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Original Article

Investigating the Impact of Acute and 28-days Oral Exposure to Decaprenyl phosphoryl-β-D-ribose 2′- epimerase (DprE1) Inhibitors on Vital Organ Function in Swiss Albino Mice

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ABSTRACT

Aim/Background: Tuberculosis (TB) is a severe airborne infectious disease caused by *Mycobacterium tuberculosis*, a contagious bacillus and the second leading cause of mortality. A pivotal obstacle in Tuberculosis (TB) therapy lays in the swift emergence of resilient TB mycobacterial variants during treatment regimens, thereby precipitating the dissemination of Multi-Drug Resistant (MDR-TB) and extensively drug-resistant *M. tuberculosis* (XDR-TB) strains. The objective of present study to evaluate toxicity of synthesized novel DprE1 inhibitors. **Materials and Methods:** Research is currently directed towards discovering novel targets possessing advantageous microbiological characteristics for treating tuberculosis. Key compounds such as imidazo-pyridine, pyrazine, pyrimidine and quinazoline are pivotal elements of therapeutic significance in this endeavour. Despite this, there remains a scarcity of drugs developed for this infectious disease. Decaprenyl-phosphoryl-β-D-ribose-2-Epimerase (DprE1) is a crucial enzyme involved in arabinose biosynthesis, a component of the mycobacterium cell wall. We had synthesized series of compounds with basic nucleus imidazo-pyridine, quinazoline-4-carboxaminde and benzothiazole substituted; these compounds were subjected for *in vitro* antitubercular assay (Risazurine microtiter assay) and DprE1 enzyme specific studies. Three compounds (each from series) were selected here for toxicity studies. Activity of these selected compounds was 5i-0.8 µmol/L, 5g-1.01 µmol/L and 3a-1.27 µmol/L. Initial screening for acute and subacute toxicity involved albino mice weighing between 25-31 g, following OECD 423 guidelines. **Results:** The research determined that a dose of 300 mg/kg was safe, with no abnormalities observed in the animals on the 7th, 14th and 28th days of the study. **Conclusion:** Necropsies revealed normal average weights and vital organs (heart, lungs, liver and kidney) in all groups compared to the control group. Histopathological examinations did not indicate any abnormalities such as swelling, elongation, shrinking, deformation, or cell death in the vital organs studied.

Keywords: Toxicity, Antitubercular Agents, DprE1 Inhibitors, NCEs.

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INTRODUCTION

Tuberculosis persists as a formidable public health challenge, contributing significantly to global mortality rates with an estimated toll of approximately one million deaths annually. The efficacy of existing pharmacotherapies has been compromised by the emergence of Multidrug-Resistant Strains (MDRTB), compounded by the prevalence of HIV co-infection. Consequently, there exists a pressing imperative to innovate and develop novel antitubercular agents, whether derived from natural sources or synthesized de novo.[1-3]

Before 1944, the therapeutic arsenal against Tuberculosis (TB) was nonexistent and over the past five decades, the market has seen minimal introduction of new drugs for TB treatment, with Bedaquiline being a notable exception. The escalating incidence of drug-resistant TB is primarily attributed to inadequate treatment practices and patient non-compliance. This phenomenon

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has engendered a perilous state wherein TB bacilli develop resistance to Isoniazid and Rifampicin, the cornerstone anti-TB medications. Compounding this predicament is the emergence of Extensively Drug-Resistant (XDR) TB, particularly prevalent in immunocompromised individuals such as those afflicted with HIV, exacerbating the crisis and necessitating the imperative development of novel therapeutic agents to curb this burgeoning TB epidemic.[4-9]

The current landscape of Tuberculosis (TB) treatment is challenged by the prolonged duration of therapy, drug-associated toxicities and the rise of Multidrug-Resistant Tuberculosis (MDR-TB), rendering existing first-line drugs such as isoniazid, rifampicin, pyrazinamide, streptomycin and ethambutol less effective.[10-12]

