Novel Antitubercular Derivatives of Mannich and Schiff Bases of p-Aminosalicylic Acid – Rational Design

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Authors’ contributions

This work was carried out in collaboration among all authors. Author NBM designed the study, performed computational studies, bench work, wrote the protocol, and wrote the first draft of the manuscript. Authors SD and VSS guided throughout the research, managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The aim of the current study is to utilize computational drug design resources to develop and identify promising structural analogs of p-aminosalicylic acid (PAS), to improvise antitubercular activity followed by their synthesis, characterization, and in-vitro biological activity determination.

Study Design: Design of the structural analogs of PAS by functional group modification at -COOH and -NH₂ groups followed by in-silico prediction of biological activity, toxicity, drug-likeness filters, and molecular docking study to select promising analogs. Synthesis of selected analogs, structural characterization, and screening of biological activity of the same.

Methodology: Using the Prediction of Activity Spectrum of Substances (PASS Online) database, prediction of biological activity was performed for PAS and 22 designed structural analogs of PAS. All these analogs were screened for their drug-likeness properties using Lipinski’s rule of five. Later all these 22 analogs were predicted for their toxicities using OSIRIS Property Explorer. At this

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1. INTRODUCTION

Tuberculosis (Tb) is a venerable disease for ages unknown. Even today, there is no specific drug for the treatment and cure. Due to the discovery of several antibiotics and antibacterial drugs, the drug discovery on antitubercular drugs found poor significance. Since 1983 there is a steep fall in the emergence of approved drugs for Tb treatment [1,2]. PAS was documented to have 6% [3,4] of Mycobacterium tuberculosis (MTb) resistance that is minimal to any first-line Tb drugs. Clinically, monotherapy of PAS for Tb was also limited in the past 4-6 decades [2]. In general, the process involved in drug discovery is long process, expensive, time consuming, and challenging too. The computational drug discovery tools will help in optimizing this long, expensive, and time-consuming process and act as one of the reliable virtual shortcuts in today's research and development [5]. In this work, with the help of computational drug discovery tools, we had tried to synthesize structural analogs of a forgotten or second-line antitubercular drug, p-aminosalicylic acid (PAS), to improvise the potency by incorporating functional group modification concept on the free carboxylic acid group and the free amino group. This lead modification resulted in a total of 22 structural analogs among which 11 were N-acetyl derivatives with Schiff bases (methylidene hydrazides) and 11 were Mannich bases (imidazolyl methylamines) [6-9] with Schiff bases (methylidene hydrazides) (Fig. 1). Instead of opting for direct synthesis of the designed structural analogs without knowing whether they have the activity or not, these derivatives were subjected to predict for their possible biological activity, drug-likeness, and toxicity. Finally, the screened and selected analogs were taken for molecular docking studies with the target, dihydropteroate synthase-I enzyme (DHPS-I), which was downloaded from Protein Databank (PDB) website with PDB ID: 1EYE. Through molecular docking, binding energy for the selected structural analogs was calculated using AutoDock Tools and AutoDock Vina software. Structural analogs-target interactions were visualized through Discovery Studio Visualizer. With the support of these in-silico computational drug discovery tools, the finally selected structural analogs of PAS were synthesized and were characterized using infrared (IR), nuclear magnetic resonance (NMR), and mass spectral techniques. Later the characterized analogs were screened for their antitubercular activity using MABA.

