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Synthesis and initial testing of novel antimalarial and antitubercular isonicotinohydrazides

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ABSTRACT

Malaria and tuberculosis (TB) though curable and preventable, remain serious public health problems globally, with devastating consequences. Co-infection of these two deadly diseases worsens the situation and particularly makes treatment very difficult. The current mainstay for malaria treatment is gradually losing their potency due to the development of resistance. *Macobacterium tuberculosis* (MTB) has developed Multi-drug Resistance (MDR) and Extensive Drug Resistance (XDR) to current antitubercular drugs due to patient incompliance resulting from long treatment regimen. A small library of isonicotinohydrazide were synthesised by incorporating 1,2,4,5-tetraoxane and hydrazine moieties. Evaluation of the compounds gave antimalarial activities in the range 0.060 ± 0.033–0.491 ± 0.012 μM against 3D7 strain of *Plasmodium falciparum*. We assessed antimycobacterial activity of selected compounds against the standard *Mycobacterium tuberculosis* reference strain H37Rv and *M. aurum* (a non-tuberculous mycobacteria) using the microplate Alamar blue (MABA) assay and found four compounds to be very potent against H37Rv but largely inactive against *Macobacterium aurum*. We followed up to estimate the minimum inhibitory concentrations of these active compounds and tested them against clinical *M. tuberculosis* strains resistant to isoniazid (INH) and rifampicin (RIF). The MICs of the active compounds against H37Rv were between 0.003 and 0.5 mg/mL however, they were largely inactive against drug resistant clinical strains except for the INH mono-resistant resistant strain which was very active with compounds **5** and **8** possessing MICs of 0.125 mg/mL.

Introduction

TB and malaria top the list of bacterial and parasitic infections respectively in the world with the highest number of cases in Africa. There is over 250 million cases of both Malaria and TB with the global death of >2 million reported annually [1].

TB is a chronic infectious bacterial disease caused by members of the *Mycobacterium tuberculosis* complex (MTBC) [2]. TB primarily affects the lungs but may affect other parts of the body such as brain, bone, and kidney. It is transmitted from person to person *via* droplets from the throat and lungs of people with the active respiratory disease. The symptoms of active TB of the lungs are coughing, sometimes with sputum or blood, chest pains, weakness, weight loss, fever, and night sweats. In 2020, about 10 million people developed TB and 1.4 million died with Africa accounting for 25 % of the global cases [3].

Tuberculosis is treatable with a six-month course of antibiotics consisting of a combination of drugs including Isoniazid (INH), rifampicin (RMP), pyrazinamide, and ethambutol. The long multi-therapy results in poor patient compliance and promotes the emergence of drug resistance. INH has been used as the mainstay for the treatment and prophylaxis of tuberculosis for more than half a century. It interferes with the biosynthesis of mycolic acids [4] and is a pro-drug oxidatively activated *in vivo* by the *katG*-encoded mycobacterial catalase-peroxidase or metal ions to generate an isonicotinoyl radical. [5]

Malaria is a potentially life-threatening disease caused by infection with *Plasmodium* parasite transmitted by an infective female Anopheles mosquito. Symptoms of malaria include fever, headache, and vomiting, and usually appear between 10 and 15 days after the mosquito bite. In 2020, a total of 241 million malaria cases were reported globally of which an estimated 228 million were in Africa representing 95% [6].

Currently, the artemisinins [7] in combination with other antimalarials such as amodiaquine, lumefantrine, mefloquine, sulfadoxime/

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pyrimethamine and primaquine [8] are the main stay for the treatment of malaria.

The two diseases affect mainly poor countries of Africa and Asia. Both diseases share common host defense pathways and co-infection between malaria and TB have been reported to generate more severe pathology than the individual diseases [9] which might be due to diverted immune response to an anti-inflammatory response. Thus, like HIV co-infection, co-infection of TB with malaria could impair TB containment and increase morbidity and mortality of TB patients [10]. Reports from studies in experimental mice suggest that malaria co-infection have exacerbated chronic TB while rendering mice less refractory to *Plasmodium* [11–12]. Unfortunately, there is currently no single drug for the treatment of both diseases. [13–14].

Incidentally, the treatment of both malaria and TB is becoming more complicated due to the emergence of strains resistant to existing drugs. New medications are required for both diseases to address the challenge of widespread drug resistance and the complexity of current treatment regimen as well as toxicity in some cases. For example, the traditional antimalarial drugs are associated with cardiovascular toxicity (chloroquine), ocular toxicity (hydroxychloroquine), myopathy, neurotoxicity, hepatotoxicity (amodiaquine) [15]. Commonly used TB drugs on the other hand led to hepatotoxicity (Rifanpicin), Retrobulbar neuritis (ethambutol), Rhabdomyolysis (pyrazamide) and psychosis (isoniazid) [16]. While shorter, simpler, less toxic, tolerable, and fewer drug-drug interactions are required for the treatment of TB, drugs applicable to different age groups, pregnant women and for the various types of parasite are needed for malaria.

Molecular hybridization is a concept of drug design and development which involves the combination of pharmacophoric moieties of different bioactive substances to yield a new hybrid compound with improved affinity and efficacy when compared with parent drugs [17]. Various molecular hybrids of natural and synthetic origins have been developed as antifungal, antimalarial, antituberculosis, anti-inflammatory and anti-cancer agents with significant improvements in their biological activities [18]. Most of the resultant hybrid drugs demonstrate superior activity and safety profiles compared to their individual constituents.

Synthetic peroxides are designed to mimic the peroxide moiety of the naturally occurring artemisinin, a very efficient antimalarial. They possess superior biological activities and pharmacokinetic and pharmacodynamic profiles. Few examples are E209 [19], N205 [20] and OZ439 [21]. Solaja and coworkers [22] have earlier demonstrated that these peroxide type molecules have activity towards both malaria and TB.

This project focused on the synthesis of isonicotinohydrazides with the concept of molecular hybridization, by incorporating 1,2,4,5- tetraoxane and hydrazine moieties as well as evaluate both antimalarial and antitubercular activities. While the antimalarials activity of the peroxides/tetraoxanes has been proven to be due to the peroxide moiety, the antitubercular activity of INH is believed to be due to the hydrazine functionality with the hope of producing a single hybrid molecule with dual activity.