The protracted duration of Tuberculosis (TB) treatment, coupled with associated toxicity and the emergence of Multidrug-Resistant Tuberculosis (MDRTB), has engendered a situation wherein current TB drugs are insufficient to combat the disease. Consequently, there exists a pressing need for the development of novel TB drugs with innovative mechanisms of action to address drug-resistant TB strains effectively. Decaprenyl phosphoryl-β-D-ribose oxidase, known as DprE1, plays a pivotal role in the biosynthesis of arabinogalactan, a constituent of the mycobacterial cell wall, rendering it an attractive target for anti-tuberculosis drug development.[13-16]

This enzyme catalyses the Flavin Adenine Dinucleotide (FAD) dependent oxidation of decaprenyl phosphoryl-β-D-ribose (DPR) to decaprenyl phosphoryl-β-D-2'- ketoerythropentafuranose (DPX).[17] the precursor for arabinogalactan and lipoarabinomannan synthesis, in conjunction with decaprenylphosphoryl-D-2-keto erythro pentose reductase. DprE1 initiates the first step of the epimerization process, where DPR is oxidized to the intermediate decaprenylphospho-2′-keto-D-arabinose (DPX), cofactored by flavin flavoenzyme a promising target for developing novel therapeutic candidates to tackle TB. The druggable yet promiscuous nature of DprE1 has led to a significant number of DprE1 inhibitors with diverse molecular scaffolds and pharmacological profiles. There have been 23 new classes of DprE1 inhibitors identified with antimycobacterial activity and their different scaffolds These inhibitors are divided into two types, according to their mechanism of action (MoA): (1) covalent binders, where five classes have been shown to irreversibly inhibit DprE1 by generating a covalent adduct with the C387 residue and (2) noncovalent inhibitors, in which 17 reported classes were experimentally confirmed to act as competitive inhibitors.[18-20] Based on a whole cell screen, 4-aminoquinolone piperidine amide was identified as noncovalent DprE1 inhibitors. This compound was modified to optimize the inhibitory activities against DprE1 as well as *M. tuberculosis* whole cell. Moreover, this series (4-aminoquinolone piperidine amide) exhibited excellent cidal properties *in vitro* against replicating as well as intracellular *M. tuberculosis*.[21-25]

Studying both acute and 28-day repetitive toxicity is significant in toxicological research for comprehensive risk assessment of pharmaceuticals. Acute toxicity studies provide immediate insight into potential hazards from single exposures, aiding in initial safety evaluations. On the other hand, 28-day repetitive toxicity studies simulate prolonged exposure, revealing cumulative effects and potential long-term risks, essential for understanding chronic toxicity and ensuring the safety of drugs over extended usage periods. Together, they form a robust framework for regulatory submissions and safeguarding public health.

Acute Toxicity

Acute toxicity delineates the deleterious manifestations induced by a substance, stemming either from a solitary exposure or from multiple exposures within a concise timeframe. Such adverse effects must manifest within a span of 14 days subsequent to substance administration to merit classification as acute toxicity. Diverging from this acute paradigm, chronic toxicity delineates the adverse health ramifications ensuing from repetitive exposures, typically at attenuated dosages, to a substance over an extended temporal domain. Ethical considerations preclude the utilization of human subjects in acute (or chronic) toxicity investigations; nonetheless, incidental human exposures proffer limited insights. Predominantly, acute toxicity data emanates from animal experimentation or, more contemporaneously, *in vitro* methodologies, augmented by extrapolations from analogical substances.[26]

Subacute Toxicity

Subacute toxicity assessments serve the dual purpose of gauging the toxicological profile of a compound following repeated dosing and facilitating the delineation of dosages pertinent for protracted subchronic investigations. Typically, these evaluations entail the administration of three to four varying doses of the test compound, commonly via dietary admixture. Rodent models conventionally employ 10 animals per sex per dose, whereas canine studies employ three doses with 3-4 animals per sex. The administration period spans 14 days, culminating in euthanasia and comprehensive analyses encompassing clinical chemistry and histopathology.[27-30]

OECD 423 guidelines group acute and subacute studies under the same chemical compound, diverging solely in the duration of exposure. Subacute systemic toxicity manifests as adverse effects ensuing from repetitive or continuous exposure spanning 24 hr to 28 days. Figure 1 represents insights of experimental steps.

