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Aromatic Hydrazide Compounds that Inhibit the Growth of Mycobacterium Tuberculosis

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Aromatic Hydrazide Compounds That Inhibit the Growth of Mycobacterium tuberculosis

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Abstract

Aims: To demonstrate the efficacy of aromatic hydrazide compounds to inhibit growth of *Mycobacterium tuberculosis*.

Study Design: To synthesize tuberculostats and test their antibacterial activity *in-vitro*.

Place and Duration of Study: University of Nebraska, Durham Science Center, 6001 Dodge Street, Omaha NE 68182, and Texas A&M Health Science Center, Department of Microbial Pathogenesis and Immunology, 8447 State Hwy 47, Medical Research and Education Building, Room #3012, Bryan, TX 7780. From March 2019 to October 2019.

Methodology: Hydrazide functional groups were formed by covalently bonding hydrazine onto a carbonyl carbon that is a substituent of a single aromatic ring. Microwave excitation was utilized for synthesis, followed by evaluation of antibacterial activity. These compounds were placed into tissue culture media at various concentrations and then *Mycobacterium tuberculosis* bacteria was added, in order to determine the level of growth inhibition. Growth inhibition of the bacteria was measured as a function of compound concentration versus growth inhibition.

Results: Compounds A, B, C, and D carry hydrazide groups as a substituent to a single aromatic ring. All four compounds show zero violations of Rule of 5, indicating favorable drug-likeness. All four compounds showed greater than 50% growth inhibition of bacteria at concentrations below 50 micrograms per milliliter. Growth inhibition was measured by colony forming units and luminescence. Polar surface area, Log P, molecular volume, and other molecular properties were determined for these four compounds.

Conclusion: These four hydrazide compounds induced substantial inhibition of bacterial growth. Microwave excitation for the synthesis of hydrazide compounds is effective. These compounds have favorable drug-likeness properties and are highly effective inhibiting the growth of *Mycobacterium tuberculosis*.

Keywords:

Tuberculosis, TB, hydrazide, Mycobacterium tuberculosis, tuberculostat.

ABBREVIATIONS

MDR-TB: Multidrug-resistant Mycobacterium tuberculosis; XDR-TB: Extensively Drug-resistant Mycobacterium tuberculosis; INH: Isoniazid; RIF: Rifampin; EMB: Ethambutol; SM: Streptomycin; PZA: Pyrazinamide.

1. INTRODUCTION

The bacteria *Mycobacterium tuberculosis* is the causative agent of tuberculosis (TB), which according to the World Health Organization there were 10 million new cases of active TB in 2017 [1]. This disease is considered a global infectious threat that is exacerbated by the growing incidence of drug-resistant forms [1]. The Multidrug-resistant TB (MDR-TB), defined by resistance to both isoniazid and rifampicin, showed 490,000 new cases in 2016 [1]. The bacteria causing TB has the capacity for dormancy, with a long generation time and a low metabolic activity profile that makes it a difficult therapeutic target [2]. The bacteria can locate in empyema pus, solid caseous material, and pulmonary cavities that make penetration of antibiotics very difficult, and/or sites of low pH that inhibit the activity of some antibiotics (i.e. aminoglycosides) [2]. The first-line antibiotic isoniazid is vital in the early stage of therapy, due to its rapid reduction of sputum associated pathogens because it is active against the bacteria growing aerobically in pulmonary cavities [2].

Drug-resistant forms of TB is an escalating global health crisis, with the appearance of extensively drug-resistant TB (XDR-TB) that resist both the fluoroquinolones group and second-line injectable agents [3]. The control and management of these resistant forms of TB are problematic due to the long duration of therapy, high costs, and various debilitating effects of second-line drugs [3]. First-line drugs include isoniazid (INH), rifampin (RIF), ethambutol (EMB), streptomycin (SM), and pyrazinamide (PZA) [2,3]. The presence of drug degrading and modifying enzymes, along with a thick, waxy, hydrophobic cell envelope is believed to contribute to the drug resistance by this bacterium [4]. Some investigators contend that up to one-third of the world's population is infected with *Mycobacterium tuberculosis*, causing up to two million deaths per year [5].

