RESEARCH ARTICLE

Design, Synthesis and Evaluation of Riboflavin-Isoniazid Conjugate

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ABSTRACT

The infectious, contagious disease tuberculosis (TB), which is caused by the bacteria Mycobacterium tuberculosis (MT), frequently lasts the entirety of a person's life and results in the formation of tubercles in various parts of the body. This condition is exceedingly difficult to treat because of the complicated connections between different side effects and drug resistance. Along with AIDS and malaria, TB is a strain on healthcare systems in developing countries. Oxidoreductase enzyme was selected for molecular docking. The docking includes the comparison of docking results of Standard ligand that is 6TEV and molecular docking results of Riboflavin- Isoniazid Conjugate. The study used a linker (Riboflavin-Succinic Acid Linker) to create the molecule known as riboflavin-isoniazid conjugate. Melting point and TLC were used to verify the compound's purity. The final compound was confirmed using FTIR, NMR and HRMS study. Conjugate was tested for anti-tubercular activity using the Microplate Almar Blue Assay (MABA).

Keywords: Mycobacterium Tuberculosis, Molecular docking, Riboflavin-Isoniazid Conjugate, Anti-Tubercular activity.

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INTRODUCTION

Mycobacterium tuberculosis (MT) causes tuberculosis (TB), a contagious, infectious disease that spreads through the air. TB sanatoriums, surgical methods, and the COVID-19 pandemic have contributed to the ongoing spread of multidrug- or rifampicin-resistant TB strains. The WHO's "Rapid communication" highlights the need for new treatments to address the ongoing issue. TB spreads through the air through coughing, speaking, singing, and sneezing, producing droplet nuclei ranging from 1-5 μm in diameter.[1-3] Dr. Robert Koch discovered the tubercle bacillus in 1882, leading to the development of TB sanatoriums in Germany and the spread across America. Edward Trudeau contributed to improved short-term cure rates through sanatorium treatment and surgical methods.[4] Mycobacterium tuberculosis (MT) was first discovered in East Africa three million years ago, with a global prevalence of 2 billion people. Early scientists identified TB as an infectious disease, disproportionately affecting Scheduled Castes and tribes, and raising human rights concerns due to stigma. Tuberculosis, caused by a multi-drug-resistant bacterium, poses a public health concern due to the lack of viable treatments.[5-7] Mycobacterium ulcerans, an infection-causing bacterium, has been present for over 150 million years. Early literary descriptions, Hebraic connections, and Andean archaeological evidence support its presence before European colonization. Streptomycin, isoniazid, rifampicin, ethambutol, and pyrazinamide were used in 1944 for tuberculosis treatment. Mycobacterium tuberculosis complex includes rod-shaped, aerobic, non-spore-forming bacteria, responsible for tuberculosis, and stains easily with carbol fuchsin dye.[8] In 1882, Robert Koch identified Mycobacterium tuberculosis as the causative agent of tuberculosis. This aerobic bacterium has a rod-like form and slow growth, with a mycolic arabinogalactan-peptidoglycan complex at its center. The cell wall proteins include PIMs, LM, and LAM. When disintegrated, the complex remains as an insoluble residue.[9] Tuberculosis causes 1.8 million deaths annually, with poor adherence leading to multidrug resistance. WHO's Direct Observed Treatment Short-course effectively combats the global TB epidemic. Mantoux skin test diagnoses tuberculosis using NTM and PPD antigens, despite logistic and performance issues, with positive results 48-72 hours post-injection. IFN production by T lymphocytes is crucial for resistance to Mycobacterium tuberculosis infection. FDA approved two

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Table 1: Molecular Docking Results of Riboflavin – Isoniazid conjugate

Sr. No.	Interactions	Observations
1	Binding Energy	-8.64
2	Conventional Hydrogen bond interaction	CYS A 345
3	Pi–SIGMA Interaction	PHE A: 230
4	Pi-Alkyl Interaction	META: 86, PRO A: 346, ALA A: 2

commercial IGRAs for detecting LTBI: QuantiFERON-TB Gold assay and T SPOT-TB test.[10] Drug Conjugation is a process of attaching pharmaceuticals or prodrugs to molecular carriers, such as polymers, proteins, and carbohydrates, for controlled drug release and targeted delivery.[11]