Previously, leveraging computational methodologies, we designed novel DprE1 inhibitors, delving into intricate molecular interactions through molecular docking analyses, synthesis and antitubercular activity. Subsequently, a diverse array of

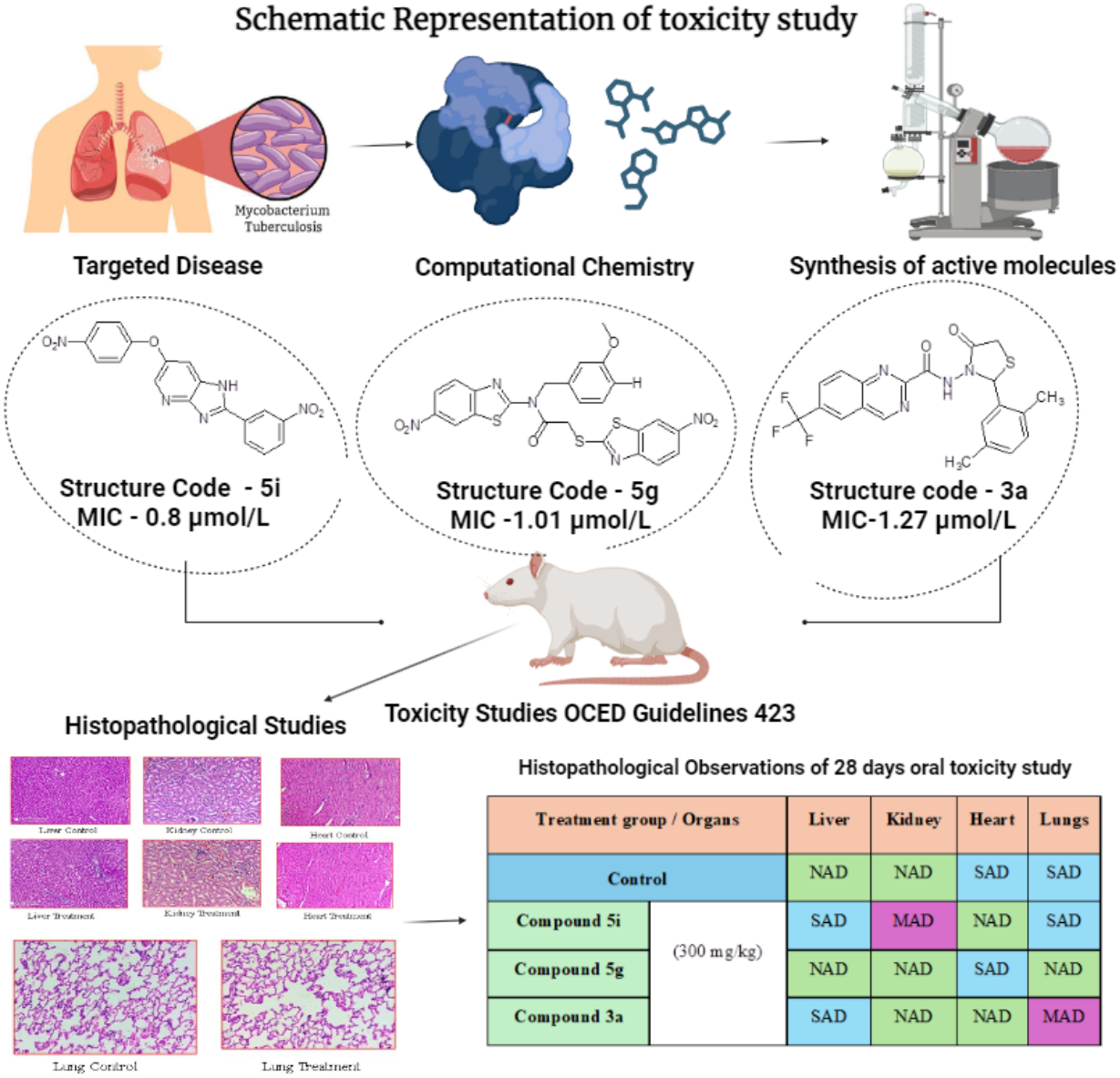
compounds featuring imidazo[4,5-b] pyridine, benzothiazole and quinazoline-2-carboxamide scaffolds were synthesized and characterized via spectral analyses. These compounds were subjected to rigorous scrutiny for *in vitro* antitubercular efficacy. Promising candidates were further subjected to enzyme-specific assays. In this current investigation, acute and subacute toxicity assessments were conducted on three representative compounds harbouring distinct chemical scaffolds.[31-33] Based on the recommendations of several expert meetings, revision was considered timely because: i) international agreement has been reached on harmonised LD_{50} cut-off values for the classification of chemical substances, which differ from the cut-offs recommended in the 1996 version of the Guideline.