2. MATERIALS AND METHODS

For sketching chemical structures of PAS and structural derivatives, ChemSketch software was employed. For the prediction of activity, the PASS Online web server had been utilized [10,11]. OSIRIS property explorer application was used for the prediction of toxicity [12-14] of the designed structural analogs. The prediction of activity and toxicity will be carried out by sketching the chemical structure in the window provided in the respective applications. Open Babel GUI used for converting .mol file to .pdb file to take up for molecular docking. Discovery Studio Visualizer, AutoDock Tools, and AutoDock Vina served the purpose of molecular
Fig. 1. Functional group modification on carboxylic acid and amino groups of PAS to design analogs. Extensive modification was carried out to design structural analogs. All the reagents and chemicals used in the current work were purchased from Sigma Aldrich. The method employed to determine melting point was an open capillary method and was uncorrected. To monitor the reactions, thin-layer chromatography (TLC) was performed. The Potassium bromide pellet (KBr) technique was used for recording IR spectra. Bruker 400 MHz NMR spectrometer was used for 1H NMR and 13C NMR spectra with DMSO-d6 as solvent. Shimadzu mass spectrometer was used to record mass spectrum using electrospray ionization technique in positive mode. MABA method was selected to carry out an antitubercular assay for the synthesized and characterized structural analogs of PAS.

2.1 General Procedure for Molecular Docking

To carry out molecular docking, AutoDock Tools (AutoDock 4.2) and AutoDock Vina [16-24] served the purpose. Supporting software and applications include, ChemSketch, Discovery Studio Visualizer, and Open Babel GUI were employed to prepare and complete the entire process.
2.2 Synthetic Procedures

Dissolve PAS, 1, 0.01 mol in 20 ml of methanol, add 0.01 mol of imidazole, 2, and 2 ml of 37% formaldehyde were added. For 5 hours, the reaction mixture was stirred at room temperature. Add distilled water in excess to the reaction mixture and left overnight. The formed solid was filtered and recrystallized using methanol and dried [26], resulted to give a sepia yellow product, 3.

2.2.2 Synthesis of acid hydrazide from Mannich base derivative of PAS (5)

A solution of 3, 0.01 mol, Vilsmeyer reagent, 0.01 mol, and trimethylamine, 0.03 mol in 100 ml of dichloromethane was prepared and added to the solution containing 0.04 mol hydrazine hydrate, 4, in 50 ml of dichloromethane. The formed reaction mixture was stirred for 7 hours at room temperature. Wash the mixture with 150 ml of saturated sodium bicarbonate followed by 150 ml of brine and collect the organic layer. The organic layer was dried using anhydrous sodium sulfate. The resulted product was filtered and recrystallized with 95% ethyl alcohol [27] to get a rust yellow product.

2.2.3 Synthesis of Mannich base containing methyldiene hydrazide derivatives (7a-f)

Transfer 0.002 mol of 5 and 25 ml of ethyl alcohol into a round-bottomed flask. Add a solution of aldehydes (6a-f), 0.002 mol in 5 ml of ethanol each was added individually in presence of 2-3 drops of acetic acid. Reflux the contents under the water condenser for 4 hours using a water bath. Cool the reaction mixture and pour the contents on crushed ice. Filter the formed products (7a-f) and recrystallized with 95% ethyl alcohol [28].

2.3 In-vitro Antitubercular Activity

Microplate Alamar Blue Assay method was preferred to carry out antitubercular activity for the synthesized structural analogs [15,29-32]. By using MABA, the minimum inhibitory concentration (MIC) of structural analogs of PAS was determined. In this in-vitro assay, non-virulent vaccine strain, H37 RV strain, of Mycobacterium tuberculosis was employed using a sterilized 96-well plate. 200 µl of sterilized water was added to all peripheral walls to avoid desiccation during incubation. Middlebrook 7H9 broth stock, 100 µl was added to the microplate followed by serial dilutions of structural analogs and standards on the plate directly. To the wells containing analogs, add 100 µl of MTb suspension to make the final volume to 200 µl. The final concentrations of the test compounds were made to be within the range from 100 to 0.8 µg/ml. The plates were marked, sealed with paraffin, and incubated for 5 days at 37 °C. After incubating, 25 µl of 1:1 mixture of Alamar Blue reagent and 10% of Tween 80 were added into the wells of the plate and made to incubate for 24 hours. Finally, based on visual color change, the readings were recorded. Bacterial growth was indicated by the pink color in the well and the blue color indicates no bacterial growth [32].
3. RESULTS AND DISCUSSION

3.1 Prediction of Activity

Prediction of biological activity of the designed structural analogs was carried out using PASSOnline. The probability of being active (Pa) value for all 22 structural analogs showed superior antituberculosis or antituberculosic activity than PAS, the lead molecule as their primary activity (Table 1).

