz, 1H, H-2'), 5.44 (t, J = 4.8 Hz, 1H, 5'-OH), 5.68 (d, J = 6.0 Hz, 1H, 3'-OH), 6.05 (dd, J = 1.8 Hz, 1H, H-1'), 8.01 (s, 1H, H-6); ^{13}C NMR (DMSO-d6) δ: 30.76 (C-5'), 58.40 (3-N-CH_2), 59.54 (5-O-CH_2), 67.63 & 67.74 (d, C-3' & 3-N-propynyl CH), 73.84 (3-N-propynyl C), 79.70 (5-O-propynyl C), 83.95 (C-4' & 5-O-propynyl CH), 88.12 & 88.35 (d, C-1'), 93.43 & 94.67 (d, C-2'), 124.09 (C-6), 132.57 (C-5), 148.72 (C-4), 158.18 (C-2). Anal. Calcd for $C_{15}H_{15}FN_2O_6.H_2O$: C 50.56, H 4.78, N 7.90. Found C 49.99, H 4.40, N 7.52 (Appendix 12).

Further elution with MeOH/$CHCl_3$ (7:93, v/v) yielded **22**, (47 mg, 41%) as a solid; M.p. 150-155°C; ^{1}H NMR (DMSO-d6) δ: 3.65-3.68 (m, 2H, H-5'), 3.83-3.86 (t, J = 3.0 Hz, 1H, H-4'), 3.95 (d, J = 7.2 Hz, 1H, H-3'), 4.22-4.29 (m, 1H, 5-O-propynyl CH), 4.63 (d, J = 2.4 Hz, 2H, 5-O-CH_2), 5.04 & 5.13 (2q, J = 2.4 Hz, & J = 1.8 Hz, 1H, H-2'), 5.38 (t, J = 4.8 Hz, 1H, 5'-OH), 5.68 (d, J = 6.6 Hz, 1H, 3'-OH), 5.90 (q, J = 1.8 Hz, 1H, H-1'), 7.86 (s, 1H, H-6), 11.70 (s, 1H, NH); ^{13}C NMR (DMSO-d6) δ: 58.30 (C-5'), 59.68 (5-O-CH_2), 67.87 & 67.76 (d, C-3'), 79.52 (5-O-propynyl C), 83.79 & 83.76 (C-4' & 5-O-propynyl CH), 87.52 & 87.49 (C-1'), 94.65 & 93.41 (d, C-2'), 125.13 (C-6), 133.23 (C-5), 149.43 (C-4), 159.80 (C-2). Anal. Calcd for$C_{12}H_{13}FN_2O_6$: C 48.01, H 4.36, N 9.33. Found C 47.79, H 4.72, N 9.65 (Appendix 13).

2.2.2.10. 5-(2-Propynyloxy)-3'-fluoro-2',3'-dideoxyuridine (24) and **5-(2-propynyloxy)-3-*N*-(2-propynyl)- 3'-fluoro-2',3'-dideoxyuridine (25)**

To a solution of 5-hydroxy-3'-fluoro-2',3'-dideoxyuridine (40 mg, 0.16 mmol) in methanol (3 mL), water (2 mL) and 1N NaOH (1 mL) propargyl bromide (0.02 mL, 0.24 mmol) was added and the reaction mixture was stirred at room temperature for 12 h. The progress of reaction was monitored by TLC in MeOH/$CHCl_3$ (10:90, v/v). The solvent was removed *in vacuo* and the crude product thus obtained was purified on a silica gel column using MeOH/$CHCl_3$ (2:98 v/v) as an eluent to give **25** (10 mg, 19%) as a solid; M.p. 115-120°C; ^{1}H NMR (DMSO-d6) δ: 2.26 (s, 1H, H-2'), 2.44 (d, 1H, J = 6.0 Hz, H-2'), 3.22 (t, 1H, J = 2.4 Hz, 5-O-propynyl CH), 3.69-3.73 (m, 3H, H-4' & H-5'), 4.27 & 4.32 (ds, 1H, 3-N-propynyl CH), 4.60 (t, 1H, J = 2.4 Hz, N-CH_2), 4.70 (d, 1H, J = 2.4 Hz, O-CH_2), 5.38 (t, 1H, J = 4.2 Hz, 5'-OH), 5.44 (d, 1H, J = 4.8, H-3'), 6.37 (q, 1H, J = 6.0 Hz, H-1'), 7.90 (s, 1H, H-6); ^{13}C NMR (DMSO-d6) δ: 30.87 (C-5'), 58.45 (3-N-CH_2), 61.37 & 61.45 (d, C-2'), 63.54 (5-O-CH_2), 73.81 (3-N-propynyl C), 79.84 (5-O-propynyl C), 85.55-85.78 (C-1', C-4' & 5-O-propynyl CH), 94.13 (3-N-propynyl CH), 96.06 & 94.91 (d, C-3'), 124.24 (C-6), 132.66 (C-5'), 148.95 (C-4), 158.13 (C-2). Anal. Calcd for $C_{15}H_{15}FN_2O_5$: C 55.90, H 4.69, N 8.69. Found C 55.73, H 4.81, N 8.32 (Appendix 14).

Further elution with MeOH/$CHCl_3$ (5:95, v/v) yielded **24**, (30 mg, 65%) as a solid; M.p. 125-130°C; ^{1}H NMR (DMSO-d6) δ: 2.33-2.42 (m, 1H, H-2'), 2.47-2.53 (m, 1H, H-2'), 3.65-3.74 (m, 3H, H-5', H-4'), 4.25 (dt, 1H, J = 3.5 Hz & J = 3.6 Hz, 5-O-propynyl CH), 4.67 (d, 2H, J = 3.0 Hz, OCH_2), 5.33 (t, 1H, J = 4.8 Hz, 5'-OH), 5.38 (dd, 1H, J = 4.8 Hz, H-3'), 6.30 (q, 1H, J = 5.4 Hz, H-1'), 7.76 (s, 1H, H-6), 11.68 (s, 1H, NH); ^{13}C NMR (DMSO-d6) δ: 58.36 (C-5'), 61.40 & 61.48 (d, C-2') 63.54 (5-O-CH_2), 84.61 & 84.64 (C-4' & 5-O-propynyl CH), 85.34 & 85.49

(d, C-1'), 94.97 & 96.12 (d, C-3'), 125.21 (C-6), 133.33 (C-5), 149.66 (C-4), 159.73 (C-2). Anal. Calcd for $C_{12}H_{13}FN_2O_5$: C 50.71, H 4.61, N 9.86. Found C 50.58, H 4.41, N 9.69 (Appendix 15).

2.2.2.11. 5-Hydroxymethyl-3-*N*-(2-propynyl)-2'-deoxyuridine (30)

To a solution of 5-hydroxymethyl-2'-deoxyuridine (100 mg, 0.38 mmol) in methanol (3 mL), water (2 mL) and 1N NaOH (1 mL) propargyl bromide (0.14 mL, 1.5 mmol) was added and the reaction mixture was stirred at room temperature for 72 h. The progress of reaction was monitored by TLC in MeOH/CHCl3 (15:85, v/v). The solvent was removed *in vacuo* and the crude product thus obtained was purified on a silica gel column using MeOH/CHCl3 (6:94 v/v) as an eluent to give 30 (50 mg, 44%) as a solid; M.p. 155-160°C; ^{1}H NMR (DMSO-d6) δ: 2.23 (m, 2H, H-2'), 3.18 (t, J = 2.4 Hz, 1H, 3-N-propynyl CH), 3.63 (m, 2H, H-5'), 3.87 (q, J = 3.6 Hz, 1H, H-4'), 4.24 (t, J = 5.4 Hz, 2H, 5-CH_2), 4.31 (q, 1H, J = 3.6 Hz, H-3'), 4.59 (q, J = 2.4 Hz, 2H, 3-N-CH_2), 5.07 (m, 2H, 5-OH & 5'-OH), 5.35 (d, J = 4.2 Hz, 3'-OH), 6.30 (t, 1H, J = 7.2, H-1'), 7.91 (S, 1H, H-6); ^{13}C NMR (CD_3OD) δ: 29.65 (C-5'), 40.13 (5-CH_2), 57.05 (3-N-CH_2), 61.43 (C-2'), 70.52 (3-N-propynyl C), 70.78 & 70.89 (C-3'&3-N-propynyl CH), 86.06 (C-4'), 87.47 (C-1'), 113.06 (C-5), 136.65 (C-6), 150.14 (C-4), 161.68 (C-2). Anal. Calcd for $C_{13}H_{16}N_2O_6$: C 52.70, H 5.44, N 4.96. Found C 52.43 H 5.61 N 4.82 (Appendix 16).

2.2.2.12. 5-Hydroxymethyl-3-*N*-(2-propynyl)-3'-O-methyluridine (31)

To a solution of 5-hydroxymethyl-3'-O-methyluridine (40 mg, 0.14 mmol) in methanol (3 mL), water (2 mL) and 1N NaOH (1 mL) propargyl bromide (0.07 mL, 0.83 mmol) was added

and the reaction mixture was stirred at room temperature for 76 h. The progress of reaction was monitored by TLC in MeOH/$CHCl_3$ (15:85, v/v). The solvent was removed *in vacuo* and the crude product thus obtained was purified on a silica gel column using MeOH/$CHCl_3$ (6:94 v/v) as an eluent to give **31** (20 mg, 44%) as a solid; M.p. 115-120^oC; ^1H NMR (DMSO-d6) δ: 3.19 (t, 1H, J = 2.4 Hz, 3-N-propynyl CH), 3.43 (s, 3H, OCH_3), 3.63 (m, 1H, H-5'), 3.71 (m, 1H, H-5'), 3.76 (t, 1H, J = 4.8 Hz, H-2'), 4.04 (q, 1H, J = 3.0 Hz, H-4'), 4.24 (d, 2H, J = 4.8 Hz, 5-CH_2), 4.30 (q, 1H, J = 5.4 Hz, H-3'), 4.60 (q, 2H, J = 3.0 Hz, N-CH_2), 5.08 (t, 1H, J = 5.4 Hz, 5-OH), 5.22 (t, 1H, J = 4.8 Hz, 5'-OH), 5.54 (d, 1H, J = 6.0 Hz, 2'-OH), 5.91 (q, 1H, J = 5.4 Hz, H-1'), 7.98 (s, 1H, H-6), 11.47 (s, 1H, NH); ^{13}C NMR (DMSO-d6) δ: 30.34 (C-5'), 57.38 (3-N-CH_2), 61.32 (5-CH_2), 73.03 (H-2'), 73.55 (3-N-propynyl C), 79.48 & 79.50 (H-3' & 3-N-propynyl CH), 83.07 (C-4'), 113.80 (C-5), 136.51 (C-6), 150.48 (C-4), 161.11 (C-2). Anal. Calcd for $C_{14}H_{18}N_2O_7.H_2O$: C 48.83, H 5.81, N 8.13. Found C 48.61, H 6.21, N 8.47 (Appendix 17).

2.2.2.13. 5-Hydroxymethyl-3-*N*-(2-propynyl)-3'-azido-2',3'-dideoxyuridine (32)

To a solution of 5-hydroxymethyl-2'deoxy-3'-azido-2',3'-dideoxyuridine(75 mg, 0.26 mmol) in methanol (3 mL), water (2 mL) and 1N NaOH (1 mL) propargyl bromide (0.04 mL, 0.39 mmol) was added and the reaction mixture was stirred at room temperature for 96 h. The progress of reaction was monitored by TLC in MeOH/$CHCl_3$ (8:92, v/v). The solvent was removed *in vacuo* and the crude product thus obtained was purified on a silica gel column using MeOH/$CHCl_3$ (2:98 v/v) as an eluent to give **32** (55 mg, 64%) as a solid; M.p. 108-110$^{\circ}C$; ^1H NMR (DMSO-d6) δ: 2.40-2.48 (m, 2H, H-2'), 3.20 (t, J = 2.4 Hz, 1H, 3-N-propynyl CH), 3.69 (m, 2H, H-5'), 3.94 (q, J = 4.2, 1H, H-4'), 4.26 (t, 2H, J = 7.8 Hz, 5-CH_2), 4.47 (m, 1H, H-3'), 4.59 (q, 2H, J = 2.4 Hz, N-CH_2), 5.08 (t, 1H, J = 5.4 Hz, 5-OH), 5.28 (t, 1H, J = 5.4 Hz, 5'-OH),

6.22 (t, 1H, J = 6 Hz, H-1'), 7.91 (S, 1H, H-6); ^{13}C NMR (DMSO-d6) δ: 30.29 (C-5'), 36.96 (5-CH_2), 56.93 (3-N-CH_2), 60.69 & 60.75 (C-3' & 3-N-propynyl CH), 61.26 (C-2'), 73.57 (3-N-propynyl C), 84.78 (C-4'), 85.14 (C-1'), 113.98 (C-5), 136.41 (C-6), 150.08 (C-4), 161.59 (C-2).Anal. Calcd for $C_{13}H_{15}N_5O_5$: C 48.60, H 5.44, N 4.71. Found C 48.48 H 5.21 N 4.83 (Appendix 18).

2.2.2.14. 5-Hydroxymethyl-3-*N*-(2-propynyl)-2',3'-dideoxyuridine (33)

To a solution of 5-hydroxymethyl-2',3'-dideoxyuridine (75 mg, 0.30 mmol) in methanol (3 mL), water (2 mL) and 1N NaOH (1 mL) propargyl bromide (0.16 mL, 1.85 mmol) was added and the reaction mixture was stirred at room temperature for 96 h. The progress of reaction was monitored by TLC in MeOH/$CHCl_3$ (10:90, v/v). The solvent was removed *in vacuo* and the crude product thus obtained was purified on a silica gel column using MeOH/$CHCl_3$ (2:98 v/v) as an eluent to give **33** (62 mg, 71%) as a solid; M.p. 80-85°C; ^{1}H NMR (DMSO-d6) δ: 1.88 (q, 1H, J = 9 Hz, H-2'), 2.05 (m, 2H, H-3'), 2.38 (q, 1H, J = 6.6 Hz, H-2'), 3.17 (t, 1H, J = 2.4 Hz, 3-N-propynyl CH), 3.62 (m, 1H, H-5'), 3.72 (m, 1H, H-5'), 4.11 (m, 1H, H-4'), 4.24 (d, 2H, J = 6 Hz, 5-CH_2), 4.59 (q, 2H, J = 2.4 Hz, N-CH_2), 5.07 (m, 2H, 5-OH & 5'-OH), 6.10 (q, 1H, J = 3.6 Hz, H-1'), 7.97 (s, 1H, H-6); ^{13}C NMR (CD_3OD) δ: 23.74 (C-5'), 28.85 (5-CH_2), 31.42 (C-3'), 56.30 (N-CH_2), 62.21 (C-2'), 69.65 (3-N-propynyl C), 81.45 (C-4' & 3-N-propynyl CH), 86.33 (C-1'), 111.55 (C-5), 136.09 (C-6), 149.32 (C-4), 161.01 (C-2);Anal. Calcd for $C_{13}H_{16}N_2O_5$: C 55.71, H 5.75, N 10.00. Found C 55.48 H 5.56 N 9.86 (Appendix 19).

2.2.3. ***In vitro*** **antimycobacterial activity assay (*Mtb*, *M. bovis*, and *M. avium*)**

Mtb (H37Ra), *M. bovis* (BCG), and *M. avium* (ATCC 25291) were obtained from the American Type Culture Collection (ATCC), Rockville, MD. These strains were cultured in Middlebrook 7H9 Broth medium supplement with glycerol, Tween 80 and Middlebrook ADC (Bovine Albumin, Dextrose and Catalase) enrichment purchased from Becton Dickinson and company, MD, USA. Antimycobacterial activity was determined using the Microplate Alamar Blue assay (MABA) (37). The cell viability reagent Alamar Blue was purchased from Bio-Rad Laboratories, Inc., USA. Test compounds were dissolved in dimethyl sulfoxide (DMSO) (purchased from Fisher Scientific, Canada) at 10 mg/mL and subsequent dilutions were made in 7H9GC medium (Difco Laboratories, Detroit, Michigan) in 96-well plates. For these experiments, initially each compound was tested at 200, 100, 50, 25 and 10 μg/mL in triplicate. The experiments were repeated three times and the mean percent inhibition from a representative experiment is provided in Table 2.1. The standard deviations of triplicates were within 10%. For combination studies detailed dose responses of the selected compounds was determined in a range from 1-200 μg/mL. Frozen mycobacterial inocula were diluted in 7H9GC medium and added to each well at a final concentration of 2.5 x 10^5 CFU/mL. Sixteen control wells consisted of eight with bacteria alone (B) and eight with medium alone (M). Plates were incubated for six days and then 20 μL of 10 x alamar blue and 12.5 μL of 20% Tween 80 were added to one M and one B well. Wells were observed for an additional 24-48 h for visual color change from blue to pink and read by spectrophotometer (Fluostar Optima, BMG Labtech, GmbH, Ortenberg, Germany) at excitation 530/525 nm and emission 590/535 nm to determine OD values. If the B well became pink by 24 h (indicating growth), reagent was added to the entire plate. If the B well remained blue, additional M and B wells were tested daily until bacterial growth could be

visualized by color change. After the addition of the reagent to the plate, cultures were incubated for 24 h and plates were observed visually for color change and read by spectrophotometer. MIC was defined visually as the lowest concentration of a compound that prevented a color change from blue to pink. Percent inhibition was calculated as 100 - (test well - Medium well) / (Bacteria well - Media well) x 100. Similar methodology was used for all (three) mycobacteria strains. Isoniazid, rifampicin and clarithromycin were used as positive controls. As negative controls, DMSO, was added to the B well at concentrations similar to those of compound wells; M wells served as negative controls. In most of the experiments, the M wells gave an OD of 2000-3000, and the B wells had OD values ranging between 40,000-60,000.

2.2.4. **Intracellular antimycobacterial activity in a human monocytic cell line**

Human monocytic cell line (THP-1) was cultured at 1 x 10^5 cells/well in a 96-well plate in complete Roswell Park Memorial Institute Medium (RPMI) (GE Healthcare Life Sciences). A 50 ng/mL concentration of phorbol myristate acetate (PMA) (Sigma-Aldrich) was added to each well, and the plates were incubated for 1 day at 37°C to allow adherence and differentiation. Cells were then infected with 5 x 10^6 CFU/mL of *Mtb* (H37Ra) was added to each well and allowed to infect the macrophages for 24 h. Non-phagocytosed mycobacteria were removed by washing three times with RPMI medium. Test compounds were added in triplicate to the infected cell culture in concentrations of 200, 100 and 50 μg/mL along with control wells of 1 μg/mL isoniazid, DMSO and media and incubated for 7 days. Cells from each well were lysed and aliquots of lysates were transferred to 7H11 plates in serial dilution. The growing colonies were counted at 2-3 weeks (38).

2.2.5. *In vitro* antimicrobial activity of test compounds in combination with standard drug(s)

For combination studies, MABA activity assays employing similar methodology was used as described above. For two drug combination studies INH was used at 0.2 µg/mL concentration. For three drug combination studies INH was used at 0.006-0.012 µg/mL and RIF was used at 0.002 µg/mL concentration. The combination effect of compounds was determined by calculating combination index (CI) with a general equation for use with any number (n) of drugs (a,b,c,…) in a combination as follows: $CI = (E_{a,\ alone} + E_{b,\ alone} + E_{c,\ alone...} + E_{n,\ alone} / E_{a,\ b,\ c,\ldots n\ combination})$ (39). CI was calculated as a sum of the individual drug effect (E) divided by the combination effect of drugs. The CI were interpreted as follows: CI < 1, synergistic (the combined effect is greater than the sum of their individual effects); CI = 1, additive (the combined effect is equal to the sum of their individual effects); CI > 1, antagonistic effects (the combined effect is less than the sum of their individual effects).

2.2.6. *In vitro* cytotoxicity assay

Cell viability was measured using the cell proliferation kit 1 (XTT; Xenometric:Endotell). A 96-well plate was seeded with Vero cells cultured in Dulbecco's modification of Eagle medium (DMEM, Gibco Thermo Fisher Scientific) supplemented with 10% heat inactivated fetal bovine serum (Gibco Thermo Fisher Scientific) at a density of 2×10^5 cells per well. Compounds were dissolved in DMSO at 10 mg/mL and subsequent dilutions were made in DMEM medium in 96-well plates. Cells were allowed to attach for 24 h, and the DMEM medium was replaced with DMEM medium containing compounds at concentrations of 300, 200, 100, 50 and 10 µg/mL. DMSO was also included as a solvent control. Plates were

incubated for 3 days at 37°C. The color reaction involved adding 10 µL XTT reagents per well and incubating for 4 h at 37°C until color change to orange (40). Plates were read on an ELISA plate reader (Fluostar Optima, BMG Labtech, GmbH, Ortenberg, Germany) at Abs $_{450\text{-}500}$ nm. Percent viability was calculated as (OD of test well) - (OD of Medium well without cells) / (OD of control solvent well) - (OD of Medium well without cells) x 100.

2.2.7. Animals and infection

The experimental animal protocol used in this study was approved by the University Animal Care and Use Committee (ACUC) for Health Sciences, and conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC). Five- to six-week-old female BALB/c mice were purchased from Charles River Laboratories and were allowed to acclimate for 1 week. Mice were challenged intravenously in the tail vein with 0.5 x 10^6 CFU/mouse of *Mtb* (H37Ra) in saline. Female balb/c mice were selected as they provide higher, and more consistent and prolonged *Mtb* infection than male counterparts (41, 42). In these studies, there mice per group were used to examine large effects of treatment with compounds **32** and **33** with a signal to noise ratio of >3 with 80% power, and the power analysis provided n=3 to be sufficient.

2.2.8. Administration of drugs

The test compounds **32** and **33,** and control drug isoniazid were suspended in 0.5% methyl-cellulose in saline. Compounds **32** and **33** were administered orally at a dose of 100 mg/kg and isoniazid was administered intraperitoneally at 25 mg/kg dose. Drug treatments were

given once daily (43). Control animals received equivalent volumes of diluent only. Drug treatment was initiated 4 days after mycobacterial challenge and continued for a total of two weeks (44, 45). Three days after the last treatment, mice were euthanized using a CO_2 chamber and lungs, liver, and spleen were removed aseptically and individually homogenized in 5 mL of saline. A 100 µL of aliquot of each organ homogenate from individual mice were plated on 7H11 selective agar plates (BD Biosciences). The plates were incubated at 37°C in ambient air for up to 4 weeks prior to counting the colonies. The number of colonies was counted manually using a magnifying glass apparatus. CFU counts per organ were determined by multiplying the number of colonies to the dilution factor. The number of CFUs represents the total CFUs from the whole organ.

2.2.9. Statistical analyses

Statistical analyses were performed using GraphPad Prism 6 Software (GraphPad Prism Software Inc., CA, USA). Data were represented as mean or mean ± SD (standard deviation). The differences in the means of CFU counts among multiple groups were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. A p-value less than 0.05 ($p \leq 0.05$) was considered to be statistically significant.

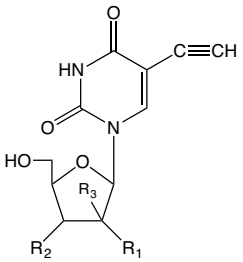
2.3. Results and Discussion

The antimycobacterial activities of 5-ethynyl (**6-10**), 5-(2-propynyloxy) (**16, 18, 20, 22, 24**), 5-(2-propynyloxy)-3-*N*-(2-propynyl) (**17, 19, 21, 23, 25**) and 5-hydroxymethyl-3-*N*-(2-propynyl) (**30-33**) nucleoside analogs were evaluated *in vitro* against *Mtb* (H37Ra), *M. bovis* (BCG) and *M. avium* by the Microplate Alamar Blue assay (MABA) at 10-200 μg/mL concentrations. Rifampicin, isoniazid and clarithromycin were used as reference standards. Results are summarized in Table 2.1.

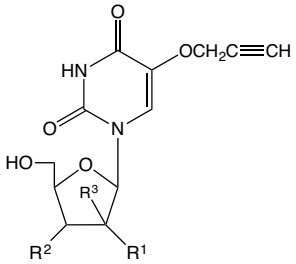
Among the newly synthesized compounds, 5-ethynyl substituted pyrimidine nucleosides (**6-10**) did not inhibit replication of any of the mycobacteria. However, corresponding nucleosides (**16, 18, 20, 22** and **24**) possessing a propynyl moiety at the C-5 position of the pyrimidine base, 5-(2-propynyloxy) uridine (**16**), 5-(2-propynyloxy)-2'-O-methyluridine (**18**) 5-(2-propynyloxy)-2'-arabinouridine (**20**) 5-(2-propynyloxy)-2'-riboflourouridine (**22**) and 5-(2-propynyloxy)-3'-flouro-2',3'-dideoxyuridine (**24**), led to modest to significant inhibition of *Mtb* (H37Ra) and *M. bovis* at 200 μg/mL, suggesting that a longer carbon chain at the C-5 position is required for the activity against *Mtb* and *M. bovis*.

In the 5-(2-propynyloxy) series of pyrimidine nucleoside analogs, compounds **16, 20** and **22** containing a ribose, 2'-arabinose and 2'-fluororibose sugar moieties had strong activity (71-99% inhibition) against both *Mtb* and *M. bovis* in contrast to compounds **18** and **24** with a 2'-methoxyribose and 3'-fluoro-2',3'-dideoxy sugar moiety that led to 38-41% inhibition. These results suggest that carbohydrate moieties also play an important role in modulating the antimycobacterial activity of this series of compounds and that the methoxy substituent at the C-2' position and the fluoro group at the C-3'-position were detrimental to the activity.

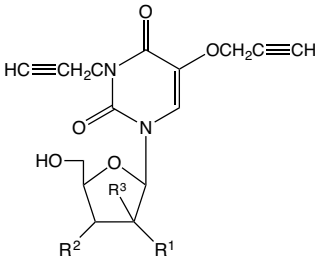
Table 2.1: *In vitro* antimycobacterial activity of compounds (**6-10, 16-25** and **30-33**) against *Mtb* (H37Ra), *M. bovis* (BCG) and *M. avium*



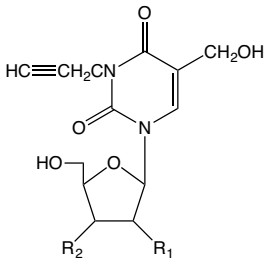
6-10



16,18,20,22,24



17,19,21,23,25



30-33

Compd	R_1	R_2	R_3	Antimycobacterial activity (Concentration µg/mL)[a]			
				Mtb (H37Ra)	*M. bovis* (BCG)	*M. avium* (ATCC25291)	Intracellular *Mtb* (H37Ra)
				% inhibition	% inhibition	% inhibition	% reduction[c]
6	OH	OH	H	0	0	0	ND[b]
7	H	OH	OH	0	0	0	ND
8	H	F	H	0	0	0	ND
9	H	N_3	H	0	0	0	ND
10	H	H	H	0	0	0	ND
16	OH	OH	H	99 (200)	89 (200)	0	61 (200)
17	OH	OH	H	26 (200)	30 (200)	0	ND
18	OCH_3	OH	H	41 (200)	38 (200)	0	ND
19	OCH_3	OH	H	32 (200)	23 (200)	0	ND
20	H	OH	OH	76 (200)	71 (200)	0	50 (200)
21	H	OH	OH	35 (200)	29 (200)	0	ND
22	F	OH	H	97 (200)	98 (200)	0	50 (200)
23	F	OH	H	35 (200)	34 (200)	0	ND
24	H	F	H	39 (200)	40 (200)	0	ND
25	H	F	H	32 (200)	29 (200)	0	ND
30	H	OH	-	31 (200)	35 (200)	0	ND
31	OH	OCH_3	-	98 (200)	99 (200)	0	59 (200)
32	H	N_3	-	98 (200)	99 (200)	0	68 (200)
33	H	H	-	99 (200)	98 (200)	0	65 (200)
Rifampicin				100 (0.5)	100 (0.5)	90 (2)	ND
Isoniazid				100 (1)	100 (1)	ND	>90 (1)
Clarithromycin				ND	ND	100 (2)	ND

[a]Antimycobacterial activity of test compounds was determined at concentrations of 200, 100, 50, 25 and 10 µg/mL. Only the 200 µg/mL concentration data are shown. Positive control drugs rifampicin at 0.5 or 2, isoniazid at 1 and clarithromycin at 2 µg/mL were used. [b]ND = not determined. [c]% reduction of the number of surviving bacteria in the human monocyte cell line (THP-1) with respect to untreated control. The experiment was performed on three separate days, in triplicate on each day and the mean percent inhibition from a representative experiment is provided. The standard deviation of three separate experiments was within 10%.

Introduction of an alkynyl moiety at the N-3 position of **16, 18, 20, 22** and **24** pyrimidine nucleosides did not result in compounds with enhanced activity as di-alkynylated 5-(2-propynyloxy)-3-*N*-(2-propynyl)uridine (**17),** 5-(2-propynyloxy)-3-*N*-(2-propynyl)-2'-O-methyluridine (**19),** 5-(2-propynyloxy)-3-*N*-(2-propynyl)-2'-arabinouridine (**21),** 5-(2-propynyloxy)-3-*N*-(2-propynyl)-2'-ribofluorouridine (**23)** and 5-(2-propynyloxy)-3-*N*-(2-propynyl)-3'-flouoro-2',3'-dideoxyuridine (**25**) derivatives showed comparatively reduced inhibition of both *Mtb* and *M. bovis* (26-35% inhibition) at 200 μg/mL. It is observed that incorporation of the alkynyl moiety at the N-3 position of the active compounds **16, 20** and **22** also diminished their activity, further denoting that inclusion of an N-3 propynyl group is not tolerated at this position for antimycobacterial effect in this series of compounds.

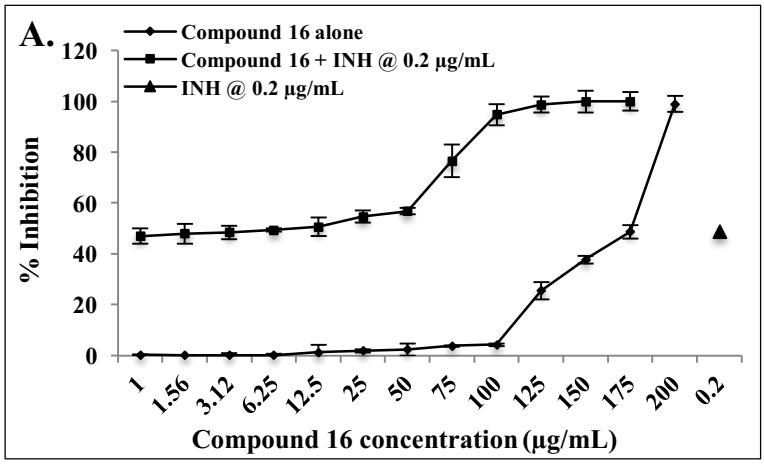
Among the 5-hydroxymethyl-substituted pyrimidine nucleosides, interestingly**,** 5-hydroxymethyl-3-*N*-(2-propynyl)-3'-O-methyluridine (**31)** and 5-hydroxymethyl-3-*N*-(2-propynyl)-3'-azido-2',3'-dideoxyuridine (**32)** and 5-hydroxymethyl-3-*N*-(2-propynyl)-2',3'-dideoxyuridine (**33)** inhibited replication of both *Mtb* and BCG 98-99% at 200 μg/mL, in contrast to compounds **17, 19, 21, 23** and **25** . It appears that a less hydrophobic hydroxymethyl substituent and a shorter chain at the C-5 position in di-alkynylated nucleosides instead of a propynyloxy side chain are preferred for antimycobacterial activity. Since, the glycosyl moieties in **31-33** were different than the sugar moieties of **17, 19, 21, 23** and **25**, the contribution of the carbohydrate portion cannot be ruled out. Therefore, superior efficacy demonstrated by **31-33** could be a result of modifications made at both C-5 of the base and C-2' of the sugar portion of **31-33**. This observation is supported by compound **30** of this series with a 2'-deoxyribose moiety, which displayed modest activity (31-35% inhibition). None of the investigated compounds **6-10**, **16-25** and **30-33** displayed activity against *M. avium*.

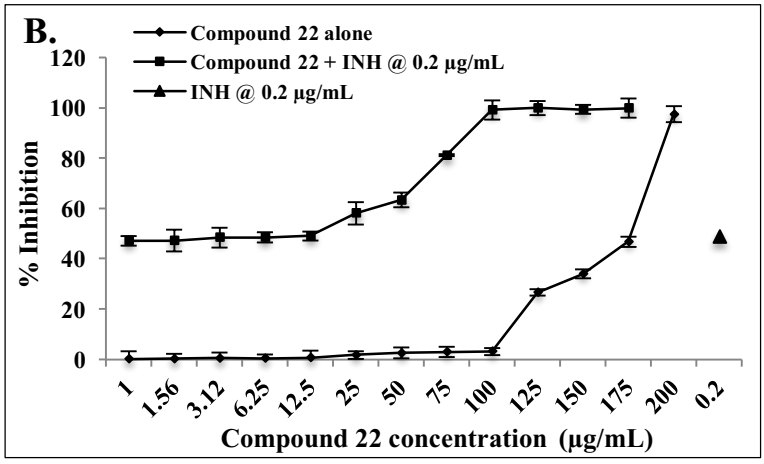
Mycobacteria are intracellular pathogens, which reside, propagate and hide within macrophages. Most individuals contain these pathogens intracellularly for years albeit in non-replicating form. Therefore, the most active compounds **16, 20, 22** and **31-33** were also examined for their activity against intracellular *Mtb* (H37Ra) using a human monocytic cell line (THP-1). In this assay, all of the test compounds provided a 59-65% reduction in the CFUs of intramacrophagic *Mtb* at 200 μg/mL. The intracellular efficacy of **16, 20, 22** and **31-33** correlates with their activity against extracellular mycobacteria. These studies suggest that this new class of compounds have potential to inhibit mycobacteria harbored within macrophages in infected people.

The active compounds **16**, **22** and **31-33** alone led to <1 log bacterial reduction at high concentrations (200 μg/mL) that might preclude their clinical use. If the MIC of the drug that is effective against a pathogen is at a concentration that can be achieved easily in serum, the drug is likely to be clinically useful, and if the effective concentration is greater than their physiologically achievable thresholds the drug may not be useful. However, the effective concentration of the drug can be lowered in combination with other agents acting at the same or different bacterial targets.

A limitation of current antituberculosis drugs is their tendency to select for resistant mycobacterial strains as a result of poor compliance and insufficient dosing, associated toxicities and their inherent ineffectiveness against already resistant strains. We therefore reasoned that even modestly active compounds such as **16, 22** and **31-33** may find therapeutic use in combination with first-line drugs if they lead to synergistic effects. Combined drug therapy is common for several chronic infections including *Mtb*. Combination not only reduces the therapeutic dose of each drug but may also lead to reduced resistance development, especially if

the combined drugs act at different mycobacterial targets. As described in figures 2.2 and 2.3, I used the MABA assay to examine the effect of the most active compounds **16, 22** and **31-33** at various concentrations in combination with isoniazid (INH) at 0.2 µg/mL (<MIC_{50}). Interestingly, combinations of the compounds **16, 22** and **31-33** at 100-150 µg/mL with INH at its <50% effective concentration exhibited >90% inhibition of *Mtb* (H37Ra) replication (Figure 2.2 A-E).