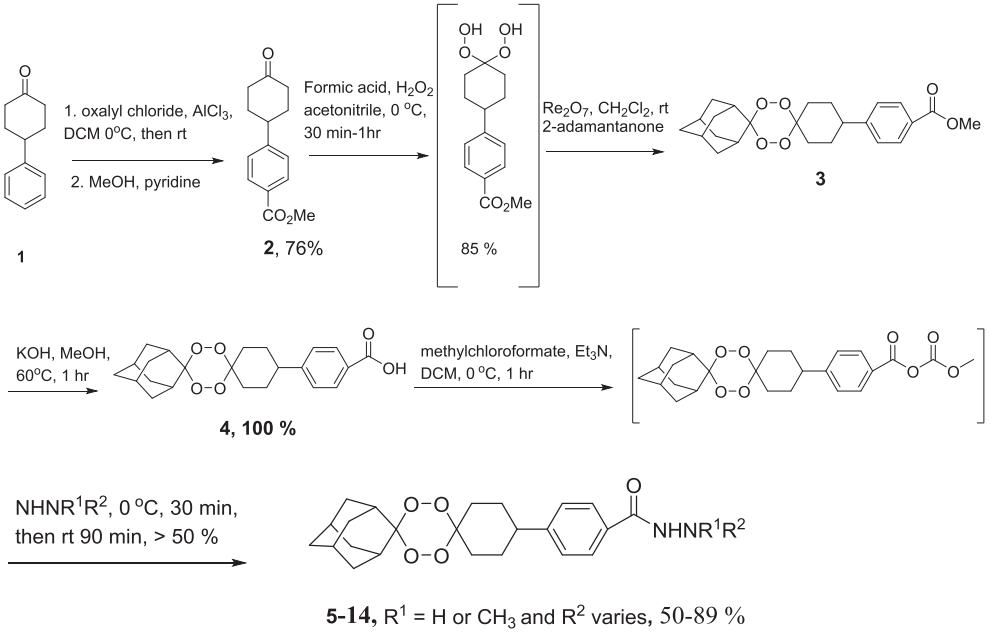
Results and discussions

Chemical synthesis of compounds

The nicotinohydrazides were synthesized in six chemical steps in moderate to high yields involving an already established synthetic route developed by O'Neill *et al.* [23]. The key intermediate methyl ester **3** was synthesized *via* a modified Friedel-Crafts (FC) reaction with 4-phenylcyclohexanone to produce methyl 4-(4-oxocyclohexyl)benzoate) **2** (Scheme 1. To prepare compound **3**, 4-phenylcyclohexanone **1** was treated with oxalyl chloride in the presence of Aluminum chloride as a catalyst to yield the acyl chloride which was further treated with pyridine and quenched *in situ* by methanol to give the methyl ester **2**. Compound **2** was then reacted with 30 % hydrogenperoxide in acetonitrile to generate the bishydroperoxide intermediate which was not isolated and condensed with 2-adamantone catalysed by Re_2O_7 to give the tetraoxaned ester **3**. The ester was then hydrolysed under acidic conditions to give the carboxylic acid **4**. The titled compounds **5**–**14** were synthesized *via* amide coupling through a mixed unhydride intermediate, which was not isolated, with selected hydrazines ($NHNR^1R^2$) including isoniazid in moderate to high yields (Table 1).

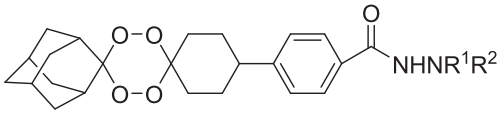
Antimalarial activity

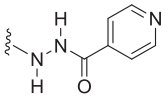
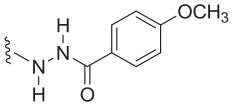
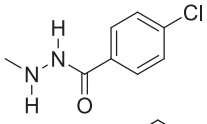
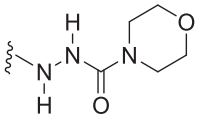
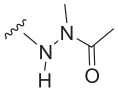
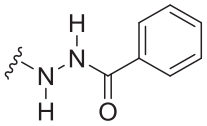
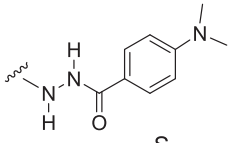
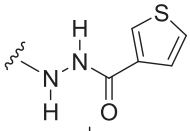
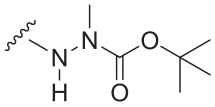
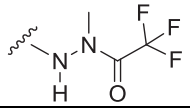
A number of the compounds synthesized were tested *in vitro* against the 3D7 strain of *P. falciparum* and they displayed activities in nanomolar range. All tetraoxane analogues displayed activities between 0.06 and 0.269 μM with the most potent possessing IC_{50} value 0.060 ± 0.033



Scheme 1. Synthetic route for the preparation of the nicotinamidehydrazides.

Table 1
Compounds Synthesised and their Percentage Yields.

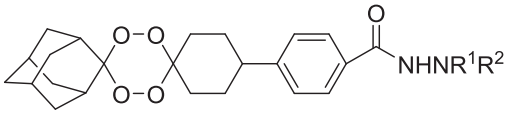


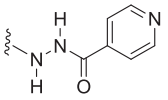
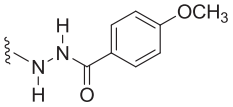
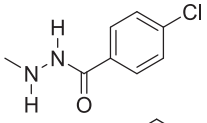
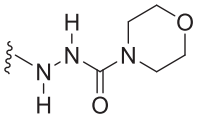
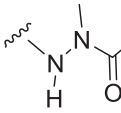
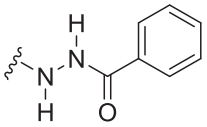
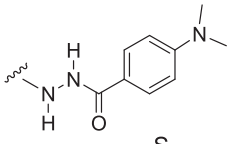
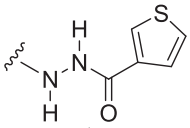
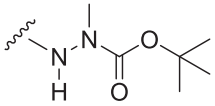
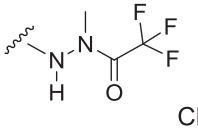
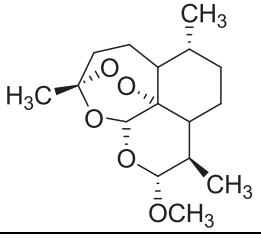
CODE	-NR^{1}R^2	Yield (%)
5		56
6		64
7		64
8		50
9		60
10		70
11		60
12		89
13		45
14		62

μM (Table 2), with Artemether as the control. The clinically used Artemether was chosen as control because, the tetraoxane class of compounds possess the same pharmacophore and have similar antimalarial activity. A representative IC_{50} curve for **6** is shown in Fig. 1 below.