Some estimates determine that 480 000 people develop MDR-TB tuberculosis each year [6]. Studies have shown that in 2013, only 20% of these individuals received recommended second-line treatment regimens, but only approximately 50% of the people that did receive such drug regimens had successful treatment outcomes [6]. However, access to new or repurposed drugs is linked with substantial challenges for the majority of patients in environments with high burdens of MDR-TB [6]. Novel drug designs that incorporate a novel alteration of proven drug carriers for the hydrazide functional group have shown tremendous success in tests against *Mycobacterium tuberculosis* [7,8,9]. The rational drug design leading to favorable structure alterations that preserve favorable drug-likeness but are very successful in suppressing the growth of methicillin-resistant *Staphylococcus aureus* [10,11,12]. Similarly, this approach for drug design has produced very successful drug designs for suppressing the growth of ampicillin-resistant *Escherichia coli* [13,14,15,16]. This approach is applied to this study, in introducing four novel aromatic hydrazide compounds that inhibit the growth of *Mycobacterium tuberculosis*.

2. METHODOLOGY

2.1 Reagents and Instrumentation

All reagents we reobtained from Aldrich-Sigma Company (P.O. Box 2060, Milwaukee, WI 53201 USA). Infrared spectroscopy can be accomplished in a Mattson Galaxy FTIR in a dimethyl sulfoxide solvent that has been previously dried over molecular sieves to remove water prior to obtaining spectra.

2.2 Molecular Modeling and Numerical Analysis

Molecular modeling (2-D and 3-D) were accomplished by ChemSketch version 12.01 ACD/Labs Release: 12.00 (90 Adelaide Street West, Toronto, Ontario M5H V9, Canada). Molecular properties were determined by utilizing Moinspiration (Liscie Udolie 2, SK-841 04 Bratislava, Slovak Republic). Statistical analysis was accomplished by Microsoft Office Excel 2007 or PAST version 2.06 (copyright Hammer and Harper 1999-2011) and/or PAST version 2.06 (copyright Hammer and Harper 1999-2011).

2.3 Synthesis of Compounds

Prior to use, the hydrazine (NH_2NH_2) must be distilled over CaO and NaOH. The anhydrous NH_2NH_2 was collected at 113°C and was stored sealed at -20ºC. Hydrazide derivatization for all compounds were treated similarly: place 120 mg of the compound having carboxylic acid group, into a pyrex open test tube with 64 microliter of $SOCl_2$. Microwave 3 to 5 minutes (avoid over-heating as that will cause degradation of the agent), allowed to cool to room temperature. Vacuum pump removal of any unreacted thionyl chloride is recommended. Add 400 microliter of anhydrous NH_2NH_2 and microwave at 45 second intervals up to five minutes (avoid excess heating during the process) [17]. Excess hydrazine was removed by vacuum pump at room temperature or alternatively the product is washed several times in ice-cold diethyl ether. The derivatives were not heated or allowed to become wet. After thorough drying, they are stored dry in airtight containers at -20°C. The presence of the hydrazine group can be confirmed by use of FTIR, in which the Thermo Scientific Nicolet iS5 is suitable. Compounds can be easily viewed by iS5, utilizing neat film produced after removal of methylene chloride solvent. The primary peaks observing for are at 944 cm^{-1} for hydrazine and 1000 cm^{-1} to 1200 cm^{-1} for C-N stretch, and around 3000 cm^{-1} to 3500 cm^{-1} for N-H stretch. Furthermore, the presence of the hydrazide group on all final products can be confirmed by the colorimetric protocol and UV-Visible spectrophotometric analysis utilizing Gibb's reagent as described previously [18]. Assignments can be achieved in deuterated chloroform in 400 megahertz instrument of C-13 NMR(ppm): Compound A) Aromatic carbons-128.1,127.2, 127.2, 137.5, 130.1, 134; carbonyl carbon 168.7; substituent carbon 20.3; Compound B) Aromatic carbons-118.3, 124.7, 129.6, 109.3, 134, 149.1; carbonyl carbon 169.1, 168.5; compound C) Aromatic carbons-128.4, 128.4, 129.7, 129.7, 133.8, 138.6; carbonyl carbon-164.5; substituent carbon 45.1; Compound D) Aromatic carbons-117.4, 117.4, 128.2, 128.2, 133.8, 140.1; carbonyl carbon 164.6.