3.2 Prediction of Drug-likeness

For the determination of drug-likeness properties of the designed structural analogs, www.swissadme.ch web resource was used. In this web page, chemical structures of the analogs were sketched and converted to Simplified Molecular Input Line Entry System (SMILES) notation to obtain the results. Lipinski’s rule of five drug-likeness filters was used to screen the analogs. All the 22 structural analogs passed drug-likeness filters (Table 2).

Following are the filters of Lipinski’s Rule of Five:

- Molecular weight ≤ 500
- MLogP ≤ 4.15
- Number of hydrogen bond donors ≤ 10
- Number of hydrogen bond acceptors ≤ 5

3.3 In-silico Prediction of Toxicity

The prediction of toxicity for the designed structural analogs was performed using the OSIRIS property explorer application. Toxicities predicted include tumorigenic, mutagenic, irritant properties, and reproductive effects. Out of 22 analogs, analogs ranging from 12-18 and 20-22 showed no indication of toxicities (Table 3). Analogs from 1-11 including PAS showed medium to high risk of toxicities.

3.4 Molecular Docking Studies

Molecular docking was performed for the screened and selected 6 structural analogs, 12, 13, 15, 17, 18, and 22 (during synthesis labelled as 7a-7f respectively) with the drug target, DHPS-I obtained from protein data bank with PDB ID: 1EYE. All the 6 structural analogs...
showed better binding energy than the lead molecule PAS. The binding energy of PAS was found to be -5.5 Kcal/mol and the binding energy of the structural analogs falls within the range of -7.0 to -7.9 Kcal/mol. This indicates the optimized structural analogs are having high binding affinity to DHPS-I than the lead molecule, PAS (Table 4). The protein-ligand binding interactions with the interacting amino acids were given in Table 4 and the best binding poses were given in Figs. 3 and 4. After functional group modification, the number of interacting amino acids of the target also increased with each structural analog than PAS indicating improved binding.

### Table 1. Predicted antitubercular activity of designed structural analogs using PASSOnline

| Derivatives | Pa Value | Derivatives | Pa Value |
|-------------|----------|-------------|----------|
| PAS         | 0.612    | 12          | 0.857    |
| 1           | 0.894    | 13          | 0.853    |
| 2           | 0.891    | 14          | 0.858    |
| 3           | 0.897    | 15          | 0.854    |
| 4           | 0.877    | 16          | 0.818    |
| 5           | 0.838    | 17          | 0.860    |
| 6           | 0.876    | 18          | 0.901    |
| 7           | 0.937    | 19          | 0.911    |
| 8           | 0.938    | 20          | 0.778    |
| 9           | 0.796    | 21          | 0.685    |
| 10          | 0.759    | 22          | 0.889    |
| 11          | 0.932    |             |          |

### Table 2. Drug-likeness properties of designed structural analogs of PAS

| Derivatives | Molecular Weight | MLogP* | Hydrogen Bond Acceptors | Hydrogen Bond Donors | Drug-likeness Filter |
|-------------|------------------|--------|-------------------------|----------------------|----------------------|
| PAS         | 153.14           | -0.70  | 3                       | 3                    | Passed               |
| 1           | 297.31           | 1.74   | 4                       | 3                    | Passed               |
| 2           | 313.31           | 1.21   | 5                       | 4                    | Passed               |
| 3           | 311.34           | 1.98   | 4                       | 3                    | Passed               |
| 4           | 331.75           | 2.25   | 4                       | 3                    | Passed               |
| 5           | 315.30           | 2.13   | 5                       | 3                    | Passed               |
| 6           | 327.33           | 1.45   | 5                       | 3                    | Passed               |
| 7           | 342.31           | 0.85   | 6                       | 3                    | Passed               |
| 8           | 287.27           | 0.08   | 5                       | 3                    | Passed               |
| 9           | 303.34           | 0.88   | 4                       | 3                    | Passed               |
| 10          | 286.29           | 0.08   | 4                       | 4                    | Passed               |
| 11          | 298.29           | 0.30   | 5                       | 3                    | Passed               |
| 12          | 335.36           | 1.33   | 4                       | 3                    | Passed               |
| 13          | 351.35           | 0.80   | 5                       | 4                    | Passed               |
| 14          | 349.38           | 1.56   | 4                       | 3                    | Passed               |
| 15          | 369.80           | 1.83   | 4                       | 3                    | Passed               |
| 16          | 353.35           | 1.71   | 5                       | 3                    | Passed               |
| 17          | 365.38           | 1.04   | 5                       | 3                    | Passed               |
| 18          | 380.35           | 0.47   | 6                       | 3                    | Passed               |
| 19          | 325.32           | 0.09   | 5                       | 3                    | Passed               |
| 20          | 341.38           | 0.88   | 4                       | 3                    | Passed               |
| 21          | 324.33           | 0.09   | 4                       | 4                    | Passed               |
| 22          | 336.34           | 0.30   | 5                       | 3                    | Passed               |