Other compounds including **5**, **9**, **10** and **11** retained antimalarial potency below 100 nM while the remaining compounds **8**, **12**, **13**, and **14** gave activities below 500 nM. It was observed that apart from the analogue prepared from isoniazid, the compounds with an aromatic ring were the most potent suggesting that the incorporation of the phenyl ring might have impacted on the antimalarial activity. Whereas the compounds with electron donating groups as substituents on the aromatic ring, **6** and **11,** were highly potent, the only analogue with an electron withdrawing group **7**, gave lower antimalarial activity (IC_{50} = 0.491 ± 0.012 μM) implying that the nature of the substituent is critical for antimalarial activity. Of the analogues without the incorporation of an aromatic group, the least lipophilic analogue containing the morpholine substituent **8** was found to be the most active followed by **13**, **12**

Table 2
In vitro antimalarial activity profiles of synthesized compounds.



CODE	-NR^{1}R^2	IC_{50} (μM)
5		0.072 ± 0.039
6		0.060 ± 0.033
7		0.491 ± 0.012
8		0.113 ± 0.029
9		0.061 ± 0.003
10		0.082 ± 0.009
11		0.099 ± 0.019
12		0.261 ± 0.030
13		0.140 ± 0.004
14		0.269 ± 0.008
Artemether		0.0078 ± 0.009 (O' Neill et al., 2010)

and **114**. Compounds **9** and **14** are similar except the methyl group in **9** is replaced by trifluoromethyl group. However, a significant drop-in antimalarial activity (from 0.061 ± 0.003 μM for **9** to 0.269 ± 0.008 μM for **14**) was observed when the electron donating methyl group was replaced by an electron withdrawing substituent.

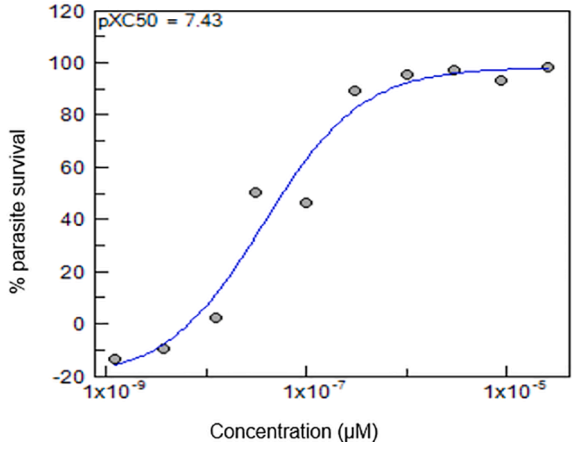


Fig. 1. IC_{50} curve for compound **6**. All data was processed using IDBS ActivityBase [24]. Raw data was converted into percent inhibition through linear regression by setting the high inhibition control as 100 % and the no inhibition control as 0 %. IC_{50} curve fitting employed a four-parameter logistic dose response curve (model 205): y = A+((B-A)/(1+(C/(x)^D))). Where A = % inhibition at bottom, B = % inhibition at top, C = EC_{50}, D = Slope, x = inhibitor concentration and y = % inhibition. If curve definition was poor B was fixed to 100.

Antitubercular activity and minimum inhibitory concentrations (MIC) of active compounds

Five of the compounds **5**, **6**, **7**, **8**, **9** was subjected to drug susceptibility testing against standard pathogenic *M. tuberculosis* strain H37Rv (H37Rv) and fast-growing mycobacteria surrogate for antitubercular drug test, *M. aurum,* using the Microplate Alamar Blue Assay (MABA). The assays were set up in duplicates to ensure confidence. The compounds in concentrations ranging from 0.25 to 8 mg/mL were used to investigate whether the compounds will exhibit growth-inhibitory activity towards the selected mycobacteria (**Table S3**). It was observed that compound **6** which was only active at the highest concentration (8 mg/mL), whereas remaining compounds **5**, **7**, **8** and **9** were impressively active against H37Rv even at the lowest concentrations. On the other hand, the compounds were largely inactive against *M. aurum,* for example, only compounds **5, 7** and **8** were active albeit at relatively high concentrations while compounds **5, 9** and **10** showed no growth-inhibitory activity against *M. aurum*.

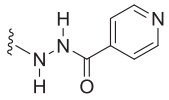
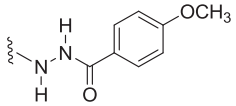
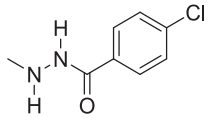
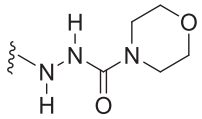
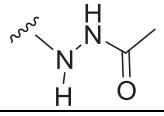
Mycobacterial minimum inhibitory concentrations (MIC) of compounds

Following the impressive performance of compounds **5**, **7**, **8** and **9**, they were subjected to further testing against H37Rv to establish the exact MIC for these compounds (Table 4).

Apart from compound **8** which displayed the highest MIC value (0.4 mg/mL) against *M. aurum*, the remaining compounds tested had MIC of 8 mg/mL or above against *M. aurum* (Table 4). Interestingly, while compound **6** possessed the most active antimalarial activity with IC_{50} of 60 nM, it gave the least anti-TB activity with MIC of 8 mg/mL. All the compounds tested showed activity against H37Rv, the standard *M. tuberculosis* strain. Compound **5** gave the best performances with MIC of 0.003 mg/mL followed by **8 (**0.125 mg/mL), **9** (0.125 mg/mL) and **7** (0.500 mg/mL) (Table 4).