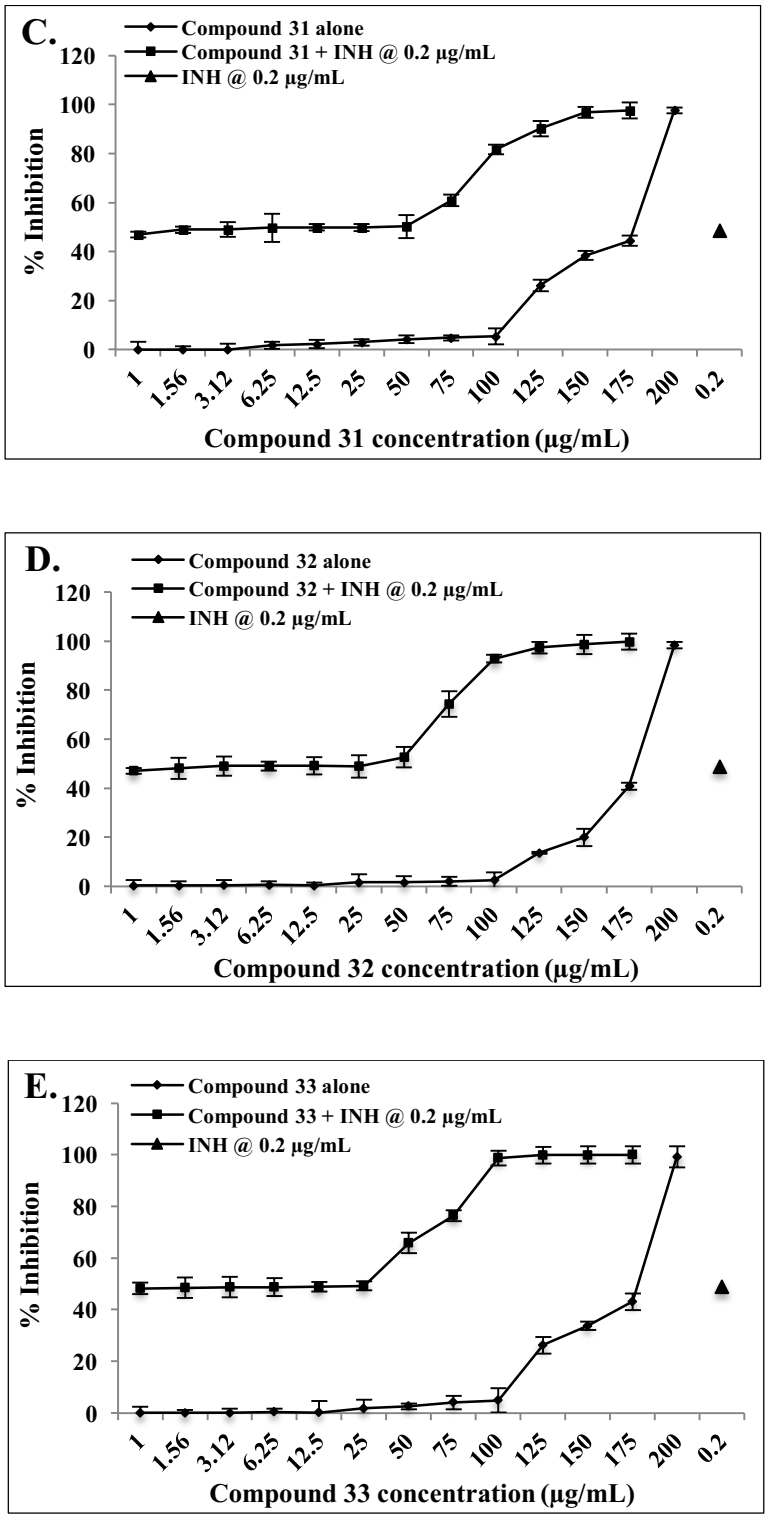


Figure 2.2: ***In vitro*** **combination effect of compounds 16, 22 and 31-33 with INH (isoniazid) against *Mtb* (H37Ra).**

The nature of interaction between test compound and INH was analyzed by calculating combination index (CI) as described in section 2.2.5. A CI value of <1 indicates synergistic effect; CI = 1 indicates additive effect, and CI > 1 indicates antagonistic effect. The interactions of compounds **16, 22** and **31-33** with INH were found to show synergism with CI values of 0.56 (**16**), 0.52 (**22**), 0.66 (**31**), 0.55 (**32**) and 0.54 (**33**), (Table 2.2). Although, compound **31** displayed synergy with INH, it demonstrated lowest inhibition of *Mtb* compared to **16**, **22**, **32** and **33**.

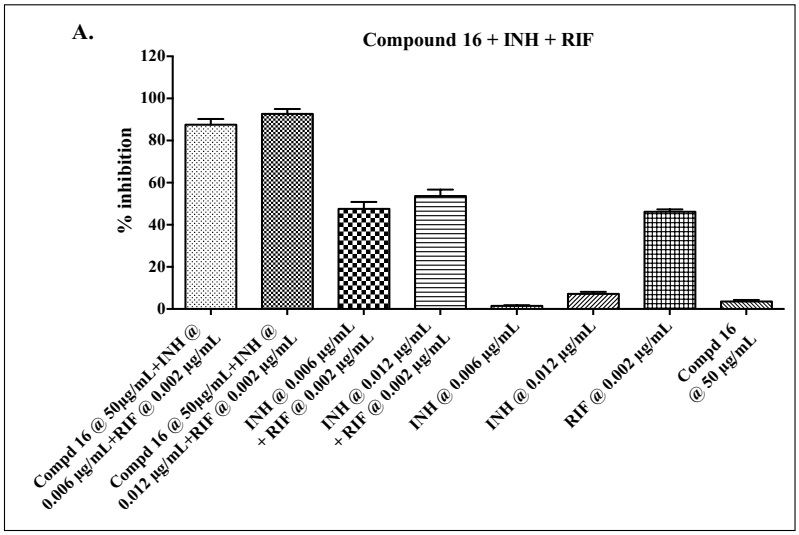
Table 2.2: Combination index (CI) for compounds **16**, **22** and **31-33** tested in combination with isoniazid

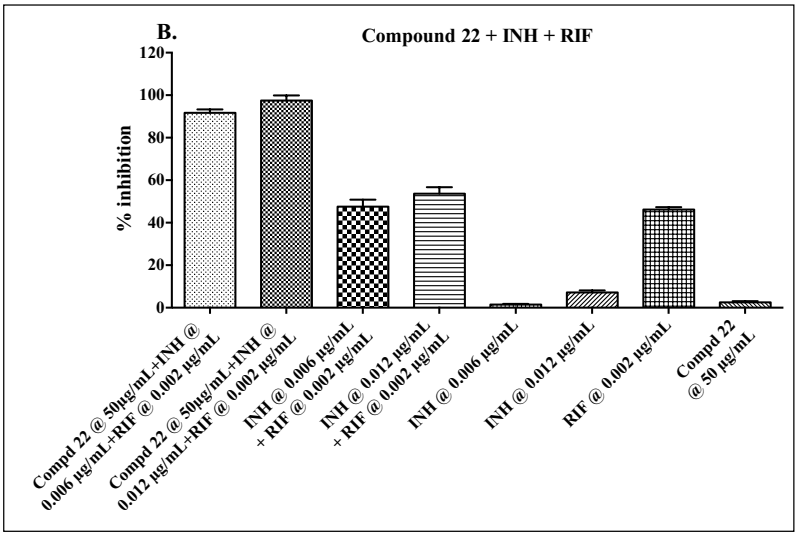
Compound	Conc. (μg/mL)	Antimycobacterial activity, % inhibition *Mtb* (H37Ra)			
		Compound alone	Combination with isoniazid @ 0.2 μg/mL	CI	Outcome
16	100	4.26	94.74	0.56	Synergistic
22	100	3.15	99.15	0.52	Synergistic
	75	1.15	81.16	0.62	Synergistic
31	100	5.37	81.61	0.66	Synergistic
32	100	2.51	92.84	0.55	Synergistic
33	100	4.89	99.80	0.54	Synergistic
Isoniazid	0.2	48.79	-	-	-

The experiment was performed on three separate days, in triplicate on each day and the mean percent inhibition from a representative experiment is provided. The standard deviation of three separate experiments was within 10%.

Encouraged by results from two-drug combinations, I tested compounds **16, 22, 32** and **33** in a three-drug combination with two first-line drugs, INH and rifampicin, at their <50% inhibitory concentrations of 0.006-0.012 and 0.002 μg/mL, respectively (Figure 2.3 A-D). It was intriguing to see that combining the novel nucleoside analogs even at 50 μg/mL concentration with both rifampicin and isoniazid together resulted in >90% inhibition at much reduced concentrations of both first-line drugs and improved the activity threshold of each of the drugs in combination. The

CIs obtained for compounds **16, 22, 32** and **33** in 3-drug combinations were <1, indicating synergistic interactions (Table 2.3).





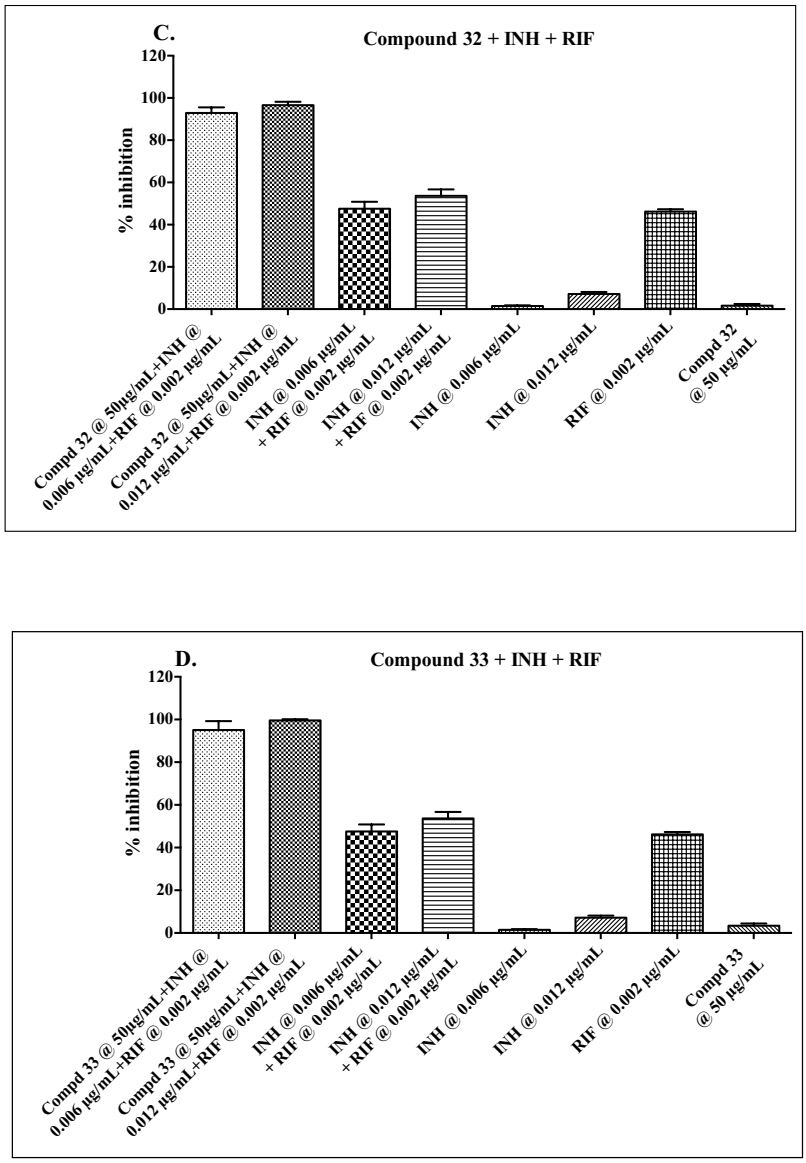


Figure 2.3: *In vitro* combination effect of compounds 16, 22, 32 and 33 with INH (isoniazid) and RIF (rifampicin) against *Mtb* (H37Ra).

Table 2.3: Combination index (CI) for compounds **16, 22, 32** and **33** tested in combination with isoniazid (INH) and rifampicin (RIF)

Compound	Conc. (μg/mL)	Antimycobacterial activity, % inhibition *Mtb* (H37Ra)					
		Compd alone	Combination with INH @ 0.012 μg/mL + RIF @ 0.002 μg/mL	CI	Combination with INH @ 0.006 μg/mL + RIF @ 0.002 μg/mL	CI	Outcome
16	50	3.56	91.30	0.62	90.19	0.57	Synergistic
22	50	2.51	97.81	0.57	93.36	0.54	Synergistic
32	50	1.53	95.64	0.57	92.94	0.53	Synergistic
33	50	3.37	99.90	0.57	94.77	0.54	Synergistic
Isoniazid	0.012	7.22	-	-	-	-	-
	0.006	1.47	-	-	-	-	-
Rifampicin	0.002	46.21	-	-	-	-	-

The experiment was performed on three separate days, in triplicate on each day and the mean percent inhibition from a representative experiment is provided. The standard deviation of three separate experiments was within 10%.

In these studies, it is intriguing to note that the inhibitions obtained upon combining the nucleoside analogs **16, 22, 32** and **33** were significantly higher than INH and rifampicin together, demonstrating an important contribution of the investigated antimycobacterial nucleosides in the three-drug combinations tested.

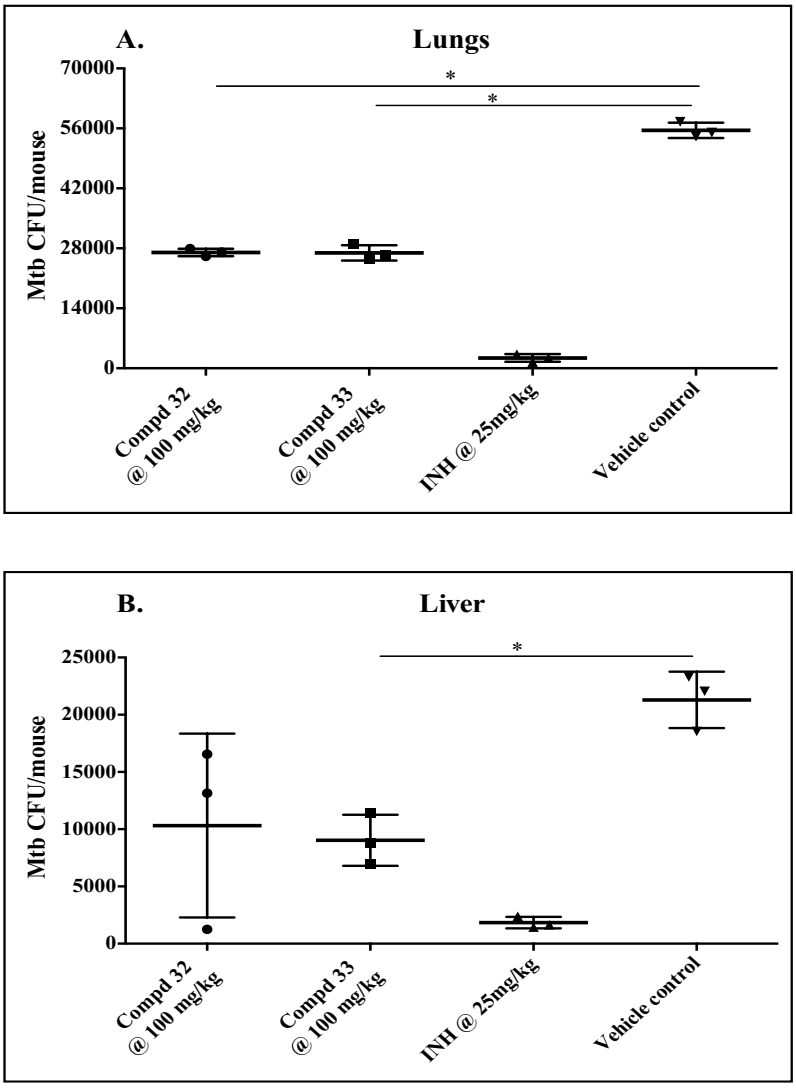
Nucleoside analogs were evaluated *in vitro* against *Mtb* (H37Ra) in combination with anti-TB drugs INH and/or RIF. They together exhibited synergy. The combined effect of nucleoside analogs with isoniazid and rifampicin were greater then their individual effects. The exact mechanism of synergy was not determined, however, possibilities include decreased plasma protein binding, preventing each drug in combination from being converted to inactive metabolites or attacking three different mycobacterial targets simultaneously: cell wall lipid synthesis, RNA synthesis and nucleic acid synthesis by the isoniazid, rifampicin and nucleoside

analogs, respectively. Rifampicin is highly lipophilic with high plasma protein binding (80%) (46). Although untested in these studies, this may interfere with the nucleoside analog binding to plasma protein and may increase the plasma concentration of the nucleoside analogs such that more drug reaches its target. The observed synergy may also be due to weakening of the cell wall by isoniazid that may improve nucleoside analog penetration. This notion is supported by a study from Medoff *et al*. in fungal and yeast infection where amphoterin B was found to alter the permeability barrier of the cell surface membrane and allow increased penetration of nucleoside analogs into the cell (47). The *in vitro* results suggested that there is a significant interaction between nucleoside analogs, isoniazid and rifampicin.

Vero cells derived from the kidney of an African green monkey are commonly used mammalian cell line in the assessment of cytotoxicity of chemotherapeutic agents in drug discovery research (32, 48, 49). The XTT assay was performed to evaluate the toxicity of compounds **6-10, 16-25** and **30-33** toward Vero cells. No toxicity was observed with these compounds for Vero cells up to the highest concentration tested (CC_{50} > 300 μg/ml) (Appendix 20).

Dideoxynucleosides with 3'-azido and 2',3'-dideoxy sugar moieties found in anti-HIV agents 3'-azidothymidine (AZT) and 2',3'-dideoxyinosine (DDI), have shown very good oral bioavailability clinically. Because they possess these structural features, and based on their *in vitro* results, we selected compounds **32** and **33** to test if modestly-active antimycobacterial nucleosides could effectively treat mice infected with *Mtb*. Effectiveness of conventional antimycobacterial drugs (e.g. ethambutol, pyrazinamide, *p*-aminosalicylic acid) at a very high dose in animals also supported this notion. Compounds **32** and **33** were tested at an oral dose of 100 mg/kg for two weeks as described in detail in the experimental section. Control drug INH

was used at 25 mg/kg intraperitoneally. Notably, both compounds caused significant reduction of the bacterial counts in the lungs, liver and spleen of BALB/c mice infected with *Mtb* strain H37Ra compared to untreated controls (Figure 2.4). Viable counts of mycobacteria were reduced approximately 50% in the lungs of all three mice by **32** and **33** compared to untreated controls. These results were statistically significant. Compound **32** also decreased bacterial loads in liver and spleen compared to controls, but these were not statistically significant. Compound **33** showed statistically significant reduction in mycobacterial loads in both spleen and liver compared to controls.



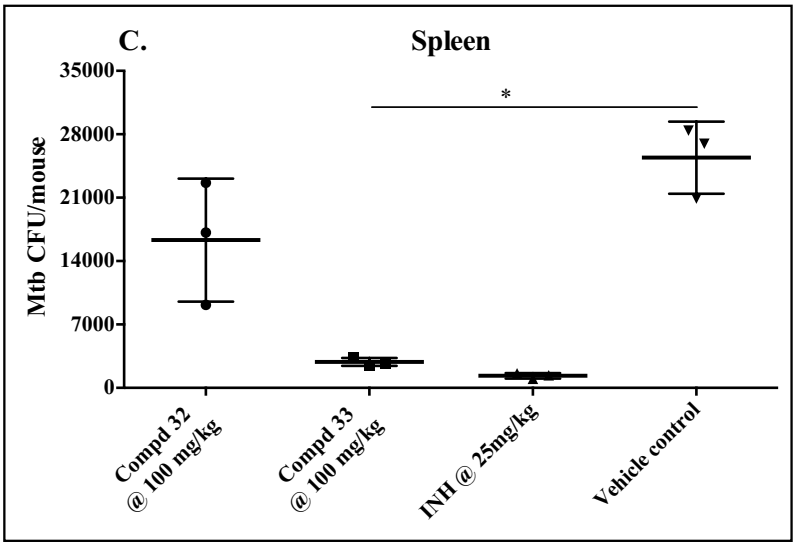


Figure 2.4: Efficacy of compounds 32 and 33 in a murine model of tuberculosis. BALB/c female mice (n = 3) were challenged with *Mtb* (H37Ra) (0.5×10^6 CFU/mouse) intravenously. Mice were treated with compound **32** (100 mg/kg) or compound **33** (100 mg/kg) orally or INH (25 mg/kg) intraperitoneally for two weeks. Control mice received vehicle diluent. Three days after the last treatment, mice were euthanized and lungs, liver and spleens were collected. Bacterial loads were determined in **(A)** lungs, **(B)** liver and **(C)** spleen by the CFU assay. All results are shown as CFUs from three individual mice and their mean ± standard deviation. Data are representative of two different repeated experiments. '*' indicate significant differences at $p \leq 0.05$.

Mice administered with compounds **32** and **33** at 100 mg/kg for two weeks showed no adverse effects in terms of behavioral changes, weight loss or post-mortem gross necroscopy. The unexpected *in vivo* effects of **32** and **33** may be ascribed to their possible biotransformation into active metabolites, efficient phosphorylation, stability, long plasma half-life, etc. These mechanisms need to be determined.

Overall, these studies demonstrate that modestly and weakly active antimycobacterial compounds can provide *in vitro* synergistic interactions at lower than optimum concentrations if combined with other agents targeting different pathways. This strategy may provide more effective regimens with reduced doses of combined drugs, lower toxicity, better compliance and reduced emergence of resistance because it capitalizes on differences in molecular structures and mechanisms of action of the combined drugs. The exact mechanism of action that allows the active compounds to inhibit mycobacterial multiplication in this study is not clear. It is postulated that the compounds are metabolically converted to phosphorylated forms by mycobacterial kinases, and that these phophoryated forms may be selectively inhibiting the mycobacterium's DNA and/or RNA synthesis by acting as substrates and/or inhibitors of metabolic enzymes of DNA/RNA synthesis. It is expected that upon treatment with investigated compounds in combination with INH and/or RIF, in addition to inhibition of the mycobacterial DNA and RNA synthesis, mycobacterial cell wall synthesis and/or protein synthesis are also being interrupted simultaneously, leading to synergistic *in vitro* antimycobacterial effects.

These studies also demonstrate that modestly active nucleosides could be effective *in vivo.* The compounds **32** and **33** emerging from these studies possessed efficacy at high doses, and their clinical use may be limited on their own. However, in combination with current anti-TB agents, the combined efficacy at lower doses may prove to be a beneficial clinical strategy.

2.4. Reference

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Chapter 3

Design, synthesis and investigation of novel conjugated compounds as a new class of antituberculosis agent*

*In this chapter I designed, synthesized and performed all of the experiments. Dr. R. Kumar (supervisor), Dr. D.Y. Kunimoto (committee member) and Dr. B. Agrawal (collaborator) contributed to the concept for these studies and the data analysis

3.1. Introduction

The growing number of cases of multi-, extremely- and totally-drug resistant TB (MDR/XDR/TDR-TB) worldwide is of utmost concern, as it is trend that could result in making TB incurable (1-7). Therefore, urgent and extraordinary actions must be taken to control this disease. The emergence of significant resistance in TB strains has had serious consequences on the availability of drugs, and the duration, cost, toxicity and success of drug therapy (8). Standard short course chemotherapy for TB takes six months with a combination of TB drugs. With 100% compliance this produces an 85-90% success rate (8, 9). However, patients with MDR-TB are prescribed with a 4-5-drug combination regimen for 2 years after a negative culture is obtained. This duration, accompanied by frequent serious side effects, leads to unsatisfactory patient compliance, and cessation of treatment (9-12). Many second-line drugs (e.g., ethionamide, PAS, cycloserine and kanamycin) are less preferred because they are less active and more toxic (9-17). To shorten the treatment and make compliance easier, the U.S. FDA in 1998 approved a new drug, rifapentine (a derivative of rifampicin), however, it is cross-resistant with rifampicin and has a higher relapse rate (13-18). Bedaquiline, despite a narrow therapeutic window and major concerns regarding its safety profile with only phase 2 data, was approved by FDA in 2013 for emergency use in combinations (19, 20). There is a paucity of clinical data on the efficacy of this agent (19, 20).

An important goal of antituberculosis drug development is that new drugs and regimens should not only have activity against wild-type TB but also be effective in shortening the current treatment to improve compliance, reduce side effects and reduce the emergence of resistant strains.

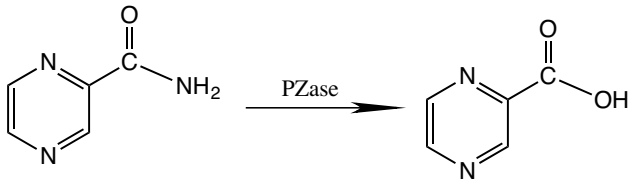
The prodrug concept has been used extensively to improve the undesirable properties of a drug (21). A prodrug is an inactive or masked form of the active drug molecule that must

undergo chemical and/or enzymatic transformation to release the active parent drug. The activated parent drug can then elicit its desired pharmacological response in the body (21). Prodrug strategies are used to overcome various barriers to drug formulation and delivery such as chemical instability, poor aqueous solubility, inadequate oral absorption, rapid pre-systemic metabolism, and toxicity (22, 23). Prodrug design can also improve cell permeability, lipophilicity, and tissue specificity of the active drugs and thereby improve drug delivery properties (24, 25). However, a main drawback of the prodrug approach is that the pro-moiety released during activation can lead to adverse effects (22-25).

Rationally, if a prodrug can be designed in such a way where a pharmacologically active drug can be coupled with a pro-moiety (conjugate) that possess additional bioactivity by acting at a different target (21-25), then in theory a maximum inhibition of mycobacterial growth can be obtained. This prodrug design could provide synergistic effects and improved drug delivery properties of both agents (21, 26). It might also reduce dosage and toxicities of both agents and provide a significant advancement in the development of novel therapeutic regimens for TB.

Pyrazinamide (PZ) is a unique antituberculosis drug, effective against latent tubercle bacilli within macrophages (27) and against MDR and XDR-TB in human disease (28). PZ enters *Mtb* cells by passive diffusion or through porins, and hydrolyzes there into its active form, pyrazinoic acid (PZA) (Figure 3.1) (27, 29). PZA has been suggested to disrupt mycobacterial cell wall membrane and transport functions (29, 30). It also decreases intracellular levels of ATP. However, the required high therapeutic doses of PZ cause liver toxicity (30). Furthermore, mutations in the *Mtb* gene *pncA* cause resistance by abrogating the pyrazinamidase (PZase) activity, a specific enzyme required for the conversion of the PZ prodrug to the active drug PZA (31). Although PZA cannot penetrate through the

mycobacterial cell wall due to its ionic nature and low lipophilicity (32), PZ-resistant *Mtb* still retains susceptibility to PZA (33-35).



PZ PZA

Figure 3.1: Activation of PZ to PZA

Various ester prodrugs of PZA have been investigated to overcome its bioavailability limitations. These prodrugs were found to be inactive or had a greater *in vitro* antimycobacterial activity than PZA. However, despite *in vitro* improved anti-mycobacterial activity of some PZA esters, they failed to provide efficacy in mice (27, 33-39).

Our laboratory has previously reported that 5-fluoro-2'-deoxyuridine (FUDR), a clinically used anticancer drug, exhibits significant inhibition of *Mtb* replication *in vitro* (40). It was proposed to function by interfering with mycobacterial DNA or RNA synthesis (41). However, it is a strong anti-proliferative agent and has serious toxicity issues (41).

Here, I have designed, synthesized and investigated novel antimycobacterial conjugates of PZA and FUDR as a new class of antituberculosis agents. Such conjugates could have reduced toxicity, reduced dosing and increased efficacy due to slow release of the parent drug PZA and the pro-moiety FUDR inside the bacterium acting at two different mycobacterial targets (Figure 3.2). Further, this approach could improve compliance and prevent the onset of resistance by circumventing PZase activity, maximally inhibiting mycobacterial growth and leading to longer plasma half-life of the parent drugs.

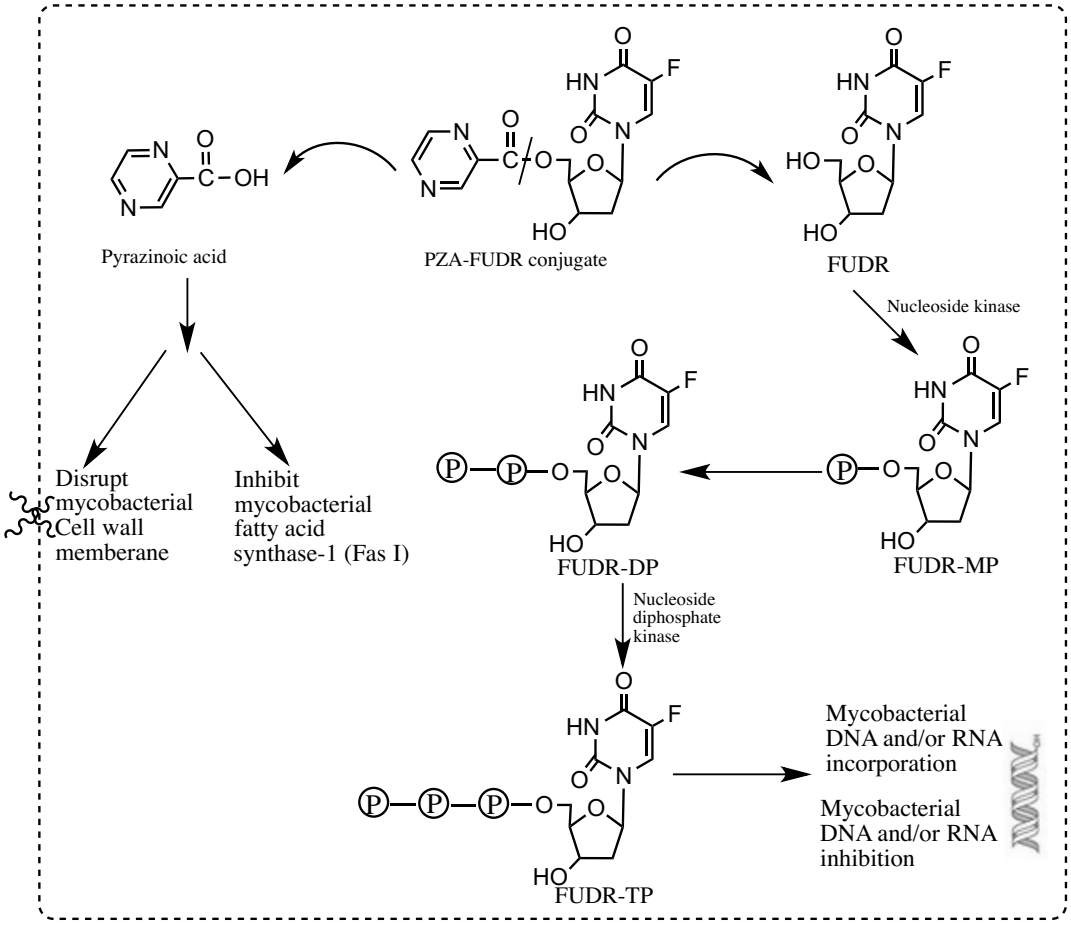


Figure 3.2: Possible mechanism of action of the novel PZA-FUDR conjugates

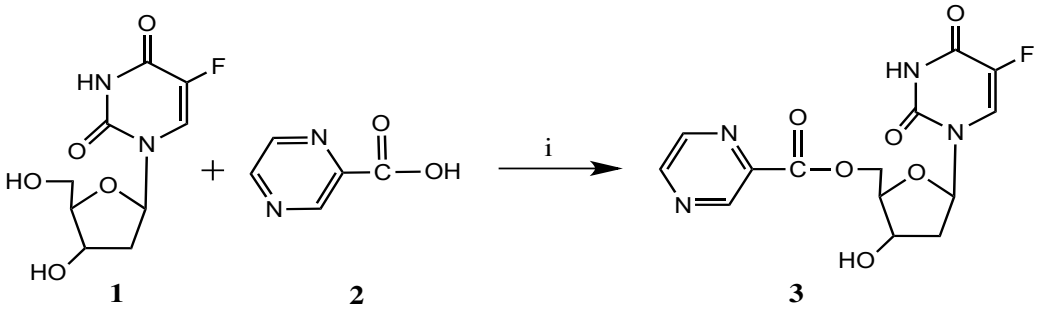
3.2. Material and Methods

3.2.1. Chemistry

The conjugation reaction between FUDR (**1**) and pyrazinoic acid (**2**) was carried out in the presence of diethyl azodicarboxylate (DEAD) and triphenylphosphine (PPh_3) in dry dioxane at 70^{o}C for 32 hours as shown in Schemes 3.1A and 3.1B. This reaction provided two major products as a result of mono-esterification of the 5'-hydroxyl functionality of FUDR and di-esterification of both 3'- and 5'- hydroxyl groups of FUDR in quantitative yields. The structures

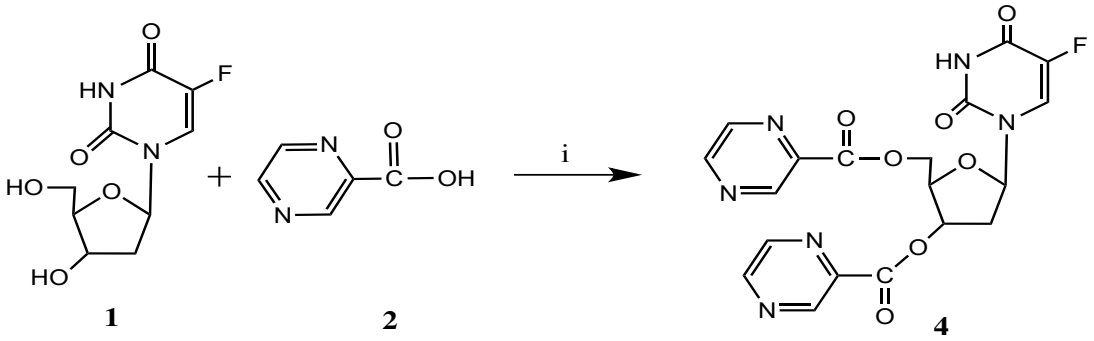
of 5-fluoro-2'-deoxyuridine-5'-O-pyrazinoate (**3**) and 5-fluoro-2'-deoxyuridine-3',5'-di-O-pyrazinoate (**4**) were confirmed by ^{1}HNMR, ^{13}CNMR and spectral studies.

All of the starting materials and reagents were purchased from Sigma Aldrich, Canada. Dioxane was dried over calcium hydride. Reactions and purity of the products were monitored by thin layer chromatography with a methanol-dichloromethane (3:97, v/v) mixture as eluent.



1 **2** **3**

Scheme 3.1A: Reagents and conditions: (i) diethyl azodicarboxylate, triphenylphosphine, dry dioxane, 70^{o}C, 32 h.



1 **2** **4**

Scheme 3.1B: Reagents and conditions: (i) diethyl azodicarboxylate, triphenylphosphine, dry dioxane, 70^{o}C, 20 h.

3.2.2. Experimental design

Melting points were determined with an electrothermal melting point apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were determined for samples in Me_2SO-d_6 on a Bruker AM 600 spectrophotometer using TMS as an internal standard. ^{13}C NMR spectra were determined, where methyl and methyne carbon resonance appear as positive peaks and methylene and quaternary carbon resonances appear as negative peaks. Chemical shifts are given in ppm relative to TMS and signals are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublets), dt (doublet of triplets) and dm (doublet of multiplet). The assignment of exchangeable proton (OH, NH) was confirmed by the addition of D_2O. All of the final compounds had >95% purity determined by microanalysis. Microanalysis results were within ± 0.4% of the theoretical values for all elements listed unless otherwise indicated. Silica gel column chromatography was carried out using Merck 7734 silica gel (100-200 μM particle size). Thin-layer chromatography (TLC) was performed with Machery-Nagel AlugramSil G/UV silica gel slides (20 μM thickness).

3.2.2.1. Synthesis of 5-fluoro-2'-deoxyuridine-5'-O-pyrazinoate (3)

FUDR (**1**, 500 mg, 2.03 mmol) was added to a solution of diethyl azodicarboxylate (DEAD) (352 mg, 2.02 mmol) and triphenylphosphine (PPh_3) (1.06 g, 4.05 mmol) in dry dioxane (35 mL) at 0^{o}C in a nitrogen atmosphere. The reaction mixture was stirred for 30 min at room temperature. Pyrazinoic acid (252 mg, 2.03 mmol) was then added and the reaction mixture was further stirred at 70^{o}C for 20 h. The progress of the reaction was monitored by TLC in MeOH/CH_2Cl_2 (1:99 v/v). Additional aliquots of PPH_3 (800 mg, 3.05 mmol) and DEAD (352 mg, 2.02 mmol) were added to the reaction mixture and the stirring of the reaction was continued

for 12 h at 70°C. The solvent was removed *in vacuo*. The residue obtained was purified on a silica gel column using MeOH/CH_2Cl_2 (3:97 v/v) as an eluent to give **3** (285 mg, 40%) as a solid. M.p. 175-180°C.^{1}H NMR (DMSO-d_6) δ: 2.17-2.15 (m, 1H, H-2'), 2.32-2.28 (m, 1H, H-2"), 4.13 (q, J = 3.6, 1H, H-3'), 4.36 (q, J = 3.6 Hz, 1H, H-4'), 4.63 and 4.50 (2q, J = 4.2 Hz and 3.6 Hz, 2H, H-5'), 5.49 (d, J = 4.2 Hz, 1H, 3'-OH), 6.26-6.23 (m, 1H, H-1'), 8.16 (d, J = 6.6 Hz, 1H, H-6), 8.80 (q, J = 1.2 Hz, 1H, aromatic), 8.94 (d, J = 2.4 Hz, 1H, aromatic), 9.26 (d, J = 1.2 Hz, 1H, aromatic), 11.87 (s, 1H, NH). ^{13}C NMR (DMSO-d6) δ: 65.70 (C-5'), 70.88 (C-3'), 84.30 (C-4'), 84.93 (C-1'), 125.21 and 125.43 (d, J = 33 Hz, C-6), 139.86 (C-Ar pyrazine), 141.39 (C=O), 143.06 (C-2'), 145.10, 146.15 and 146.17, 148.93 (C-Ar pyrazine), 149.56 (C-4), 157.35 and 157.52 (d, J = 26 Hz, C-5), 163.99 (C-2). Anal. Calcd for $C_{14}H_{13}FN_4O_6$ (M.W.352.28): C 47.73, H 3.72, N 15.90. Found C 47.40, H 3.96, N 15.64 (Appendix 21).