Vitamin-drug conjugation aims to explore targeting moieties for new drug synthesis, improving drug stability, pharmacokinetics, and toxicity profiles. This approach can enhance efficacy, bioavailability, and reduce drug resistance. Drugs that are conjugated with vitamins are transported to specific target sites. overcome obstacles such as non-selectivity, systemic toxicity, and medication resistance to several drugs. As a result of conjugates' ability to identify tumor-associated antigens, malignant cells are more susceptible to ligand-targeted therapies, whereas healthy cells are exposed to fewer medicines. A new strategy called drug conjugation can potentially improve bioavailability and efficacy while lowering medication resistance.[12-13]

Examples include Vitamin-B12-Ampicillin conjugates, which show improved activity against Escherichia coli.[12] Riboflavin has an important Role in the treatment of Tuberculosis due to its immune activation and potential antimicrobial target. Literature survey reveals that the conjugation of Riboflavin to various drugs or polymers enhances the anticancer activity.[13-19] Isoniazid remains a critical drug in TB treatment due to its targeted inhibition of mycobacterial cell wall synthesis, immune system interactions, and role in combination regimens for effective disease control. Isoniazid shows the drug resistance due to mutations in genes such as katG and inhA, which gives reduction in its activation or target binding. Based on the literature survey, conjugation of Riboflavin to Isoniazid can

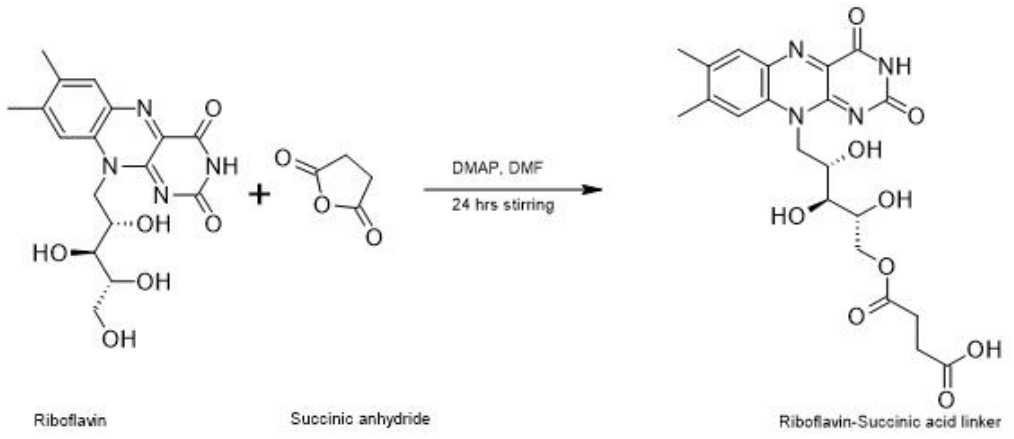


Figure 1: Reaction Scheme for Riboflavin-Succinic acid linker

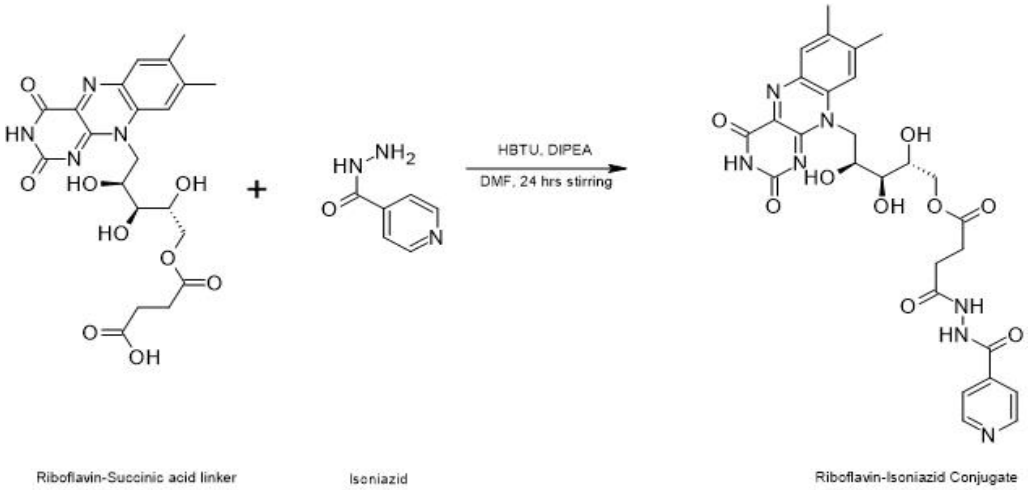


Figure 2: Reaction Scheme for Riboflavin-Isoniazid Conjugate

Table 2: Results of the Antitubercular Activity for Riboflavin-Isoniazid Conjugate