The electronic effect of the substituent on the phenyl ring was observed to influence the antitubercular activity. While the activity of hydrazide with an electron donating substituent on the phenyl ring, **6,** was low, the analogue with an electron withdrawing group, **7,** gave a superior activity. Though not overly surprising, it was observed that the hybrid with isoniazid incorporated gave the most active antitubercular result suggesting that the pyridyl nitrogen might be critical for activity.

Table 4
Minimum inhibitory concentrations of test compounds with *M. aurum* and H37Rv.

CODE	-NR^1R^2	MIC (mg/mL) *M. aurum*	H37Rv
5		8.000	0.003
6		> 8.000	8.000
7		8.000	0.500
8		0.400	0.125
9		> 8.000	0.125

The least lipophilic compounds (**Table S**): **5**, **8**, **9** was observed to be the most active anti-TB agents, suggesting that high lipophilicities may not necessarily enhance anti-TB activity. The lipophilicities were calculated using CHEMDRAW18.2.

Activity of compounds against drug susceptible and resistant clinical Mycobacterium tuberculosis isolates

After the MICs of the tested compounds was determined, further testing was carried out on the most potent compounds **(5, 7, 8,** and **9)** against clinical *Mycobacterium tuberculosis* complex isolates resistant to first-line anti-TB drugs. The isolates were isoniazid mono-resistant (INH^{mono}), rifampicin mono-resistant (RIF^{mono}) and multi-drug resistant (MDR) strains. We observed highest activity of the compounds against the INH^{mono} isolate even though the MICs were relatively higher compared to the drug susceptible strain H37Rv. Compounds **5** and **8** were the most active against the INH^{mono} strain with MICs < 0.250 mg/mL whereas compound **9** was the least active against INH^{mono} strain with MIC 1 mg/mL (Table 5). The four compounds were largely inactive against the RIF^{mono} and MDR strains with MICs at least 8 mg/mL.

Comparing antimalarial and antitubercular results

From the antimalarial testing, compound **6** was the most active with IC_{50} of 0.060 ± 0.033 μM but was not active when tested for antitubercular activity. Compound **5** on the other hand gave the best antitubercular activity with MIC of 0.003 mg/mL and 0.072 ± 0.039 μM antimalarial activity. Generally, however, only a few of the compounds tested showed activity against both malaria and TB. This could be

Table 5
MIC's of active compounds with clinical resistant isolates in comparison to H37Rv.

COMPOUNDS	MIC (mg/mL)			
	INH^{mono}	RIF^{mono}	MDR	H37Rv
5	< 0.250	> 8	> 8	<0.0039
7	0.5	8	> 8	0.5
8	< 0.250	> 8	8	0.125
9	1.0	>8	> 8	0.125

because of binding of the compounds to different targets for both diseases. A compound may have high affinity and bind effectively to the target for say malaria hence returning good activity but the binding of the same compound to a different target in TB might be poor, hence having low activity.

Materials and methods

Synthesis of target compound

Preparation of methyl 4-(4-oxocyclohexyl)benzoate 2

A solution of oxalyl chloride 4.51 mL (53.3 mmol) in DCM (50 mL) was added to a suspension of 4-phenylcyclohexanone **1** (7 g, 40 mmol) and $AlCl_3$ (16.07 g, 120 mmol) in DCM (150 mL) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h then at room temperature for 2 h. A mixture of methanol (10 mL) and pyridine (8.1 mL) was added drop wise to the reaction mixture and left to stand overnight. The reaction mixture was then washed with water, 3 N HCl, $NaHCO_3$ solution, dried over $NaSO_4$, filtered and concentrated. Purification by flash column chromatography using Hexane:EtOAc, 4:1 (Rf = 0.5) gave methyl 4-(4-oxocyclohexyl)benzoate in 65 %. ^{1}H NMR (400 MHz, $CDCl_3$-d_6) δ_H 8.00 (d, 2H, J = 8.3 Hz, Ar), 7.32 (d, 2H, J = 8.3 Hz, Ar), 3.92 (s, 2H, OCH_3), 3.10 (tt, 1H, J = 11.4 Hz, 3.0 Hz, CH), 2.53 (dd, 4H, J = 9.6 Hz, 5.0 Hz, CH_2), 2.28–2.20 (m, 2H, CH_2), 2.03–1.90 (m, 2H, CH_2); ^{13}C NMR(100 MHz, $CDCl_3$-d_6) δ_C 211.0, 167.3, 150.5, 130.4, 129.0, 127.2, 52.5, 43.2, 41.6, 34.2; MS (ES +), $[M + Na]^+$ (100) 255.1 HRMS calculated for 255.0997 $C_{14}H_{16}O_3Na$, found 255.0989.

Preparation of methyl 4-((1r,3r,5r,7r)-dispiro[adamantane-2,3′-[1–2,4–5]tetraoxane-6′,1′′-cyclohexan]-4′′-yl)benzoate **3.**

To a solution of methyl 4-(4-oxocyclohexyl)benzoate **2** (4 g, 17 mmol) in acetonitrile (75 mL) at 0 °C was added formic acid (8 mL) and 30 % H_2O_2 (16 mL). The resulting reaction mixture was stirred for 30 min at 0 °C, warmed to room temperature and diluted with water (30 mL). The resulting mixture was extracted in DCM (3 × 50 mL), dried over $MgSO_4$ and concentrated to give the crude *gem*-bishydroperoxide which was used without further purification. The *gem*-bishydroperoxide was dissolved in CH_2Cl_2 (50 mL) and added to a stirring solution of the required adamantanone (1.5 eq) and rhenium (VII) oxide (0.02 eq) in CH_2Cl_2 (50 mL) at room temperature. The reaction mixture was stirred for 1 h, filtered through a plug of silica and concentrated. Purification by flash column chromatography with DCM:EtOAc, 9:1 (Rf = 0.8) gave the compound in 48 % as a white powder. ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ_H 7.97 (d, J = 7.6 Hz, 2H), 7.31 (d, J = 7.6 Hz, 2H), 3.91 (s, 3H), 2.69 (t, J = 10.6 Hz, 1H), 2.56 (s, 2H), 2.16 – 1.54 (m, 20H); ^{13}C NMR (126 MHz, $CDCl_3$-d_6) δ_C 167.2, 151.1, 129.7, 128.1, 126.7, 110.6, 107.2, 52.0, 47.1, 43.6, 39.3, 37.0, 36.3, 33.2, 27.5, 27.1 MS (ES +), $[M + Na]^+$ (100) 437.5 HRMS calculated for 437.19 $C_{24}H_{30}O_6Na$, found 437.1906