3.2.2.2. Synthesis of 5-fluoro-2'-deoxyuridine-3',5'-di-O-pyrazinoate (4)

FUDR (**1**, 750 mg, 3.05 mmol) was added to a solution of diethyl azodicarboxylate (DEAD) (795 mg, 4.56 mmol) and triphenylphosphine (PPh_3) (1.99 g, 7.59 mmol) in dry dioxane (35 mL) at 0°C in a nitrogen atmosphere. The reaction mixture was stirred for 30 min at room temperature. Pyrazinoic acid (**2**, 756 mg, 6.1 mmol) was then added and the reaction mixture was stirred for another 5 h at 70°C. Additional aliquots of PPH_3 (800 mg, 3.05 mmol) and DEAD (530 mg, 3.04 mmol) were added to the reaction mixture followed by stirring overnight at 70°C. The progress of the reaction was monitored by TLC in MeOH/CH_2Cl_2 (1:99 v/v). The reaction was not completed and therefore more aliquots of PPH_3 (800 mg, 3.05 mmol) and DEAD (530 mg, 3.04 mmol) were added to the reaction mixture followed by stirring for 3 h at 70°C. The reaction mixture was concentrated *in vacuo* and purified on silica gel column using

MeOH/CH_2Cl_2 (4:96 v/v) as solvent to yield **4** (900 mg, 65%) as a solid. M.p. 245-250°C.^{1}H NMR (DMSO-d_6) δ: 2.50-2.45 (t, J = 16.8, 1H, H-2'), 3.02-2.97 (m, 1H, H-2'), 4.65 (s, 1H, H-4'), 4.89 and 4.83 (2q, J= 4.2 Hz, 2H, H-5'), 5.83 (s, 1H, H-3'), 6.33 (d, J = 7.8 Hz, 1H, H-1'), 8.38 (d, J = 7.2 Hz, 1H, H-6), 8.75 (d, J = 16.2 Hz, 2H aromatic), 8.94 (dd, J = 10.2, J = 1.8 Hz, 2H, aromatic), 9.28 (d, 2H, aromatic), 11.88 (s, 1H, NH), ^{13}C NMR (DMSO-d6) δ: ^{13}C NMR (DMSO-d6) δ: 63.20 (C-5'), 66.81 (C-2'), 74.34 (C-3'), 79.98 (C-4'), 84.61 (C-1'), 125.41 and 125.64 (d, J = 35 Hz, C-6), 139.77 (C-Ar pyrazine), 141.29 (C-Ar pyrazine), 142.96 and 142.98 (C=O), 145.02, 145.13, 146.04, 146.22, 148.72, 148.73 (C-Ar pyrazine), 149.50 (C-4), 157.53 and 157.70 (d, J = 26 Hz, C-5), 162.97 (C=O), 163.77 (C-2). Anal. Calcd for $C_{19}H_{15}FN_6O_7$ (M.W.458.36): C 49.79, H 3.30, N 18.34. Found C 49.69, H 3.39, N 18.61 (Appendix 22).

3.2.3. *In vitro* antimycobacterial activity assay (*Mtb*, *M. bovis*, and *M. avium*)

Mycobacterium tuberculosis (*Mtb*) strain H37Ra, *M. bovis* strain BCG, and *M. avium* strain ATCC 25291 were obtained from the American Type Culture Collection (ATCC), Rockville, MD. These strains were cultured in Middlebrook 7H9 Broth medium supplement with glycerol, Tween 80 and Middlebrook ADC (Bovine Albumin, Dextrose and Catalase) enrichment purchased from Becton Dickinson and company, MD, USA. Antimycobacterial activity was determined using the Microplate Alamar Blue assay (MABA). The cell viability reagent Alamar Blue was purchased from Bio-Rad Laboratories, Inc., USA. Test compounds were dissolved in dimethyl sulfoxide (DMSO) at 10 mg/mL and subsequent dilutions were made in 7H9GC medium (Difco Laboratories, Detroit, Michigan) in 96-well plates. For these experiments, each conjugate was initially tested at 100, 50, 10 and 1 μg/mL in triplicate, followed by a detailed dose response study at 25, 12.5, 6.25, 3.12, 1.56 and 0.78 μg/mL. The

experiments were repeated three times and the mean percent inhibitions from triplicates of a representative experiment are shown in Table 3.1. The standard deviations were within 10%. Frozen mycobacterial inocula were diluted in 7H9GC medium and added to each well at a final concentration of 2.5 x 10^5 CFU/mL. Sixteen control wells included eight with bacteria alone (B) and eight with medium alone (M). Plates were incubated for six days and then 20 μL of 10 x Alamar Blue and 12.5 μL of 20% Tween 80 were added to one M and one B well. Wells were observed for an additional 24-48 h for visual color change from blue to pink and read by spectrophotometer (Fluostar Optima, BMG Labtech, GmbH, Ortenberg, Germany) at excitation 530/525 nm and emission 590/535 nm to determine OD values. If the B well became pink by 24 h (indicating growth), reagent was added to the entire plate. If the B well remained blue, additional M and B wells were tested daily until bacterial growth could be visualized by color change. After the addition of the reagent to the plate, cultures were incubated for 24 h and plates were observed visually for color change and read by spectrophotometer. MIC was defined visually as the lowest concentration of a compound that prevented a color change from blue to pink. Percent inhibition was calculated as: 100 - (Test well − Medium well) / (Bacteria well − Medium well) x 100. Isoniazid and rifampicin purchased from Aldrich Chemical Company Inc. USA were used as positive controls. As negative controls, DMSO, was added to the B well at concentrations similar to those of compound wells; M wells served as negative controls. In most of the experiments, the M wells gave an OD of 3000-5000, and the B wells had OD values ranging between 40,000-60,000.

3.2.4. *In vitro* antimycobacterial activity of conjugates 3 and 4 in combination with standard drug(s)

For drug combination studies, MABA assays employing similar methodology were used as described above. Isoniazid and rifampicin were used at (0.20-0.10 μg/mL) and (0.015-0.0075 μg/mL) concentrations, respectively. The combination effect of compounds was determined by calculating combination index (CI) as described in section 2.2.5 on page 80.

3.2.5. *In vitro* cytotoxicity assay

Cell viability was measured using the cell proliferation kit 1 (XTT; purchased from Xenometric:Endotell). A 96-well plate was seeded with Vero cells cultured in Dulbecco's modification of Eagle medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) at a density of 2 x 10^5 cells per well. Conjugate **3** and **4** were dissolved in DMSO at 10 mg/mL and subsequent dilutions were made in DMEM medium in 96-well plates. Cells were allowed to attach for 24 h, and the DMEM medium was replaced with DMEM medium containing conjugates at concentrations of 200, 100, 50, 10 and 1 μg/mL. DMSO was also included as a solvent control. Plates were incubated for 3 days at 37°C. The color reaction involved adding 10 μL XTT reagents per well and incubating for 4 h at 37°C until color change to orange (43). Plates were read on an ELISA plate reader (Fluostar Optima, BMG Labtech, GmbH, Ortenberg, Germany) at Abs $_{450\text{-}500}$ nm. Percent viability was calculated as (OD of test well) – (OD of Medium well without cells) / (OD of control solvent well) – (OD of Medium well without cells) x 100.

3.2.6. Animals and infection

The animal experimental protocol used in this study was approved by the University Animal Care and Use Committee (ACUC) for Health Sciences, and conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC). Five- to six- week old female BALB/c mice were purchased from Charles River Laboratories and were allowed to acclimate for 1 week. Mice were challenged intravenously in the tail vein with 0.5 x 10^6 CFU/mouse of *Mtb* (H37Ra) in saline. In order to compare the designed conjugates with parent drugs and their combinations, a 90% power and minimum signal to noise ratio of 2.5 was needed and therefore five mice per group were used.

3.2.7. Administration of drugs and *in vivo* activity evaluation

From day 4 onwards, the mice infected with *Mtb* were treated orally with conjugates **3**, **4**, or control drugs FUDR, isoniazid, rifampicin or pyrazinamide. The test conjugates and control drugs were suspended in 0.5% methylcellulose in saline. The dosages of compounds used were: **3** at 75 mg/kg; **4** at 75, 25, 12.5 and 6.25 mg/kg; FUDR at 25 mg/kg; isoniazid at 0.5 mg/kg; rifampicin at 2 mg/kg; pyrazinamide at 100 mg/kg. Drug treatments were given once daily (44). Control animals received equivalent volumes of diluent only. Drug treatments were initiated 4 days after mycobacterial challenge and continued for a total of two weeks (7 days a week) (45, 46). Four days after the last treatment, mice were euthanized using a CO_2 chamber and their lungs, liver, and spleen were removed aseptically and individually homogenized in 5 mL of saline. A 100 μL of aliquot of each organ homogenate from individual mice were plated on 7H11 selective agar plates (BD Biosciences). The plates were incubated at 37^{o}C for up to 4 weeks prior to counting the colonies. The number of colonies was counted manually using a magnifying

glass apparatus. CFU counts per organ were determined by multiplying the number of colonies to the dilution factor. The number of CFUs represents the total CFUs from the whole organ.

3.2.8. Statistical analyses

Statistical analyses were performed using GraphPad Prism 6 Software (GraphPad Prism Software Inc., CA, USA). Data were represented as mean or mean ± SD (standard deviation). The differences in the means of CFU counts among multiple groups were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. A p-value less than 0.05 ($p \leq 0.05$) was considered to be statistically significant.

3.3. Result and discussion

The antimycobacterial activities of the designed pyrazinoate-FUDR conjugates **3** and **4** were determined *in vitro* against *Mtb* (H37Ra), *M. bovis* (BCG) and *M. avium*, and in the BALB/c mouse model of *Mtb* infection. Their antimycobacterial effects were also examined *in vitro* and *in vivo* combinations with first-line antituberculosis drugs INH and RIF working at different mycobacterial targets, to investigate if enhanced inhibition of *Mtb* replication can be achieved. The data are described in tables 3.1-3.3 and figures 3.3-3.7.

Conjugates **3** and **4** inhibited the growth of *Mtb* with an MIC_{100} of 50 μg/mL (Table 3.1). They exhibited better efficacy (MIC_{50} = ~ 6.25-12.5 μg/mL) than the parent drugs FUDR, PZ or PZA where no inhibition of *Mtb* was obtained at 10 μg/mL. It is likely that they have facilitated passage through the bacterial cell wall and exert their antimycobacterial effects after intramycobacterial hydrolysis to their parent molecules. However, it is also possible that the conjugates have intrinsic antimycobacterial activity since alkyl pyrazinoates have been shown to be inhibitory to *Mtb* due to their intrinsic activity (27, 29). Conjugates **3** and **4** showed diminished activity against *M. bovis* and no activity against *M. avium.*

Table 3.1: *In vitro* antimycobacterial activities of the conjugates **3** and **4** against *Mtb*, *M. bovis* and *M. avium*

Compound	Antimycobacterial activity (concentration μg/mL)[a]		
	Mtb (H37Ra)	*M. bovis* (BCG)	*M. avium* (ATCC25291)
	% inhibition	% inhibition	% inhibition
3	100 (100, 50), 89 (10), 50 (6.25)	100 (100), 50 (50)	0
4	100 (100, 50), 49 (12.5)	100 (100), 50 (50)	0
FUDR	100 (100, 50) 0 (10)	100 (100), 50 (50), 0 (10)	0
PZ (Pyrazinamide)	20 (100)	ND[b]	ND
PZA (Pyrazinoic acid)	0 (100)	0 (100)	0 (100)
INH (Isoniazid)	100 (1)	100 (1)	ND
RIF (Rifampicin)	100 (0.5)	100 (0.5)	90 (2)
Clarithromycin	ND	ND	100 (2)

[a]Antimycobacterial activity was determined at 100, 50, 10 and 1 μg/mL followed by a detail dose response studies at 25, 12.5, 6.25, 3.12, 1.56 and 0.78 μg/mL. [b]ND = not determined. PZ is not active against *M. bovis* and *M. avium* due to lack of pyrazinamidase activity (47, 48). Positive control drugs rifampicin at 0.5 or 2, isoniazid at 1 and clarithromycin at 2 μg/mL were used. The experiment was performed on three separate days, in triplicate on each day and the mean percent inhibition from a representative experiment is provided. The standard deviation of three separate experiments was within 10%.

Interestingly, when conjugate **3** was evaluated in combination with INH at its <50% effective concentration (0.20 μg/mL), **3** led to >99% inhibition of *Mtb* at 6.25 μg/mL, compared to **3** alone (50% inhibition at 6.25 μg/mL). This interaction was found to be an additive effect with a CI value of 1.0 (Table 3.2). A similar additive effect (99% inhibition) with a CI of 1.0 was obtained by **3** at 6.25 μg/mL (50% inhibitory concentration) in combination with RIF at 0.002 μg/mL (~50% inhibitory concentration) (Table 3.3).

Table 3.2: *In vitro* antimycobacterial activity of the conjugates **3** and **4** in combination with isoniazid

Compound	Conc. (μg/mL)	Antimycobacterial activity *Mtb* (H37Ra)			
		Compound alone	Combination with INH @ 0.2 μg/mL		Outcome
		% inhibition	%inhibition	CI[a]	
3	6.25	49.6	99.8	1.0	Additive
	3.12	22.8	68.0	1.0	Additive
	1.56	11.1	58.0	1.0	Additive
	0.78	2.5	50.1	1.0	Additive
4	12.5	49.4	99.1	0.9	Additive
	6.25	22.0	97.8	0.7	Synergistic
	3.12	9.0	90.0	0.6	Synergistic
	1.56	5.0	55.0	0.9	Synergistic
Isoniazid	0.20	46.5	-	-	

[a]CI < 1 (Synergistic), 1 (Additive), The experiment was performed on three separate days, in triplicate on each day and the mean percent inhibition from a representative experiment is provided. The standard deviation of three separate experiments was within 10%.

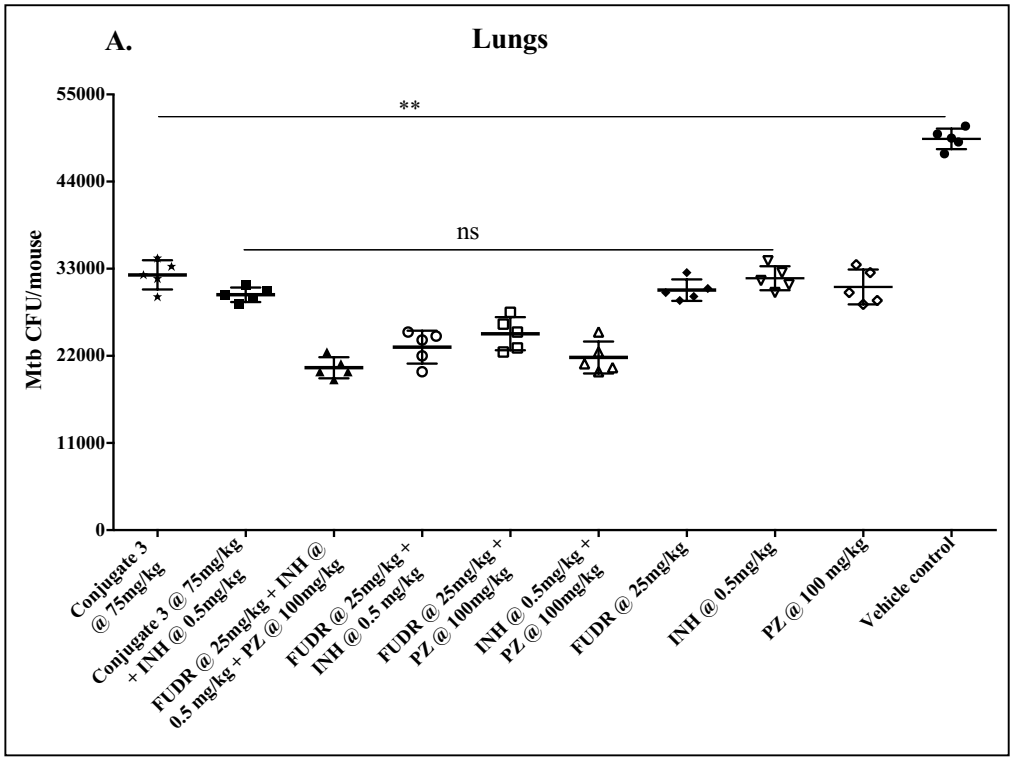
In contrast, *in vitro* combination of conjugate **4** at 6.25 μg/mL with INH at its <50% effective concentration (0.20 μg/mL) and RIF at its ~50% effective concentration (0.002 μg/mL) were found to be synergistic, with CIs of 0.7 and 0.8, respectively (Tables 3.2 and 3.3). It was interesting to note that **4** at a lower concentration (1.56 μg/mL) also demonstrated synergy with INH (CI = 0.9) and an additive interaction with RIF (CI = 1) (Tables 3.2 and 3.3). These results suggest that conjugate **4** alone despite its lower activity (22% inhibition at 6.25 μg/mL) than conjugate **3** (50% inhibition at 6.25 μg/mL) has higher antimycobacterial effects when combined with INH or RIF. Although the reasons for this observation are not clear, it is possible that a more optimal drug milieu is being generated in the combination.

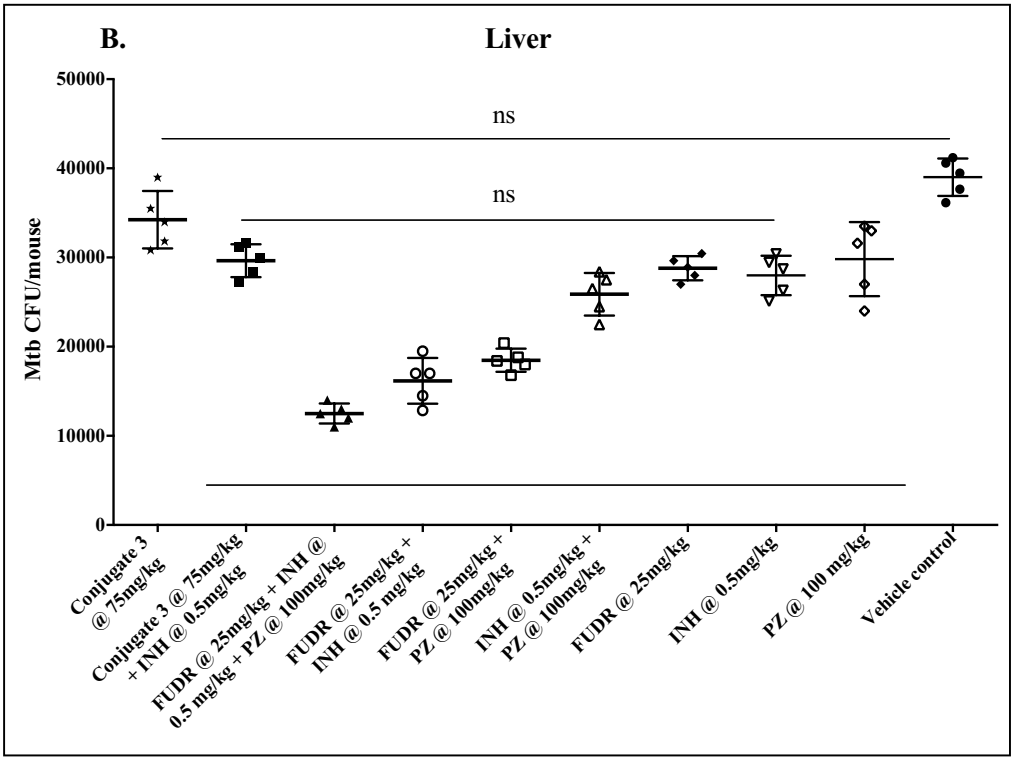
Table 3.3: *In vitro* antimycobacterial activity of the conjugates **3** and **4** in combination with rifampicin

Compound	Conc. (μg/mL)	Antimycobacterial activity *Mtb* (H37Ra)			
		Compound alone	Combination with RIF @ 0.002 μg/mL		Outcome
		% inhibition	% inhibition	CI[a]	
3	6.25	49.6	99.0	1.0	Additive
	3.12	22.8	75.6	1.0	Additive
	1.56	11.1	61.2	1.0	Additive
	0.78	2.5	52.0	1.0	Additive
4	12.5	49.4	95.0	1.0	Additive
	6.25	22.0	90.1	0.8	Synergistic
	3.12	9.0	89.4	0.6	Synergistic
	1.56	5.0	55.0	1.0	Additive
Rifampicin	0.002	48.6	-	-	

[a]CI < 1 (Synergistic), 1 (Additive). The experiment was performed on three separate days, in triplicate on each day and the mean percent inhibition from a representative experiment is provided. The standard deviation of three separate experiments was within 10%.

Conjugate **3** upon oral administration at 75 mg/kg for 2-wks, showed significant inhibition (~ 30%) of *Mtb* in both lungs and spleen of the *Mtb* (H37Ra) infected mice compared to the control vehicle group (Figures 3.3 and 3.4), but it did not provide better inhibition of *Mtb* than parent drugs FUDR and PZ alone or their combination (Figure 3.3). In combination studies, although **3** (at 75 mg/kg) with a low dose of INH (at 0.5 mg/kg) led to improved inhibition of *Mtb* than **3** alone, the obtained effect was not greater than combinations of FUDR + INH + PZ, FUDR + INH, INH + PZ and INH or PZ alone (Figure 3.3). Conjugate **3** displayed a similar phenomenon in the *in vivo* infection model when tested with RIF at 2 mg/kg combination (Figure 3.4).


A.
Lungs
Mtb CFU/mouse
55000
44000
33000
22000
11000
0
**
ns
Conjugate 3 @ 75mg/kg
Conjugate 3 @ 75mg/kg + INH @ 0.5mg/kg
FUDR @ 25mg/kg + INH @ 0.5 mg/kg + PZ @ 100mg/kg
FUDR @ 25mg/kg + INH @ 0.5 mg/kg
FUDR @ 25mg/kg + PZ @ 100mg/kg
INH @ 0.5mg/kg + PZ @ 100mg/kg
FUDR @ 25mg/kg
INH @ 0.5mg/kg
PZ @ 100 mg/kg
Vehicle control


B.
Liver
Mtb CFU/mouse
50000
40000
30000
20000
10000
0
ns
ns
Conjugate 3 @ 75mg/kg
Conjugate 3 @ 75mg/kg + INH @ 0.5mg/kg
FUDR @ 25mg/kg + INH @ 0.5 mg/kg + PZ @ 100mg/kg
FUDR @ 25mg/kg + INH @ 0.5 mg/kg
FUDR @ 25mg/kg + PZ @ 100mg/kg
INH @ 0.5mg/kg + PZ @ 100mg/kg
FUDR @ 25mg/kg
INH @ 0.5mg/kg
PZ @ 100 mg/kg
Vehicle control

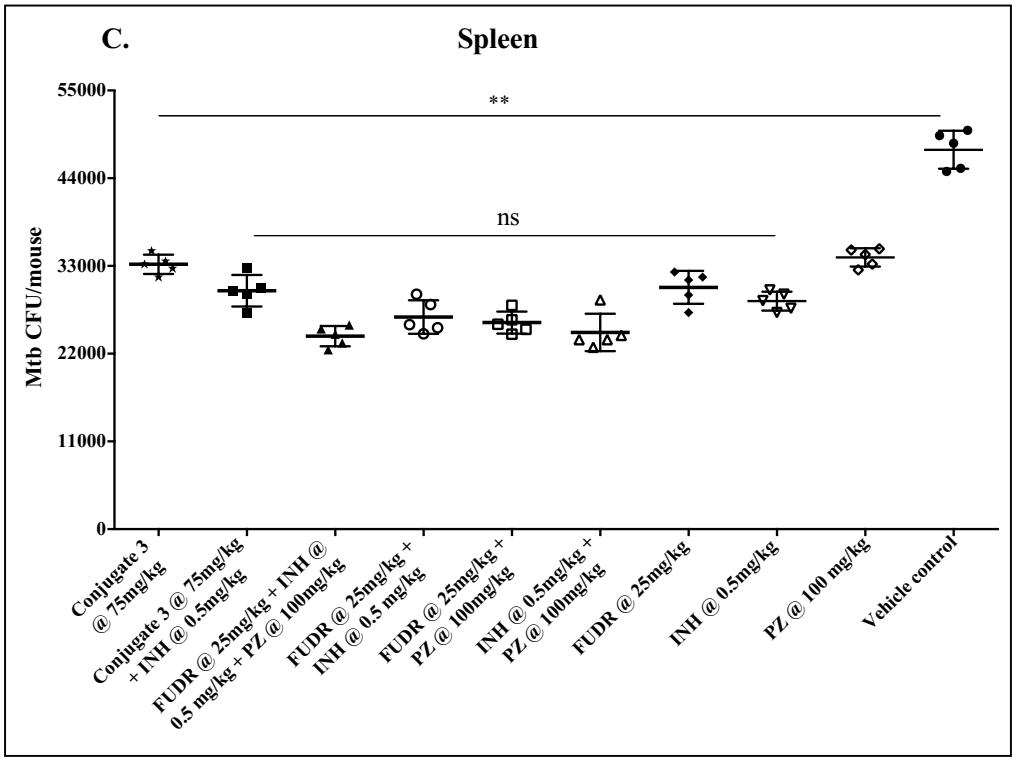
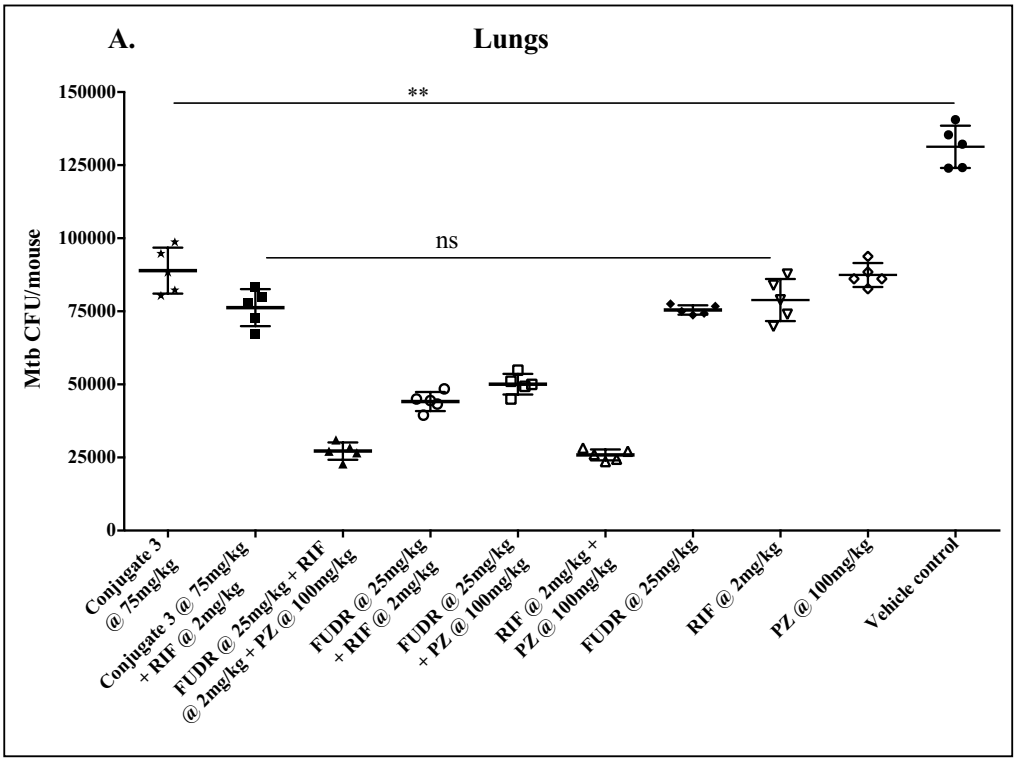
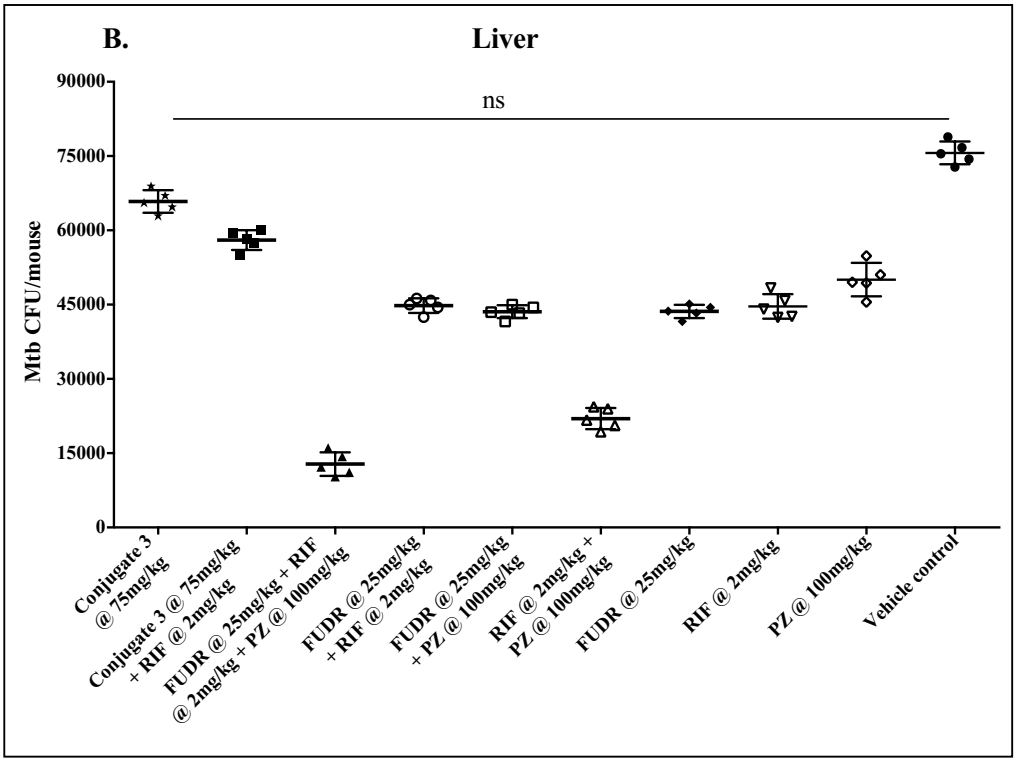


Figure 3.3: ***In vivo*** **activity of conjugate 3 alone and in combination with isoniazid against** ***Mtb*** **(H37Ra)**. BALB/c female mice (n = 5) were challenged with H37Ra (0.5×10^6 CFU/mouse) intravenously. Mice were treated with conjugate **3** (75 mg/kg), conjugate **3** (75 mg/kg) + INH (0.5 mg/kg), FUDR (25 mg/kg) + INH (0.5 mg/kg) + PZ (100 mg/kg), FUDR (25 mg/kg) + INH (0.5 mg/kg), FUDR (25 mg/kg) + PZ (100 mg/kg), INH (0.5 mg/kg) + PZ (100 mg/kg), FUDR (25 mg/kg), INH (0.5 mg/kg) or PZ (100 mg/kg) orally for two weeks. Control mice received vehicle diluent. Four days after the last treatment, mice were euthanized and lungs, liver and spleens were collected. Bacterial loads were determined in **(A)** lungs, **(B)** liver and **(C)** spleen by the CFU assay. All results are shown as mean ± standard deviation of CFU from five individual mice. Data are representative of two different repeated experiments. '**' indicates significant differences at $p \leq 0.01$.


A.
Lungs
Mtb CFU/mouse
150000
125000
100000
75000
50000
25000
0
**
ns
Conjugate 3 @ 75mg/kg
Conjugate 3 @ 75mg/kg + RIF @ 2mg/kg
FUDR @ 25mg/kg + RIF @ 2mg/kg + PZ @ 100mg/kg
FUDR @ 25mg/kg + RIF @ 2mg/kg
FUDR @ 25mg/kg + PZ @ 100mg/kg
RIF @ 2mg/kg + PZ @ 100mg/kg
FUDR @ 25mg/kg
RIF @ 2mg/kg
PZ @ 100mg/kg
Vehicle control


B.
Liver
Mtb CFU/mouse
90000
75000
60000
45000
30000
15000
0
ns
Conjugate 3 @ 75mg/kg
Conjugate 3 @ 75mg/kg + RIF @ 2mg/kg
FUDR @ 25mg/kg + RIF @ 2mg/kg + PZ @ 100mg/kg
FUDR @ 25mg/kg + RIF @ 2mg/kg
FUDR @ 25mg/kg + PZ @ 100mg/kg
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PZ @ 100mg/kg
Vehicle control

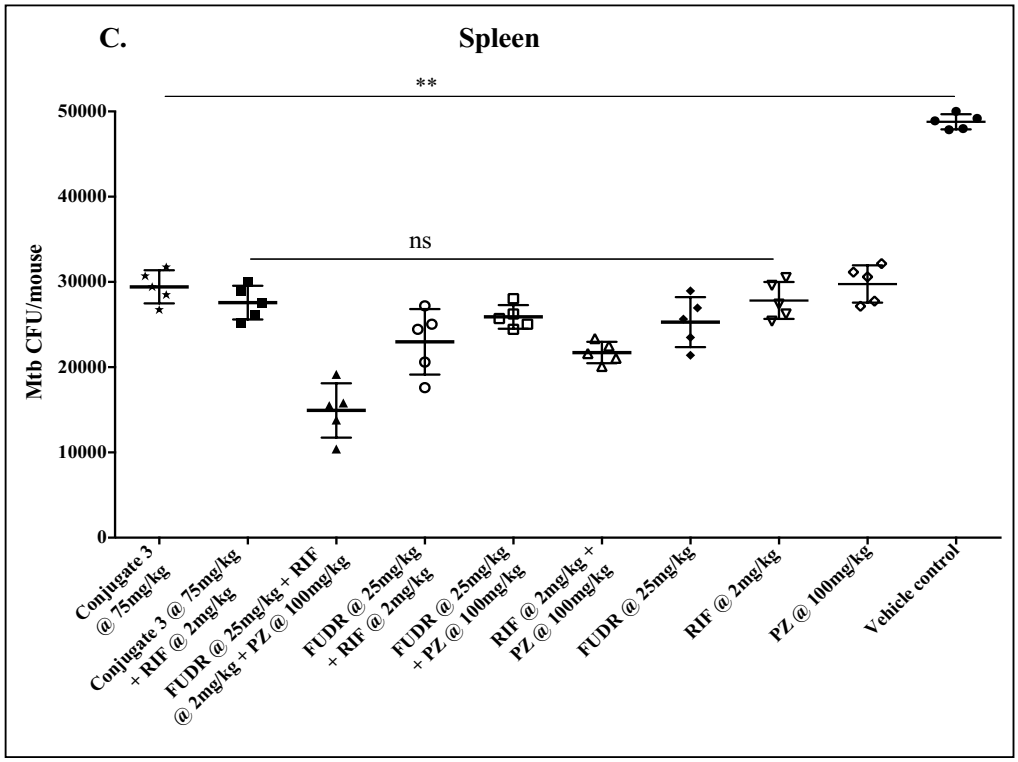


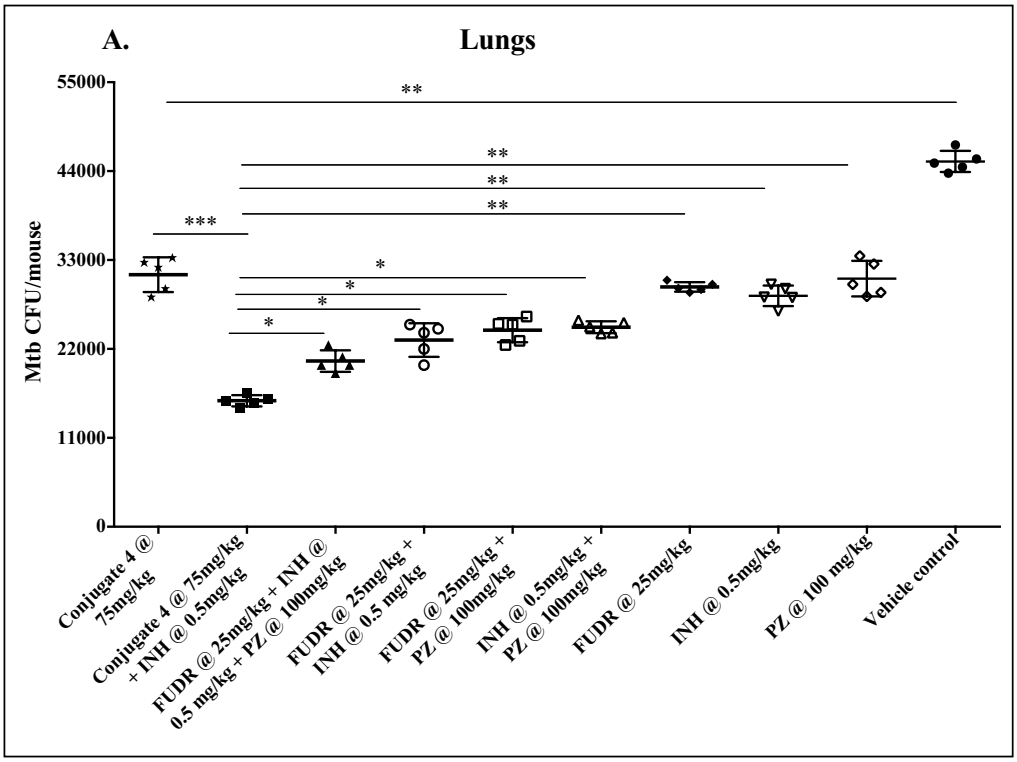
Figure 3.4: *In vivo* activity of conjugate 3 alone and in combination with rifampicin against *Mtb* (H37Ra). BALB/c female mice (n = 5) were challenged with H37Ra (0.5×10^6 CFU/mouse) intravenously. Mice were treated with conjugate **3** (75 mg/kg), conjugate **3** (75 mg/kg) + RIF (2 mg/kg), FUDR (25 mg/kg) + RIF (2 mg/kg) + PZ (100 mg/kg), FUDR (25 mg/kg) + RIF (2 mg/kg), FUDR (25 mg/kg) + PZ (100 mg/kg), RIF (2 mg/kg) + PZ (100 mg/kg), FUDR (25 mg/kg), RIF (2 mg/kg) or PZ (100 mg/kg) orally for two weeks. Control mice received vehicle diluent. Four days after the last treatment, mice were euthanized and lungs, liver and spleens were collected. Bacterial loads were determined in **(A)** lungs, **(B)** liver and **(C)** spleen by the CFU assay. All results are shown as mean ± standard deviation of CFU from five individual mice. Data are representative of two different repeated experiments. '**' indicates significant differences at $p \leq 0.01$.