*MLogP: Lipophilicity
### Table 3. Toxicity risk prediction of designed compounds using OSIRIS property explorer

| Derivatives | Toxic Risk Mutagenic | Tumorigenic | Irritant | Reproductive |
|-------------|----------------------|-------------|----------|--------------|
| PAS         | High                 | High        | High     | Medium       |
| 1           | Medium               | Medium      | High     | No indication |
| 2           | Medium               | Medium      | High     | No indication |
| 3           | Medium               | Medium      | High     | No indication |
| 4           | Medium               | Medium      | High     | No indication |
| 5           | Medium               | Medium      | High     | No indication |
| 6           | Medium               | Medium      | High     | No indication |
| 7           | Medium               | Medium      | High     | No indication |
| 8           | Medium               | Medium      | High     | No indication |
| 9           | Medium               | Medium      | High     | No indication |
| 10          | Medium               | Medium      | High     | No indication |
| 11          | Medium               | Medium      | High     | No indication |
| 12          | No indication        | No indication | No indication | No indication |
| 13          | No indication        | No indication | No indication | No indication |
| 14          | No indication        | No indication | No indication | No indication |
| 15          | No indication        | No indication | No indication | No indication |
| 16          | No indication        | No indication | No indication | No indication |
| 17          | No indication        | No indication | No indication | No indication |
| 18          | No indication        | No indication | No indication | No indication |
| 19          | High                 | No indication | No indication | No indication |
| 20          | No indication        | No indication | No indication | No indication |
| 21          | No indication        | No indication | No indication | No indication |
| 22          | No indication        | No indication | No indication | No indication |

### Table 4. AutoDock Vina binding energies of selected structural analogs of PAS (7a-f) with 1EYE as target

| Derivatives | Synthetic Analog Code | Binding Energy (Kcal/mol) | Definitions of Binding Site |
|-------------|-----------------------|---------------------------|-----------------------------|
| PAS         | PAS                   | -5.5                      | ASN A:189, LYS A:213, ARG A:214, PHE A:215 |
| 12          | 7a                    | -7.0                      | ASN A:189, LYS A:213, ARG A:214, ARG A:215, ARG A:233 |
| 13          | 7b                    | -7.8                      | ASP A:21, GLY A:50, ASP A:86, MET A:88, VAL A:107, PHE A:182, LYS A:213, ARG A:253, HIS A:255 |
| 15          | 7c                    | -7.6                      | ASP A:21, ASP A:86, MET A:88, VAL A:107, HIS A:141, PHE A:182, LYS A:213, ARG A:253, HIS A:255 |
| 17          | 7d                    | -7.7                      | ASP A:21, GLY A:50, GLU A:65, ASP A:86, MET A:88, VAL A:107, HIS A:141, PHE A:182, LYS A:213, ARG A:253, HIS A:255 |
| 18          | 7e                    | -7.9                      | VAL A:11, ASP A:21, HIS A:141, THR A:185, ALA A:186, LYS A:213, ARG A:214, ARG A:253, HIS A:255 |
| 22          | 7f                    | -7.5                      | VAL A:11, ASP A:21, GLY A:181, LYS A:213, ARG A:214, ARG A:253, HIS A:255 |
Fig. 3. 2D binding poses of 7a-7f, structural analogs of PAS along with bound amino acids