Type	Concentration (µg/ml)									
Std/Conjugate	100	50	25	12.5	6.25	3.125	1.6	0.8	0.4	0.2
Std 1(Isoniazid)	S	S	S	S	S	S	S	R	R	R
Std 2(Ethambutol)	S	S	S	S	S	S	S	R	R	R
Std 3(Pyrazinamide)	S	S	S	S	S	R	R	R	R	R
Std 4(Rifampicin)	S	S	S	S	S	S	S	S	R	R
Std 5(Streptomycin)	S	S	S	S	S	S	S	S	R	R
Sample (RIC)	S	S	S	S	S	R	R	R	--	--

* R- Resistant S- Sensitive RIC- Riboflavin-Isoniazid Conjugate

improve the targeted drug delivery. Nano formulations containing the Riboflavin-Isoniazid conjugate can overcome bacterial resistance due to improved penetration and controlled release. The synergistic effects can be observed due to an enhanced immune system, along with a reduction in the side effects of the isoniazid due to antioxidant effect of Riboflavin[20]

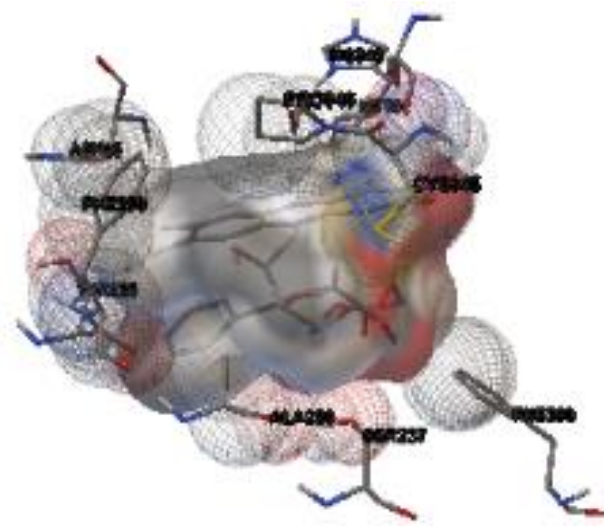
MATERIALS

Riboflavin, Succinic Anhydride, 4-dimethyl aminopyridine (DMAP), Dimethyl formamide (DMF), Diethyl Ether, Silica gel, Acetone, Methanol, Aq. Ammonia, Iodine crystals, Hexafluorophosphate Benzotriazole Tetramethyl Uronium (HBTU), N, N-Diisopropylethylamine (DIPEA), Isoniazid, Dimethyl formamide, Hydrochloric acid, Dichloromethane. All the materials and chemicals were purchased from Research-Lab Fine Chem Industries, Mumbai.

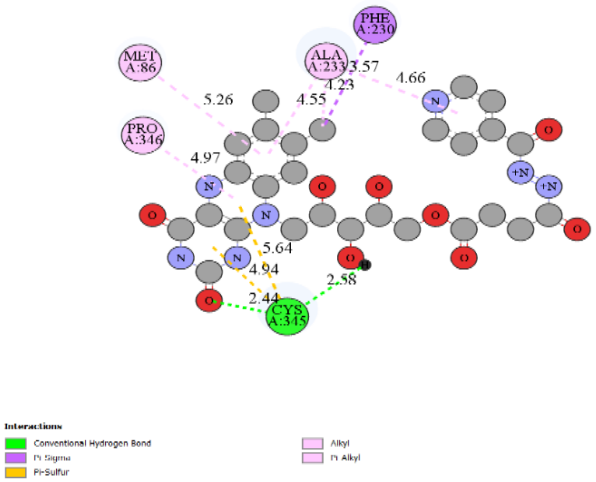
METHODS

Synthesis of Riboflavin-Succinic Acid Linker

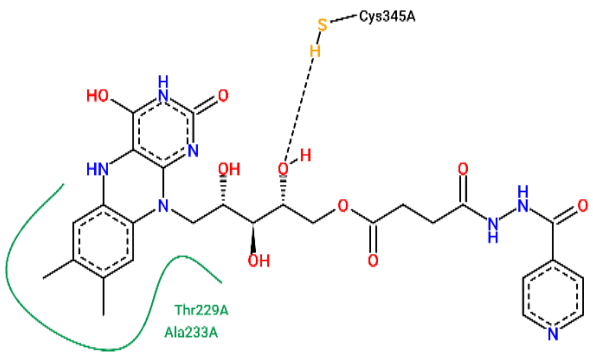
Succinic Anhydride (0.53g, 5.2 mmol), 4-