2.4 Bacterial Culture

2.4.1 Strain of bacteria

Mycobacterium tuberculosis strain $mc^2$7000 expressing a codon-optimized click beetle red gene (CBR) was used. The bacteria were grown to an optical density (OD) ~ 1.0 and then diluted to OD = 0.5 in media for survival assays (OD read at 600 nm wavelength).

2.4.2 Media for *In vitro* evaluation of bacterial growth inhibition

M. tuberculosis was grown in Middlebrook 7H9 supplemented with albumin dextrose complex (M-ADC, per liter, 50 grams bovine serum albumin fraction V, 20 grams dextrose, 8.5 grams NaCl,) (Difco), 0.05% Tween 80 (M-ADCTw), 100 microgram/milliliter pantothenate, and kanamycin at a final concentration of 10 µg/ml to select for plasmid maintenance in the strains.

2.4.3 *In vitro* evaluation of compounds

All compounds were dissolved to a final concentration of 5 mg/ml in aqueous solution. All compound solutions were sterilized by passage through 0.22 µm-syringe filters. Survival by Optical Density: Four clear 96-well flat-bottom plates were filled with 108 µl per well of M-ADCTw media supplemented with 10 µg/ml kanamycin to maintain luminescence in the strain. Each tested compound, as well as isoniazid, was added in duplicate wells at 72 µl per well at a final concentration of 2 mg/ml. Twofold serial dilutions were carried out six times for all compounds and isoniazid. The last row of wells was maintained without any antibiotic. A 10 µl per well of M. tuberculosis (bacteria at an equal concentration in 10 µL for all wells) was added to 96-well plates to give a final volume of 100 µl per well. Plates were incubated at 37°C for 3 days, 7 days and 14 days.

2.4.4 Measurement of survival by luminescence

Four solid white 96-well flat-bottom plates were prepared as before. Plates were incubated at 37°C for 7, and 15 days. Luminescence measurements were taken for day 7, and 15 in the presence of tested compounds. Bacterial Lumine scence was measured immediately after the addition of 20 µl of 5 mM D-luciferin in 0.45 M sodium citrate buffer pH 5.0 (Gold Biotechnology) using an EnVision (Perkin Elmer) plate reader [19]. Photon collection time was one second per well.

2.4.5 Measurement of survival by Colony Forming Units (CFU)

M. tuberculosis cultures incubated with different concentrations of compounds A-D in clear 96-well flat-bottom plates were obtained at days 3, 7 and 15. Using OD at 600 nm

as a guide for bacterial growth, *M. tuberculosis* treated with different concentrations were serially diluted in 1x PBS and plated in triplicates in volumes of 20 µl on selective MADC plates. These plates were incubated for 3 to 4 weeks for bacterial colony growth and CFU/ml was calculated for each compound concentration.