Preparation of 4-((1r,3r,5r,7r)-dispiro[adamantane-2,3′- [1–2,4–5] tetraoxane-6′,1′′-cyclohexan]-4′′-yl)benzoic acid **4.**

A solution of the tetraoxane ester **3** (3.86 mmol) in 10 % w/v potassium hydroxide/methanol (12.6 mL) was stirred at reflux for 90 min. The solution was allowed to cool to room temperature and concentrated under reduced pressure. The resulting residue was taken up in water (15 mL) and washed with diethyl ether (3 × 12 mL). The aqueous layer was acidified with concentrated hydrochloric acid and a white precipitate formed. Diethyl ether (18 mL) was added to dissolve the precipitate and the aqueous phase extracted with diethyl ether (2 × 12 mL). The combined organic phases were washed with brine (10 mL), dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give a white solid. Recrystallization from ethanol gave the carboxylic acid as a white solid in 91% yield. ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ_H 7.97 (d, J = 8.0 Hz, 2H), 7.26 (d, J = 8.0 Hz, 2H), 2.62 (dd, J = 15.6, 7.7 Hz, 1H), 2.06–1.42 (m, 22H); ^{13}C NMR (126 MHz, $CDCl_3$-d_6) δ_C 171.3, 152.2, 130.8, 127.4, 127.1, 110.6, 106.8, 47.1, 44.1, 39.2, 37.0, 33.3, 29.7, 27.1. MS (ES-); $[M - H]^-$ (100) 399.2 HRMS calculated for 399.1808 $C_{23}H_{27}O_6$, found 399.1808.

General procedure for the amide formation

To a solution of the acid (2.33 mmol) in dry DCM (30 mL) was added triethylamine (0.7 mmol, 1.5 eq) and ethylchloroformate (2.33 mmol, 1.0 eq). The reaction was stirred for 60 min at 0 °C. (2.33 mmol, 1.0 eq) of the required amide was added, and after stirring for 30 min, the reaction mixture was warmed to room temperature and stirred for a further 90 min. The reaction mixture was then diluted with water and extracted with DCM (3 × 30 mL). The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4 and concentrated. Purification by flash column chromatography afforded the required amide.

Preparation of N'-(4-((1r,3r,5r,7r)-dispiro[adamantane-2,3′-[1–2,4–5]tetraoxane-6′,1′′-cyclohexan]-4′′-yl)benzoyl)isonicotinohydrazide **5**.

This product was prepared according to the general procedure for preparing amides as a white solid (0.23 g, 0.44 mmol, 56 %) from (0.1 g 0.24 mmol) of **4**. Purification was done by column chromatography using DCM:EtOAc, 9:1 Mpt: 148–150 °C; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ_H 8.70 (d, J = 5.4 Hz, 2H), 8.00 (s, 1H), 7.98 (s, 1H), 7.77 (d, J = 8.1 Hz, 2H), 7.68 (d, J = 2.0 Hz, 2H), 7.29 (d, J = 8.1 Hz, 2H), 2.71 – 2.61 (m, 1H), 2.10 – 1.60 (m, 22H); ^{13}C NMR (126 MHz, $CDCl_3$-d_6) δ_C 165.1, 162.75, 151.1, 150.5, 138.4, 128.8, 127.6, 127.4, 120.9, 110.6, 107.3, 43.5, 39.0, 34.0, 37.0, 36.0, 33.3, 27.7, 27.0. MS (ES +), $[M + Na]^+$ (100) 543.2 HRMS calculated for 543.1943 $C_{29}H_{33}O_6N_3Na$, found 543.1896.

Preparation of 4-((1r,3r,5r,7r)-dispiro[adamantane-2,3′- [1–2,4–5] tetraoxane-6′,1′′-cyclohexan]-4′′-yl)-N'-(4-methoxybenzoyl)benzohydrazide **6**.

This product was prepared according to the general procedure for preparing amides as a white solid (0.21 g, 0.39 mmol, 64 %) from (0.1 g 0.24 mmol) of **4**. Purification was done by flash column chromatography with Hex: EtOAc,4:1(R_f = 0.8) Mpt: 179–181 °C; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ_H 9.40 (s, 1H), 9.36 (s, 1H), 7.77 (d, J = 8.8 Hz, 2H), 7.72 (d, J = 8.2 Hz, 2H), 7.23 (d, J = 8.2 Hz, 2H), 6.86 (d, J = 8.8 Hz, 2H), 3.78 (s, 3H), 2.66 – 2.53 (m, 1H), 2.05 – 1.49 (m, 22H); ^{13}C NMR (126 MHz, $CDCl_3$-d_6) δ_C 164.1, 162.9, 150.7, 129.2, 127.5, 127.3, 123.6, 114.0, 110.6, 107.3, 55.5, 45.9, 43.6, 37.0, 33.2, 29.7, 27.1. MS (ES +), $[M + Na]^+$ (100) 571.6 HRMS calculated for 571.6318 $C_{31}H_{36}O_7N_2Na$, found 571.6285.

Preparation of 4-chloro-N'-(4-((1r,3r,5r,7r)-dispiro[adamantane-2,3′- [1–2,4–5]tetraoxane-6′,1′′-cyclohexan]-4′′-yl)benzoyl)benzohydrazide **7**.