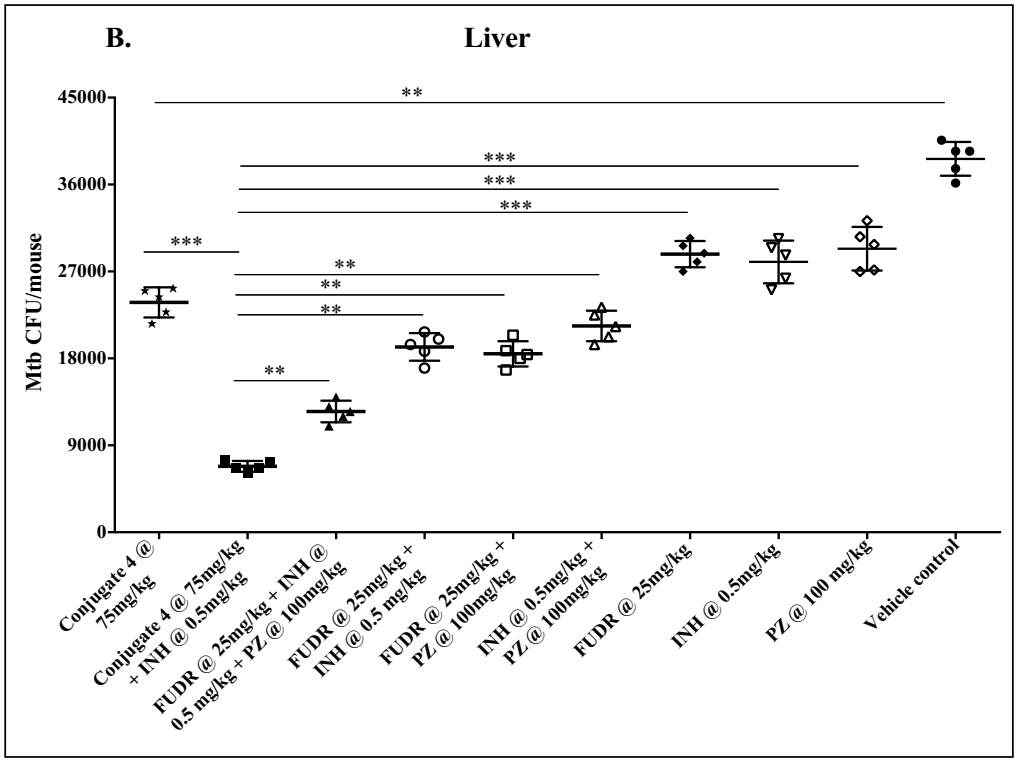
Conjugate **4** alone at 75 mg/kg for 2-wks oral dose, in contrast to **3**, provided significant inhibition (~30-40%) of *Mtb* in all organs (lungs, liver and spleen) compared to the control vehicle group. It led to superior inhibition of *Mtb* in liver and spleen but not lungs, compared to parent drugs FUDR and PZ (Figure 3.5). Intriguingly, conjugate **4** (75 mg/kg) demonstrated excellent inhibition of *Mtb* in all organs (66-82%) when combined with a low dose of INH (0.5 mg/kg) compared to not only the control vehicle group but also when compared to groups treated with FUDR + INH + PZ (48-68%), FUDR + INH (44-50%), FUDR + PZ (46-52%), INH + PZ (44-48%) and FUDR (26-36%), INH (28-37%) or PZ (24-32%) alone (Figure 3.5).

Since, conjugate **4** provided potent inhibition in combination with INH as compared to conjugate **3,** I further investigated effects of conjugate **4** at lower doses. Interestingly, conjugate **4** alone, also exhibited significant reduction of *Mtb* loads in all organs at lower doses of 25, 12.5 and 6.25 mg/kg compared to the vehicle control group (Figure 3.6). Although, it appears that the conjugate **4** showed enhanced activity at lower doses compared to high dose (75 mg/kg), a direct quantitative comparison is difficult because they were done in two different experiments. However, it is possible that at high dose (75 mg/kg) conjugate **4** is leading to negative feedback due to released high amounts of parent moieties resulting in reduced antimycobacterial activity. It has been shown earlier that FUDR can inhibit thymidylate synthetase (TS) and generate increased intracellular pool of dUMP, which eventually competes with 5-FdUMP for binding with TS, and results in reduced anticancer activity of the FUDR at high doses (49). *Mtb* encodes TS and similar negative feedback may be contributing to the reduced antimycobacterial effects of **4**.

Remarkably, **4** at 25, 12.5 and 6.25 mg/kg in combination with INH (0.5 mg/kg), also displayed a higher reduction of *Mtb* loads (81-87%, at 25 mg/kg; 64-82%, at 12.5 mg/kg; 61-

78%, at 6.25 mg/kg) in all organs at all doses compared to INH alone (37-40%) (Figure 3.6).





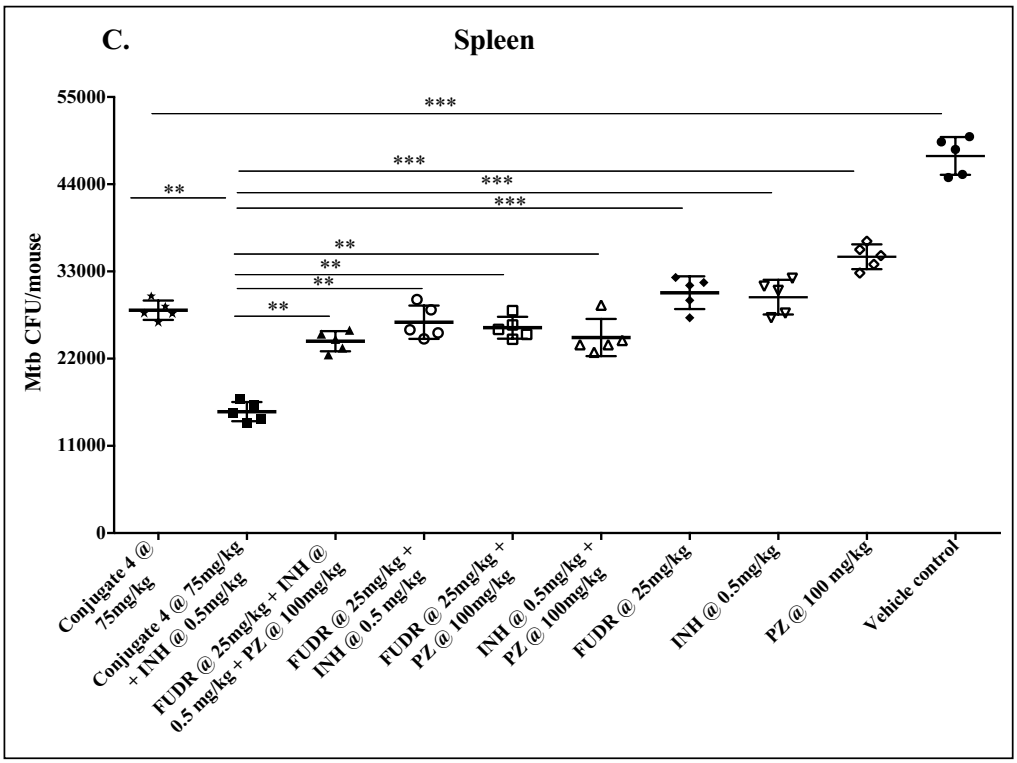
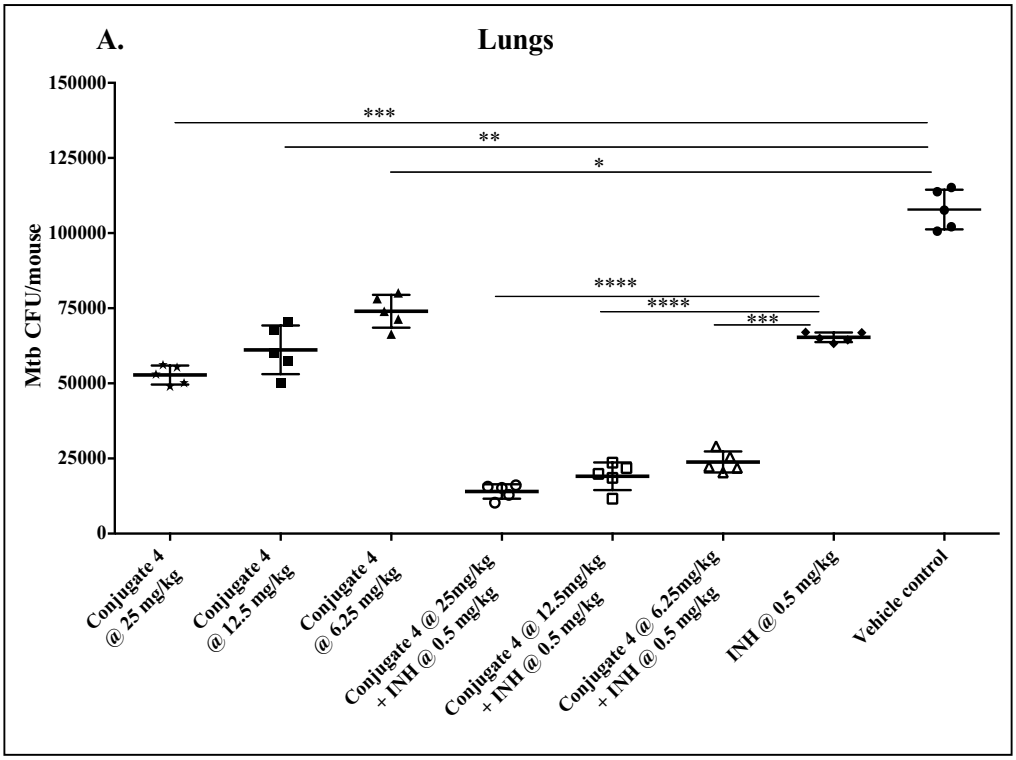


Figure 3.5: *In vivo* activity of conjugate 4 alone and in combination with isoniazid against *Mtb* (H37Ra). BALB/c female mice (n = 5) were challenged with H37Ra (0.5×10^6 CFU/mouse) intravenously. Mice were treated with conjugate **4** (75 mg/kg), conjugate **4** (75 mg/kg) + INH (0.5 mg/kg), FUDR (25 mg/kg) + INH (0.5 mg/kg) + PZ (100 mg/kg), FUDR (25 mg/kg) + INH (0.5 mg/kg), FUDR (25 mg/kg) + PZ (100 mg/kg), INH (0.5 mg/kg) + PZ (100 mg/kg), FUDR (25 mg/kg), INH (0.5 mg/kg) or PZ (100 mg/kg) orally for two weeks. Control mice received vehicle diluent. Four days after the last treatment, mice were euthanized and lungs, liver and spleens were collected. Bacterial loads were determined in **(A)** lungs, **(B)** liver and **(C)** spleen by the CFU assay. All results are shown as mean ± standard deviation of CFU from five individual mice. Data are representative of two different repeated experiments. '*', '**', and '***' indicate significant differences at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$ respectively.


A.
Lungs
Mtb CFU/mouse
150000
125000
100000
75000
50000
25000
0
***
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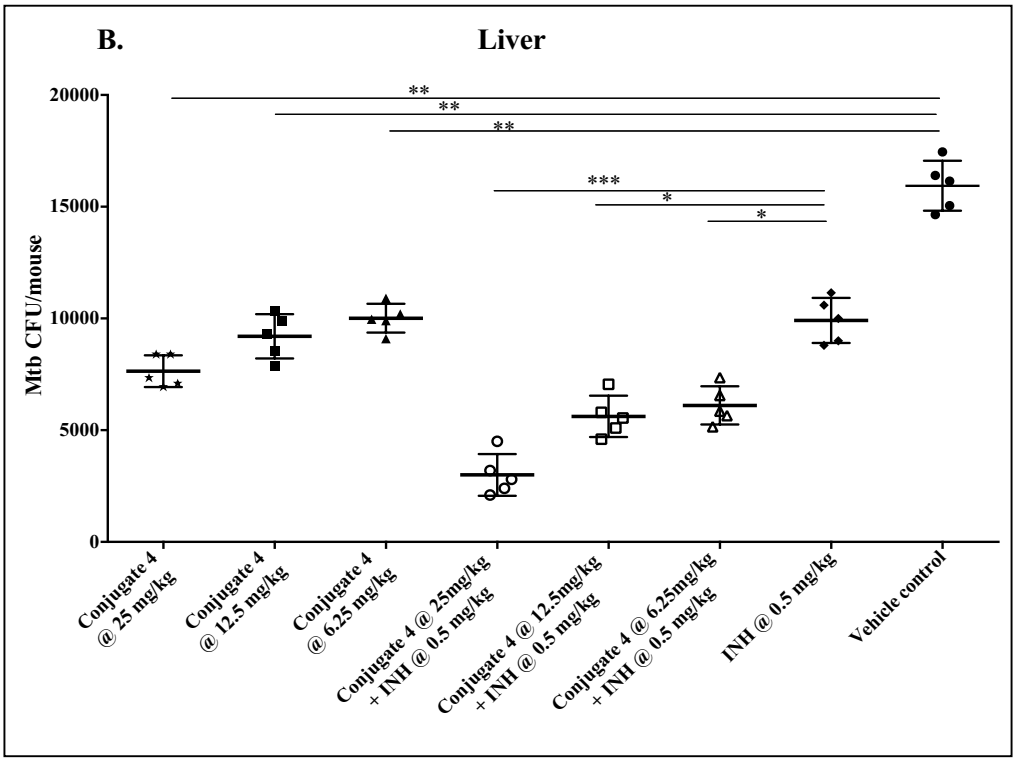
Conjugate 4 @ 25 mg/kg
Conjugate 4 @ 12.5 mg/kg
Conjugate 4 @ 6.25 mg/kg
Conjugate 4 @ 25mg/kg + INH @ 0.5 mg/kg
Conjugate 4 @ 12.5mg/kg + INH @ 0.5 mg/kg
Conjugate 4 @ 6.25mg/kg + INH @ 0.5 mg/kg
INH @ 0.5 mg/kg
Vehicle control


B.
Liver
Mtb CFU/mouse
20000
15000
10000
5000
0
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Conjugate 4 @ 25 mg/kg
Conjugate 4 @ 12.5 mg/kg
Conjugate 4 @ 6.25 mg/kg
Conjugate 4 @ 25mg/kg + INH @ 0.5 mg/kg
Conjugate 4 @ 12.5mg/kg + INH @ 0.5 mg/kg
Conjugate 4 @ 6.25mg/kg + INH @ 0.5 mg/kg
INH @ 0.5 mg/kg
Vehicle control

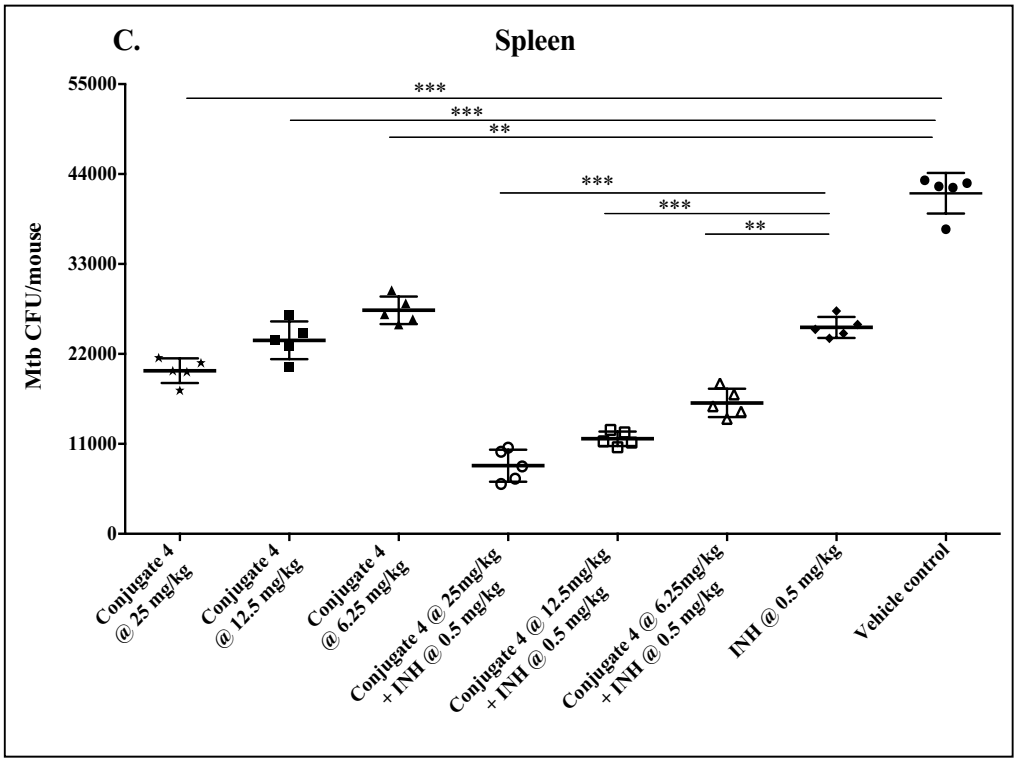


Figure 3.6: *In vivo* activity of conjugate 4 alone and in combination at lower doses with isoniazid against *Mtb* (H37Ra). BALB/c female mice (n = 5) were challenged with H37Ra (0.5 × 10^6 CFU/mouse) intravenously. Mice were treated with conjugate **4** (25 mg/kg), conjugate **4** (12.5 mg/kg), conjugate **4** (6.25 mg/kg), conjugate **4** (25 mg/kg) + INH (0.5 mg/kg), conjugate **4** (12.5 mg/kg) + INH (0.5 mg/kg), conjugate **4** (6.25 mg/kg) + INH (0.5 mg/kg) or INH (0.5 mg/kg) orally for two weeks. Control mice received vehicle diluent. Four days after the last treatment, mice were euthanized and lungs, liver and spleens were collected. Bacterial loads were determined in **(A)** lungs, **(B)** liver and **(C)** spleen by the CFU assay. All results are shown as mean ± standard deviation of CFU from five individual mice. Data are representative of two different repeated experiments. '*', '**', '***' and '****' indicate significant differences at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$ respectively.

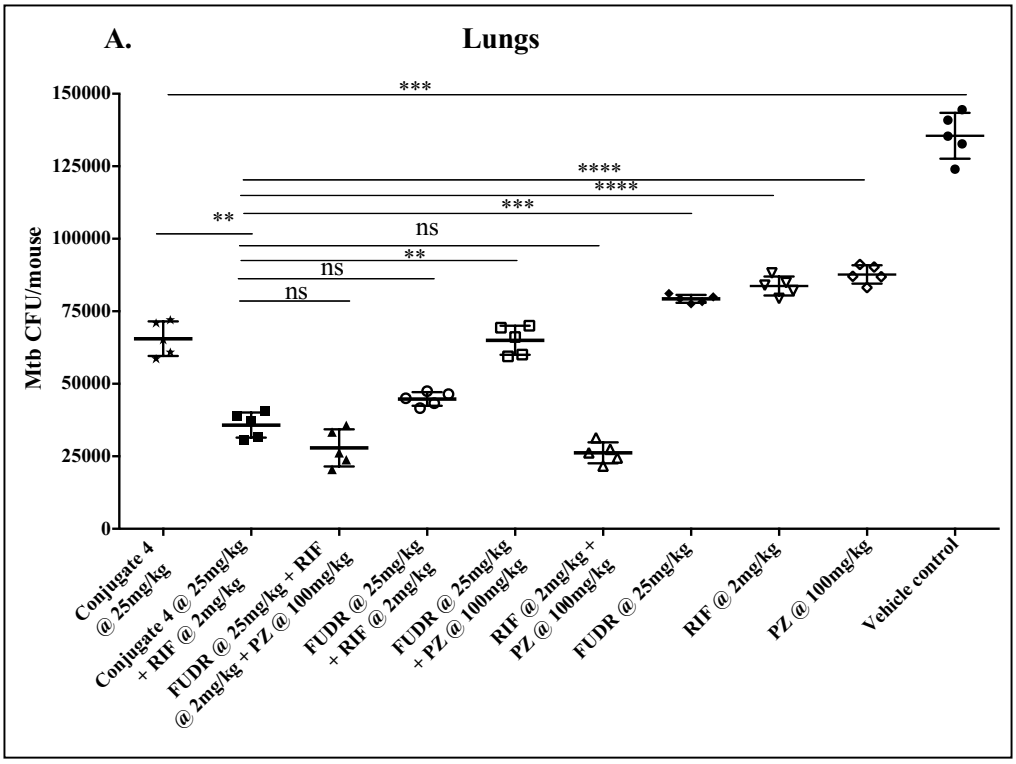
The effect of conjugate **4** at 25 mg/kg was also investigated in combination with RIF at 2 mg/kg where it showed a significantly higher inhibition of *Mtb* (66-72%) in lungs, liver and spleen than groups treated with RIF (2 mg/kg) (33-36%), PZ (100 mg/kg) (30-33%) alone or FUDR (25 mg/kg) (35-37%) (Figure 3.7). Although the antimycobacterial effect exerted by the combination of **4** + RIF was not superior compared to the three-individual drugs (FUDR + RIF + PZ) or combinations of 2 individual drug (RIF + PZ), it is important to note that the conjugate **4** was administered at the concentration of 25 mg/kg compared to PZ at 100 mg/kg when used in combination.

Conjugate **4** at 25 mg/kg demonstrated superior antimycobacterial effects in combination with INH compared to RIF combination. Although the exact reason is not known, it can be postulated that there is enhanced delivery of conjugate **4** in the mycobacteria due to disruption of bacterial cell wall by INH.

The data obtained in our mouse model studies clearly correlate with the *in vitro* combination studies where **4** displayed synergistic effects and **3** had an additive interaction with both first-line antimycobacterial drugs INH and RIF.

Compounds **3** and **4** were non-toxic to Vero cells up to a concentration of 200 μg/ml (CC_{50} > 200 μg/ml) in the *in vitro* assay (Appendix 2). Also, none of the mice became sick, lost weight, died, or showed any abnormalities upon gross necroscopy in all of the treatment groups with **3** and **4**.

These results suggest that the designed conjugates certainly have enormous potential in reducing doses, toxicity and improving compliance of current drugs, opening new and exciting possibilities for the treatment of MDR/XDR/TDR-TB.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Mtb CFU/mouse
150000
125000
100000
75000
50000
25000
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Conjugate 4 @ 25mg/kg
Conjugate 4 @ 25mg/kg + RIF @ 2mg/kg
FUDR @ 25mg/kg + RIF @ 2mg/kg + PZ @ 100mg/kg
FUDR @ 25mg/kg + RIF @ 2mg/kg
FUDR @ 25mg/kg + PZ @ 100mg/kg
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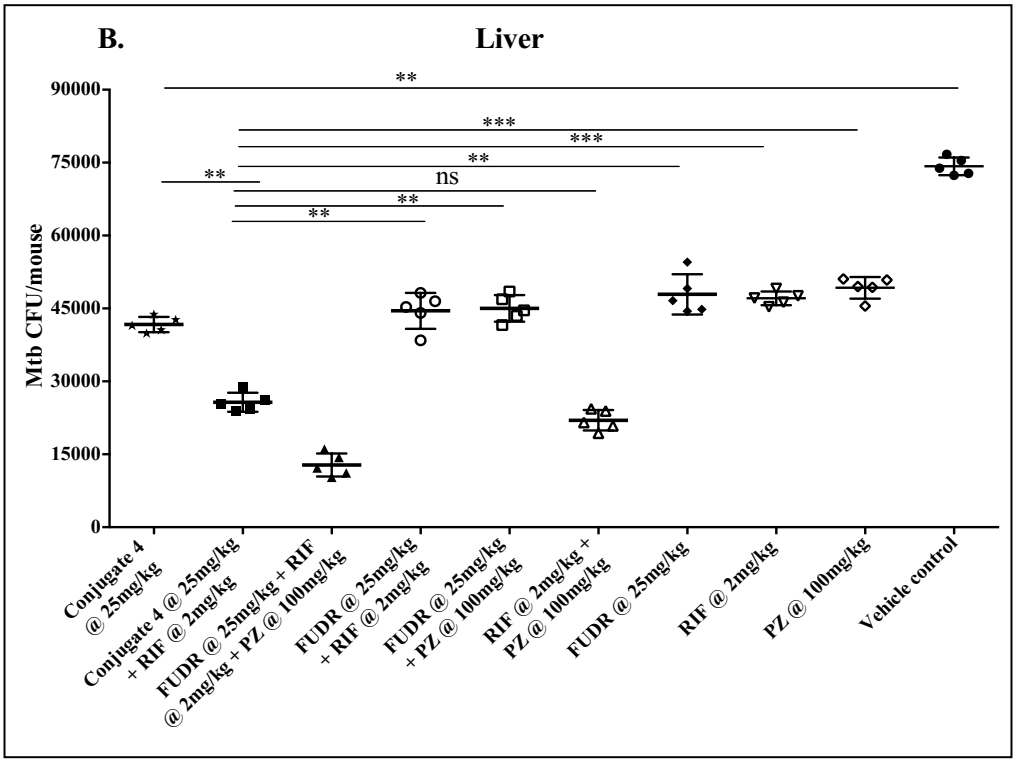
RIF @ 2mg/kg
PZ @ 100mg/kg
Vehicle control


B.
Liver
Mtb CFU/mouse
90000
75000
60000
45000
30000
15000
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Conjugate 4 @ 25mg/kg
Conjugate 4 @ 25mg/kg + RIF @ 2mg/kg
FUDR @ 25mg/kg + RIF @ 2mg/kg + PZ @ 100mg/kg
FUDR @ 25mg/kg + RIF @ 2mg/kg
FUDR @ 25mg/kg + PZ @ 100mg/kg
RIF @ 2mg/kg + PZ @ 100mg/kg
FUDR @ 25mg/kg
RIF @ 2mg/kg
PZ @ 100mg/kg
Vehicle control

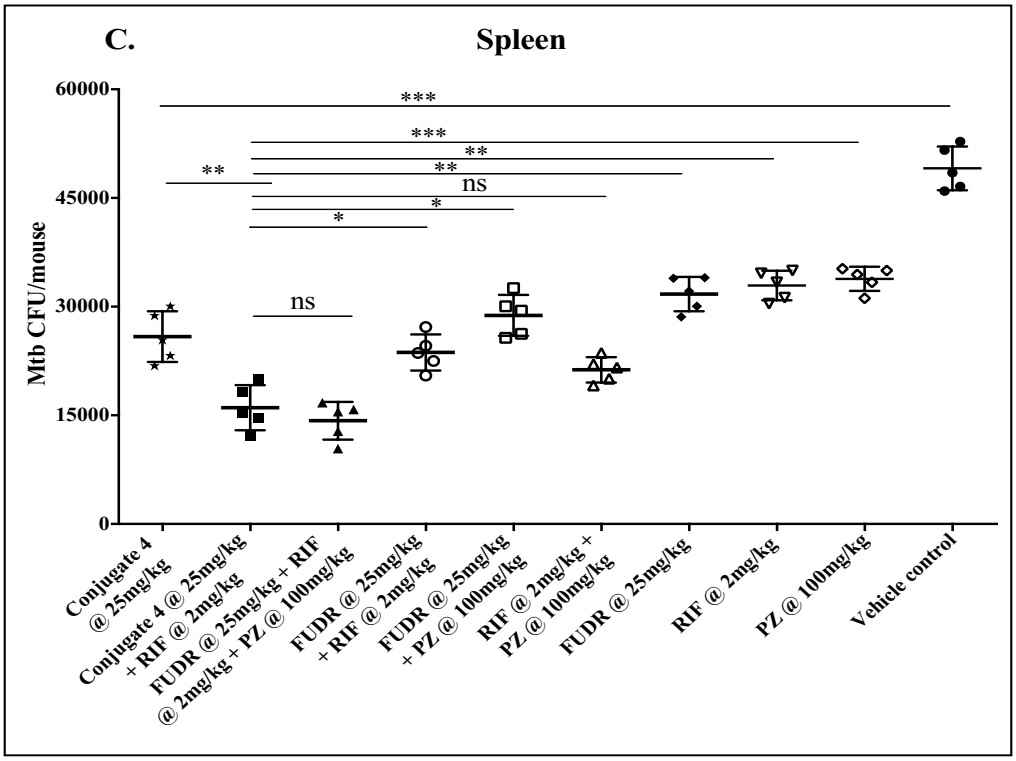

Figure 3.7: *In vivo* activity of conjugate 4 alone and in combination with rifampicin against *Mtb* (H37Ra). BALB/c female mice (n = 5) were challenged with H37Ra (0.5×10^6 CFU/mouse) intravenously. Mice were treated with conjugate **4** (25 mg/kg), conjugate **4** (25 mg/kg) + RIF (2 mg/kg), FUDR (25 mg/kg) + RIF (2 mg/kg) + PZ (100 mg/kg), FUDR (25 mg/kg) + RIF (2 mg/kg), FUDR (25 mg/kg) + PZ (100 mg/kg), RIF (2 mg/kg) + PZ (100 mg/kg), FUDR (25 mg/kg), RIF (2 mg/kg) or PZ (100 mg/kg) orally for two weeks. Control mice received vehicle diluent. Four days after the last treatment, mice were euthanized and lungs, liver and spleens were collected. Bacterial loads were determined in **(A)** lungs, **(B)** liver and **(C)** spleen by the CFU assay. All results are shown as mean ± standard deviation of CFU from five individual mice. Data are representative of two different repeated experiments. '*', '**', '***' and '****' indicate significant differences at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and $p \leq 0.0001$ respectively.

Although the exact mechanism of action of the investigated conjugates has not been determined, it is expected that the anti-mycobacterial nucleoside could undergo phosphorylation by host or bacterial kinases to the triphosphate form inhibiting DNA or RNA synthesis of the mycobacteria, whereas pyrazinoic acid (PZA) would disrupt the cell membrane or inhibit fatty acid synthetase I (Fas I) of mycobacteria (21).

An expectation of new TB drugs is that they should have shorter, simpler, more effective, less toxic and more easily tolerated therapeutic regimens. In these studies, I have identified novel conjugates of a nucleoside antimycobacterial agent with PZA, which demonstrated significant inhibition of *Mtb* at a low treatment dose when used alone and when combined with a third TB drug INH or RIF, as compared to PZ. This promising activity in *Mtb*-infected mice is likely due to the additive and/or synergistic action with INH or RIF of the released nucleoside analog and PZA from **3** and **4** by *Mtb* esterases (28). This innovative strategy led to marked reduction in effective doses of all agents. Moreover, such PZA conjugates may possess added advantages of i). bypassing pyrazinamidase activity, which is the target for resistance development against pyrazinamide, and ii). inhibiting latent *Mtb* within phagocytes. Considering the advantages of conjugates **3** and **4**, and their potent *in vivo* activity by the oral route, these conjugates may serve as new useful candidates for treating active, resistant and latent TB infection.

3.4. References

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Chapter 4

Design, synthesis and investigation of a novel co-drug incorporating anti-TB agent p-amino salicylic acid (PAS) and anti-HIV nucleoside drug AZT for treating TB and/or TB-HIV co-infection*

*In this chapter I designed, synthesized and performed all of the experiments. Dr. R. Kumar (supervisor), Dr. D.Y. Kunimoto (committee member) and Dr. B. Agrawal (collaborator) contributed to the concept for these studies and the data analysis

4.1. Introduction

Tuberculosis (TB) is the leading opportunistic infection, with high mortality, among people with human immunodeficiency virus (HIV) infection (1, 2). HIV is the single most important determinant of the widely observed increases in TB in both developing and industrialized countries (3-5). It is associated with a high TB attack rate, which leads to rapid disease progression and high mortality (3-5). Among mycobacterial infections, *Mycobacterium tuberculosis* (*Mtb*) and *Mycobacterium avium (M. avium)* infections are most prevalent in HIV-positive patients (6, 7). Out of 37 million people living with HIV, one-third of them are infected with latent TB (8). TB and HIV co-infection have formed a deadly syndemic that greatly increases the risk of reactivation of TB. The incidence of active TB in HIV-patients is approximately 26 times higher than in non-HIV individuals (9). In 2014 alone, 1.2 million (12%) of the 9.6 million people who developed TB worldwide were HIV-positive (8). Also in 2014, TB accounted for 390,000 deaths due to HIV-associated TB (8). It was reported that 60-70% of HIV-infected people developed active TB in their life time (8-10).

Concurrent treatment of TB and HIV is complicated by overlapping drug-toxicities and drug-drug-interactions between anti-retrovirals (ARTs) and anti-TB agents (11). Antiretroviral drugs including nucleosides and non-nucleoside reverse transcriptase inhibitors (NNRTIs) and first-line anti-TB drugs, isoniazid (INH), rifampicin (RIF) and pyrazinamide (PZ), together cause drug-induced hepatitis and peripheral neuropathy (12, 13). The most serious drug-drug interactions have been between rifampicin and NNRTIs and protease inhibitors (14, 15). Rifampicin-induced cytochrome P450 enzyme activity leads to suboptimal plasma concentration of antiretroviral NNRTIs and protease inhibitors that cause the risk of treatment failure and emergence of drug-resistance (14-17).

Treatment of drug-resistant TB in HIV-patients further poses significant challenges (18). Treatment of multi-drug resistant (MDR) TB has been limited due to incompetent treatment regimens and poor adherence of the treatment (19). Among HIV-infected individuals, treating extensively drug-resistant (XDR) TB is even more difficult and increases mortality (19, 20). However, despite the complexities of simultaneously treating both diseases, each of which requiring multi-drug therapy, ARTs play a crucial role in saving lives of patients with TB and HIV co-infection.

The anti-HIV drug 3'-azido-2', 3'-dideoxythymidine (AZT), is a nucleoside RT (reverse transcriptase) inhibitor and is a potent antiretroviral agent. Initially, AZT was administered frequently to maintain its therapeutic levels (21). High doses of AZT were associated with severe toxicities that include anemia, neutropenia, hepatotoxicity, myopathy, and bone marrow suppression (21, 22). Several clinical trials have now demonstrated that AZT remains accumulated intracellularly in its tri-phosphate form for a long period of time which suggests that antiviral activity of AZT is retained despite its low and less frequent dosing (21-25). However, AZT has a short plasma half-life of about one hour (21). In addition, the majority of AZT undergoes 5'-glucuronidation in liver microsomes during its first phase of metabolism (21, 26-28). But first-pass metabolism does not involve the cytochrome P450 enzyme (21, 26-28).

Para-amino salicylic acid (PAS) is a second-line anti-TB drug (29). Although, it was withdrawn because of its frequent and unpleasant gastrointestinal intolerance, it has been re-introduced for the treatment of MDR- and XDR-TB (30). PAS gets incorporated into the folate pathway by dihydropteroate synthase (DHPS) and dihydrofolate synthase (DHFS) and generates a hydroxyl-dihydrofolate antimetabolite that inhibits dihydrofolate reductase (DHFR) enzyme activity inside mycobacteria (30, 32). PAS also inhibits the mycobacterial DHPS enzyme directly

by mimicking the natural substrate para-amino benzoic acid (PABA) of DHPS (31). However, PAS is administered in high doses (8-12 gm/day in two or three divided doses) (32-34) since its absorption is accompanied by first-pass rapid acetylation in the gut followed by the liver, thereby increasing the concentration of metabolic product acetyl-PAS in the blood (30, 32-35). When PAS is administered in high doses, the acetylation process gets saturated due to limited availability of the enzyme acetyl-co-A. As a result, lower acetyl-PAS formation takes place and a minimum effective concentration of PAS is maintained (30). In addition to undesired acetylation, PAS has a short plasma half-life of 2 hours (30, 35, 36). Further, PAS undergoes metabolism to a major byproduct, meta-aminophenol, which is highly toxic (30, 36).

Co-trimoxazole is recommended to HIV-infected individuals to prevent secondary bacterial or parasitic infections (37-40). Several clinical studies have shown that co-trimoxazole preventive therapy also reduces the incidence of TB among HIV-infected adults and children (39-42). The *in vitro* antimycobacterial activity of co-trimoxazole against *Mtb* supports this clinical observation (43-44). Interestingly, co-trimoxazole has also been reported to inhibit the growth of MDR strains of TB (45). Co-trimoxazole is a mixture of sulphamethoxazole (SMX) and trimethoprim (TMP) where sulphamethoxazole competes with PABA and trimethoprim inhibits the DHFR enzyme essential for DNA and RNA synthesis (46-48). In contrast to co-trimoxazole, antimycobacterial drug PAS alone targets the enzyme activity of both DHPS and DHFR, inhibiting mycobacterial replication (30, 31).

Co-drug is a novel drug design strategy to chemically bind two or more drugs to improve therapeutic efficiency or decrease side effects. It has been shown that co-drugs possess several benefits over the parent active drugs, including enhanced bioavailability, prolong half-life and reduced toxicity.