3. RESULTS AND DISCUSSION

The study and design of new drugs for contending with the presence and spread of tuberculosis worldwide is an important endeavor that deserved the utilization of substantial resources [6]. Realizing that isoniazid, having a pyridine ring with a covalently bonded hydrazide functional group (-C(=O)$NHNH_2$), is among the most effective tuberculostats and a first-line agent for the treatment of TB it is possible to generate novel constructs to enhance the favorable drug-likeness of this drug. The pharmaceutical industry faces an ever-increasing challenge to produce safer and more effective medicine [20]. New drugs not only need to be active, but also drug-like and suitable for clinical development. Drug-like properties include solubility, permeability, metabolic stability and transporter effects are of critical importance for the success of drug candidates [20]. Properties affect oral bioavailability, metabolism, clearance, toxicity, as well as in vitro pharmacology [20]. Various rules/guidelines have been developed in an attempt to improve the predictions of drug-likeness for potential drug candidates. A notable and successful set of guidelines are known as the 'rule of 5' [21]. The 'rule of 5' is based on a distribution of calculated properties among several housand drugs [21]. The 'rule of 5' states that: poor absorption or permeation is more likely when [21]: 1) There are more than 5 H-bond donors (expressed as the sum of -OH and -NHn); 2) The molecular weight is over 500; 3) The Log P is over 5; and 4) There are more than 10 H-bond acceptors (expressed as the sum of Nitrogen and Oxygen). These properties are utilized to influence the design of new structures to carry the bioactive hydrazide group.

Shown in Fig. 1 are the molecular structures of compounds A, B, C, and D. Immediately seen is the presence of the aromatic ring, onto which are the substituents affecting their properties. Compound A contains the hydrazide group (-C(=O)$NHNH_2$), an aromatic ring, and methyl (-CH_3) substituent. Compound B contains the hydrazide groups, aromatic ring, and substituent (-OC(=O)CH_3). Compound C contains an aromatic ring, hydrazide group, and substituent (-CH_2-Cl). Compound D contains the aromatic ring, hydrazide group, and nitro substituent (-NO_2).

These structures in comparison to the first-line tuberculostat isoniazid, shown in Figure 2. All compounds A, B, C, and D along with isoniazid are small molecular weight structures (molecular weight below 500 Daltons according to the 'rule of 5'). Isoniazid retains a pyridine ring and hydrazide functional group. The molecular properties such as polar surface area; Log P, etc are determined and presented in Table 1.

The molecular properties of compounds A, B, C, D, and isoniazid are shown in Table 1 for comparison. For the partition coefficient Log P, values range from hydrophilic-0.97 for isoniazid to more lipophilic 0.90 for compound C. Polar surface area ranges from

55.12 Angstroms 2 for compounds A and C, to 100.94 Angstroms 2 for compound D. The evaluation of the nine properties including number of oxygen, nitrogen, hydroxyl (-OH), and amine groups (-NHn) show that all structures have zero violations of the 'rule of 5'. This is a very favorable outcome; indicating compounds A, B, C, and D have favorable drug-likeness including good absorption/permeation.