This product was prepared according to the general procedure for preparing amides as a white solid (0.22 g, 0.40 mmol, 64 %) from (0.1 g 0.24 mmol) of **4**. Purification was done by flash column chromatography with Hex: EtOAc,4:1(R_f = 0.8) Mpt:148–150 °C; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ_H 8.01 (s, 1H), 8.00 (s, 1H), 7.83 (d, J = 8.5 Hz, 2H), 7.75 (d, J = 8.3 Hz, 2H), 7.38 (d, J = 8.5 Hz, 2H), 7.32 (d, J = 8.3 Hz, 2H), 2.74 – 2.48 (m, 1H), 2.11 – 1.57 (m, 22H); ^{13}C NMR (126 MHz, $CDCl_3$-d_6) δ_C 170.1, 166.1, 152.0, 138.8, 130.4, 129.0, 128.8, 127.02, 110.6, 107.3, 43.8, 37.0, 33.2, 29.7, 27.1 MS (ES +), $[M + Na]^+$ (100) 576.1 HRMS calculated for 576.0944 $C_{14}H_{16}O_3Na$, found 576.0943.

Preparation of N'-(4-((1r,3r,5r,7r)-dispiro[adamantane-2,3′-[1–2,4–5]tetraoxane-6′,1′′-cyclohexan]-4′′-yl)benzoyl)morpholine-4-carbohydrazide **8**.

This product was prepared according to the general procedure for preparing amides as a white solid (0.26 g, 0.49 mmol, 50 %) from (0.1 g 0.24 mmol) of **4**. Purification was done by flash column chromatography with Hex: EtOAc,4:1 (R_f = 0.7) Mpt: 136–138 °C; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ_H 9.21 (s, 1H), 9.18 (s, 1H), 7.75 (d, J = 8.1 Hz, 2H), 7.21 (d, J = 8.1 Hz, 2H), 3.58 – 3.54 (m, 4H), 3.40 – 3.35 (m, 4H), 2.62 – 2.53 (m, 1H), 2.05–1.51 (m, 22H); ^{13}C NMR (126 MHz, $CDCl_3$-d_6) δ_C 166.2, 157.0, 150.3, 129.5, 127.6, 127.1, 110.3, 107.1, 66.3, 47.0, 43.9, 43.6, 39.2, 37.0, 36.3, 33.2, 27.4, 26.9. MS (ES +), $[M + Na]^+$ (100) 551.1 HRMS calculated for 550.6099 $C_{28}H_{37}O_7N_3Na$, found 550.6143

Preparation of N'-acetyl-4-((1r,3r,5r,7r)-dispiro[adamantane-2,3′-[1–2,4–5]tetraoxane-6′,1′'-cyclohexan]-4′'-yl)benzohydrazide **9**.

This product was prepared according to the general procedure for preparing amides as a white solid (0.20 g, 0.43 mmol, 60 %) from (0.1 g 0.24 mmol) of **4**. Purification was done by flash column chromatography with Hex: EtOAc,4:1 ($R_f = 0.8$). Mpt: 138–140 °C; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ_H 9.61 (d, $J = 3.9$ Hz, 1H), 9.47 (d, $J = 3.9$ Hz, 1H), 7.76 (d, $J = 7.9$ Hz, 2H), 7.27 (d, $J = 7.9$ Hz, 2H), 2.74 – 2.48 (m, 1H), 2.11 (s, 3H), 2.06 – 1.57 (m, 22H); ^{13}C NMR (126 MHz, $CDCl_3$-d_6) δ_C 167.6, 164.3, 150.6, 129.0, 127.6, 127.2, 110.3, 107.7, 43.5, 39.3, 37.0, 33.2, 27.1, 20.8. MS (ES +), [M + Na] $^+$ (100) 479.53 HRMS calculated for 479.5318 $C_{25}H_{32}O_6N_2Na$, found 479.5423

Preparation of N'-benzoyl-4-((1r,3r,5r,7r)-dispiro[adamantane-2,3′-[1–2,4–5]tetraoxane-6′,1′'-cyclohexan]-4′'-yl)benzohydrazide **10**.

This product was prepared according to the general procedure for preparing amides as a white solid (0.19 g, 0.37 mmol, 70 %) from (0.1 g 0.24 mmol) of **4**. Purification was done by flash column chromatography with Hex: EtOAc,1:1 ($R_f = 0.6$). Mpt: 158–160 °C; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ_H 9.59 (s, 1H), 9.54 (s, 1H), 8.01 (d, $J = 7.5$ Hz, 2H), 7.90 (d, $J = 6.7$ Hz, 2H), 7.82 (s, 1H), 7.46 (d, $J = 7.2$ Hz, 2H), 7.32 (d, $J = 7.5$ Hz, 2H), 2.76 – 2.53 (m, 1H), 2.19 – 1.60 (m, 22H); ^{13}C NMR (126 MHz, $CDCl_3$-d_6) δ_C 170.3, 152.1, 150.9, 130.4, 128.8, 127.6, 127.3, 127.0, 110.6, 107.3, 47.0, 43.8, 43.6, 39.3, 37.0, 33.2, 27.1. MS (ES +), [M + Na] $^+$ (100) 541.6 HRMS calculated for 541.6056 $C_{14}H_{16}O_3Na$, found 541.6166

Preparation of 4-(dimethylamino)-N'-(4-((1r,3r,5r,7r)-dispiro[adamantane-2,3′- [1–2,4–5]tetraoxane-6′,1′'-cyclohexan]-4′'-yl)benzoyl) benzohydrazide **11**.

This product was prepared according to the general procedure for preparing amides as a white solid (0.23 g, 0.41 mmol, 60 %) from (0.1 g 0.24 mmol) of **4**. Purification was done by flash column chromatography with Hex: EtOAc,1:1 ($R_f = 0.4$). Mpt: 165–167 °C; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ_H 9.42 (s, 1H), 9.20 (s, 1H), 7.79 (d, $J = 8.5$ Hz, 2H), 7.69 (d, $J = 8.3$ Hz, 2H), 7.31 (d, $J = 8.3$ Hz, 2H), 6.73 (d, $J = 8.5$ Hz, 2H), 3.05 (s, 6H), 2.72 – 2.52 (m, 1H), 2.09 – 1.59 (m, 22H); ^{13}C NMR (126 MHz, $CDCl_3$-d_6) δ_C 152.97, 128.70, 127.29, 127.19, 127.09, 111.43, 111.01, 110.48, 107.24, 77.16, 76.91, 76.65, 43.51, 39.96, 36.87, 33.07, 29.59, 26.98, 14.00. MS (ES +), [M + Na] $^+$ (100) 585.1 HRMS calculated for 584.6715 $C_{32}H_{39}O_6N_3Na$, found 584.6672

Preparation of 4-((1r,3r,5r,7r)-dispiro[adamantane-2,3′- [1–2,4–5] tetraoxane-6′,1′'-cyclohexan]-4′'-yl)-N'-(2-(thiophen-3-yl)acetyl)benzohydrazide **12**.