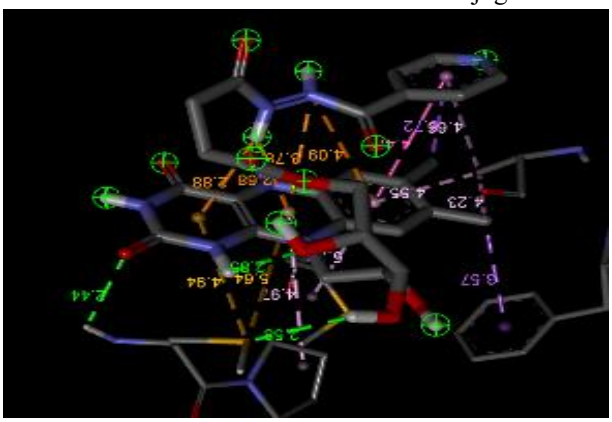
Binding site Interaction



2D view of Riboflavin-Isoniazid conjugate



Binding View of Riboflavin-Isoniazid conjugate



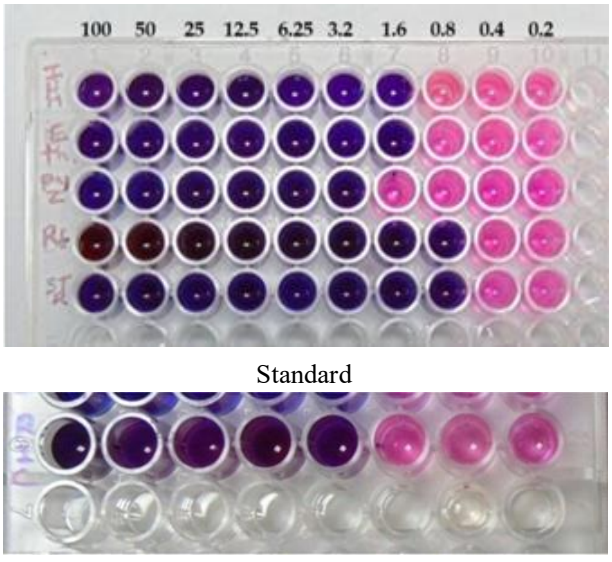
3D view of Riboflavin-Isoniazid conjugate

Figure 3: Molecular Docking Results of Riboflavin –Isoniazid conjugate

dimethylaminopyridine (0.64g, 5.2 mmol), and riboflavin (1g, 2.6 mmol) were stirred in 50 mL of DMF for 24 hours at 70°C. Filtered to remove any remaining material, the reaction mixture was slowly added to the diethyl ether while being vigorously stirred. The precipitate was collected, dried under low pressure, and then the product was recrystallized using acetone. Utilizing TLC and melting point (RF Value: 0.8, M.P.: 204–2060C), the resultant compound was verified. The FTIR spectra of Riboflavin-Succinic Acid Linker revealed clearly peaks around 1150 cm^{-1}that depict the C–O stretching of the ester bond. The FT-IR spectrum of Riboflavin- Succinic Acid Linker shows characteristic –C=O stretching of ester bond at 1694 cm^{-1}, which confirms the riboflavin linker to the ester-terminated succinic anhydride. The Aromatic C-C stretch is also seen at 1409 cm^{-1}. The peaks at 1694 and 3616 cm^{-1} show dimer C=O and Ar-OH stretching, respectively.

Synthesis of Riboflavin - Isoniazid Conjugate

The Riboflavin-succinic acid linker (70 mg, 0.15 mmol) was dissolved in 20 mL of dry DMF, and the reaction mixture was cooled to 0°C. HBTU (96 mg, 0.253 mmol) and DIPEA (0.08 mL, 0.000619 mmol) were added to the above reaction mixture with continuous stirring in dark conditions. Isoniazid (23 mg, 0.169 mmol) was added after 15 minutes. The mixture was stirred for 24 hours. TLC was used to track the completion of the reaction. The reaction was diluted with 10 ml DCM and quenched with 10 ml 0.1N HCl after 24 hours. DCM was used to extract the organic layer, which was then washed with brine (10 ml) and water. Rotating evaporators were then used to concentrate the organic layer. TLC and melting point measurements (Rf Value: 0.9, M.P.: 237–2400C) were used to confirm the final compound.