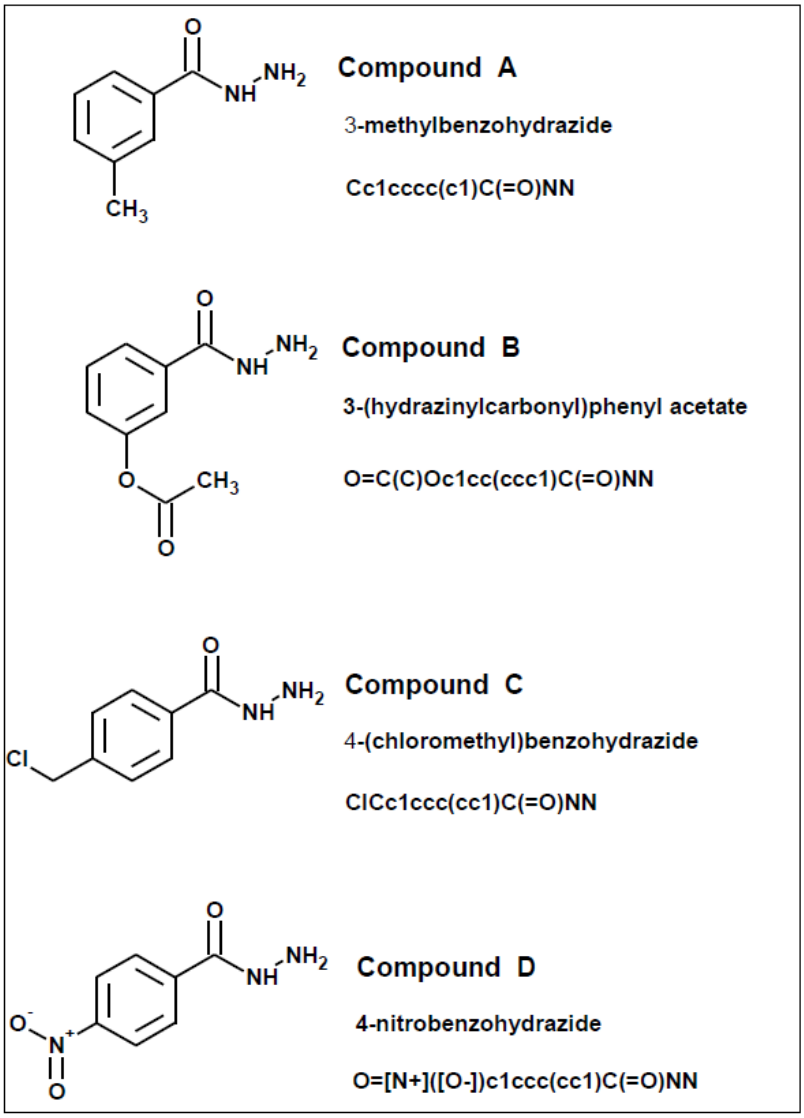

Fig. 1. Molecular structures of aromatic hydrazide compounds for growth inhibition of *Mycobacterium tuberculosis*. Each compound contains an aromatic ring and hydrazide functional group (-C(=O)NHNH$_2$). Each compound has a substituent covalently bonded to the aromatic ring

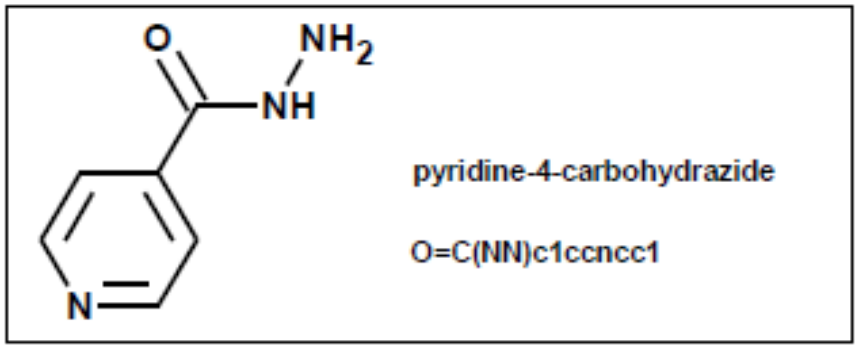

Fig.2. Molecular structure of isoniazid. This drug contains a pyridine ring in addition to the hydrazide functional group (-C(=O)NHNH2). Isoniazid is a first-line tuberculostat

Table 1. Molecular properties of compounds

Compounds	Log P	Polar surface area (Angstroms^2)	Number of atoms	Molecular weight	Number of O & N	Number Of –NH$_n$ -OH	Violations of rule of 5	Rotatable bonds	Molecular volume (Angstroms^3)
A	0.74	55.12	11	150.18	3	3	0	1	143.28
B	-0.15	81.43	14	194.19	5	3	0	3	171.25
C	0.90	55.12	12	184.63	3	3	0	2	157.06
D	0.28	100.94	13	181.15	6	3	0	2	150.05
Isoniazid	-0.97	68.01	10	137.14	4	3	0	1	122.56

In terms of statistical Pearson r correlation: polar surface area is very highly positively correlated to number of oxygen/nitrogen atoms (r = 0.9955); number of atoms are highly correlated to rotatable bonds (r = 0.9449) and molecular volume (r = 0.9160); molecular weight is highly correlated to rotatable bonds (r = 0.9321) and molecular volume (r = 0.9376).