In this study, I have designed, synthesized and investigated a novel co-drug of PAS and AZT, in which PAS was linked via its carboxyl moiety to the 5'-position of AZT. It is rationalized that the designed PAS-AZT co-drug would provide improved treatment outcomes in TB-HIV co-infections due to improved delivery and sustained release of both the anti-HIV and the anti-TB agents. This could increase plasma-half life, reduce doses and toxicities, and lead to better patient compliance with these drugs (21, 49). It was expected that intracellular cleavage of the co-drug in the presence of host cell esterases (21) would simultaneously release AZT and PAS. AZT would then be converted to AZT-TP by intracellular cellular kinases where it would inhibit retroviral RNA-dependent DNA polymerase and terminate the viral DNA chain elongation. Meanwhile, released PAS would inhibit mycobacterial replication by blocking the mycobacterial folate pathway (Figure 4.1) (21, 50). An additional advantage of this approach is that the co-drug may efficiently enter into the mycobacterial cell as well as releasing PAS directly into the presence of mycobacterial esterases without undesired metabolic conversion of PAS into acetyl-PAS outside the bacterial cell.

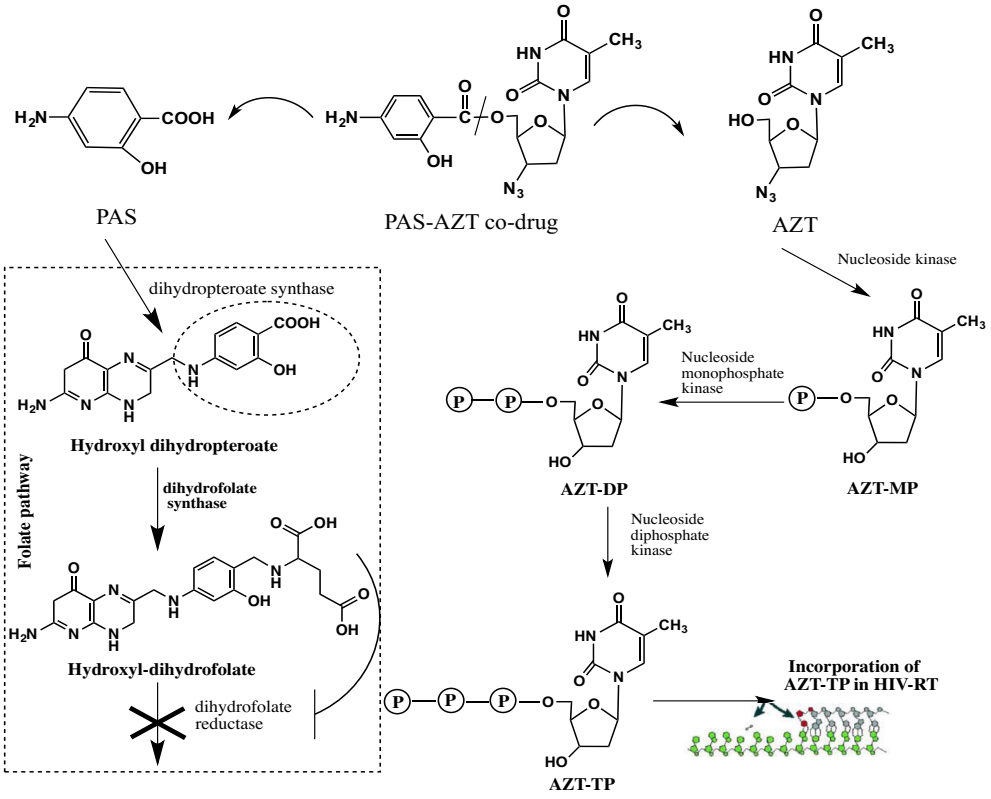


Figure 4.1: Possible mechanism of action of the novel PAS-AZT co-drug

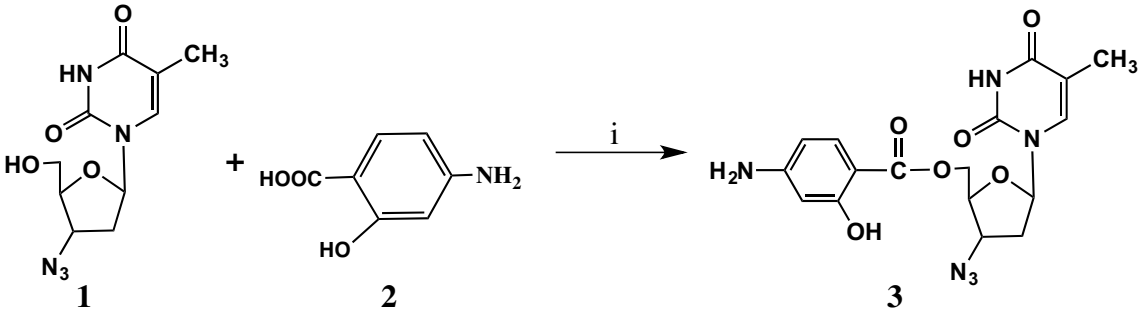
Herein I have described the synthesis of the novel co-drug 5'-O-para-aminosalicylate-AZT and its *in vitro* and *in vivo* antimycobacterial activities alone and in combination with first-line drugs isoniazid and rifampicin.

4.2. Materials and Methods

4.2.1. Chemistry

The synthesis of co-drug was carried out by the reaction of 3'-azidothymidine (AZT, **1)** and para-aminosalicylic acid (PAS, **2)** in the presence of 4-dimethylaminopyridine (DMAP) and dicyclohexylcarbodiimide (DCC) in dimethylformamide (DMF) at 25^{o}C as outlined in Scheme 4.1. Reaction progress was slow and six days later the desired product 5'-O-para-aminosalicylated-AZT **(3)** was obtained in 83% yield. The structure of **3** was confirmed by

^{1}HNMR and ^{13}C NMR spectral studies. The starting material (AZT, PAS, DMAP and DCC) used in the reaction were purchased from Aldrich chemical company, Inc. USA.



Scheme 4.1. Reagent and conditions: (i) 3'-Azidothymidine, p-aminosalicylic acid, 4-dimethylaminopyridine, dicyclohexylcarbodiimide, dimethylformamide, 25^{o}C, 6 days.

4.2.2. Experimental design

The melting point of **3** was determined with an electrothermal melting point apparatus. ^{1}H NMR and ^{13}C NMR spectra were determined in Me_2SO-d_6 on a Bruker AM 600 spectrophotometer using TMS as an internal standard. Chemical shifts are given in ppm relative to TMS and signals are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublets), dt (doublet of triplets) and dm (doublet of multiplet). The assignment of exchangeable protons (OH, NH) was confirmed by the addition of D_2O. The final compound had >95% purity as determined by microanalysis. Microanalysis results were within ± 0.4% of the theoretical values for all elements listed unless otherwise indicated. Silica gel column chromatography was carried out using Merck 7734 silica gel (100-200 μM particle size). Thin-layer chromatography (TLC) was performed with Machery-Nagel AlugramSil G/UV silica gel slides (20 μM thickness).

4.2.2.1. Preparation of 5'-O-para-aminosalicylate-AZT

To a solution of 3'-azidothymidine (400 mg, 1.49 mmol) in anhydrous dimethylformamide (35 mL), were added para-aminosalicylic acid (229 mg, 1.49 mmol), 4-dimethylaminopyridine (154 mg, 0.747 mmol) and dicyclohexylcarbodiimide (0.462 mg, 2.24 mmol). The reaction mixture was stirred at room temperature for 24 h. The progress of the reaction was monitored by TLC in MeOH/dichloromethane (6:94, v/v). Additional aliquots of dicyclohexylcarbodiimide (155 mg, 0.751 mmol) and para-amino salicylic acid (115 mg, 0.751 mmol) were added to the reaction mixture at an interval of 24 h up to 6 days. After that the reaction mixture was concentrated in vacuo. The residue thus obtained was purified on a silica gel column using MeOH/dichloromethane (1.5:98.5,v/v) as an eluent to provide 5'-O-para-aminosalicylate of AZT (500 mg, 83% yield) as a solid; M.p 175-180°C; ^{1}H NMR (DMSO-d_6) δ1.735 (d, J = 0.6 Hz, 3H, CH_3), 2.42-2.47 (m, 1H, H-2') and 2.52-2.54 (m. 1H, H-2'), 4.16 (q, J = 4.8, 1H, H-3'), 4.48 and 4.58 (2q, J = 4.2 Hz and 3.6 Hz, 2H, H-5'), 4.58-4.68 (m, 1H, H-4'), 6.18-6.26 (m, 4H, H-1', NH_2, 2H aromatic), 7.46 (d, J = 1.2 Hz, 1H aromatic), 7.56 (d, J = 9 Hz, 1H, H-6), 10.70 (s, 1H, OH), 11.42 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 12.39 (CH_3), 36.24 (C-5'), 60.33 (C-3'), 63.36 (C-2'), 81.07 (C-4'), 83.81 (C-1'), 98.98 (C-Ar), 99.61 (C-NH_2 Ar), 107.19 (C-Ar), 110.44 (C-OH Ar), 131.63 (C-Ar), 136.18 (C-6), 150.83 (C-5), 156.76 (C=O), 163.44 (C-Ar), 164.06 (C-4), 169.43 (C-2). Anal. Calcd for $C_{17}H_{18}N_6O_6$ (M.W.352.28): C 50.75, H 4.51, N 20.89. Found C 50.65, H 4.60, N 20.49 (Appendix 24).

4.2.3. *In vitro* antimycobacterial activity against *Mtb*, *M. bovis*, and *M. avium*

Mtb (H37Ra), *M. bovis* (BCG), and *M. avium* (ATCC 25291) were obtained from the American Type Culture Collection (ATCC), Rockville, MD. These strains were cultured in Middlebrook 7H9 Broth medium supplement with glycerol, Tween 80 and Middlebrook ADC (Bovine Albumin, Dextrose and Catalase) enrichment purchased from Becton Dickinson and company, MD, USA. The cell viability reagent Alamar Blue was purchased from Bio-Rad Laboratories, Inc., USA. Antimycobacterial activity was determined using the microplate Alamar Blue Assay (MABA). Test compound was dissolved in dimethyl sulfoxide (DMSO) (Fisher Scientific, Canada) at 10 mg/mL and subsequent dilutions were made in 7H9GC medium (Difco Laboratories, Detroit, Michigan) in 96-well plates. For these experiments, initially co-drug **3** was tested at 100, 50, 10, 1, 0.5, 0.25 and 0.12 μg/mL in triplicate. Further, a detailed dose response study of co-drug **3** was performed at 0.75, 0.37, 0.18 and 0.09 μg/ml concentrations. Experiments were repeated three times and the mean percent inhibition from triplicates of a representative experiment is reported in Table 4.1. The standard deviations were within 10%. Briefly, frozen mycobacterial inocula were diluted in 7H9GC medium and added to each well at a final concentration of 2.5 x 10^5 CFU/mL. Sixteen control wells consisted of eight with bacteria alone (B) and eight with medium alone (M). Plates were incubated for six days and then 20 μL of 10 x Alamar Blue and 12.5 μL of 20% Tween 80 were added to one M and one B well. Wells were observed for an additional 24-48 h for visual color change from blue to pink and read by spectrophotometer (Fluostar Optima, BMG Labtech, GmbH, Ortenberg, Germany) at excitation 530/525 nm and emission 590/535 nm to determine OD values. If the B well became pink by 24 h (indicating growth), reagent was added to the entire plate. If the B well remained blue, additional M and B wells were tested daily until bacterial growth could be visualized by color

change. After the addition of the reagent to the plate, cultures were incubated for 24 h and plates were observed visually for color change and read by spectrophotometer. MIC was defined visually as the lowest concentration of a compound that prevented a color change from blue to pink. Percent inhibition was calculated as 100 - (Test well − Medium well) / (Bacteria well − Medium well) x 100. Similar methodology was used for all (three) mycobacteria strains. Isoniazid, rifampicin and clarithromycin purchased from Aldrich Chemical Company Inc. USA were used as positive controls. As a negative control, DMSO, was added to the B well at concentrations similar to those of test compound wells; M wells served as negative controls. In most of the experiments, the M wells gave an OD of 4000-5000, and the B wells had OD values ranging between 35,000-50,000.

4.2.4. *In vitro* antimycobacterial activity in combination with isoniazid and rifampicin

For drug combination studies, MABA assays employing similar methodology were used as described above. Isoniazid and rifampicin were used at (0.20 μg/mL) and (0.002 μg/mL) concentrations, respectively. The combination effect of co-drug **3** was determined by calculating combination index (CI) as described in section 2.2.5 on page 80.

4.2.5. *In vitro* cytotoxicity study

Cell viability was measured using the cell proliferation kit 1 (XTT; purchased from Xenometric:Endotell). A 96-well plate was seeded with Vero cells cultured in Dulbecco's modification of Eagle medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) at a density of 2 x 10^5 cells per well. Co-drug **3** was dissolved in DMSO at 10 mg/mL and subsequent dilutions were made in DMEM medium in 96-well plates. Cells were

allowed to attach for 24 h, and the DMEM medium was replaced with DMEM medium containing co-drug **3** at concentrations of 200, 100, 50, 10 and 1 μg/mL. DMSO was also included as a solvent control. Plates were incubated for 3 days at 37°C. The color reaction involved adding 10 μL XTT reagents per well and incubating for 4 h at 37°C until color change to orange (52). Plates were read on an ELISA plate reader (Fluostar Optima, BMG Labtech, GmbH, Ortenberg, Germany) at Abs $_{450\text{-}500}$ nm. Percent viability was calculated as (OD of test well) – (OD of Medium well without cells) / (OD of control solvent well) – (OD of Medium well without cells) x 100.

4.2.6. Animals and infection

The experimental animal protocol used in this study was approved by the University Animal Care and Use Committee (ACUC) for Health Sciences, and conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC). Five-to-six-week old female BALB/c mice were purchased from Charles River Laboratories and were allowed to acclimate for 1 week. Mice were challenged intravenously in the tail vein with 0.5 x 10^6 CFU/mouse of *Mtb* (H37Ra) in saline. In order to compare the designed conjugates with parent drugs and their combinations, a 90% power and minimum signal to noise ratio of 2.5 was needed and therefore five mice per group were used.

4.2.7. Administration of drugs and *in vivo* activity evaluation

The co-drug **3,** isoniazid, rifampicin and PAS were suspended in 0.5% methylcellulose in saline. The test drugs were administered orally at following doses: co-drug **3** at 25 mg/kg, isoniazid at 0.5 mg/kg, rifampicin at 2 mg/kg, PAS at 500 mg/kg and AZT at 25 mg/kg. Drug

treatments were given once daily (53). Control animals received equivalent volumes of diluent only. Drug treatment was initiated 4 days after *Mtb* challenge and continued for a total of two weeks (5 days a week) (54,55). Four days after the last treatment, mice were euthanized using a CO_2 chamber and lungs, liver, and spleen were removed aseptically and individually homogenized in 5 ml of saline. A 100 µl of aliquot of each organ homogenate from individual mice were plated on 7H11 selective agar plates (BD Biosciences). The plates were incubated at 37°C in ambient air for up to 4 weeks prior to counting the colonies. The number of colonies was counted manually using a magnifying glass apparatus. CFU counts per organ were determined by multiplying the number of colonies to the dilution factor. The number of CFUs represents the total CFUs from the whole organ.

4.2.8. Statistical analyses

Statistical analyses were performed using GraphPad Prism 6 Software (GraphPad Prism Software Inc., CA, USA). Data were represented as mean or mean ± SD (standard deviation). The differences in the means of CFU counts among multiple groups were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. A p-value less than 0.05 ($p \leq 0.05$) was considered to be statistically significant.

4.3. Results and Discussion

The antimycobacterial activity of the newly designed co-drug **3** was determined *in vitro* against three strains of mycobacteria, *Mtb* (H37Ra), *M. bovis* (BCG) and *M. avium.* The co-drug **3** was also evaluated in a mouse model of *Mtb* infection. The antimycobacterial effects of **3** were also investigated in combinations with first-line anti-TB drugs INH and RIF *in vitro* and *in vivo* to investigate possible interactions.

Compound **3** demonstrated *in vitro* activity against all three mycobacterial species tested. In the case of *Mtb*, it showed 100% inhibition at 100, 50 and 10 μg/mL and 76% inhibition at 1 μg/mL. For *M. bovis,* it provided a similar inhibition pattern. Against *M. avium*, **3** was found to be less inhibitory, showing 87% inhibition at 100 μg/mL and 71% inhibition at 50 μg/mL. Among parent drugs, AZT did not inhibit mycobacterial replication up to a concentration of 100 μg/mL while PAS inhibited the growth of *Mtb* (MIC_{100} = 0.12 μg/mL), *M. bovis* (MIC_{100} = 0.25 μg/mL) and *M. avium* (MIC_{100} = 1 μg/mL) (Table 4.1). These results suggest that PAS-AZT co-drug is not as effective as PAS alone. These results are not surprising since PAS may not be freely available in high concentration due to conjugation with AZT. It is also possible that the inhibition of mycobacterial replication by **3** could be due to its intrinsic antimycobacterial effect. However, these studies indicate that co-drug is able to enter into the mycobacterial cell.

Table 4.1: *In vitro* antimycobacterial activities of co-drug **3** against *Mtb*, *M. bovis* (BCG) and *M. avium*

Compound	Antimycobacterial activity (concentration μg/mL)[a]					
	Mtb (H37Ra)		*M. bovis* (BCG)		*M. avium* (ATCC25291)	
	% inhibition	MIC_{100}[b]	% inhibition	MIC_{100}	% inhibition	MIC_{100}
3	100 (100, 50, 10), 76 (1)	10	100 (100, 50, 10), 65 (1)	10	87 (100), 71 (50)	>100
AZT (Azidothymidine)	0 (100)	-	0 (100)	-	0 (100)	-
PAS (p-aminosalicylic acid)	100 (1, 0.5, 0.25, 0.12)	0.12	100 (1, 0.5, 0.25)	0.25	100 (100, 50, 10, 1)	1
INH (Isoniazid)	100 (1)	ND[c]	100 (1)	ND	ND	-
RIF (Rifampicin)	100 (0.5)	ND	100 (0.5)	ND	90 (2)	ND
Clarithromycin	ND	-	ND	-	100 (2)	ND

[a]Antimycobacterial activity was determined at 100, 50, 10, 1, 0.5, 0.25 and 0.12 μg/mL followed by a detail dose response studies of **3** at 0.75, 0.37, 0.18 and 0.09 μg/ml. [b]Concentration of compounds exhibiting 100% inhibition in mycobacterial growth. [c]ND = not determined. Positive control drugs rifampicin at 0.5 or 2, isoniazid at 1 and clarithromycin at 2 μg/mL were used. The experiment was performed on three separate days, in triplicate on each day and the mean percent inhibition from a representative experiment is provided. The standard deviation of three separate experiments was within 10%.

The effect of co-drug **3** was examined at four concentrations (0.75, 0.37, 0.18 and 0.09 μg/mL) in combination with <50% inhibitory concentrations of INH (0.2 μg/mL) or RIF (0.002 μg/mL). In combination studies, **3** at 0.75 μg/mL (99%) and 0.37 μg/mL (94%) led to synergistic inhibition of *Mtb* when compared to **3** alone (46% inhibition at 0.75 μg/mL and 22% inhibition at 0.37 μg/mL) and INH alone (47% at 0.2 μg/mL). Similarly, **3** at 0.75 μg/mL and 0.37 μg/mL provided synergy in combination with RIF with >99% and 91% inhibition of *Mtb* compared to RIF alone (48.5% at 0.002 μg/mL) (Table 4.2).

Table 4.2: *In vitro* antimycobacterial activity of the co-drug **3** in combination with isoniazid and rifampicin

Compound	Conc. (μg/mL)	Antimycobacterial activity *Mtb* (H37Ra)					
		Compound alone	Combination with INH @ 0.2 μg/mL		Combination with RIF @ 0.002 μg/mL		Outcome
		% inhibition	% inhibition	CI[a]	% inhibition	CI	
3	0.75	46.4	99.0	0.9	99.8	0.9	Synergistic
	0.37	22.4	94.0	0.7	91.3	0.8	Synergistic
	0.18	10.0	80.7	0.7	85.0	0.7	Synergistic
	0.09	3.0	56.1	0.9	74.8	0.7	Synergistic
INH (Isoniazid)	0.20	47.1	-	-	-	-	-
RIF (Rifampicin)	0.002	48.5	-	-	-	-	-

[a]CI < 1 (Synergistic). The experiment was performed on three separate days, in triplicate on each day and the mean percent inhibition from a representative experiment is provided. The standard deviation of three separate experiments was within 10%.

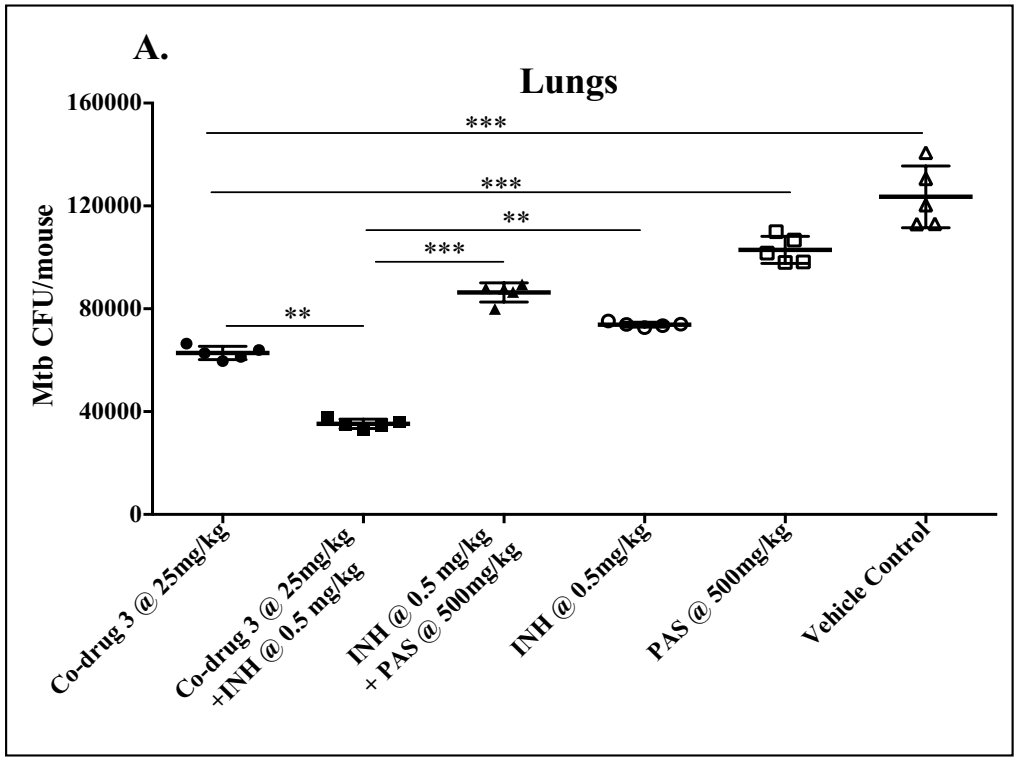
The combination effect was calculated using combination index (CIs). The CI values obtained for **3** at 0.75, 0.37, 0.18 and 0.09 μg/mL in combination with INH were 0.9, 0.7, 0.7 and 0.9, whereas with RIF the CIs were 0.9, 0.8, 0.7 and 0.7 (Table 4.2). The interactions of co-drug **3** with INH and RIF were found to be synergistic.

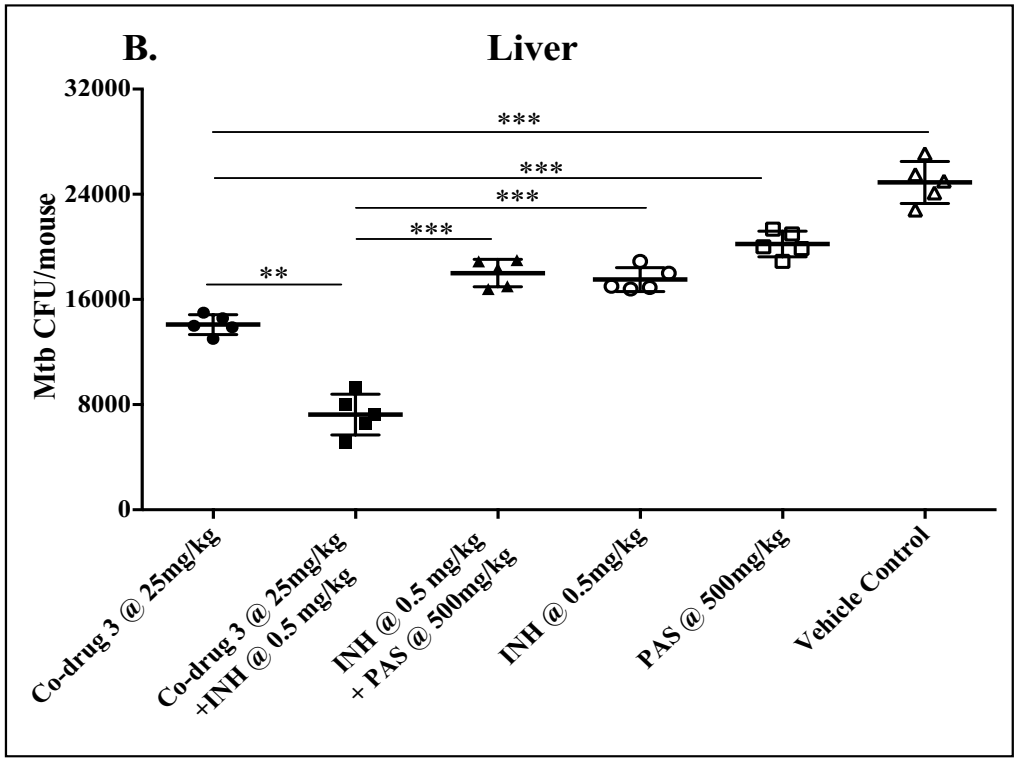
Co-drug **3** was tested in mice in an *Mtb* infection model by administrating it through the oral route. It significantly reduced mycobacterial loads in lungs, liver and spleen compared to the parent drug PAS and vehicle control group (Figures 4.2 and 4.3). AZT was not included in these experiments since it did not show any activity against *Mtb* in *in vitro* assays. It was interesting to note that compound **3** provided significant inhibition of *Mtb* in lungs (50-51%), liver (43-44%) and spleen (47-52%) at 25 mg/kg as compared to PAS where low inhibition of *Mtb* at 500 mg/kg was obtained in all organs (15-29%). The superior inhibition obtained by **3** could be due to its

increased intramacrophagic penetration and efficient delivery of the anti-TB drug, PAS, to the target site. PAS has been shown to be less effective in mice because of its poor penetration of the macrophages, which drastically compromises its ability to kill intracellular mycobacteria (56, 57). Further, PAS inactivation has been reported due to extensive extracellular acetylation (31). To achieve therapeutically effective concentrations of PAS, it is administered at a very high dose (31).

The effect of PAS-AZT co-drug **3** (at 25 mg/kg) was also evaluated in combination with a low dose of INH (at 0.5 mg/kg). Intriguingly, enhanced inhibition of mycobacterial growth was obtained in lungs, liver and spleen (50-71%) when compared to INH alone (30-40%) or a combination of two individual drugs (PAS + INH) (28-31%) (Figure 4.2). However, significant reduction in *Mtb* loads was noted in lungs and liver only. These mouse studies correlate with the *in vitro* combination studies where **3** displayed synergistic interaction with INH.

In these studies, a combination of INH and PAS provided lower antimycobacterial effects compared to INH alone. The reason for this antagonistic effect is not clear, however, it is possible that INH has undergone *in vivo* acetylation and thus effective concentrations of INH were not available. Acetylation of INH in the presence of a high dose of PAS has been reported earlier (58, 59).


A.
Lungs
Mtb CFU/mouse
160000
120000
80000
40000
0
***
***
**
***
**
Co-drug 3 @ 25mg/kg
Co-drug 3 @ 25mg/kg +INH @ 0.5 mg/kg
INH @ 0.5 mg/kg + PAS @ 500mg/kg
INH @ 0.5mg/kg
PAS @ 500mg/kg
Vehicle Control


B.
Liver
Mtb CFU/mouse
32000
24000
16000
8000
0
***
***
***
***
**
Co-drug 3 @ 25mg/kg
Co-drug 3 @ 25mg/kg +INH @ 0.5 mg/kg
INH @ 0.5 mg/kg + PAS @ 500mg/kg
INH @ 0.5mg/kg
PAS @ 500mg/kg
Vehicle Control

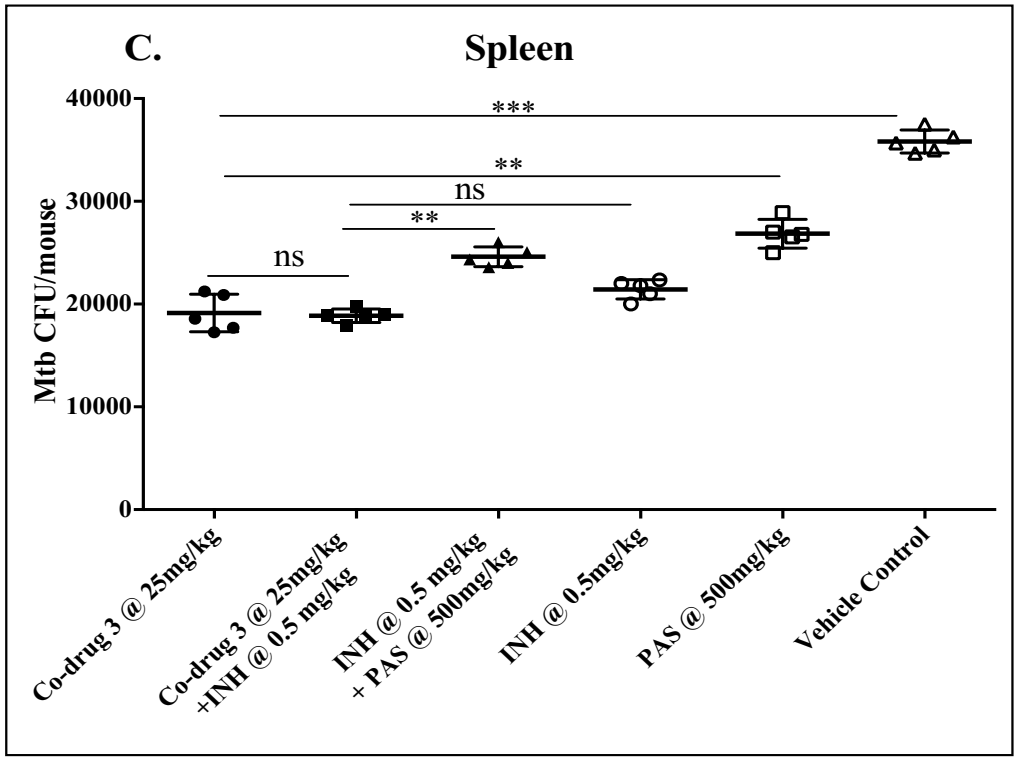
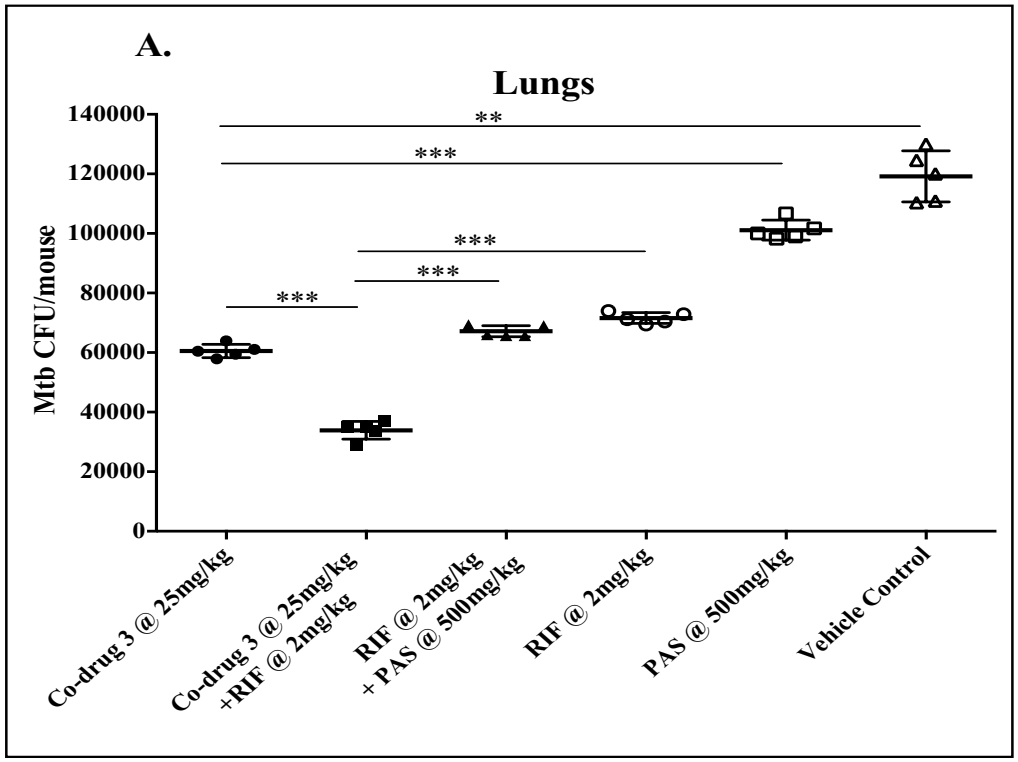


Figure 4.2: *In vivo* antimycobacterial activity of co-drug 3 alone and in combination with isoniazid. BALB/c female mice (n = 5) were challenged with H37Ra (0.5×10^6 CFU/mouse) intravenously. Mice were treated with co-drug **3** (25 mg/kg), co-drug **3** (25 mg/kg) + INH (0.5 mg/kg), PAS (500 mg/kg) + INH (0.5 mg/kg), INH (0.5 mg/kg) or PAS (500 mg/kg) orally for two weeks. Control mice received vehicle diluent alone. Four days after the last treatment, mice were euthanized and lungs, liver and spleens were collected. Bacterial loads were determined in **(A)** lungs, **(B)** liver and **(C)** spleen by the CFU assay. All results are shown as mean ± standard deviation of CFU from five individual mice. Data are representative of two different repeated experiments. '**' and '***' indicate significant differences at $p \leq 0.01$ and $p \leq 0.001$, respectively.

When the combination effect of compound **3** (at 25 mg/kg) was determined with a low dose of RIF (at 2 mg/kg), notably, a significantly higher reduction of mycobacterial CFUs in lungs, liver and spleen (49-72%) was obtained compared to RIF alone (31-40%) and a combination of two individual drugs (PAS + RIF) (31-44%) (Figure 4.3). These results correlated with the *in vitro* combination studies.

The result obtained *in vivo* in combination of **3** with isoniazid or rifampicin was highly encouraging. Further, it should be noted that the effective concentration of PAS was remarkably reduced in the conjugated form compared to the concentrations of the two individual drugs in the combinations of PAS + INH (Figure 4.2) or PAS + RIF (Figure 4.3) where PAS was administered simultaneously at a dose of 500 mg/kg.