Experimental Methodology

The three test compounds depicted in Figure 2 were synthesized within the synthetic chemistry laboratory at SVKM's NMIMS School of Pharmacy and Technology Management, Shirpur. Each compound embodies a distinct chemical nucleus, namely imidazo[4,5-b]pyridine, benzothiazole and quinazoline-2-carboxamide. Reagents and materials of analytical grade were procured from Rankem (India) and Sigma-Merck (USA). Albino mice sourced from the in-house animal facility were utilized in the study.

Animals

Swiss albino mice weighing between 25 and 30 g were sourced from the In-House Animal Facility Centre at SVKM's NMIMS School of Pharmacy and Technology Management, Shirpur Campus. These animals were maintained under standard ambient



Treatment group / Organs		Liver	Kidney	Heart	Lungs
Control		NAD	NAD	SAD	SAD
Compound 5i	(300 mg/kg)	SAD	MAD	NAD	SAD
Compound 5g		NAD	NAD	SAD	NAD
Compound 3a		SAD	NAD	NAD	MAD

Figure 1: Systematic Representation of Toxicity Study as per OECD 423.

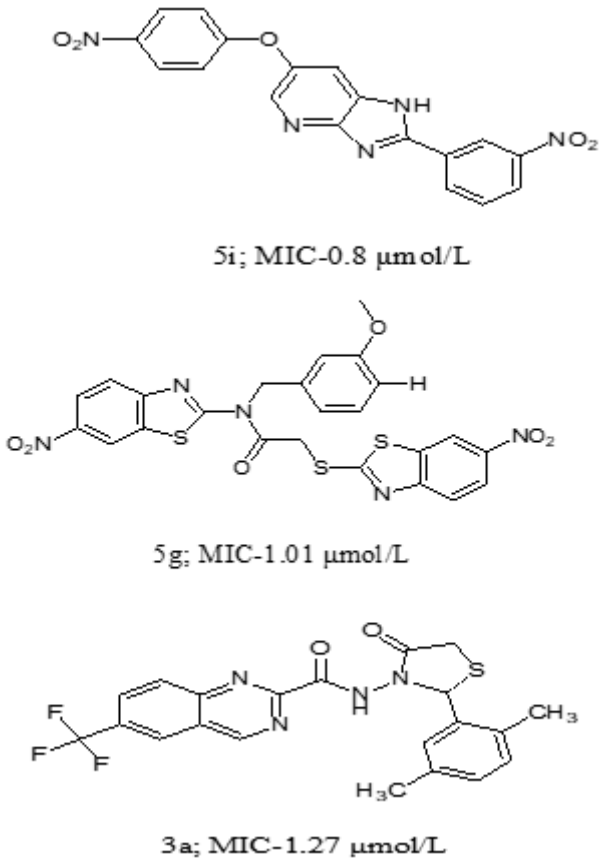


Figure 2: Selected Synthesized Compounds for Toxicity Studies.

conditions and provided with a standard diet along with access to clean drinking water. The ethical handling and usage of animals in this study were approved by the institutional animal ethical committee, in accordance with the OECD 423 guidelines for the care and utilization of animals in toxicity testing of chemicals. OEDC guidelines and specifications there under were followed for several parameters like selections of animals, initiation of studies etc.

Administration of doses

The test substance was orally administered in a single dose via gavage, utilizing a stomach tube or an appropriate intubation canula. Animals underwent a fasting regimen prior to dosing, with food withheld for 3-4 hr (for mice, while water remained accessible). Post-fasting, animals were weighed and the test substance was administered. Following substance administration, food deprivation continued for an additional 1-2 hr.