Notably the overall molecular properties of all five drugs are very highly positively correlated to all others with Pearson r values greater than 0.9800 for all five compounds. This shows clearly that although numerical values of properties vary noticeably, overall, compounds A, B, C, and D correlate very highly to the first-line tuberculostat isoniazid. In addition, one-way ANOVA analysis for all properties for all five compounds indicate they are statistically the same (P=.99) [22,23]. This supports the contention that these five compounds are highly similar, although varied in specific molecular properties.

In addition, previous studies have identified the molecular properties contributing to successful penetration into the central nervous system and through the blood-brain barrier [24,25,26]. This bacteria can travel to the meninges, which are the membranes surrounding the brain and spinal cord resulting in a life-threatening condition known as meningeal tuberculosis [27]. Meningeal tuberculosis is also known as tubercular meningitis or TB meningitis and drugs are necessary for treatment that also effectively penetrate the central nervous system (CNS). Whereas, previous studies have shown that drugs having polar surface area less than 90 Angstroms 2 and molecular weight below 400 [24,25,26] enter the CNS; compounds A, B, C, and isoniazid fulfill this requirement. New drug designs A, B, and C would be considered effective medicaments, along with first-line isoniazid, for treatment of CNS associated TB infection.

Measurement of Mycobacterial tuberculosis growth inhibition was assayed in culture in-vitro, for compounds A, B, C, D, and isoniazid, with outcomes presented in Fig.3 (CFU/ml for bacterial viability) and Figure 4 (bioluminescence marker for bacterial activity and viability). Mycobacterial tuberculosis exposed to compounds A, B, C, D and isoniazid showed a 99% reduction in viability for concentrations less than 50 µg/ml in as little as 3 days of exposure. The bacteriostatic activity of the four new compounds were comparable to that of isoniazid (Figure 3). Using bioluminescence as a marker for bacterial activity and viability, we confirm the results observed with CFU counts showing that the four new compounds had similar efficacies to that of isoniazid in their bacteriostatic activity (Fig. 4). The outcome shows clearly that compounds A, B, C, and D are effective tuberculostats that are comparable in efficiency to isoniazid.

The efficiency of bacterial growth inhibition was determined by calculating the number of colony-forming units (CFU). The CFU unit is a measure of viable bacteria cells in a sample. Tracked over a period of day 3, day 7 and day 15, the CFU of bacteria is reduced to well below 50% survival at concentrations at less than 50 micrograms per milliliter. All four compounds A, B, C, and D significantly reduce survival at a concentration of 25 micrograms per milliliter for day 3, day 7, and day 15. Compounds A, B, C, and D function similarly to the first-line tuberculostat isoniazid. Negligible bacterial growth occurs above 50 micrograms per milliliter. The growth inhibition for all compounds measured by CFU is very strong, measurable, and consistent over the 15 days of observation. By varying the molecular structure as a carrier for the bioactive hydrazide group, but by rational design, it has clearly shown its use for producing additional tuberculostats with variation in molecular properties (i.e. Log P and polar surface area). These compounds retained favorable drug-likeness as evaluated by the ‘rule of 5’. An assemblage of new tuberculated is achieved and with retention of effective growth inhibition and drug-likeness.

Inhibition of bacterial growth as measure by luminescence (see Methodology), is tracked over a period of day 7 and day 15. The bacteria are reduced to well bout 50% survival at concentrations at 50 micrograms per milliliter. All four compounds A, B, C, and D drastically reduce survival at a concentration of 50 micrograms per milliliter for day 7 and day 15. Compounds A, B, C, and D are effective similarly to the first-line luberculostat isoniazid. Very small bacterial growth occurs above 50 micrograms per milliliter. The growth inhibition for all compounds measured by luminescence is very strong, measurable, and consistent over the 15 days of observation.