A.
Lungs
Mtb CFU/mouse
140000
120000
100000
80000
60000
40000
20000
0
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***
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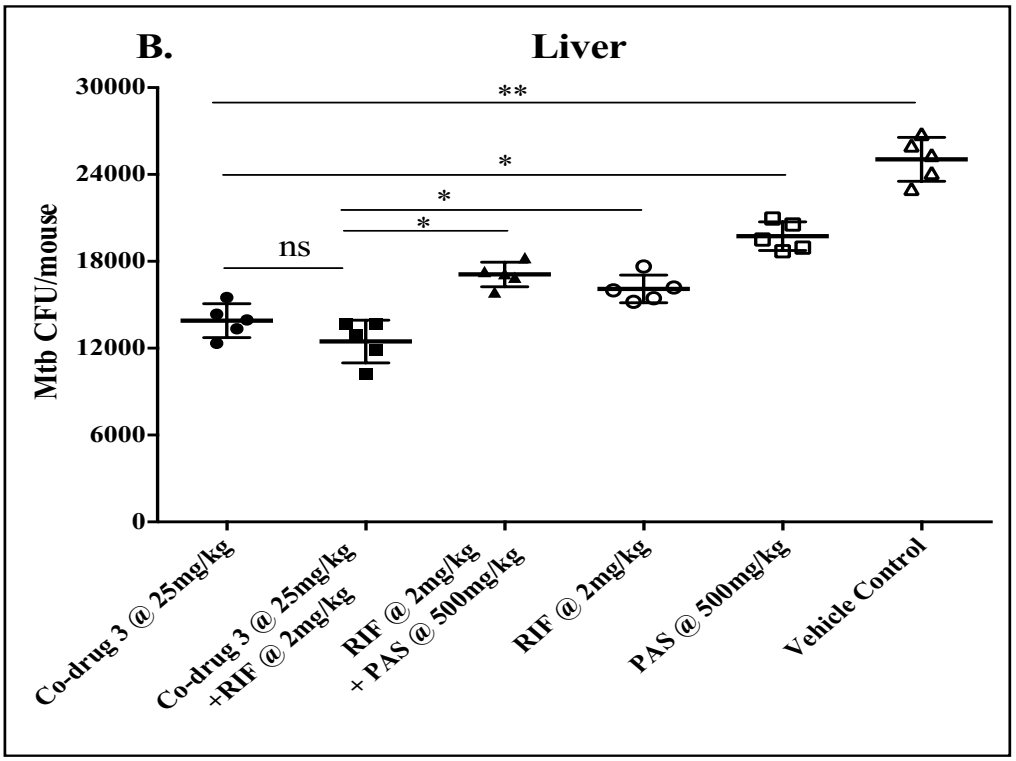
Co-drug 3 @ 25mg/kg
Co-drug 3 @ 25mg/kg +RIF @ 2mg/kg
RIF @ 2mg/kg + PAS @ 500mg/kg
RIF @ 2mg/kg
PAS @ 500mg/kg
Vehicle Control


B.
Liver
Mtb CFU/mouse
30000
24000
18000
12000
6000
0
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*
*
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ns
Co-drug 3 @ 25mg/kg
Co-drug 3 @ 25mg/kg +RIF @ 2mg/kg
RIF @ 2mg/kg + PAS @ 500mg/kg
RIF @ 2mg/kg
PAS @ 500mg/kg
Vehicle Control

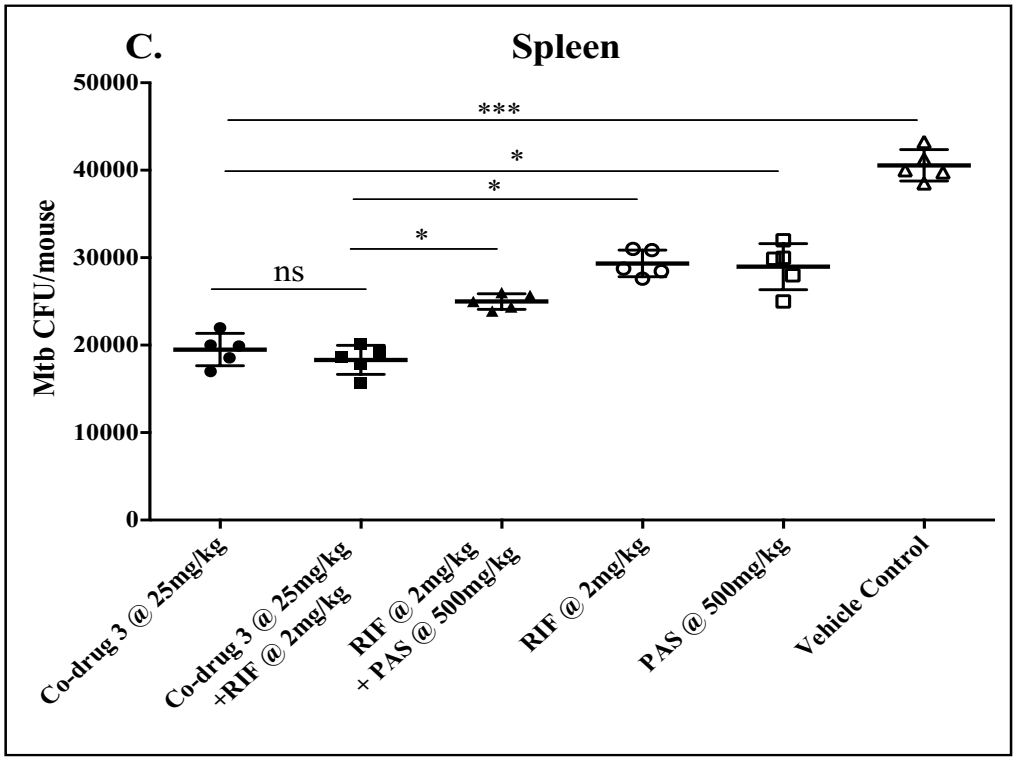

Figure 4.3: *In vivo* antimycobacterial activity of co-drug 3 alone and in combination with rifampicin. BALB/c female mice (n = 5) were challenged with H37Ra (0.5×10^6 CFU/mouse) intravenously. Mice were treated with co-drug **3** (25 mg/kg), co-drug **3** (25 mg/kg) + RIF (2 mg/kg), PAS (500 mg/kg) + RIF (2 mg/kg), RIF (2 mg/kg) or PAS (500 mg/kg) orally for two weeks. Control mice received vehicle diluent alone. Four days after the last treatment, mice were euthanized and lungs, liver and spleen were collected. Bacterial loads were determined in **(A)** lungs, **(B)** liver and **(C)** spleen by the CFU assay. All results are shown as mean ± standard deviation of CFU from five individual mice. Data are representative of two different repeated experiments. '*', '**' and '***', indicate significant differences at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively.

Clinically, PAS was used at a very high dose (8-12 gm/day), which caused gastrointestinal intolerance. The free acidic carboxyl group in PAS was responsible for GI irritation. Clinical use of PAS was discontinued in 1960's. The emergence of MDR- and XDR-TB and unavailability of effective drugs imposed the use of PAS and in 1994 it was reintroduced as GR-PAS in a granular, slow release and gastro-resistant form by Jacobus Pharmaceutical (USA), for the treatment of drug-resistant-TB (60). Therefore, PAS once again became a drug of choice for treatment of MDR- and XDR-TB. Although, GR-PAS possesses improved GI tolerance, it is still administered at high doses (4 g twice or thrice daily) to achieve the plasma concentrations of active drug PAS exceeding its MIC (≥1 ug/ml) (61).

Here, I have investigated a co-drug where PAS was linked to an anti-HIV nucleoside, AZT, via an ester bond between the carboxyl group of PAS and the hydroxyl group of AZT. This allows the co-drug to be absorbed un-hydrolyzed, eliminating the problems of catabolism and GI toxicity.

AZT is a well-known clinically effective anti-HIV drug. No toxicity of AZT was seen in mice, when it was administered at an oral dose of 20-40 mg/kg daily for a period of >90 days (62). In humans, AZT was also found to be safe and well tolerated when given 300 mg orally every 12 hours or 200 mg orally every 8 hours (63). In this study, newly designed PAS-AZT co-drug **3** exhibited significant inhibition of disseminated *Mtb* in lungs, liver and spleen at an oral dose of 25 mg/kg upon two weeks (once daily) treatment. In contrast, the antituberculosis drug, PAS provided a weak antimycobacterial effect when administered at a 500 mg/kg dose employing the same schedule and route. Strikingly, co-drug **3** led to a remarkable inhibition of mycobacterial growth at a dose that was 20 times lower than that of the parent drug PAS. These results suggest that AZT can serve as a good carrier for PAS to enhance its therapeutic potential

by delivering an effective amount of PAS into mycobacterial cell precluding its undesired metabolic conversion into acetyl-PAS, toxic byproducts, and associated GI toxicity.

The XTT assay was performed to determine *in vitro* toxicity of **3** using Vero cells. No cytotoxic effects were observed up to a concentration of 200 μg/ml (CC_{50} > 200 μg/ml) (Appendix 25). Also, none of the mice became sick, lost weight or died in any treatment group with co-drug **3**.

These studies suggest that designed co-drug **3** has strong potential in reducing doses, toxicity and improving compliance of current anti-TB regimens. The investigated co-drug could be a beneficial addition in the treatment of MDR-, XDR- and TDR-TB where it could be used in combination with current anti-TB drugs to provide more effective and less toxic therapeutic regimens. The discovered co-drug could also be developed as a useful candidate for the treatment of TB-HIV co-infection.

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Chapter 5

General discussion

In recent years, the incidence of TB caused by mycobacteria has been increasing and posing a major global health challenge. TB is the most common infection among people living with HIV/AIDS. A single individual with active TB can infect 10-14 more individuals per year. The emergence and rapid spread of extensively-drug-resistance TB has put this disease on top priority. Therefore, there is an unmet medical need to discover new classes of antimycobacterial agents. Successful treatment for TB requires long-term therapy with multiple drugs, which leads to problems of toxicity, compliance and drug resistance. In this thesis, I have designed and synthesized new classes of antimycobacterial agents and novel drug conjugates followed by evaluating their antituberculosis activity alone and in combination with known drugs in *in vitro* and *in vivo* models.

5.1. Investigation of C-5 alkynyl (alkynyloxy or hydroxymethyl) and/or N-3 propynyl substituted pyrimidine nucleoside analogs as a new class of antimicrobial agents

Pyrimidine nucleosides modified at C-5 and N-3 and/or both C-5 and N-3 positions of the base possessing various deoxyribose, ribose, arabinose and dideoxyribose carbohydrate moieties were designed, synthesized and examined against various mycobacteria (*Mtb, M. bovis* and *M. avium*) alone and in combination with existing drugs in *in vitro* assays. Among newly synthesized compounds, 5-ethynyl substituents did not contribute to antimicrobial activity whereas 5-propynyloxy and 5-hydroxymethyl substituents led to modest to significant inhibition of mycobacterial replication. These results suggest that a longe r carbon chain at the C-5 position is required for the activity against mycobacteria. In 5-ethynyl, 5-(2-propynyloxy) and 5-hydroxymethyl series of pyrimidine nucleosides analogs, the activity was also dependent on the nature of substituents present in carbohydrate portion as compounds containing a ribose, 2'-arabinose, 2'-fluororibose and 3'-azido, 2', 3'-dideoxy sugars had stronger activity than compounds with a 2'-methoxyribose and 3'-fluoro-2', 3'-dideoxy sugar

moiety. Incorporation of a propynyl moiety at the N-3 position of the 5-alkynylyl pyrimidine nucleoside analogs did not improve antimycobacterial activity. These results suggest that inclusion of an N-3 propynyl group is not tolerated at this position for antimycobacterial effect in this series of compounds. Combination drug therapy is used for several chronic diseases. An effective treatment for mycobacterial infections usually requires a combination of two or more antimycobacterial agents. To understand the potential and possible interactions of new class of pyrimidine analogs with current antitubercular drugs their combinations were investigated. Although these compounds were moderately effective, they displayed synergistic effects against *Mtb* when combined with the antitubercular drugs isoniazid and/or rifampicin. The synergistic interaction between the investigated nucleoside analogs and antituberculosis drugs isoniazid and rifampicin at lower inhibitory concentrations could be attributed to their action at two and/or three different mycobacterial targets. *Mtb* is an intracellular bacterium that replicates and survives within the macrophages for a long time. The effect of most active compounds was determined against intracellular *Mtb* (H37Ra) in a human monocytic cell line (THP-1). The active analogs inhibited replication of intramacrophagic *Mtb*. These results suggest that pyrimidine nucleosides investigated are able to cross cellular membranes, and also have potential to inhibit mycobacteria harbored in the macrophages. I also noted a significant inhibition of mycobacterial load in mice infected with *Mtb* by the investigated class of compounds despite their weak *in vitro* activity. Notably, promising compounds were effective orally, which is an important feature from drug development perspective. These *in vivo* effects could be attributed due to their possible biotransformation into active metabolites, efficient phosphorylation, stability, long plasma half-life, etc. Overall, these studies demonstrate that modestly and weakly active antimicrobial compounds can be highly effective if they are combined with other agents acting by different mechanisms. The original and important information obtained will

promote more informed, rational design and discovery of novel agents for mycobacterial infection.

5.2. Design, synthesis and investigation of novel conjugates as a new class of anti-tuberculosis agents

Besides the development of new class of agents that can act by different mechanisms, current research must include improvement of the drugs already known to be active. Chemically coupling two or more drugs acting at different targets could be an attractive approach to address the problems of toxicity, compliance and drug resistance with multiple drug therapy. In this study, I have designed novel antimycobacterial conjugates with pyrazinamide and the pyrimidine nucleoside compound FUDR. Mono-pyrazinoated and di-pyrazinoated conjugates of FUDR were synthesized and evaluated *in vitro* against *Mtb*, *M. bovis* and *M. avium* and *in vivo* in an infection model of *Mtb* (H37Ra) alone and in combination with other first-line drugs isoniazid and rifampicin.

The investigated antimycobacterial nucleoside conjugated pyrazinoates showed synergistic and/or additive inhibition of *Mtb* in *in vitro* combinations. The mono-pyrazinoated conjugate of FUDR displayed additive interactions with the first-line antituberculosis drugs isoniazid and rifampicin whereas di-pyrazinoated conjugate exhibited synergy with both drugs.

In the mouse model of *Mtb* infection, mono-pyrazinoated compound led to modest inhibition of mycobacteria in lungs and spleen but di-pyrazinoated conjugate exhibited higher reduction of *Mtb* in lungs, liver and spleen. Similarly, di-pyrazinoate compound demonstrated higher inhibition of *Mtb* in all organs in combination with INH and rifampicin, compared to mono-pyrazinoated conjugate. Notably, the di-pyrazinoated conjugate provided

synergistic and/or additive *in vivo* effect with isoniazid and rifampicin compared to 2 and 3 drug combinations of FUDR, pyrazinamide, isoniazid and rifampicin. Further it was noteworthy that the investigated di-pyrazinoated conjugate provided remarkable inhibition of mycobacterial growth upon oral administration at 25 mg/kg dose compared to a dose of pyrazinamide at 100 mg/kg alone or in various combinations.

This promising activity of the di-pyrazinoated conjugate in *Mtb*-infected mice could be ascribed to combined effects of the released parent moieties FUDR and pyrazinoic acid in adequate concentrations inside the mycobacteria by *Mtb* esterases. Further, linking pyrazinoic acid to the FUDR could have led to overcome the limitations of the ionic nature of pyrazinoic acid and facilitated its entry through mycobacterial cell wall. The conjugate was designed in such a way that upon hydrolysis in the bacterial cell released free pyrazinoic acid and FUDR would act at two different mycobacterial targets to exert synergistic effects. However, it is likely that the conjugate itself may also possess direct antimycobacterial effects as alkynyl pyrazinoates have been reported to be inhibitory to *Mtb* due to their intrinsic activity.

It is expected that the designed conjugates comprising nucleoside and pyrazinoic acid may bypass pyrazinamidase activity, which is the target for resistance development against pyrazinamide. This is one of the key features of the investigated conjugates and may have important implications for the treatment of drug-resistant *Mtb* strains. The results obtained so far suggest that the investigated novel conjugates can provide more effective regimens with lowered doses, reduced toxicities and improved compliances, opening new possibilities for the treatment of drug- resistant TB.

5.3. Design, synthesis and investigation of a novel co-drug incorporating anti-TB drug p-aminosalicylic acid (PAS) and anti-HIV nucleoside drug AZT for treating TB and/or TB-HIV co-infection

Treatment of mycobacterial infections among HIV-infected individuals has been quite difficult. The major challenges associated with co-treatment of TB and HIV includes drug inefficacy, drug-drug interactions, drug intolerance and toxicities. In this study, I have investigated a novel co-drug approach to develop treatments for TB and/or TB-HIV co-infection and improve the therapeutic efficacy of known anti-TB drugs. I have designed, synthesized and evaluated the novel co-drug 5'-para-aminosalicylate-AZT (PAS-AZT), where PAS was linked via an acid moiety to the 5'-position of AZT. This conjugation was carried out in order to enhance the overall therapeutic potential of PAS by preventing its undesired metabolism, reduce toxicity and required doses, increase plasma half-life, and improve intra-macrophagic delivery.

In *in vitro* assays, the co-drug exhibited potent inhibition of various mycobacteria [*Mtb* (H37Ra), *M. bovis* (BCG) and *M. avium*]. Although, the co-drug provided less inhibition of *Mtb* compared to the parent drug PAS, it demonstrated very good activity at lower concentrations. A possible reason for this reduced effect could be that the free PAS is not available in high concentrations due to its slow release from the PAS-AZT co-drug in presence of *Mtb* esterases. In addition, it is possible that the obtained *in vitro* effects were as a result of intrinsic antimycobacterial activity of the co-drug. Overall, however, these studies indicate that the co-drug was able to cross highly lipophilic cell wall and enter into the mycobacterial cell.

Interestingly, co-drug showed significant reduction in *Mtb l*oads in lungs, liver and spleen in mice infected with *Mtb* where PAS was not very effective. Intriguingly, investigated co-drug provided remarkable inhibition of mycobacterial growth upon oral

administration and at a dose that was 20 times lower than that of the parent anti-TB drug PAS. These observations of the significant *in vivo* efficacy of the PAS-AZT co-drug at a low dose indicate that AZT is serving as an excellent carrier for PAS to improve its gastric environment stability, plasma half-life, and reduced toxicity. It is also reasonable to speculate that AZT might be contributing to the enhanced intra-macrophagic delivery and penetration of PAS through the mycobacterial cell wall due to its known lipophilic nature.

In order to determine the interactions of the designed co-drug with other first-line anti-TB agents, I have examined its effects in *in vitro* and *in vivo* combination studies with isoniazid and rifampicin. Interestingly, synergistic interactions were observed with both isoniazid and rifampicin in *in vitro* assays. These observations were reproduced in an *Mtb* infected mouse model. The co-drug reduced mycobacterial loads significantly in all organs in combination with rifampicin or isoniazid compared to rifampicin alone, isoniazid alone or their combinations. The oral efficacy of the investigated PAS-AZT co-drug is advantageous from the drug development perspective.

These studies will provide new insights in the treatment of TB and/or TB-HIV co-infection. The investigated co-drug may lead to optimum drug concentrations due to sustained release of both anti-HIV and anti-TB agents and increased drug plasma half-life, and thereby reduced drug toxicities, improved drug delivery, reduced undesired drug metabolism and provide improved patient compliance.

This research importantly reveals that existing anti-TB and anti-HIV drugs could be readily modified to develop improved regimens for TB including MDR and XDR-TB and TB-HIV co-infection treatment.

5.4. Conclusion

This work has elucidated novel approaches and therapeutic regimens for the treatment of TB and TB-HIV co-infection. These studies have led to the discovery of new classes of pyrimidine nucleoside analogs, conjugates and co-drug as novel antimycobacterial candidates with significant activity alone and in combination with existing first-line anti-tuberculosis drugs *in vitro* and in a mouse model of *Mtb.* Therefore, they could be used alone and in combination to augment current therapy. I believe that structurally different new classes of compounds discovered in these studies will act at different targets and/or by multiple mechanisms.

Overall, these research findings will advance our knowledge, provide new and valuable information and contribute significantly in the treatment of active, latent and MDR/XDR/TDR-TB, and TB-HIV co-infection.

5.5. Future directions

This research has led to the identification of novel antimycobacterial agents. Following studies with the newly revealed compounds, conjugates and/or regimens will be warranted:

i). *In vitro* and *in vivo* activity against virulent and drug-resistant mycobacterial strains: The antimycobacterial activity of the active compounds, conjugates and co-drug against virulent (H37Rv), laboratory and clinical isolates of mono- and multi-drug resistant of *Mtb* should be investigated in bacterial cell culture and in the infection models. ii). Drug resistance studies: Development of resistance against the discovered compounds should be explored upon their continuous and long-term exposure to mycobacteria. iii). Precise mechanism of action: Inhibition of the mycobacterial DNA and RNA synthesis by the newly

identified nucleoside compounds should be studied to understand their mode of action as substrates and/or inhibitors. To determine whether the test compounds inhibit *Mtb* RNA and DNA synthesis, they should be cultured with *Mtb* along with radiolabeled thymidine triphosphate or uridine triphosphate. To delineate whether they act as substrates of mycobacterial DNA or RNA polymerase, get incorporated into the DNA or RNA chain, and lead to inhibition of bacterial growth, purified *Mtb* DNA polymerase and *Mtb* RNA polymerase should be used in nucleic acid chain extension assays. By using radiolabelled tri-phosphate derivatives of the test compounds, it can be determined whether they are being incorporated in the growing chain, or whether they are only inhibiting chain growth by inhibiting the enzymes. iv). Stability and partition co-efficient evaluation: The stability of the discovered conjugates in buffers with different pHs should be delineated. Also their stability under physiological conditions should be studied to determine their conversion and degradation to parent drugs and metabolites. Partition coefficient studies in an n-octanol/phosphate buffer system using a software (ACD/LogP) for the test conjugates should be evaluated to know their liphophilic and hydrophilic characterstics, and diffusion across cell membranes. v). *In vivo* pharmacodynamic and pharmacokinetic properties: These studies are important to delineate drug absorption, distribution, metabolism, elimination and plasma concentration. Plasma samples collected at different time points from orally treated mice should be analyzed by HPLC to determine the C_{max} (maximum concentration), T_{max} (time of C_{max}), AUC (area under the curve), drug half-life, volume of distribution, and drug clearance. vi). Detailed toxicity studies: Although, no toxicities of the discovered molecules and conjugates were observed during the treatment period, their long-term toxicity studies at effective and 5-10x doses should be carried out followed by pathological examination of various organs.

5.6. Limitation

The limitation of these studies is the lack of understanding of pharmacodynamic and pharmacokinetic properties, mechanism of action and mechanism of synergy, development of resistance and detailed toxicity. My work provides a strong basis to investigate these issues in future to move towards clinical testing.

Using anti-TB drugs pyrazinamide and p-aminosalicylic acid, and the anti-HIV drug AZT as model compounds, I have shown that conjugation of pyrimidine nucleosides with existing antimycobacterial drugs could provide unique and potent molecules, and their combinations with other anti-TB agents can further lead to enhanced effects. Therefore, it will be interesting to explore similar conjugates and co-drugs of other anti-HIV nucleosides or anti-TB nucleosides and anti-TB drugs in order to exploit the full potential of the discovered approaches and to investigate additional therapeutic regimens for the treatment of TB and TB-HIV co-infection.

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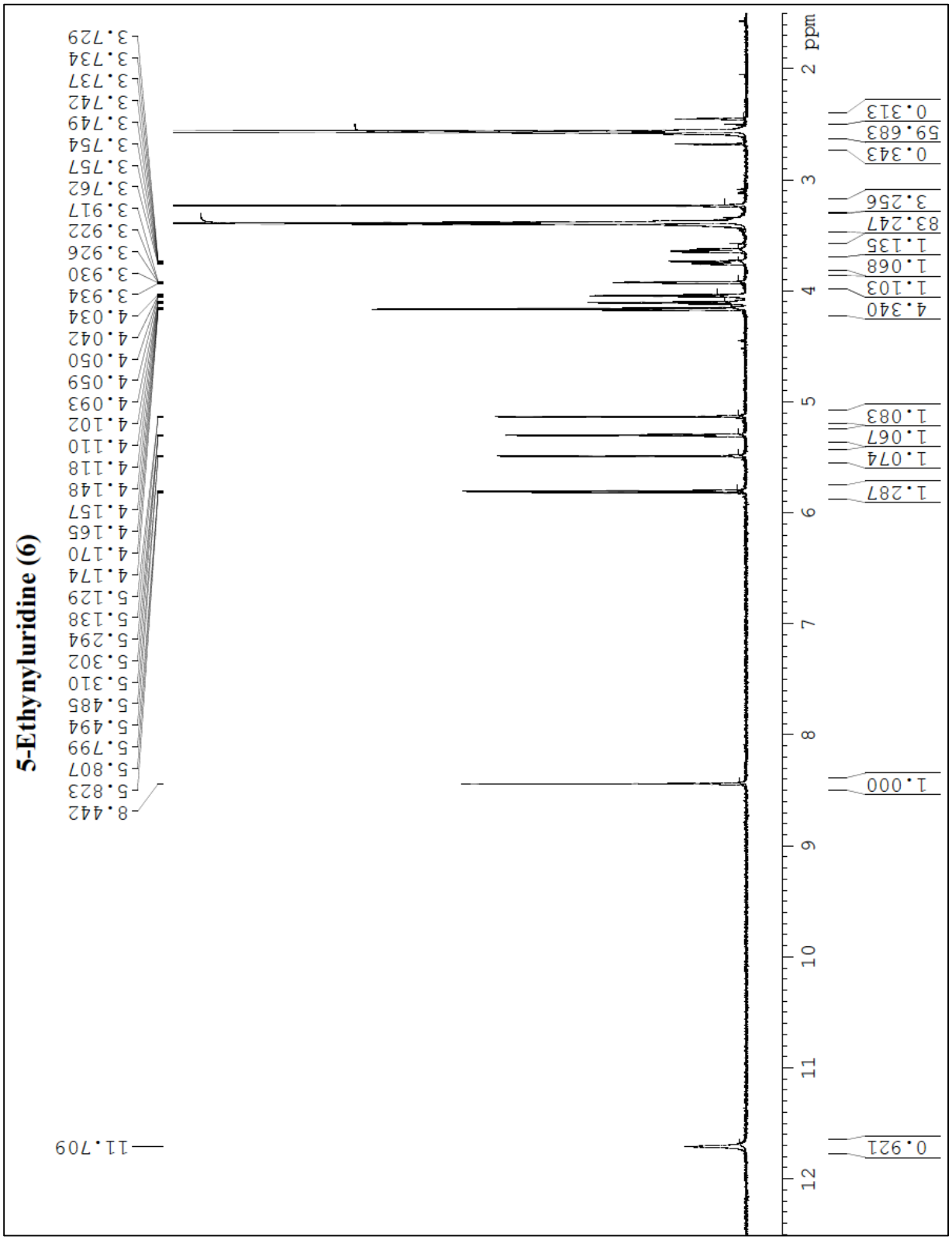
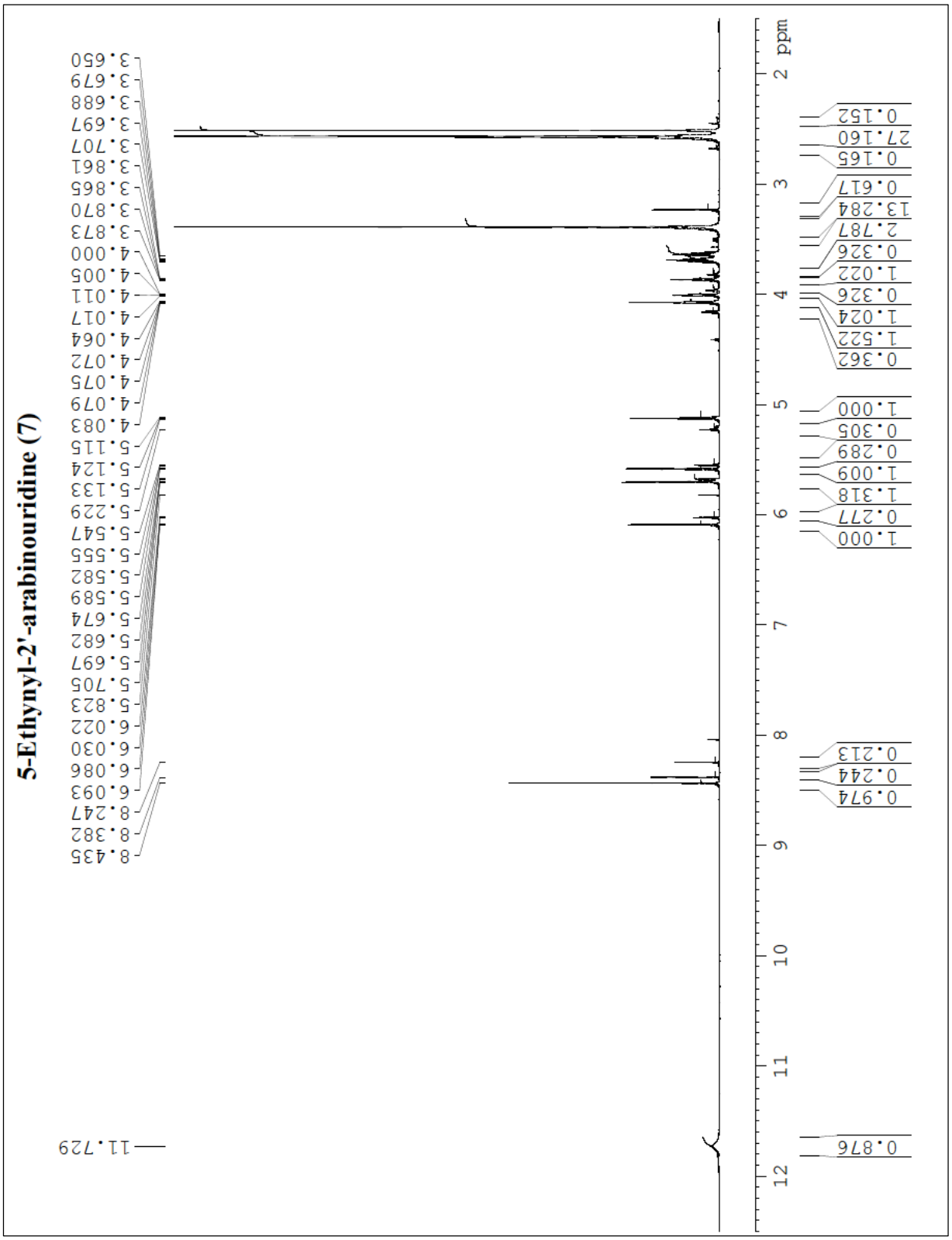
Appendices

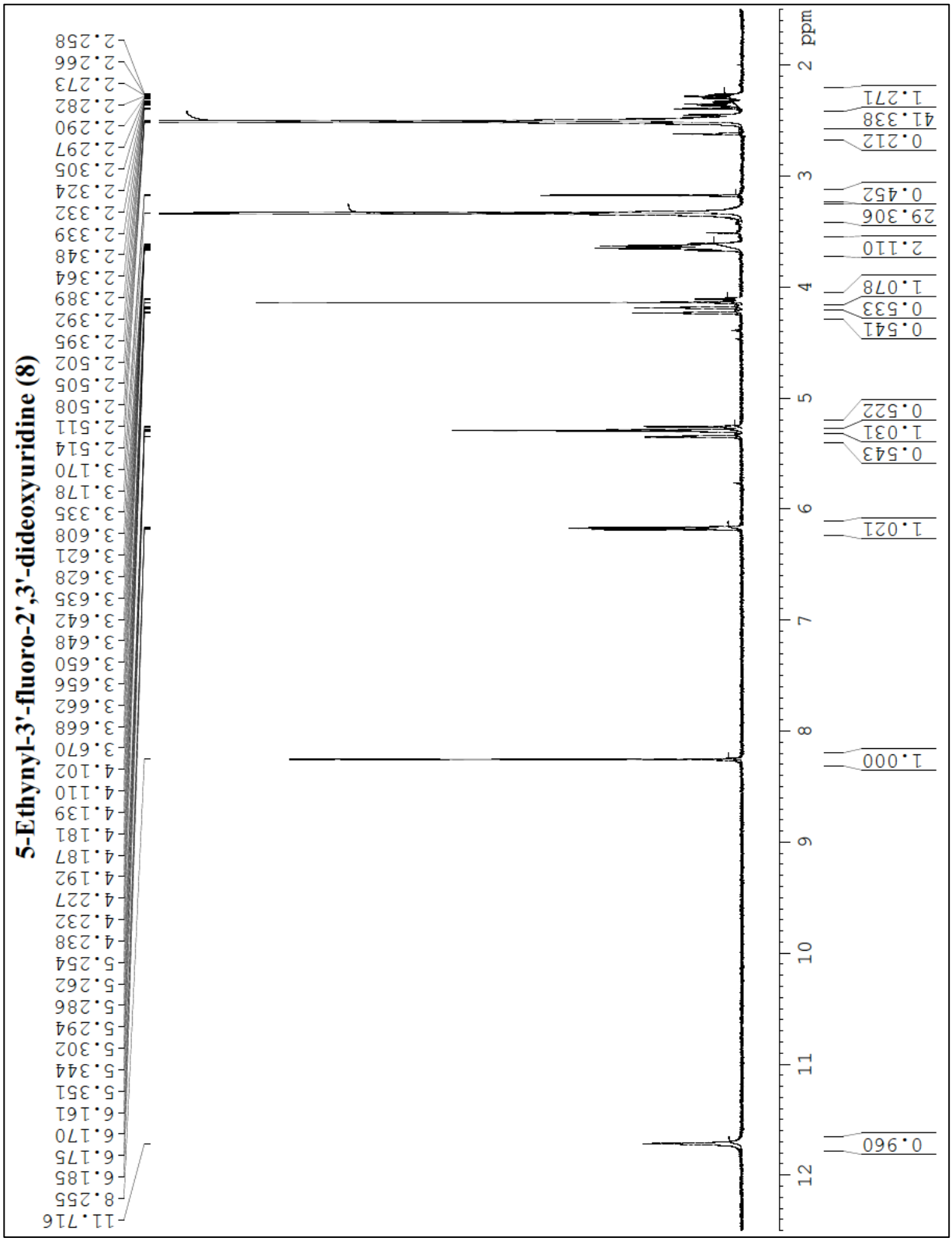
Appendix 1: NMR spectra of 5-Ethynyluridine (**6**)



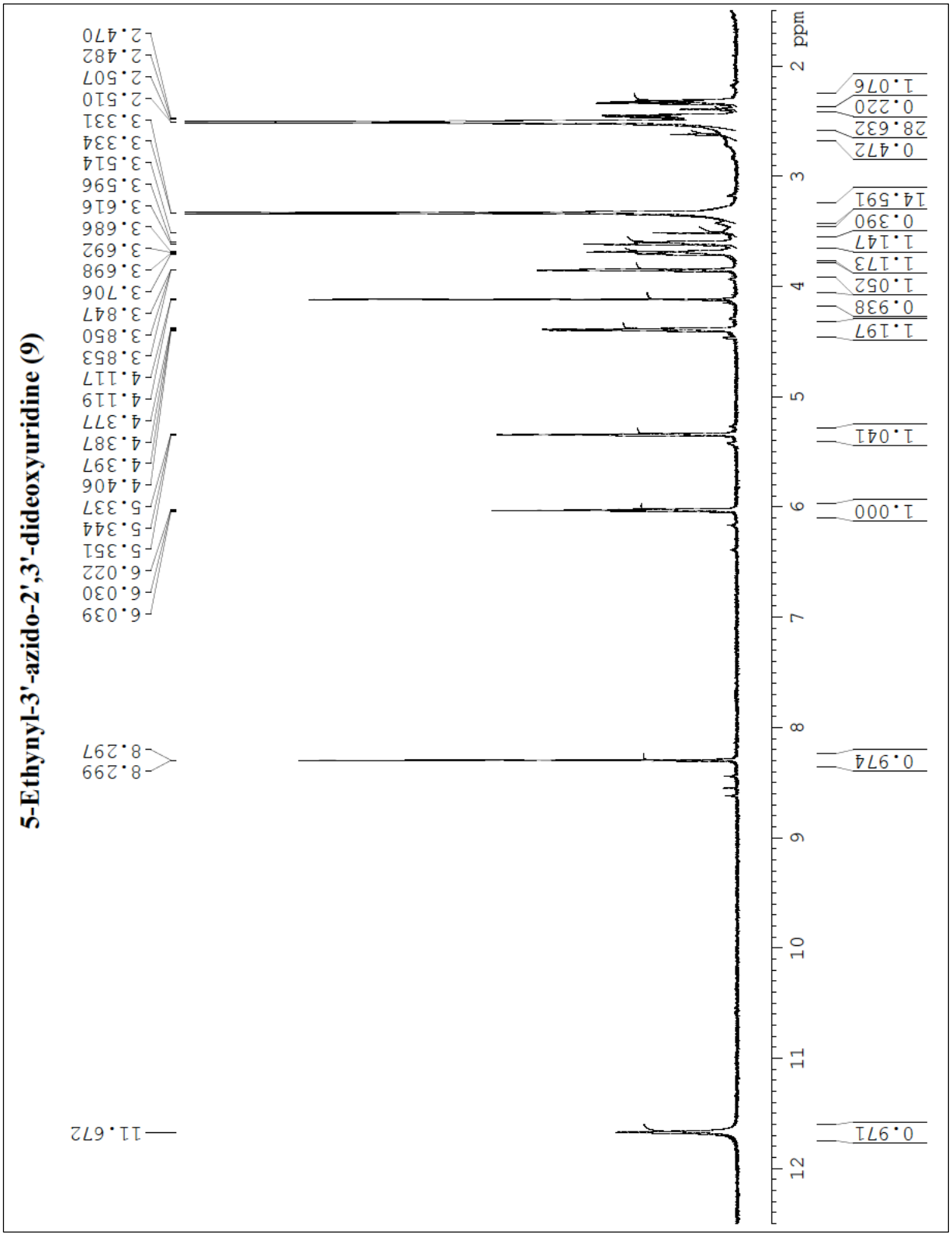
Appendix 2: NMR spectra of 5-Ethynyl-2’-arabinouridine (**7**)



