

## **Reviewer 2**

The manuscript presents the design, synthesis, and biological evaluation of novel hybrid compounds with potential antitubercular activity. The topic is relevant and aligns well with the scope of the journal, particularly in the context of developing new therapeutic strategies against *Mycobacterium tuberculosis*. The study combines synthetic chemistry with biological assays, which is a strength. However, several important issues should be addressed before the manuscript can be considered for publication.

1. First, the abstract requires clarification. The acronym “INH” should be explicitly defined upon first use to ensure accessibility to a broader scientific audience. While this may seem minor, clarity at this level is essential for proper comprehension and indexing.

**Response: The abstract has been revised accordingly, the acronym “INH” has now been explicitly defined as *isoniazid (INH)* at its first occurrence in the abstract.**

2. Second, the cytotoxicity results raise important concerns regarding the therapeutic window of the proposed compounds. The authors report that the hybrid molecules are non-cytotoxic at low concentrations but exhibit cytotoxic effects at higher concentrations. However, the manuscript does not adequately relate these findings to the concentrations required for antitubercular activity. A critical discussion is needed to determine whether the effective antimycobacterial concentrations fall within a safe, non-cytotoxic range. Without this comparison, it is difficult to assess the true pharmacological potential of the compounds. Including selectivity index (SI) values would significantly strengthen the biological evaluation.

**Response: We agree with the reviewer that our original discussion did not address whether the effective antimycobacterial concentrations fall within a safe, non-cytotoxic range. In line with the reviewer’s recommendation, we have now modified Table 2, which presents the selectivity index (SI), thus providing a comparative measure of cytotoxicity versus antimycobacterial activity.**

3. Third, the mechanistic rationale requires deeper analysis. The antitubercular activity of isoniazid (INH) is known to depend on its activation by the KatG enzyme and its subsequent inhibition of the enoyl-acyl carrier protein reductase (InhA). Resistance mechanisms frequently involve mutations in either *katG* or *inhA*. In this context, the manuscript does not sufficiently address how the synthesized hybrid compounds interact with these key enzymatic targets. It remains unclear whether these molecules retain dependence on KatG activation, directly inhibit InhA, or potentially bypass classical resistance mechanisms. The inclusion of molecular docking, enzymatic inhibition assays, or discussion supported by literature would greatly enhance the mechanistic understanding and impact of the study.

**Response: We agree with the reviewer that our original manuscript did not address the interactions between the compounds and the key enzymatic targets associated with isoniazid activity. Investigating this aspect—for example, by determining MIC values in strains overexpressing InhA, would provide valuable insight into whether these molecular hybrids operate via similar mechanisms. We feel that such studies would require substantial additional experimental work beyond the scope of the current study. However, based on the MIC values of some of the compounds in clinical strains with mutations in KatG (Table 2), we provide evidence that the hybrids are likely dependent on KatG.**

4. Additionally, the manuscript would benefit from improvements in the discussion section. The current version is largely descriptive and does not critically compare the obtained results with previously reported compounds. A more in-depth structure–activity relationship (SAR) analysis is recommended to highlight the advantages or limitations of the hybrid design.

**Response:** The discussion section has been revised to include a more in-depth SAR analysis with direct comparison to previously reported Isoniazid-based hydrazone derivatives. The updated version now critically evaluates the influence of electronic effects, substitution patterns, and the role of the INH moiety, highlighting both the advantages (enhanced potency in series 6, surpassing INH) and limitations (reduced activity in series 4 without INH) of the hybrid design.

5. From a methodological perspective, the experimental section appears generally adequate, but more detail is needed in the biological assays to ensure reproducibility. Information regarding controls, number of replicates, and statistical analysis should be clearly stated.

**Response:** As described previously, the cytotoxicity of the five most active compounds was evaluated in THP-1 cells using the resazurin assay. Untreated cells and cells exposed to the compound solvent, dimethyl sulfoxide (DMSO), served as viability controls (i.e., non-cytotoxic conditions), while 20% sodium dodecyl sulfate (SDS) was used as a cytotoxic control. Isoniazid (INH) was included as an internal reference compound, and rifabutin (RFB) was employed as an additional control in cytotoxicity assays. All experiments were performed in duplicate and independently repeated three times. Dose–response curves were generated, where applicable, and analyzed by nonlinear regression using GraphPad Prism 10.

6. Finally, the English language is generally understandable but would benefit from careful editing to improve grammar, sentence structure, and overall readability, particularly in the discussion section.

**Response:** Thank you. The entire manuscript has been revised thoroughly.