This product was prepared according to the general procedure for preparing amides as a white solid (0.15 g, 0.29 mmol, 89 %) from (0.1 g 0.24 mmol) of **4**. Purification was done by flash column chromatography with Hex: EtOAc,1:1 ($R_f = 0.7$). Mpt: 150–152 °C; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ_H 8.01 (d, $J = 8.2$ Hz, 2H), 7.30 (dd, $J = 8.2$, 6.1 Hz, 2H), 7.19 (d, $J = 4.7.0$ Hz, 2H), 7.04 (d, $J = 4.7$ Hz, 1H), 3.63 (s, 2H), 2.74 – 2.48 (m, 1H), 2.06 – 1.19 (m, 22H); ^{13}C NMR (126 MHz, $CDCl_3$-d_6) δ_C 170.7, 156.7, 151.9, 133.4, 130.4, 128.4, 127.0, 126.6, 123.6, 110.6, 107.3, 62.4, 43.7, 37.0, 35.8, 33.2, 29.7, 27.1. MS (ES +), [M + Na] $^+$ (100) 565.7 HRMS calculated for 565.6542 $C_{14}H_{16}O_3Na$, found 565.6519.

Preparation of *tert*-butyl 2-(4-((1r,3r,5r,7r)-dispiro[adamantane-2,3′- [1–2,4–5]tetraoxane-6′,1′'-cyclohexan]-4′'-yl)benzoyl)hydrazine-1-carboxylate **13**.

This product was prepared according to the general procedure for preparing amides as a white solid (0.29 g, 0.55 mmol, 45 %) from (0.1 g 0.24 mmol) of **4**. Purification was done by flash column chromatography with Hex: EtOAc,4:1 ($R_f = 0.7$). Mpt: 155–157 °C; ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ_H 8.04 (s, 1H), 8.02 (s, 1H), 7.33 (d, $J = 8.1$ Hz, 2H), 7.26 (d, $J = 8.1$ Hz 2H), 2.74 – 2.48 (m, 1H), 2.06 – 1.61 (m, 22H), 1.26 (s, 9H); ^{13}C NMR (126 MHz, $CDCl_3$-d_6) δ_C 171.3, 171.1, 152.0, 130.4, 126.9, 110.5, 107.2, 68.1, 43.7, 36.9, 33.1, 29.6, 27.0, 22.6. MS (ES +), [M + Na] $^+$ (100) 537.6 HRMS calculated for 537.6126 $C_{28}H_{38}O_7N_2Na$, found 537.6133.

Preparation of *tert*-butyl 2-(4-((1r,3r,5r,7r)-dispiro[adamantane-2,3′- [1–2,4–5]tetraoxane-6′,1′'-cyclohexan]-4′'-yl)benzoyl)hydrazine-1-carboxylate **14**.

This product was prepared according to the general procedure for preparing amides as a white solid (0.21 g, 0.40 mmol, 62 %) from (0.1 g 0.24 mmol) of **4**. Purification was done by flash column chromatography with Hex: EtOAc, 4:1 ($R_f = 0.8$). ^{1}H NMR (500 MHz, $CDCl_3$-d_6) δ_H 8.04 (s, 1H), 8.03 (s, 1H), 7.33 (d, $J = 7.9$ Hz, 2H), 7.26 (d, $J = 7.9$ Hz, 2H), 2.71 – 2.62 (m, 1H), 2.13 – 1.59 (m, 22H); ^{13}C NMR (126 MHz, $CDCl_3$-d_6) δ_C 171.2, 152.2, 130.5, 127.0, 110.6, 107.3, 47.0, 43.8, 39.3, 33.2, 29.7, 27.5. MS (ES +), [M + Na] $^+$ (100) 533.5 HRMS calculated for 533.4961 $C_{14}H_{16}O_3Na$, found 533.4955.

P. Falciparum (3D7) in vitro assay

Chloroquine-sensitive *P. falciparum strain* 3D7 cultures were maintained in a 5 % suspension of human red blood cells cutured in in RPMI 1640 medium (pH 7.3). These were supplemented with 0.5 % Albumax II (Gibco Life Technologies, San Diego, CA), 12 mM sodium bicarbonate, 0.2 mM hypoxanthine, and 20 mg/L gentamicin at 37 °C, in 1 % O2, 3 % CO_2 atmosphere and a nitrogen balance [25]. Fluorescence assay was used to quantify growth inhibition, utilizing the binding of SYBR Green [25] to double stranded DNA, which after excitation at 485 nm emitted a fluorescent signal at 528 nm. Mefloquine was the control used for assay quality monitoring. Compound bioactivity was expressed as IC_{50}. The most potent hits from each series identified by the primary screening were reconfirmed by testing in a [^{3}H]-Hypoxanthine incorporation assay.10 mM solutions of the compounds in DMSO were prepared and tested in a 10-point 1 in 3 dilution series from a top concentration of 25 μM.