Number of animals and Doses

Each experimental group comprised three animals for acute toxicity investigations. In the case of the three test compounds, nine animals were allocated into three distinct groups. Additionally, one-fourth of the total groups served as controls, receiving only drinking water and food. Test animals were administered a dose of 300 mg/kg in accordance with OECD 423 guidelines. According to guidelines, when available information suggests that mortality is unlikely at the highest starting dose level (2000 mg/kg body weight), then a limit test should be conducted. When there is no information on a substance to be tested, for animal welfare reasons it is recommended to use the starting dose of 300 mg/kg body weight.[33-38]

Acute toxicity

Healthy female Swiss albino mice, aged 6-8 weeks and weighing 25-30 g, were assigned to experimental groups comprising three individuals each. Test compounds, rendered soluble in water, were administered orally at a single dose of 300 mg/kg, while water served as the control. Prior to oral dosing, mice underwent an 8 hr fasting period with access to drinking water. Post-dosing, the mice's general behaviour was closely monitored immediately for 8 hr and subsequently at least twice daily for up to the 7th and 14th days. Observations encompassing specific changes and incidences of mortality were meticulously documented.

Subacute toxicity

Female mice, weighing between 25 and 30 g on average, were randomly distributed into three groups (n=3). These mice received daily oral doses of Compounds 5i, 5g and 3a at a dosage of 300 mg/kg/day via gavage. Continuous monitoring for signs of toxicity was performed on all mice throughout the duration

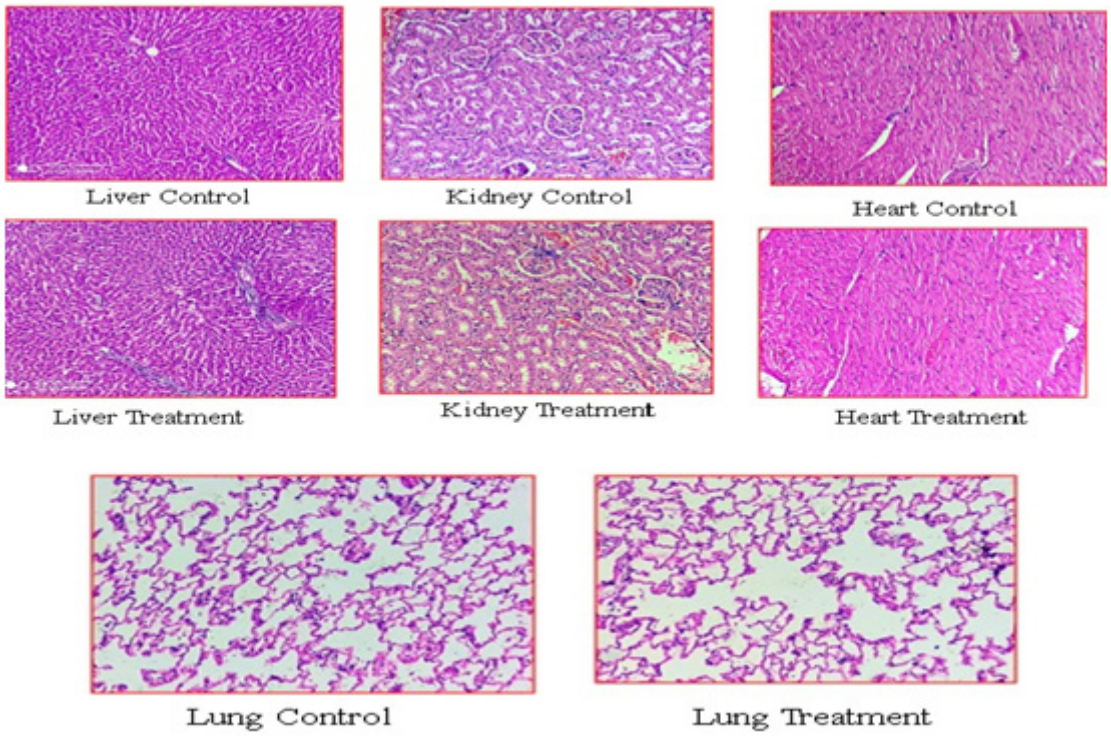


Figure 3: Histopathological Observations of Sub acute Toxicity Studies (5i).