By varying the molecular structure by rational design, indicated here, is indeed an effective way to develop new potential drugs. Variation in molecular properties (i.e. Log P and polar surface area) does occur. These compounds retained favorable drug-likeness characteristics when evaluated by the ‘rule of 5’. An assemblage of new tuberculostats is achieved and with retention of effective growth inhibition of bacteria and favorable drug-likeness properties.

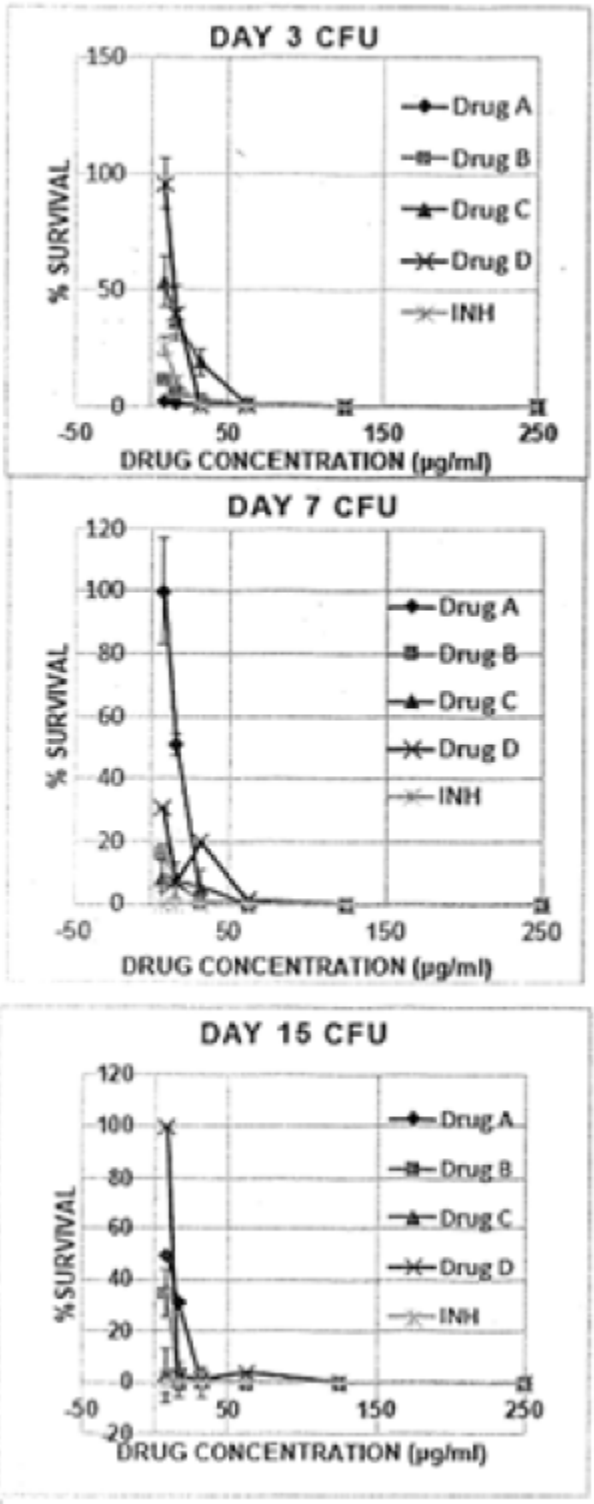

Fig. 3. Inhibition of bacterial growth is measured by colony forming units (CFU) from day 3, 7 and 15. Counting with colony-forming units counts only viable cells. Compounds A, B, C, D, as well as isoniazid (INH) reduce the percent of viable colony forming units well below 40% at concentrations below 50 micrograms/milliliter. Scale of drug concentration reads from zero to 250 micrograms/milliliter