In vitro cell assay data analysis

All data were processed using IDBS ActivityBase. Raw data was converted into per cent inhibition through linear regression by setting the high inhibition control as 100% and the no inhibition control as 0%. Quality control criteria for passing plates were as follows: z'> 0.5, S:B > 3, %$CV_{(no\ inhibition\ control)}$ < 15. Z' was calculated with;

$$1 - \frac{3 \times (StDev\_high \pm StDev\_low)}{ABS\ (Mean\_high - Mean\_low)}$$

Curve fitting was calculated with the formula;

$$y = A + \frac{B - A}{1 + (C/x)^D}$$

where A = % inhibition at bottom, B = % inhibition at the top, C = EC_{50}, D = slope, x = inhibitor concentration and y = % inhibition. B was fixed to 100 if curve definition was poor [26]

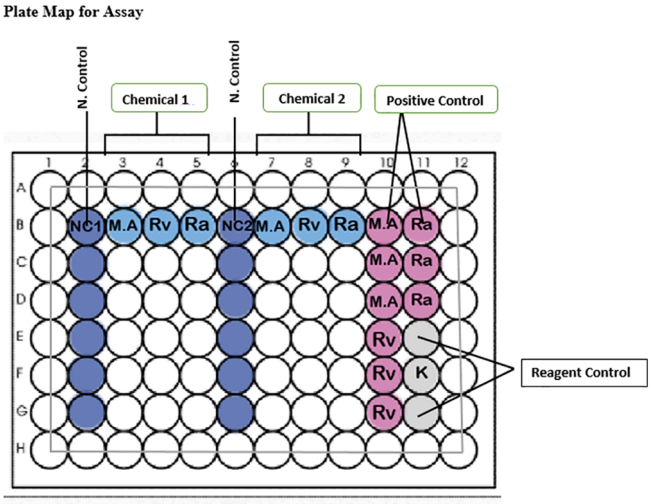
Alamar blue assay

Stock Preparation of test compounds

Ten (10) mg of powdered compound was dissolved in 125 μL of dimethyl sulfoxide (DMSO) to give a concentration of 80 mg/ml. A 1 in 10 dilution was done for the stock by adding 675 μL of DMSO to 75 μL of stock solution resulting in an 8 mg/mL working concentration. The concentration of test compound in each well down the column after a twofold Serial dilution from Column B2 to G2 on a 96 well plate is as shown below.

mg/mL → 4 mg/mL → 2 mg/mL → 1 mg/mL → 0.5 mg/mL → 0.250 mg/mL

3.4.2 Preparation of 96 well plates



Preparation of 7H9 culture media.

For mycobacteria species, H37*Rv* and *M. aurum* Middlebrook 7H9 medium (Becton Dickson company Sparks MD) supplemented with 0.2 % glycerol, 1.0 g casitone per litre, 10 % heat inactivated Fetal Bovine Serum (FBS), and 0.05 % tween 80 (sterile- filtered through 0.2 μL Millipore filters). For every 500 mL of medium solution, 2.35 g of middlebrook 7H9 is added to 450 mL of distilled water and supplemented with 0.5 g casitone and 2 mL glycerol. The 7H9 broth with glycerol and casitone (7H9-GC) was autoclaved at 121 °C for 10 min, allowed to cool and stored at 4° C. The growth media (7H9-GC supplemented with FBS and filter-sterile Tween 80 (7H9-GCFT)) were constituted fresh just before use. Five (5) mL FBS and 250 μL 10 % tween were added to 45 mL of 7H9GC medium, to give 7H9 GCFT medium (medium for the assay).

Inoculation and incubation

Suspension of mycobacterial isolates (H37*Rv* and *M.aurum*) were prepared by adding a loopful of mycobacteria colonies in 500 μL of 7H9GC medium and homogenized by vortexing. The turbidity of the suspension was adjusted to 1 McFarland. A 1 in 20 dilution was made by adding 200 μL of the suspension to 3800 μL of 7H9GC medium. Plates were inoculated by adding 10 μL of the suspension to test wells and positive control wells with exception to the negative control wells and incubated at 37 °C for 7 days.

Reading of DST plates

The plates were checked for mycobacterial growth after 7 days. The calorimetric method microplate blue assay was used to quantify the products. Alamar blue reagent was added to the first negative and positive control wells (i.e., B_2/B_6 and $B_{10}/B_{11}/E_{10}$) and incubated for 24 h and observed for color change (from blue to pink) in the positive control well. Alamar blue reagent was added to the remaining wells and incubated for 24hrs to observe for change in color. Wells with no growth/no color change remained blue whereas wells with mycobacteria growth turned pink. The assay was repeated once to ensure consistency.

The Minimum Inhibition Concentration (MIC) is the lowest concentration of drug that prevents a visible growth of bacteria for which there is a color change from blue to pink (inhibits>99 % of growth of bacterial culture). For therapy guidance, isolates with MIC of 0.2 or 0.5 μg/mL for INH and 0.4 μg/mL for rifampicin are reported as immediate resistant.

Quality control

Quality control during drug susceptibility testing (DST) assay was ensured at different levels. The outer wells were filled with sterile distilled water to minimize errors by evaporation. Each 96 well plate contains 9 positive control wells and two columns negative control, as well as 3 wells for media control to check the purity and identification of each of the isolates and test drugs as well as the medium used. The whole assay was repeated to ensure consistency and confidence.

Testing of active compounds against drug resistant clinical MTBC isolates

The compounds that showed activity against H37Rv were screened against a well characterized drug resistant clinical MTBC isolates (**supplementary Table S1** using the microplate Alamar blue assay described above.

Conclusions

Small number of isonicotinohydrazides were designed and synthesized. Several of them exhibited antimalarial activity in nanomolar range with **6** being the most active possessing an IC_{50} value of 0.060 ± 0.033 μM against the 3D7 strain of the malaria parasite**.** A selection of the hybrid molecules were further tested for their antitubercular activity first by *in vitro* test using varying concentrations followed by an MIC measurement using *M.aurum* and H37Rv strains of microbacterium tuberculosis. Of the five hybrid molecules tested, **5** was the most potent with MIC of 0.003 mg/mL. Evaluation of the compounds against clinical *M. tuberculosis* strains resistant to isoniazid (INH) and rifampicin (RIF) showed that they were largely inactive against drug resistant clinical strains except for the INH mono-resistant resistant strain which was very active with compounds **5** and **8** returning MICs of 0.125 mg/mL. Of the compounds tested, only a few showed activities against malarial and TB. The observed pattern could be because of the binding affinity of the compounds to the respective targets for both diseases.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rechem.2022.100287.

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