of the study. Upon completion of the 28-day experimental period, including the control group, mice from each cohort were anesthetized using a chloroform chamber. Subsequently, blood samples were obtained via cardiac puncture for subsequent blood chemistry and hematologic analyses. Additionally, the heart, lungs, liver and kidneys were harvested and their respective wet weights were promptly recorded. Remarkably, no mortality events were recorded during the study, prompting the administration of doses of 500 mg/kg and 200 mg/kg to another cohort of healthy animals (n=3) in a dose-escalation paradigm.

RESULTS

Acute Toxicity

Following OECD 423 guidelines, an oral dose of 300 mg/kg was administered, with mice exhibiting typical behaviour. Throughout the study, encompassing observations on days 0, 7 and 14, all animals, including those in the control group, displayed normal vitality and remained alive. Continuous vigilance for signs of poisoning, such as lethargy, piloerection and ocular squinting, was upheld for each mouse across all groups, ensuring appropriate hydration and nutrition throughout the study duration. Upon study completion, necropsies were performed, with the average weights of all animals, including controls, meticulously recorded. Organ weights were promptly documented post-isolation to mitigate potential errors. Comprehensive tabulation of results is presented in Tables 1 and 2.

Subacute Toxicity and Histopathological Investigations

The oral dose of 300 mg/kg was administered to three groups and one control (n=3 for each group). Each animal was observed for 28 days. There were neither obvious signs of any kind of abnormalities nor death was recorded. No anatomical changes or pathological changes in organs examined in liver, heart, kidneys and lungs shown in Figure 3. These livers appeared in normal colour. Histograms of organs were examined critically for cell destructions, elongation of cells, cell damage, shrinkage of cells etc. No abnormality was observed in any of organ (Table 3). As no death and abnormality was observed with 300 mg/kg dose, latter, 500 mg/kg oral dose was administered to different groups. Each animal in both groups was observed for abnormal behaviour. On the 4th day two animals to which 500 mg/kg oral dose was administered were found dead. In histopathological observations it was observed that in cells were damaged and moderate level of cell destruction was noted.

DISCUSSION

The research conducted here to access acute and subacute toxicity on albino mice to evaluate the safety of synthesized newly synthesized DprE1 inhibitors for tuberculosis treatment. The study administered a safe dose of 300 mg/kg to the mice and closely monitored them for any abnormalities or toxic effects. In acute Toxicity studies the observations were the mice, including those in the control group, displayed normal behaviour and vitality throughout the study period, during the study no abnormalities

Table 1: **Effect of oral administration of synthesized compounds on necropsy of mice.**

Treatment groups	Treatment	Weight (g)		
		0 Day	7 Day	14 Day
Control	-	25.30±0.25	26.10±0.62	27.40±0.78
Compound 5i	Dose-300 mg/kg	25.42±1.03	26.90±0.85	27.70±0.90
Compound 5g		25.77±1.05	26.29±1.17	27.66±1.0
Compound 3a		26.21±0.90	26.54±1.00	27.87±1.14

Table 2: **Effect of oral administration of synthesized compounds on necropsy of mice.**

Treatment groups	Treatment	Weight (g)			
		Liver	Kidney	Heart	Lungs
Control	-	4.82±0.30	0.97±0.48	0.68±0.35	0.81±0.45
Compound 5i	Dose-300 mg/kg	4.59±0.42	0.87±0.55	0.72±0.43	0.88±0.38
Compound 5g		4.30±0.54	0.77±0.62	0.79±0.58	0.74±0.42
Compound 3a		4.47±0.39	0.89±0.76	0.61±0.41	0.91±0.58

Table 3: **Histopathological observations of 28 days oral toxicity study.**

Treatment group / Organs		Liver	Kidney	Heart	Lungs
Control		NAD	NAD	SAD	SAD
Compound 5i	Dose-(300 mg/kg)	SAD	MAD	NAD	SAD
Compound 5g		NAD	NAD	SAD	NAD
Compound 3a		SAD	NAD	NAD	MAD

NAD: No Abnormality Detected, SAD: Slight Abnormality Detected, MAD: Moderate Abnormality Detected.

were observed in the animals on the 7th, 14th and 28th days of the study. After necropsies, it revealed normal average weights and intact vital organs in all experimental groups compared to controls. The acute toxicity assessment confirmed the safety of the 300 mg/kg dose with no signs of toxicity observed.