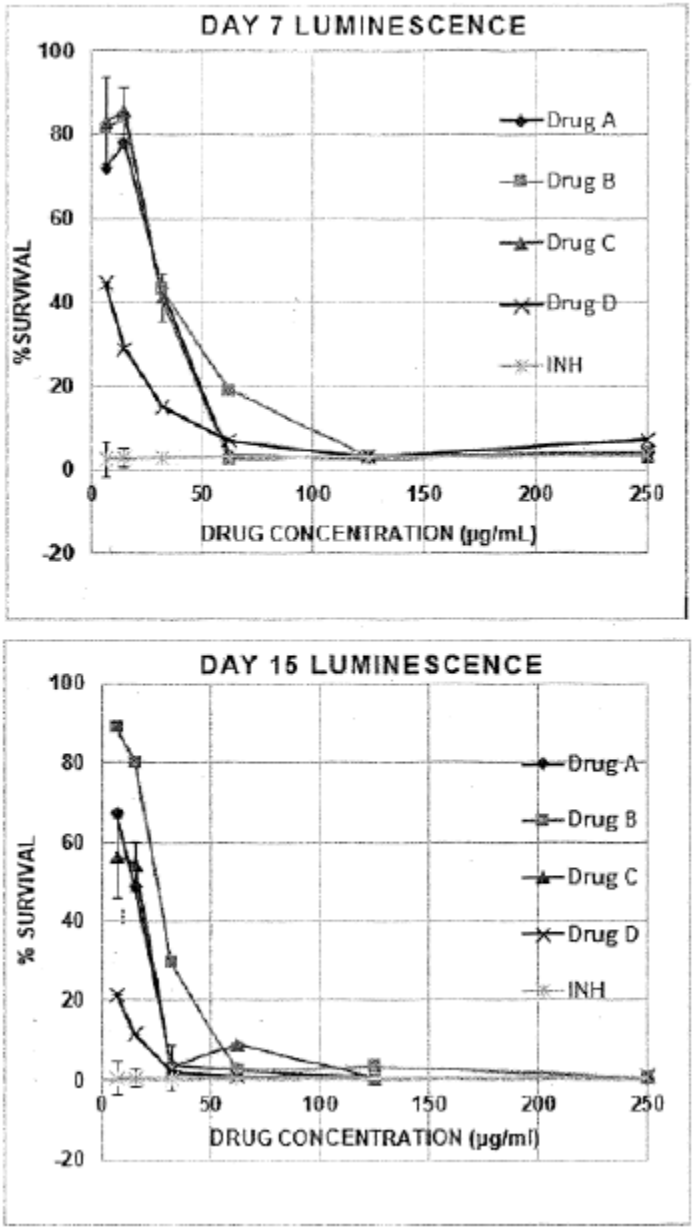

Fig. 4. Inhibition of bacterial growth by luminescence on day 7 and 15. Measured by luminescence, all compounds A, B, C, D, as well as isoniazid (INH) reduce percent survival below 50% at a concentration as low as 50 micrograms per milliliter. Scale of drug concentration reads from zero to 250 micrograms/milliliter

4. CONCLUSION

Compounds A, B, C, and D are similar to isoniazid in having the hydrazide group. For the four novel compounds presented in this study, the hydrazide group is covalently bonded to an aromatic ring having various substituents. The aromatic ring and substituents influence the molecular properties of the four compounds including drug-likeness properties influencing drug oral absorption. However, compounds A, B, C, and D show zero violations of the 'rule of 5', indicating favorable drug-likeness as well as

absorption/permeation. One-way ANOVA analysis showed compounds A, B, C, and D properties are statistically the same as first-line tuberculostat isoniazid (P=.99). Compounds A, B, and C show potential for penetration into the CNS, based on polar surface area and molecular weight. Compounds A, B, C, D, as well as isoniazid induced drastic growth inhibition at 25 micrograms per milliliter and more than 50% inhibition of growth at less than 50 micrograms per milliliter. Further studies and development of new tuberculostat drugs are necessary to fight the threat of drug resistant tuberculosis.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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