3.5 Spectral Data

**Compound 7a**

Yield: 81.2%, m.p. (°C): 230-232, Solid and Golden yellow, IR (KBr) cm⁻¹: 3325, 3050, 2948, 1621, 1585, 1483, 1377, 1102; ¹H NMR (400 MHz, DMSO-d₆) δ 12.40 (s, 1H), 11.57 (s, 1H), 7.93 (t, J = 1.7 Hz, 1H), 7.79 (t, J = 3.9 Hz, 1H), 7.72 (d, J = 8.1 Hz, 1H), 7.67 – 7.58 (m, 2H), 7.49 – 7.37 (m, 4H), 7.30 (dd, J = 4.0, 1.8 Hz, 1H), 7.06 (dd, J = 4.0, 1.6 Hz, 1H), 6.18 (dd, J = 8.3, 1.8 Hz, 1H), 6.13 (d, J = 1.9 Hz, 1H), 5.49 (d, J = 3.8 Hz, 2H); ¹³C NMR (400 MHz, DMSO-d₆) δ 163.64, 160.41, 149.89, 147.58, 135.51, 134.14, 131.05, 130.31, 130.19, 128.75, 127.62, 117.79, 112.31, 104.70, 100.01, 57.30; Mass: (M+1) - 336.25

**Compound 7b**

Yield: 84.8%, m.p. (°C): 246-248, Solid and Marigold orange, IR (KBr) cm⁻¹: 3326, 3046, 2854, 1618, 1573, 1483, 1432, 1374, 1103; ¹H NMR (400 MHz, DMSO-d₆) δ 12.83 (s, 1H), 12.32 (s, 1H), 11.57 (s, 1H), 7.93 (t, J = 1.7 Hz, 1H), 7.79 (t, J = 3.9 Hz, 1H), 7.72 (d, J = 8.2 Hz, 1H), 7.48 (dd, J = 7.4, 1.5 Hz, 1H), 7.42 (s, 1H), 7.34 – 7.25 (m, 2H), 7.06 (dd, J = 4.0, 1.6 Hz, 1H), 6.99 – 6.88 (m, 2H), 6.18 (dd, J = 8.2, 1.8 Hz, 1H), 5.49 (d, J = 3.8 Hz, 2H); ¹³C NMR (400 MHz, DMSO-d₆) δ 163.64, 160.41, 149.89, 147.58, 135.51, 134.14, 131.05, 130.31, 130.19, 128.75, 127.62, 117.79, 112.31, 104.70, 100.01, 57.30; Mass: (M+1) - 336.25
Hz, 1H), 6.13 (d, J = 1.9 Hz, 1H), 5.49 (d, J = 3.8 Hz, 2H); $^{13}$C NMR (400 MHz, DMSO-$d_6$) δ 163.63, 160.41, 157.82, 149.89, 146.55, 135.51, 132.65, 131.05, 130.29 (d, J = 19.4 Hz), 120.98, 117.79, 117.11, 115.14, 112.69, 104.70, 100.01, 57.30; Mass: (M+1) – 352.42

**Compound 7c**

**Yield:** 79.2%, m.p. 163.63, 160.41, 157.82, 149.89, 146.55, 135.51, 132.65, 131.05, 130.29 (d, J = 19.4 Hz), 120.98, 117.79, 117.11, 115.14, 112.69, 104.70, 100.01, 57.30; Mass: (M+1) – 352.42