In subacute toxicity studies mice received daily oral doses of the compounds for 28 days, with continuous monitoring for signs of toxicity. No mortality or abnormalities were recorded during the entire study and histopathological examinations did not show any pathological manifestations in the vital organs examined. A dose of 500 mg/kg was administered to another cohort, resulting in two deaths and histopathological observations indicating cell damage and moderate cell destruction. The subacute toxicity assessment reaffirmed the safety of the 300 mg/kg dose, highlighting the absence of toxicity or abnormalities in the vital organs studied.

The results of the acute and subacute toxicity assessments provide crucial insights into the safety profile of the synthesized DprE1 inhibitors for tuberculosis treatment. The study demonstrated that the 300 mg/kg dose was well-tolerated by the mice, with no toxic effects observed. These findings are significant as they indicate the potential of these compounds as safe and effective antitubercular agents. The absence of abnormalities in the vital organs and the confirmation of normal weights further support the biocompatibility of the tested dose regimen. Overall, these results highlight the promising safety profile of the novel DprE1 inhibitors, emphasizing their potential for further development as tuberculosis treatment options.

CONCLUSION

In this study, acute and subacute toxicity assessments were conducted on albino mice within the weight range of 25-31 g, adhering to the OECD 423 guidelines. The investigation determined a safe dose of 300 mg/kg based on comprehensive parameter evaluations. Notably, no abnormalities were observed in the animals on the 7th, 14th and 28th day of the study. Subsequent necropsies revealed normal average weights and intact vital organs in all experimental groups compared to controls. Histopathological analyses further confirmed the absence of any pathological manifestations such as swelling, elongation, shrinking, deformation, or cellular necrosis in the vital organs examined. These findings underscore the safety and biocompatibility of the tested dose regimen in this murine model. Toxicity studies play a crucial role in process of drug discovery and development. Our future toxicity studies will be emphasized and designed according to OECD guidelines.

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ETHICAL APPROVAL

The IAEC Committee approved the experimental protocol.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

TB: Tuberculosis; **NCE:** New Chemical Entity; **MDR-TB:** Multidrug resistant tuberculosis; **XDR-TB:** Extensively drug-resistant tuberculosis; **DprE1:** Decaprenyl-phosphoryl-β-D-ribose 2′-epimerase; **FAD:** flavin adenine dinucleotide; **DPR:** dependent oxidation of decaprenyl phosphoryl-β-D-ribose; **DPX:** decaprenyl phosphoryl-β-D-2'- ketoerythropentafuranose; **IAEC:** Institutional Animal Ethical Committee.

SUMMARY

The research paper focuses on evaluating the toxicity of synthesized novel DprE1 inhibitors for the treatment of Tuberculosis (TB). The study aimed to combat the emergence of drug-resistant TB strains by identifying new targets with favourable microbiological properties. The compounds tested, including Compound 5i, 5g and 3a showed promising antitubercular activity and inhibition of the DprE1 enzyme. Acute and subacute toxicity assessments were conducted on albino mice, with a safe dose of 300 mg/kg identified. The all three test compounds were found safe/non-toxic at the given dose. The results indicated no abnormalities in the animals, with necropsies revealing normal weights and vital organs. Histopathological examinations did not show any abnormalities in the organs studied. Overall, the study highlights the safety and biocompatibility of the tested dose regimen in this murine model, emphasizing the potential of these compounds as antitubercular agents.

Toxicological studies in the 21st century play a pivotal role in drug research and development by assessing the safety and potential risks of new compounds. They provide essential data to predict adverse effects determine safe dosage levels and understand mechanisms of toxicity. This knowledge is crucial for regulatory approvals, ensuring public health and minimizing harm. Furthermore, advanced methodologies in toxicology contribute to optimizing drug efficacy, guiding formulation strategies and fostering innovation with safer and more effective pharmaceutical products.

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