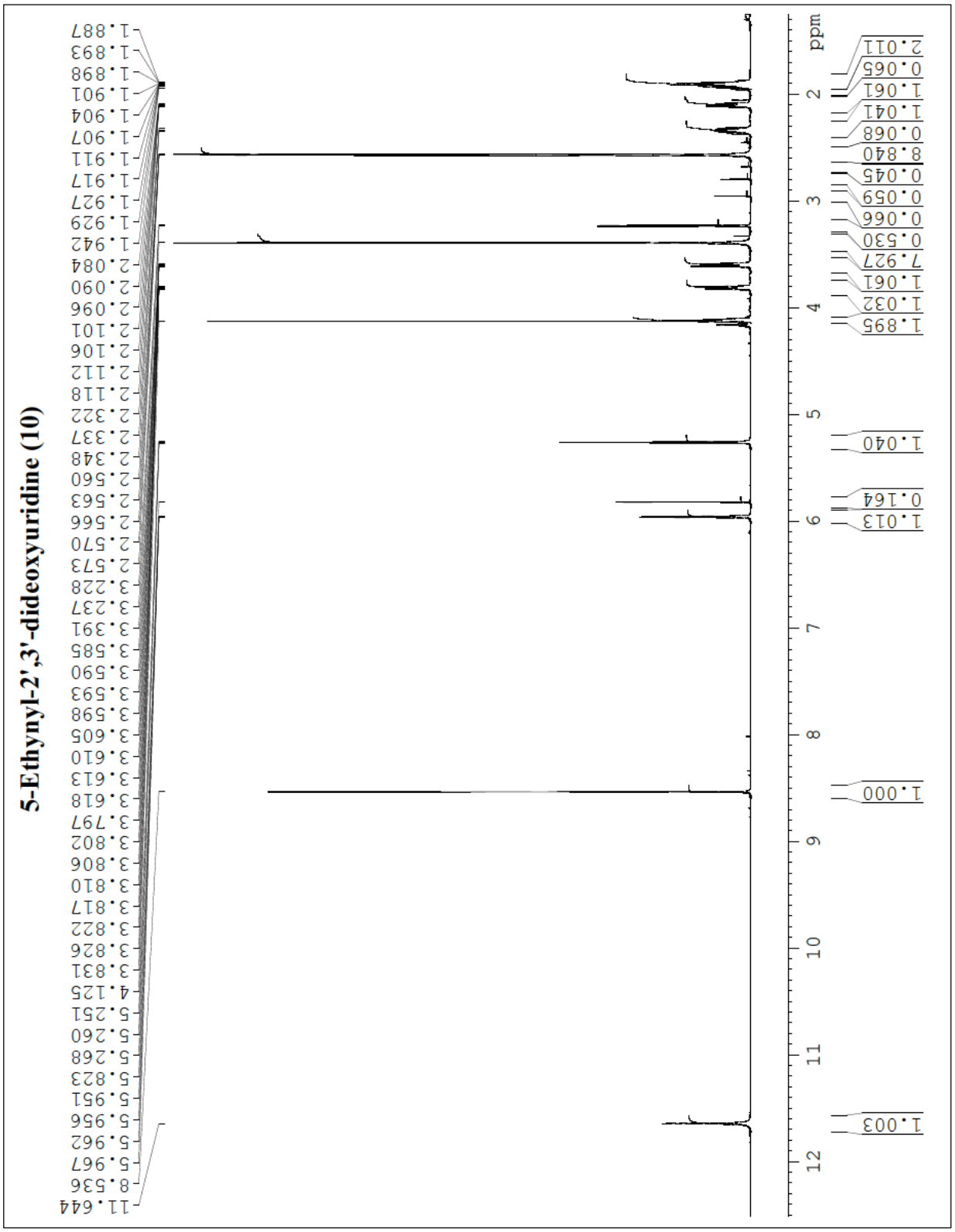
Appendix 3: NMR spectra of 5-Ethynyl-3’-fluoro-2’,3’-dideoxyuridine (**8**)



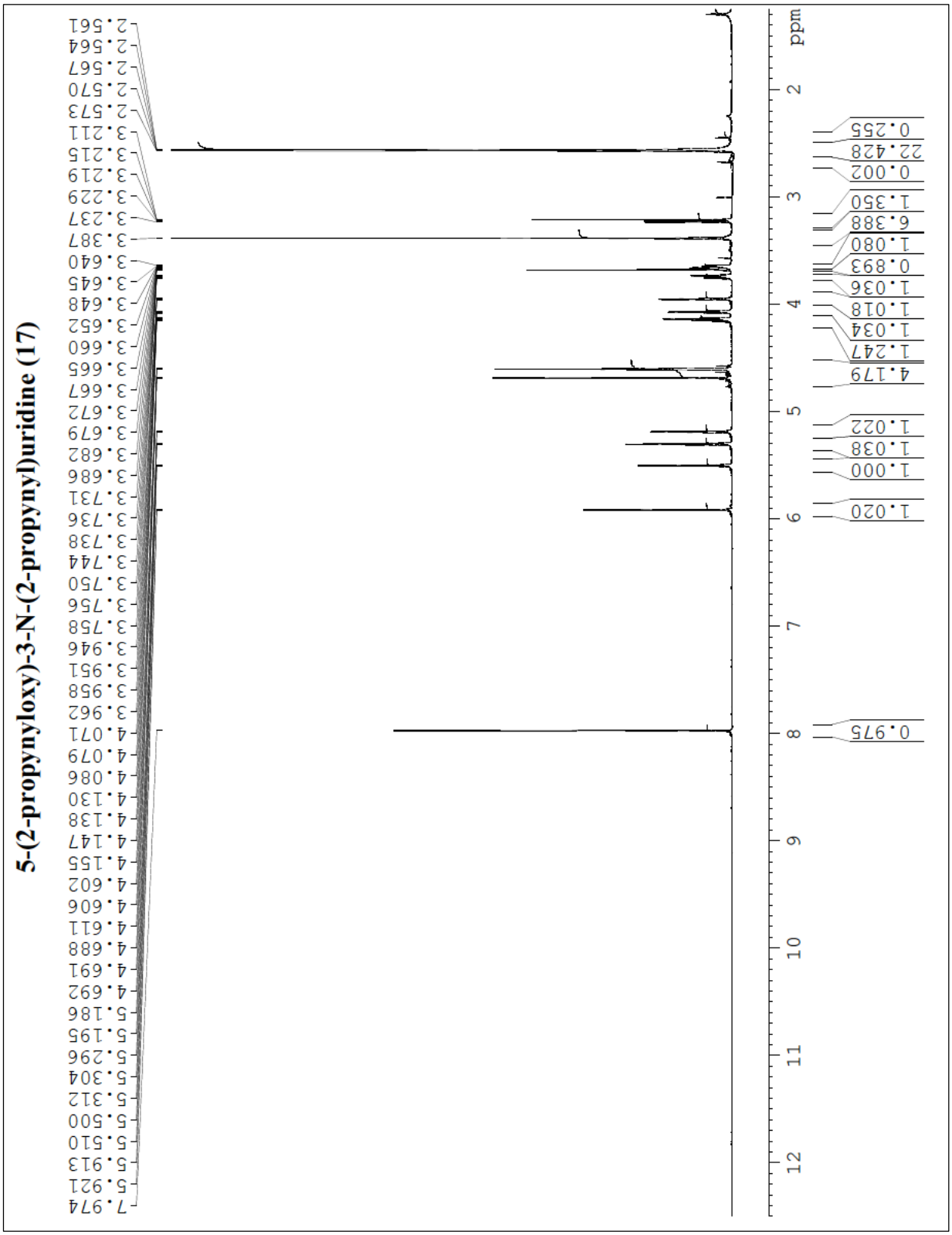
Appendix 4: NMR spectra of 5-Ethynyl-3'-azido-2',3'-dideoxyuridine (**9**)



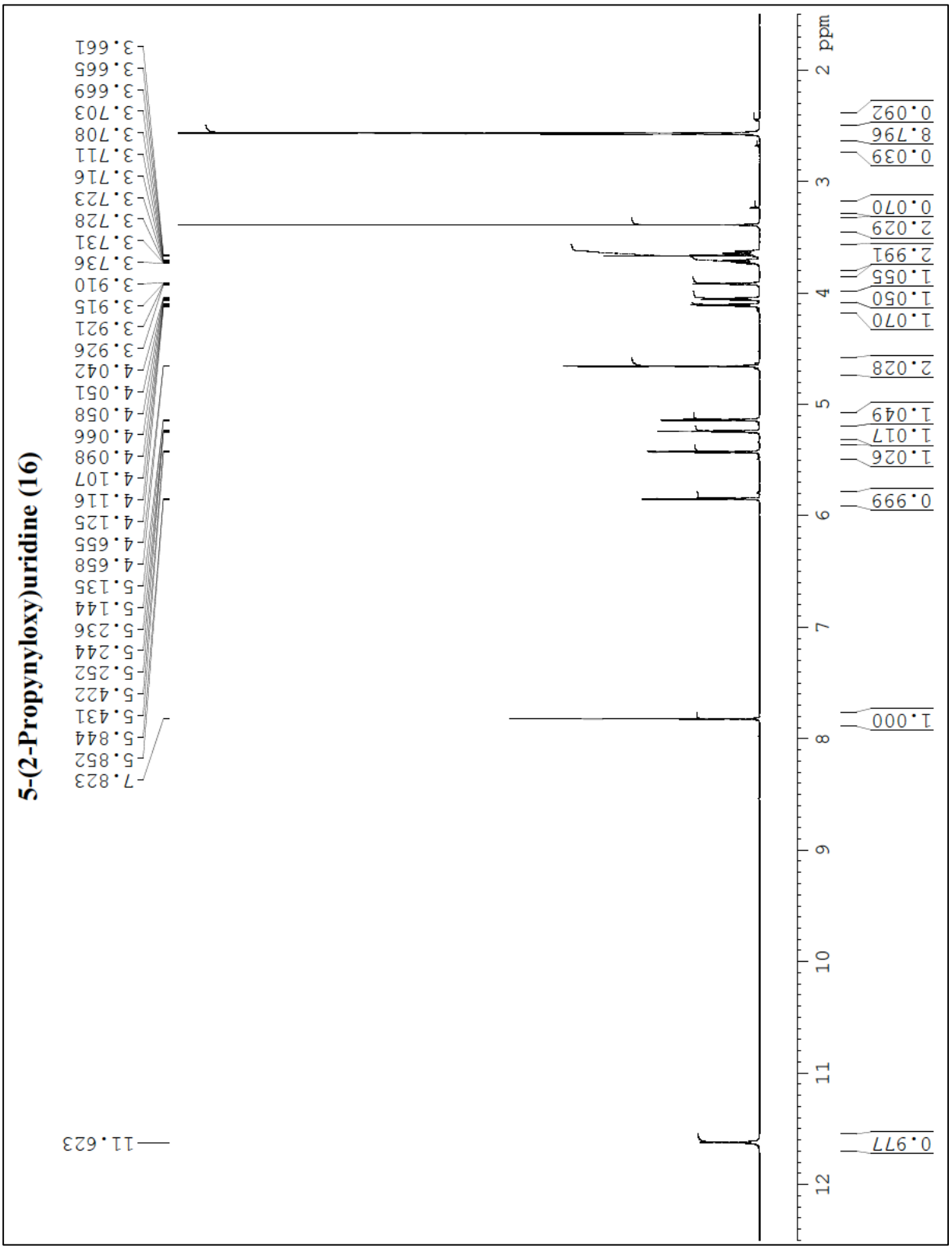
Appendix 5: NMR spectra of 5-Ethynyl-2',3'-dideoxyuridine (**10**)



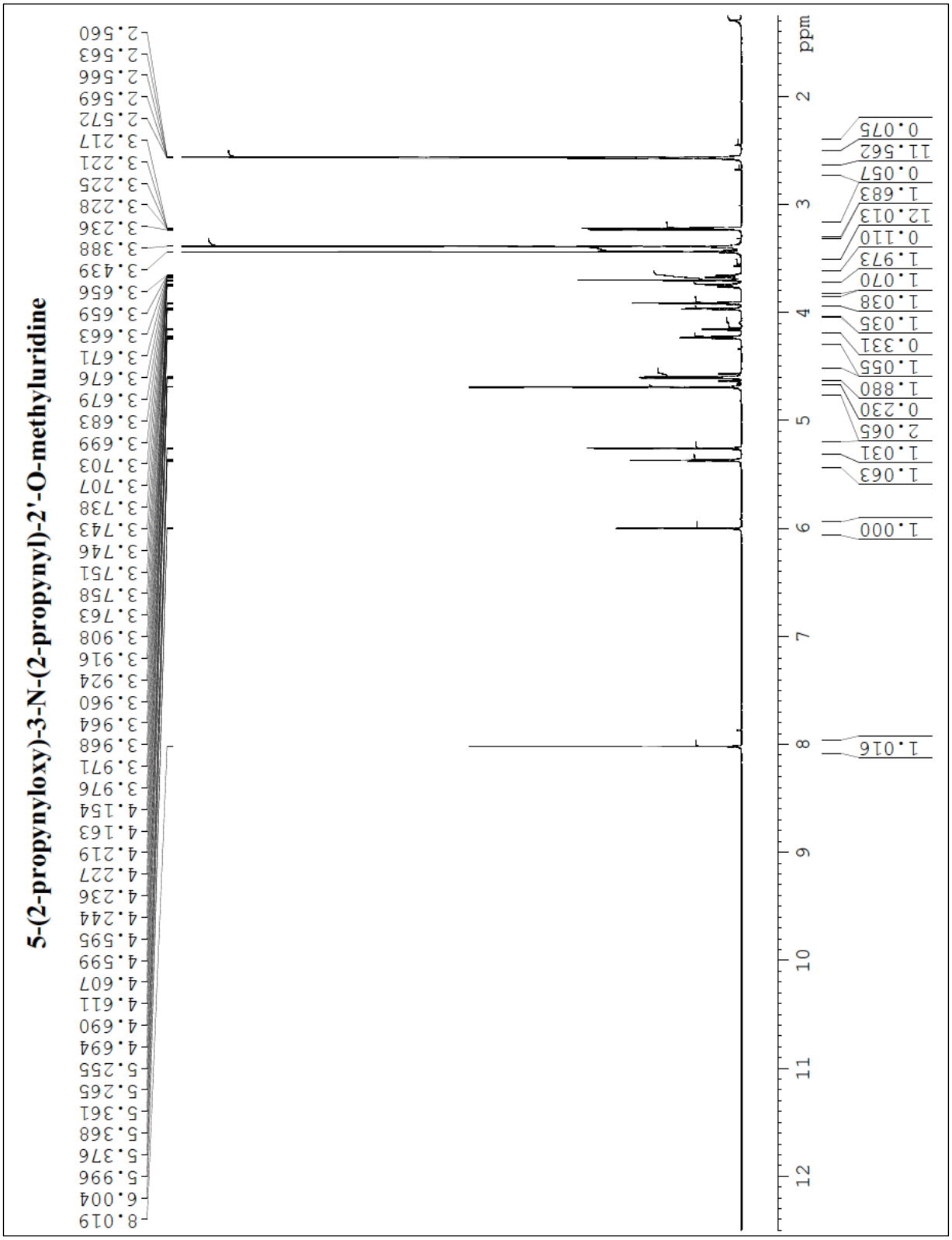
Appendix 6: NMR spectra of 5-(2-propynyloxy)-3-*N*-(2-propynyl)uridine (**17**)



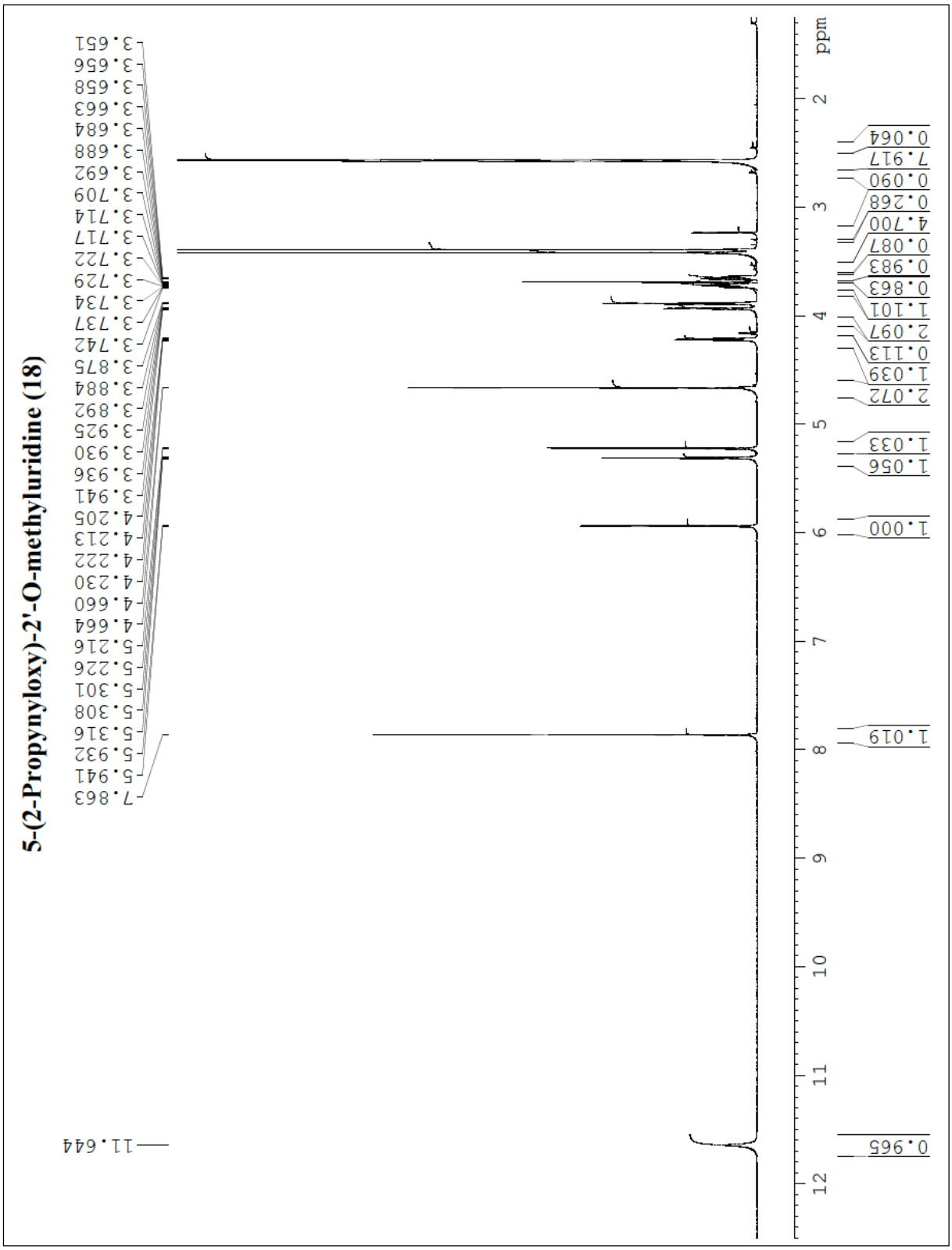
Appendix 7: NMR spectra of 5-(2-Propynyloxy)uridine (**16**)



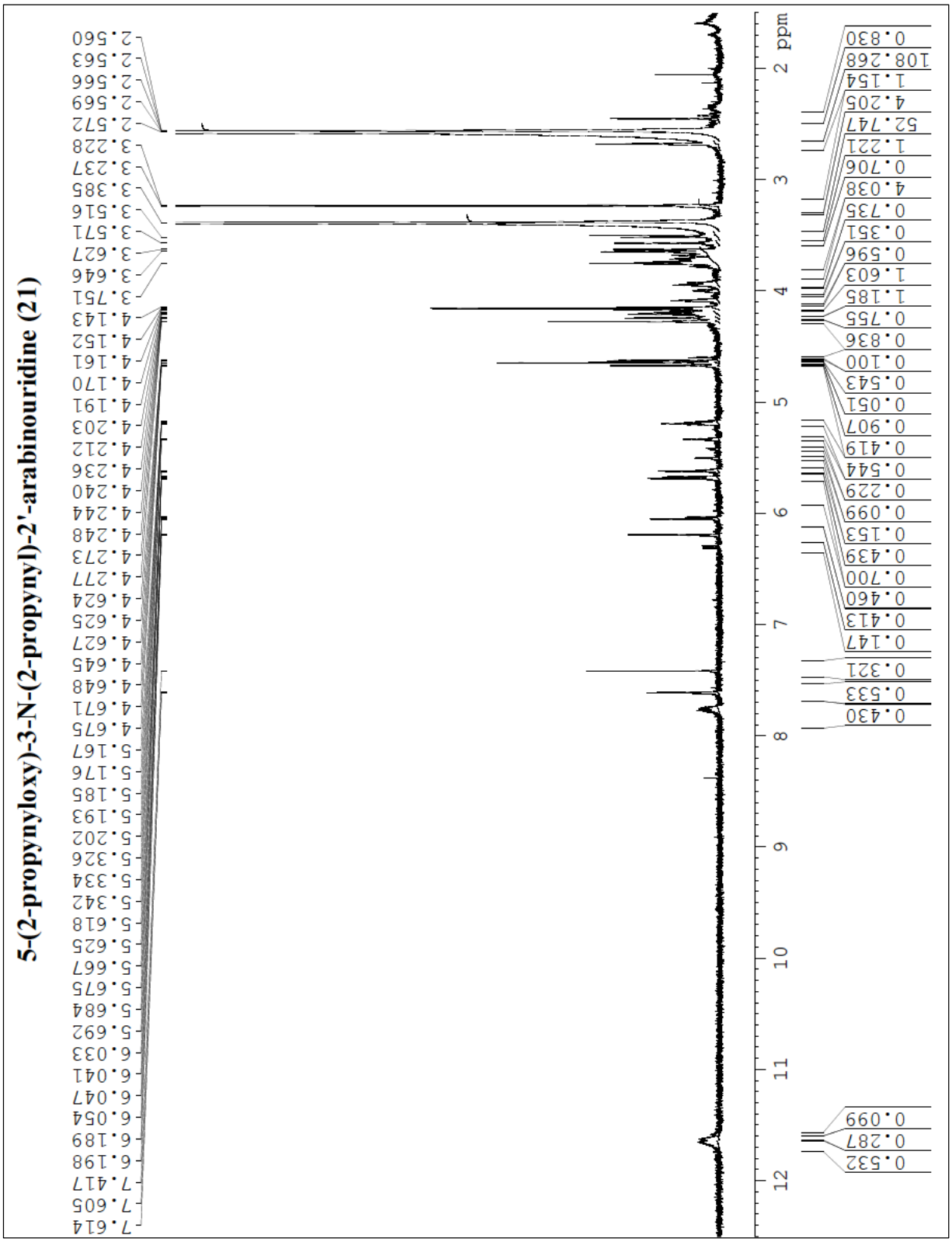
Appendix 8: NMR spectra of 5-(2-Propynyloxy)-3-*N*-(2-propynyl)-2'-O-methyluridine (**19**)



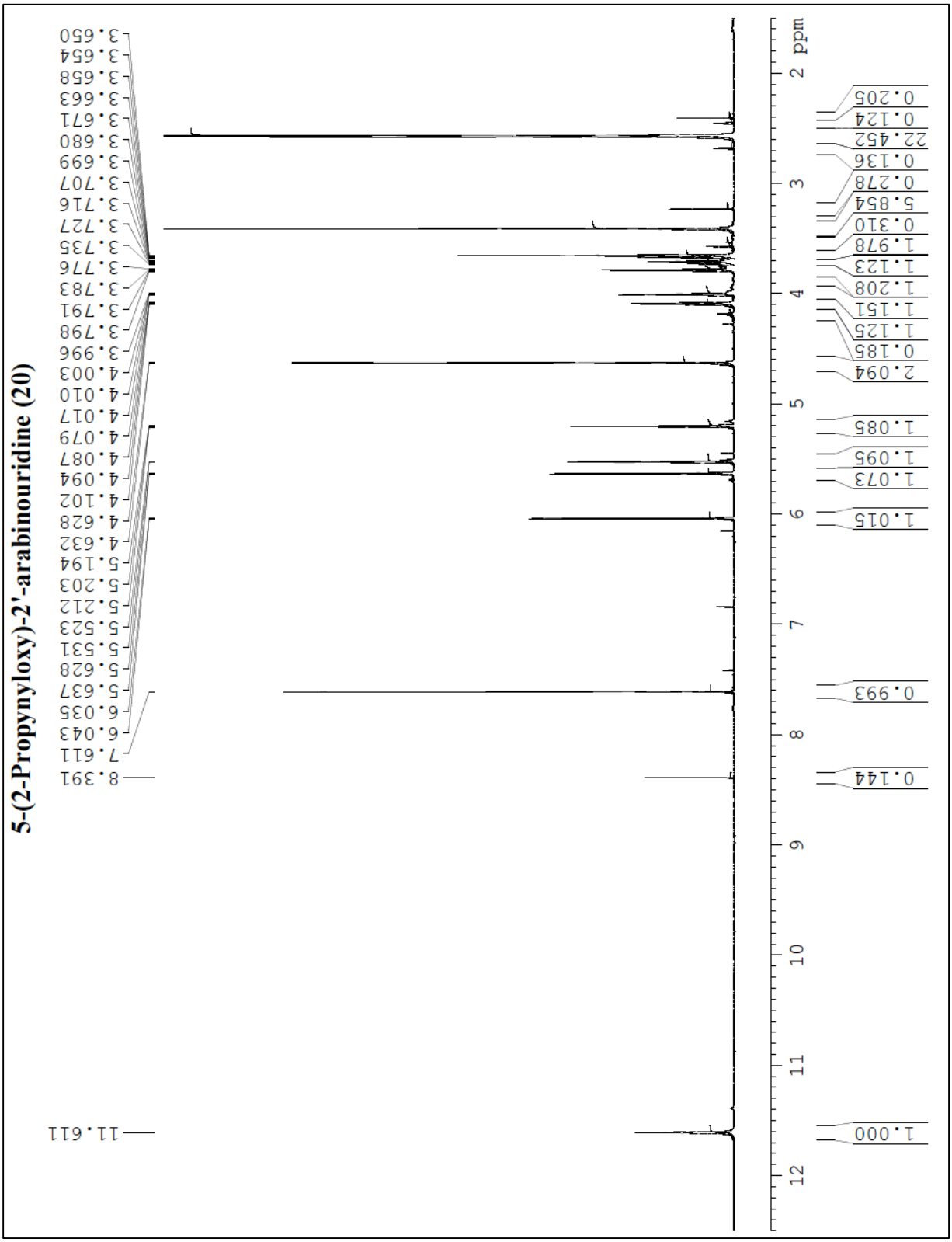
Appendix 9: NMR spectra of 5-(2-Propynyloxy)-2'-O-methyluridine (**18**)



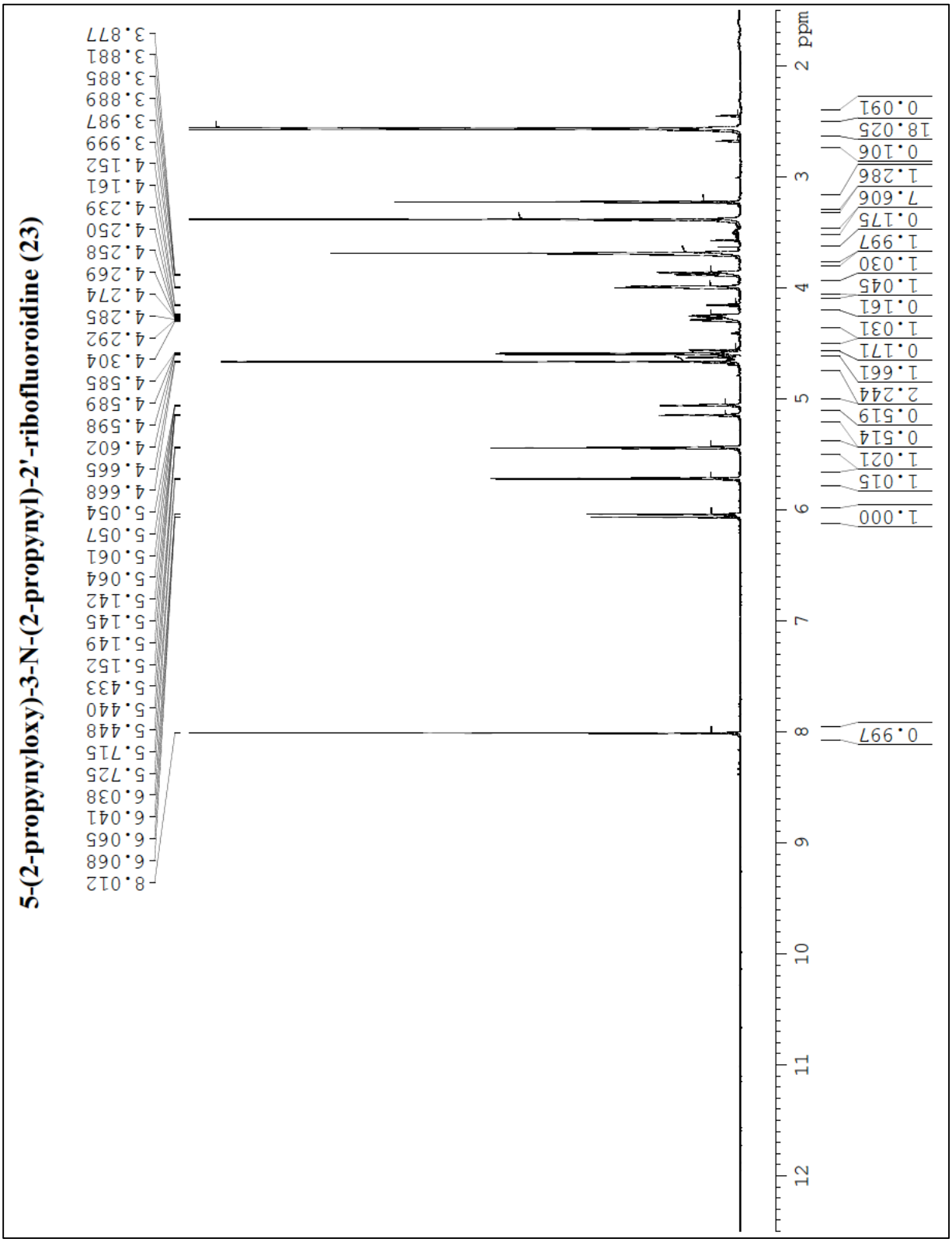
Appendix 10: NMR spectra of 5-(2-Propynyloxy)-3-*N*-(2-propynyl)-2’-arabinouridine (**21**)



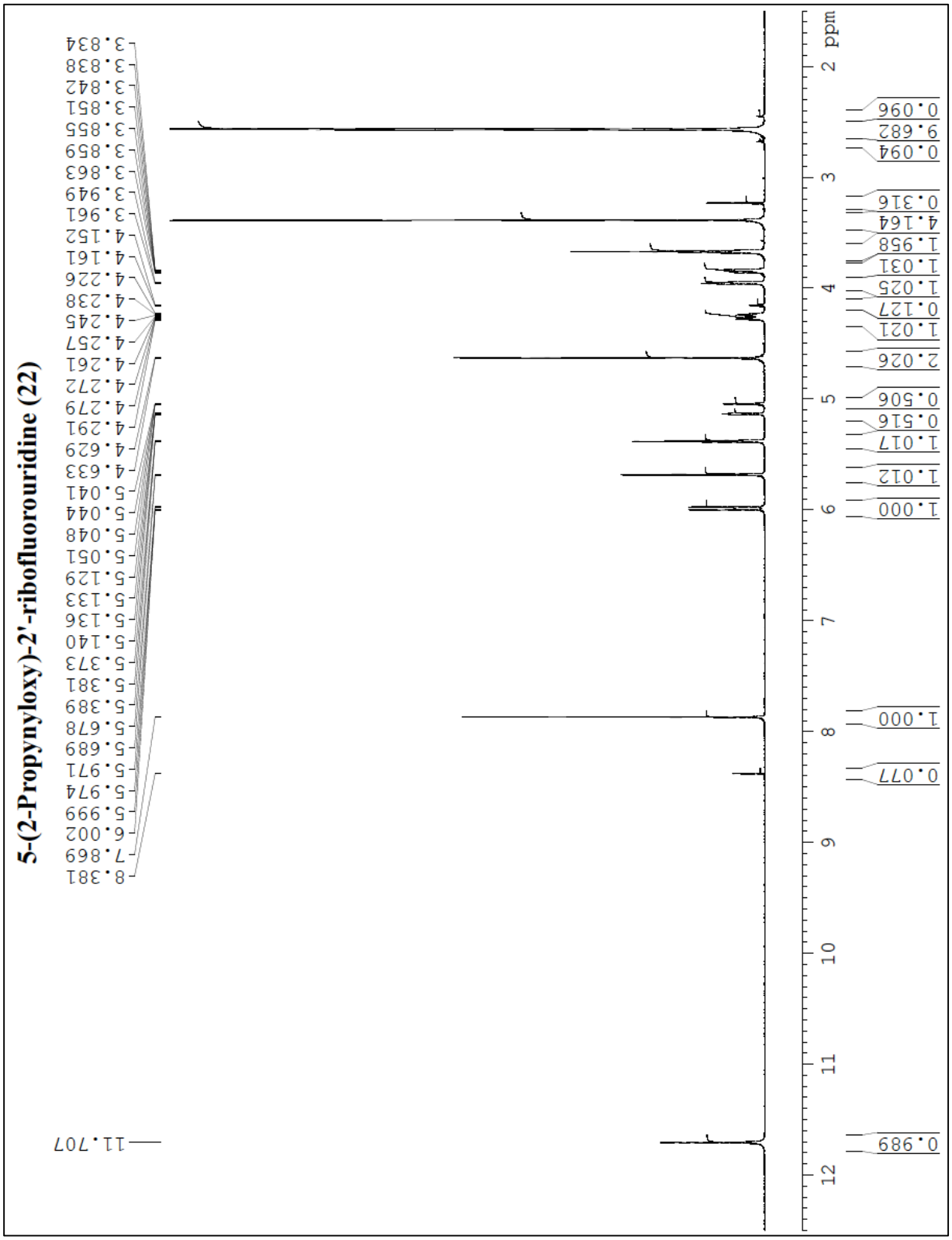
Appendix 11: NMR spectra of 5-(2-Propynyloxy)-2'-arabinouridine (**20**)



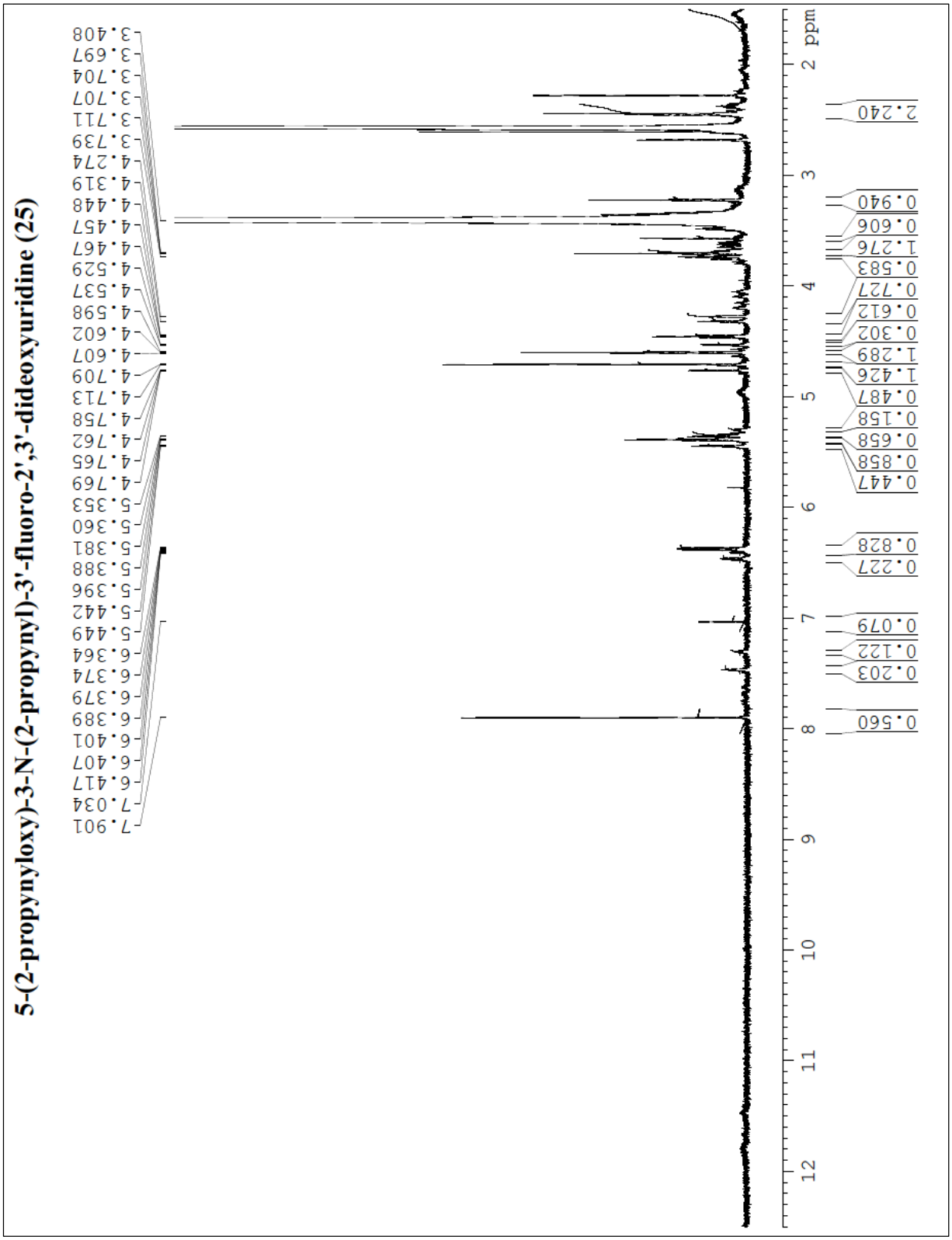
Appendix 12: NMR spectra of 5-(2-Propynyloxy)-3-*N*-(2-propynyl)-2'-ribofluorouridine (**23**)