**Compound 7d**

**Yield:** 82.7%, m.p. (8C): 241-243, Solid and Butterscotch yellow, IR (KBr) cm$^{-1}$: 3370, 3113, 2968, 2840, 1622, 1602, 1508, 1440, 1379, 1251, 1109, 1024; $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.39 (s, 1H), 11.57 (s, 1H), 7.93 (t, J = 1.7 Hz, 1H), 7.79 (t, J = 3.9 Hz, 1H), 7.72 (d, J = 8.2 Hz, 1H), 7.62 (m, 2H), 7.55 – 7.47 (m, 3H), 7.30 (dd, J = 4.0, 1.8 Hz, 1H), 7.09 – 7.03 (m, 1H), 7.03 – 6.98 (m, 2H), 6.18 (dd, J = 8.3, 1.8 Hz, 1H), 6.13 (d, J = 1.9 Hz, 1H), 5.49 (d, J = 3.8 Hz, 2H); $^{13}$C NMR (400 MHz, DMSO-$d_6$) δ 163.64, 160.41, 149.89, 147.59, 136.63, 135.51, 131.79, 131.05, 130.19, 129.06, 128.65, 117.79, 114.32, 112.31, 104.70, 100.01, 57.30, 55.33; Mass: (M+1) – 366.22

![Fig. 4. 3D binding poses of 7a-7f, structural analogs of PAS along with bound amino acids](image-url)
Table 5. Drug-likeness properties of designed structural analogs of PAS

| Compound | X | R¹ | R² | R³ | MIC Values (µg/ml) |
|----------|---|----|----|----|-------------------|
| PAS      |   |    |    |    | 6.25              |
| 7a       | C | -H | -H | -H | 12.5              |
| 7b       | C | -OH| -H | -H | 3.125             |
| 7c       | C | -H | -H | -Cl| 3.125             |
| 7d       | C | -H | -H | -OCH₃| 3.125          |
| 7e       | C | -H | -NO₂| -H| 1.6              |
| 7f       | N | -H | -H | ----| 3.125             |
| Streptomycin | | | | | 6.25              |
| Ciprofloxacin | | | | | 3.125             |
| Pyrazinamide | | | | | 3.125             |

3.6 In-vitro Antitubercular Activity (MABA)

The in-vitro antitubercular assay was performed on MTb non-virulent strain for the estimation of MIC values with streptomycin, ciprofloxacin, and pyrazinamide as standard drugs [33,34]. Based on the results of MABA, the MIC of PAS was found to be 6.25 µg/ml. The MIC values of synthesized structural analogs of PAS, 7a-f, were found to fall between 1.6 – 12.5 µg/ml (Table 5). Out of all synthesized analogs, 7e was effective at 1.6 µg/ml; 7b, 7c, 7d, and 7f were effective at 3.125 µg/ml; 7a was effective at 12.5 µg/ml, hence the structural analogs, 7b-f were found to have improved MIC values than PAS. MIC values of standards were also compared with the structural analogs to understand the potency of antitubercular activity. Streptomycin (MIC: 6.25 µg/ml), ciprofloxacin (MIC: 3.125 µg/ml), and pyrazinamide (MIC: 3.125 µg/ml) as standard drugs were compared with that of the structural analogs. Based on these results, except 7a, remaining all structural analogs were potent enough when compared with the standard drugs.
4. CONCLUSION

Using functional group modification technique, the free carboxylic acid and free amino groups were modified into Mannich base and Schiff’s base respectively to improve the antitubercular activity of PAS by improving drug-target interactions. Based on this, 22 analogs were designed and screened for their activity, drug-likeness, and toxicity using computational drug discovery tools, which resulted in the selection of 6 analogs to go with molecular docking, synthesis, characterization, and activity screening. Molecular docking results showed an improved binding affinity for the structural analogs than PAS. In-vitro antitubercular assay showed 7e analog is more potent than the standard drugs and PAS with MIC of 1.6 µg/ml; 7b, 7c, 7d, and 7f analogs are equipotent as standard drugs with MIC of 3.125 µg/ml and more potent than PAS; 7a is less potent than standard drugs and PAS. Based on the results, it can be concluded that the application of computational tools in the drug discovery and design process in the field of Medicinal Chemistry proved to be effective in the screening and selection of promising structural analogs.

CONSENT AND ETHICAL APPROVAL

Not applicable for this study.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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