Standard

Sample (Riboflavin - Isoniazide Conjugate)

Figure 4: Anti-TB activity using Alamar Blue Dye for A. standard, B. Sample

RESULTS AND DISCUSSION

Molecular Docking

AutoDdock was used to dock compounds into the 6TEV's active sites. The energies associated with the docking and binding of the ligands foretell potential binding modes and the mechanism of enzyme inhibition. The ideal pose was selected based on binding energies and the positive contacts formed between the substance and the amino acid residues of the 6TEV active site. Each ligand interacted via hydrogen bonds with HIS A:146 and CYS A:345 in the complex structures. This proves that the hydrogen-bonded amino acids are necessary for the inhibitory activity. The results of Molecular Docking are shown in Fig.3 From the docking result, it is found that binding energy of Riboflavin- Isoniazid Conjugate is -8.64 which is lower than the binding energy of Standard Protein (-6.11). The least binding energy exhibits the highest activity. Molecular docking parameters are given in table 1

Characterisation

The FTIR spectra of Riboflavin- Isoniazid Conjugate revealed clearly peaks around 1521 and 1689 cm^{-1} that depict the formation of amide bond. The FT-IR spectrum of Riboflavin- Isoniazid Conjugate show characteristic –C=O stretching of amide bond at 1689 cm^{-1}which confirms riboflavin conjugates to the amide terminated isoniazid. The free –OH of riboflavin and terminal NH_2 of Isoniazid with each other by forming Amide (=$CONH_2$). The Ar C-C stretch is also seen at 1458 cm^{-1}. The peak at 1689 and 3613 cm^{-1} show dimer C=O and Ar-OH stretching respectively. Hence, the FTIR spectrum confirmed the conjugate formation of riboflavin to surface of Isoniazid. 1H NMR (DMSO-d_6, 300 MHz), δ_H(ppm): δ 6.99 [s, 1H,Ar-CH], δ 3.19,3.43, 3.11, 2.88 [s, 1H, Aliphatic H], δ 2.31,1.2 [s, 1H, NH], δ 3.00 [s, 1H, OH], δ 8.8 [s, 1H, Ar- H]. HRMS (+ESI) m/z [M + H]+ calculated for $C_{27}H_{29}N_7O_9$,589; found, 589.

Antitubercular Activity

The Riboflavin–Isoniazid conjugate was tested in a concentration range of 0.8 µg/mL-100 µg/mL using serial dilution in 96-well plates containing Middlebrook 7H9 broth. After incubation at 37 °C for five days, a mixture of Alamar Blue reagent and Tween 80 was added to each well, and the plates were further incubated. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of the compound that prevented color change from blue to pink, indicating inhibition of bacterial growth. Riboflavin-Isoniazid Conjugate exhibited a MIC value of 6.2 µg/mL, indicating a potent level of inhibitory activity against the M. tuberculosis H37Rv strain. Results

of the Antitubercular Activity for Riboflavin - Isoniazid Conjugate are given in Table 2. From the results of antitubercular activity, it is clear that the synthesized Riboflavin- Isoniazid Conjugate shows antitubercular activity i.e., the microbial growth is not occurred from 6.2 µg/ml to 100 µg/ml concentration, which is denoted by letter "S" and shows in purple colour. From the concentration 0.8 µg/ml to 3.2 µg/ml, Riboflavin-Isoniazid Conjugate does not show activity, i.e., the microbial growth is occurred in this concentration denoted by letter "R" and shows in light pink colour. Results of Anti-TB activity are given in fig. 4

CONCLUSION

Riboflavin vitamin was chosen for conjugation with the isoniazid drug due to its importance in cell growth and function. The compound was docked to 6TEV's 3D structure using AutoDock, and compounds were synthesized with satisfactory yield. Purity was evaluated using melting point, TLC, and recrystallization in ethanol. The compounds were characterized using Infrared, Nuclear magnetic resonance, and Mass spectrometry. The synthesized molecule Riboflavin-isoniazid effectively prevents mycolic acid production in bacterial cell walls. Its binding affinity is higher towards protein, allowing it to easily carry the drug isoniazid to the infected site. Riboflavin-Isoniazid Conjugate exhibited a MIC value of 6.2 µg/mL, indicating a potent level of inhibitory activity against the M. tuberculosis H37Rv strain. Further study of the Structure Activity Relationship of this compound will provide new insights into pharmacokinetic and antioxidant studies.

Acknowledgments

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