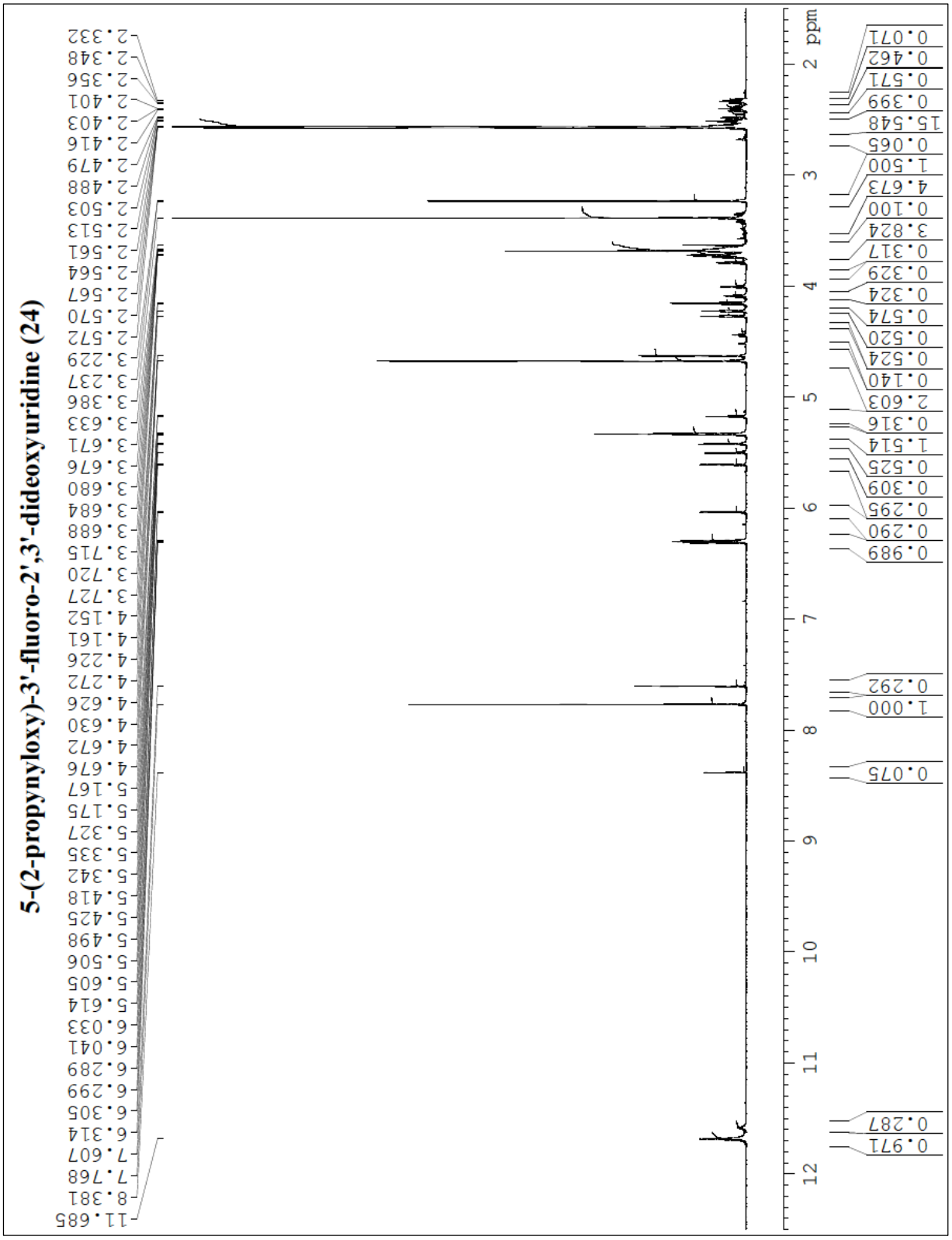
Appendix 13: NMR spectra of 5-(2-Propynyloxy)-2'-ribofluorouridine (**22**)



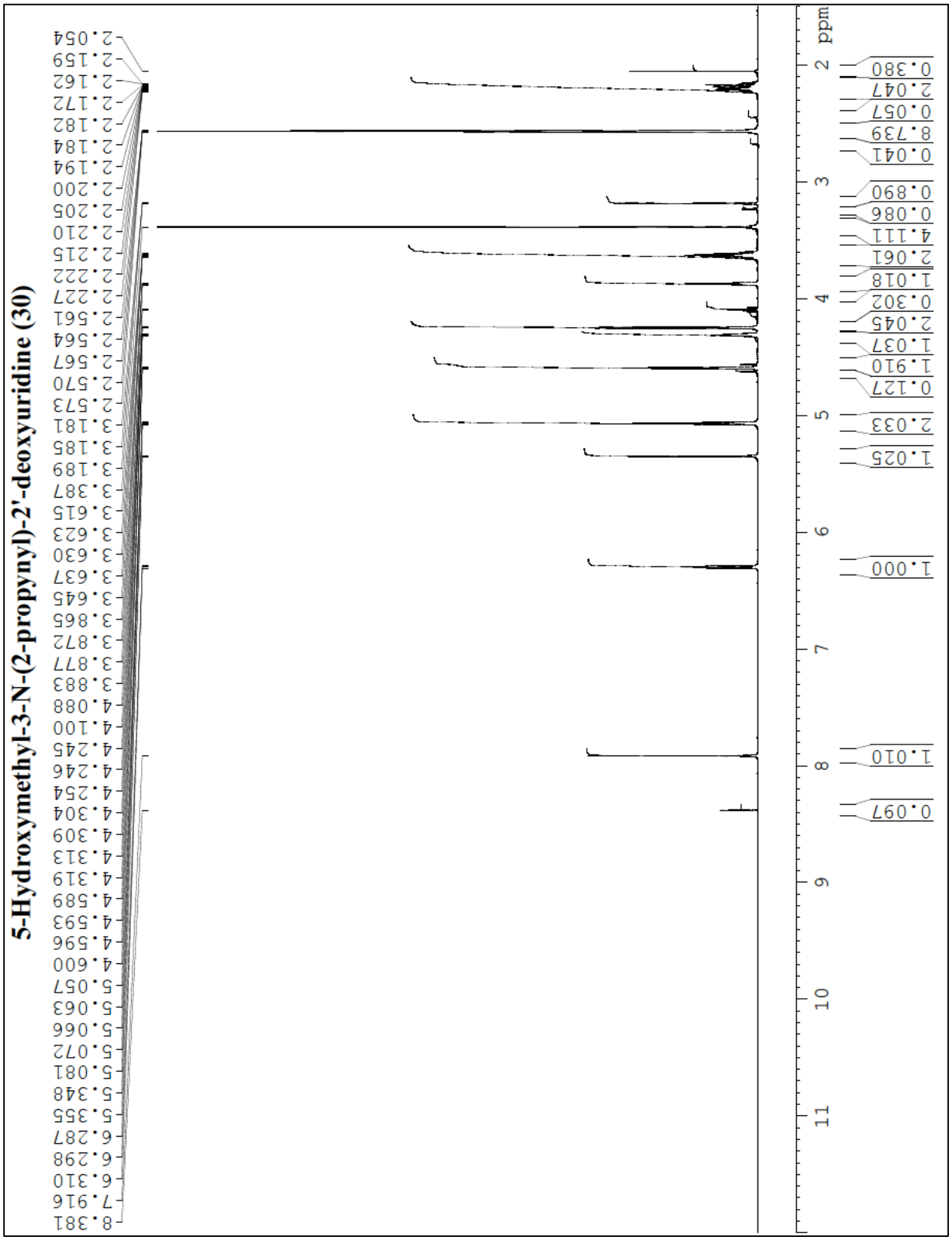
Appendix 14: NMR spectra of 5-(2-propynyloxy)-3-*N*-(2-propynyl)-3'-fluoro-2',3'-dideoxyuridine (**25**)



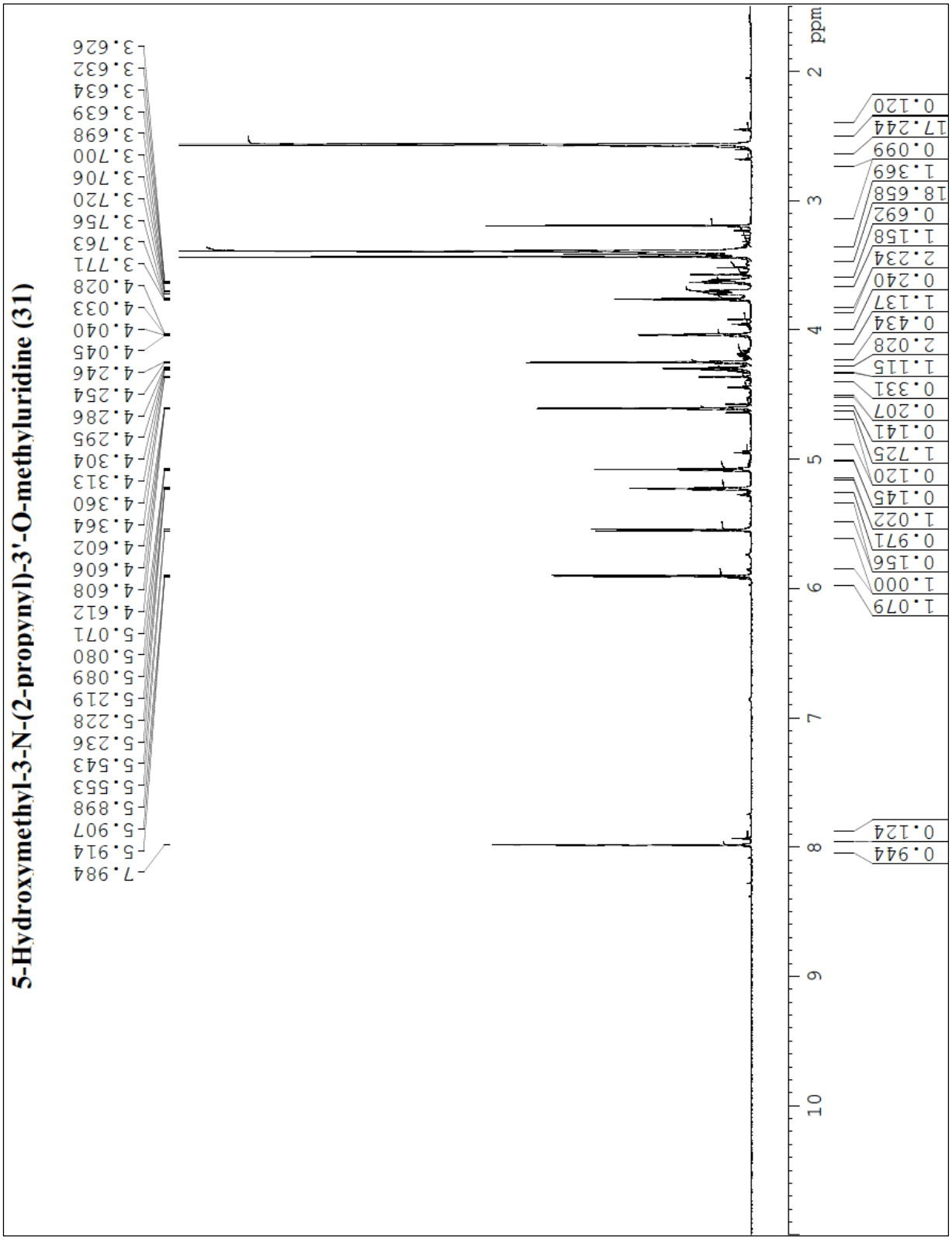
Appendix 15: NMR spectra of 5-(2-Propynyloxy)-3’-fluoro-2’,3’-dideoxyuridine (**24**)



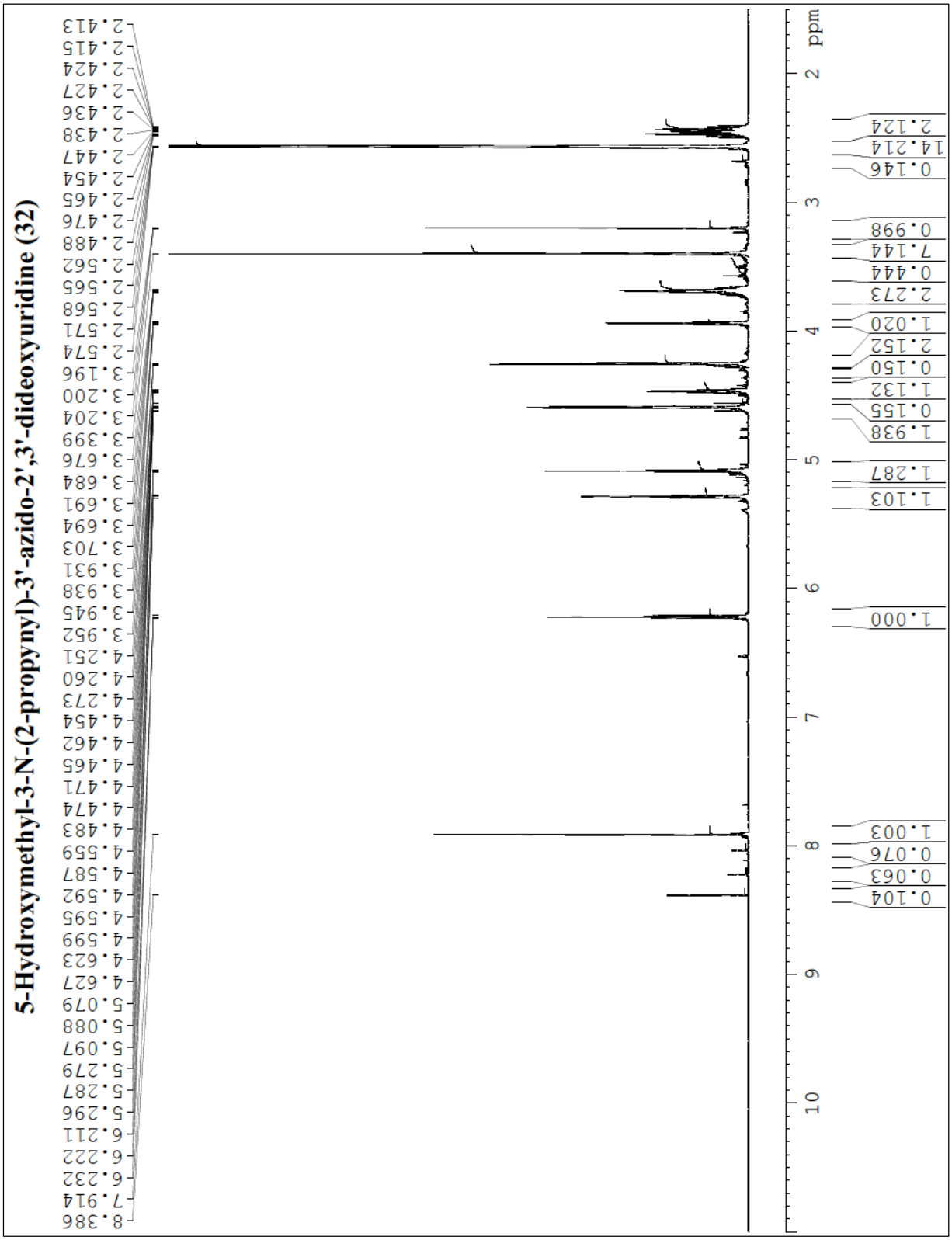
Appendix 16: NMR spectra of 5-Hydroxymethyl-3-*N*-(2-propynyl)-2'-deoxyuridine (**30**)



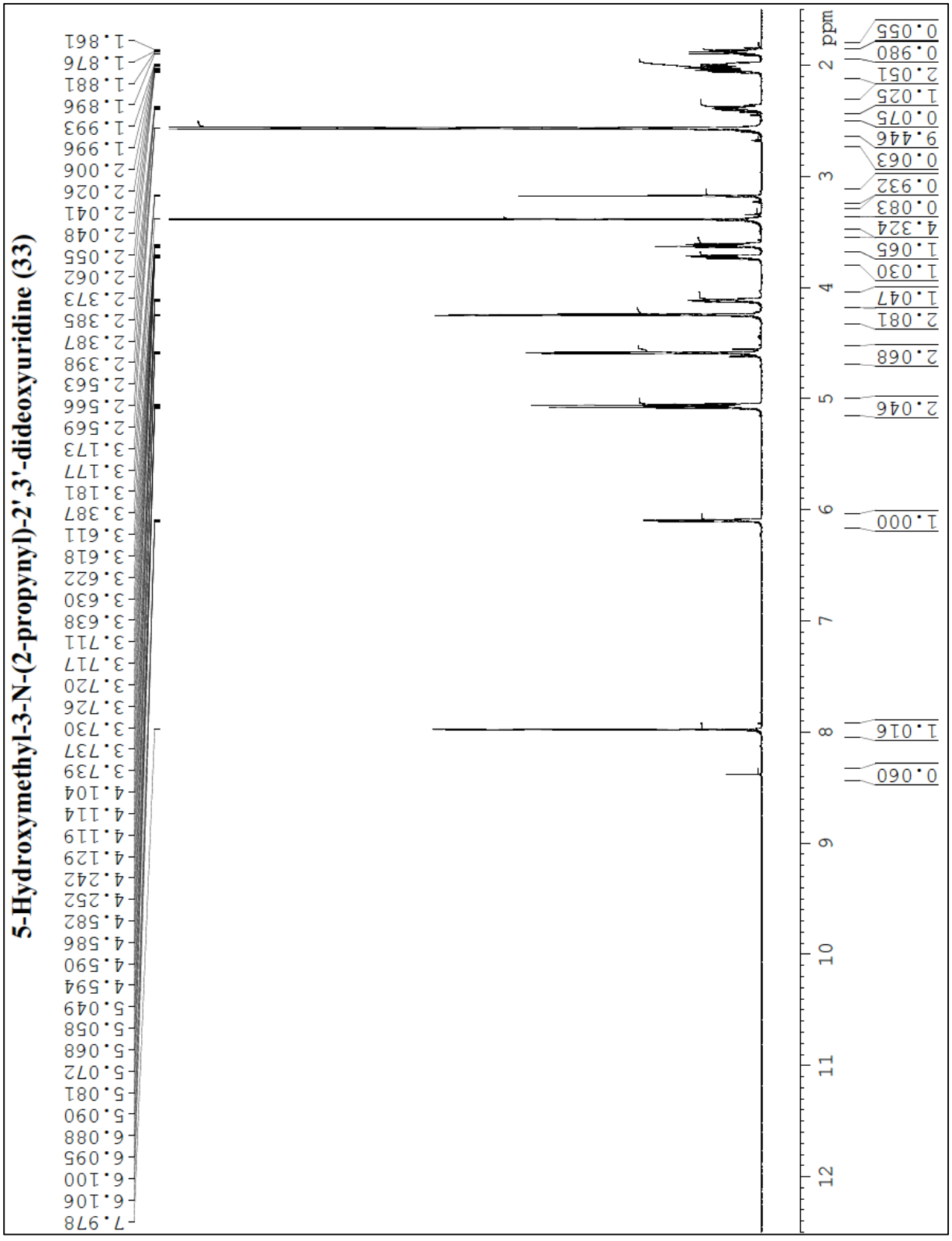
Appendix 17: NMR spectra of 5-Hydroxymethyl-3-*N*-(2-propynyl)-3’-O-methyluridine (**31**)



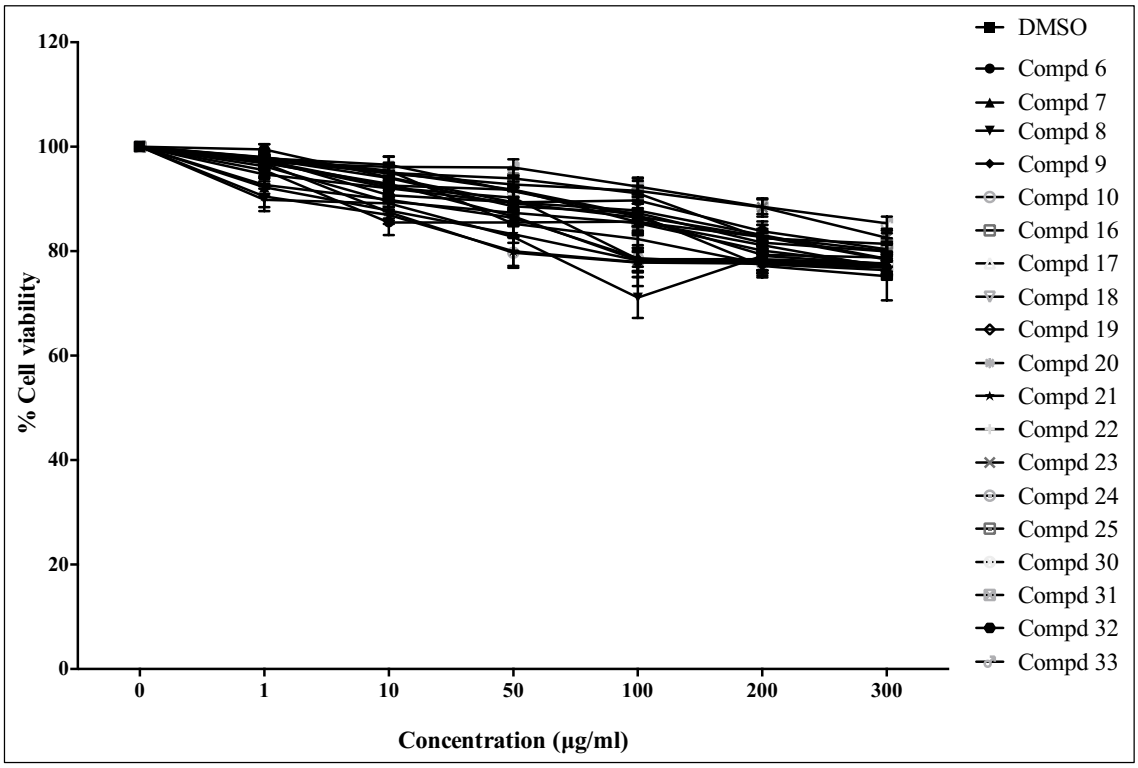
Appendix 18: NMR of 5-Hydroxymethyl-3-*N*-(2-propynyl)-3'-azido-2',3'-dideoxyuridine (**32**)



Appendix 19: NMR spectra of 5-Hydroxymethyl-3-*N*-(2-propynyl)-2',3'-dideoxyuridine (**33**)

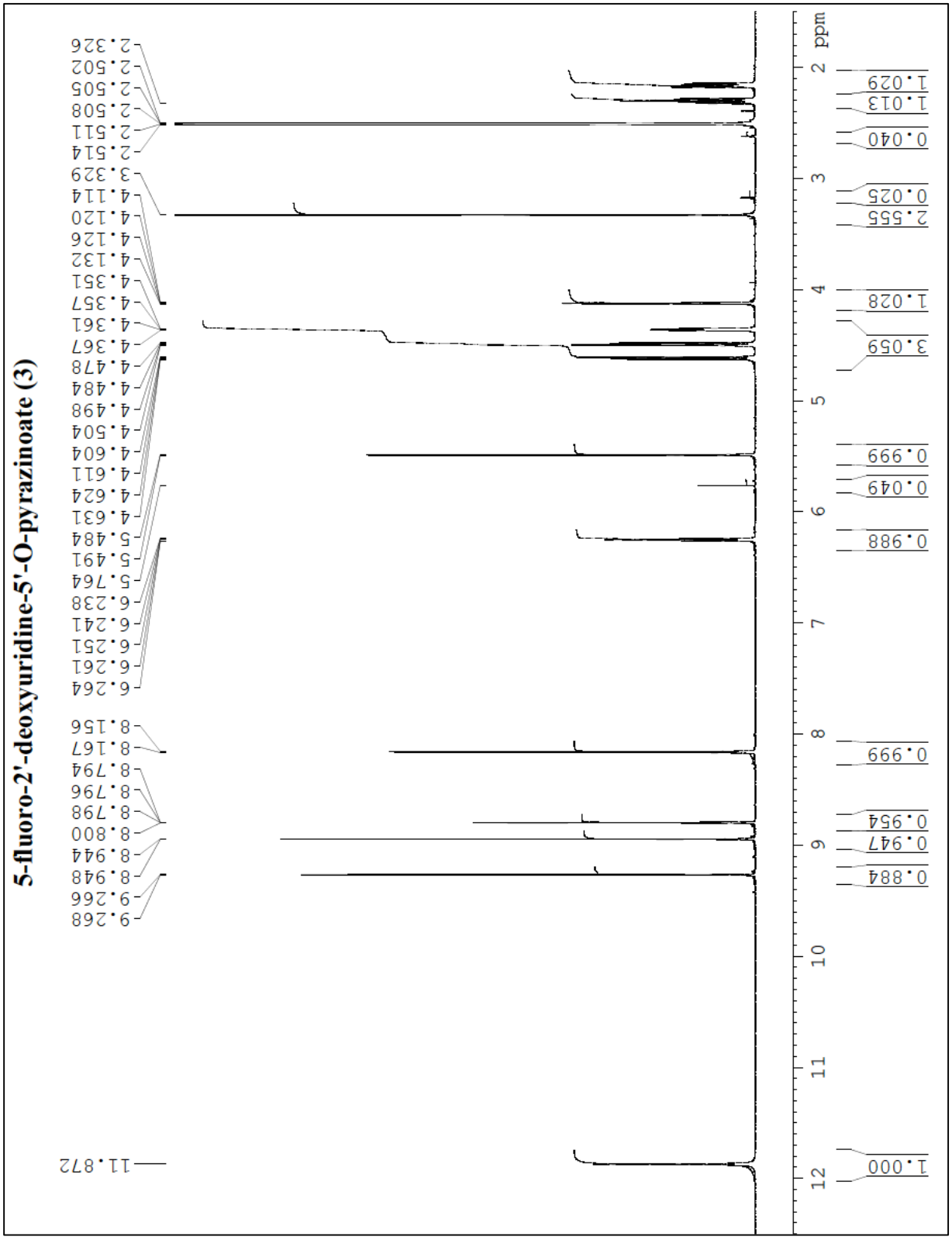


Appendix 20: *In vitro* toxicity of Compounds (**6-10**, **16-25** and **30-33**) on Vero cells

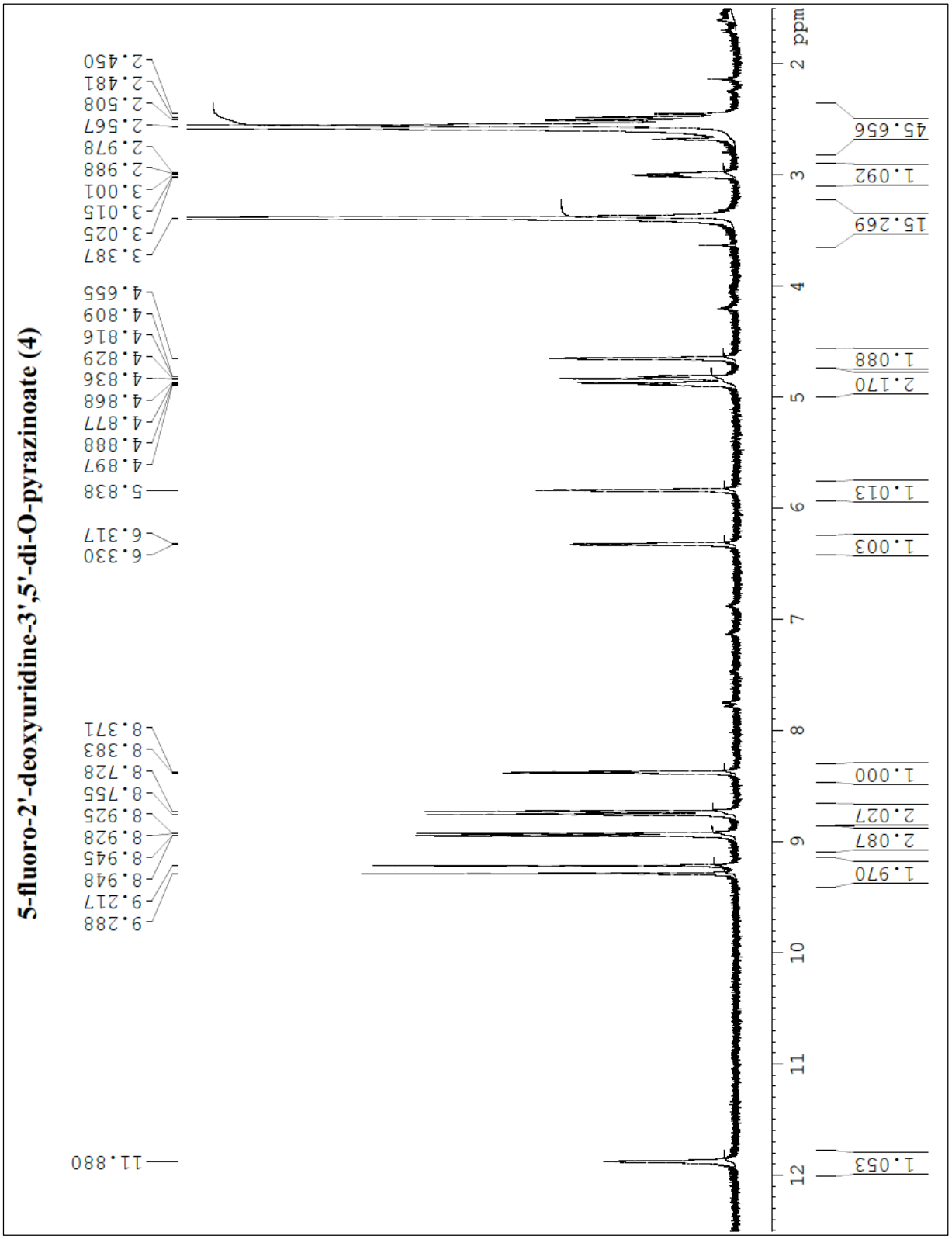


Toxicity study of test compounds was determined at concentrations of 300, 200, 100, 50, 10 and 1 µg/mL. DMSO was used as solvent control. All data represent mean ± SD (standard deviation).

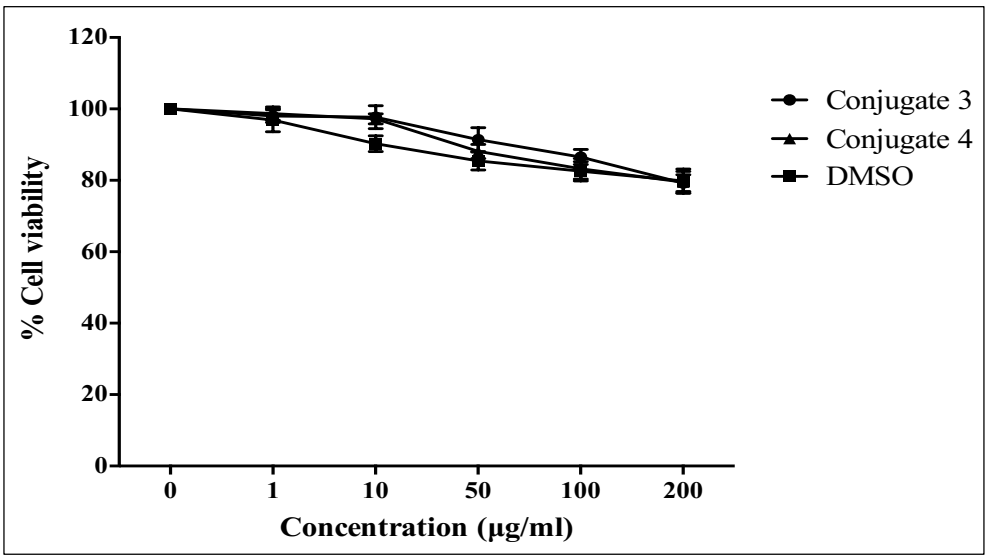
Appendix 21: NMR spectra of 5-fluoro-2’-deoxyuridine-5’-O-pyrazinoate (**3**)



Appendix 22: NMR spectra of 5-fluoro-2’-deoxyuridine-3’,5’-O-pyrazinoate (**4**)

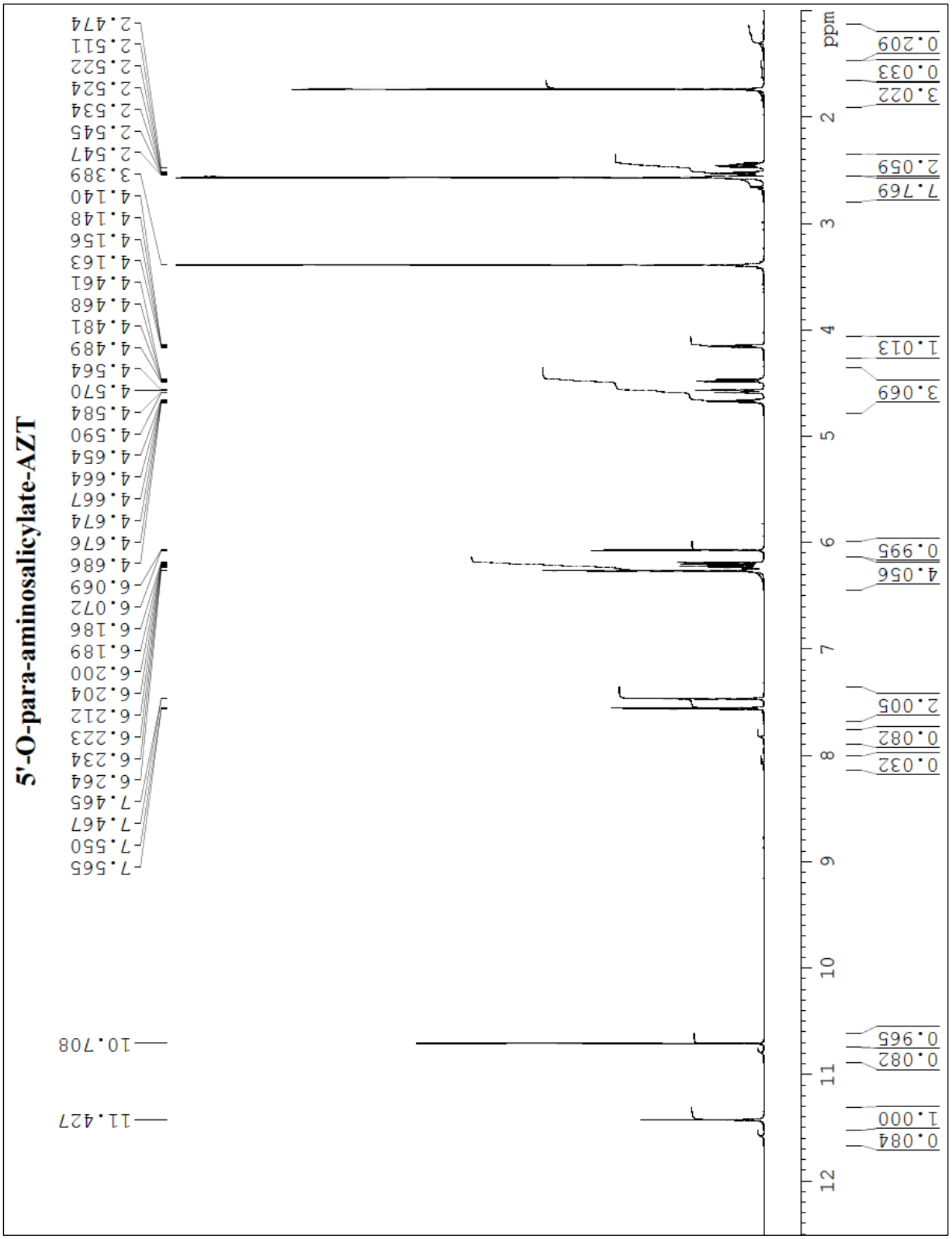


Appendix 23: *In vitro* toxicity of Conjugates **3** and **4** on Vero cells

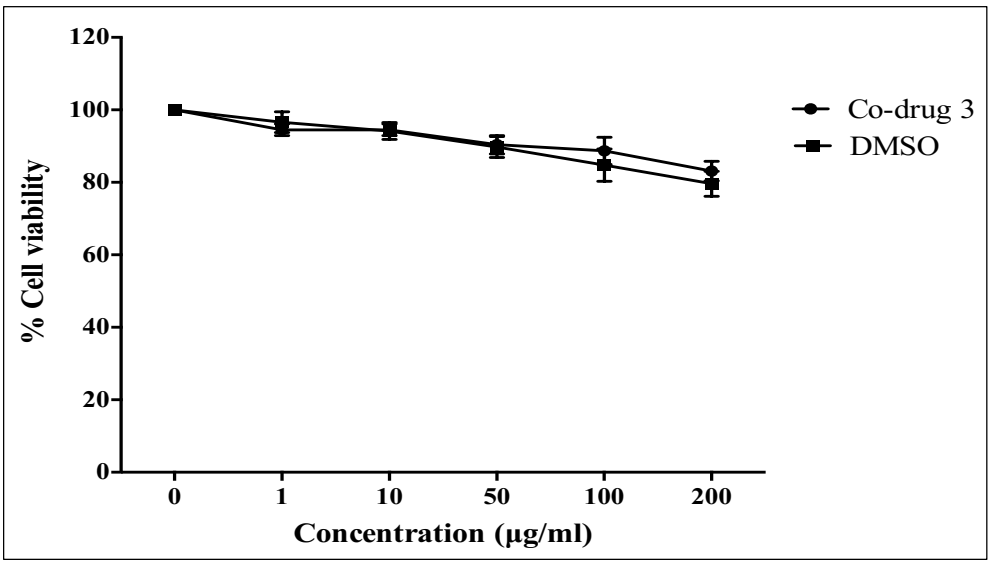


Toxicity study of conjugates **3** and **4** were determined at concentrations of 200, 100, 50, 10 and 1 µg/mL. DMSO was used as solvent control. All data represent mean ± SD (standard deviation).

Appendix 24: NMR spectra of 5’-O-para-aminosalicylate-AZT



Appendix 25: *In vitro* toxicity of Co-drug **3** on Vero cells



Toxicity study of co-drug **3** was determined at concentrations of 200, 100, 50, 10 and 1µg/mL. DMSO was used as solvent control. All data represent mean ± SD (standard deviation).