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Enabling the (3+2) cycloaddition reaction in assembling newer anti-tubercular lead acting through the inhibition of Gyrase ATPase domain: Lead optimization and structure activity profiling

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Abstract

DNA Gyrase, the sole type II topoisomerase present in *Mycobacterium tuberculosis* is absent in humans and is a well validated target for anti-tubercular drug discovery. In this study a moderately active inhibitor of *Mycobacterium tuberculosis* GyrB - the pharmaceutically unexploited domain of DNA gyrase was reengineered using a combination of molecular docking and medicinal chemistry strategies to obtain a lead series displaying considerable *in vit*ro enzyme efficacy and bactericidal property against *Mycobacterium tuberculosis* H_{37}Rv strain. A biophysical investigation using differential scanning flourimetry experiments re-ascertained the affinity of these molecules towards the GyrB domain. Furthermore the molecules were completely devoid of hERG toxicity until 30μM, evaluated in a zebra fish model with a good selectivity index to be worked out from a pharmaceutical point of view as potential candidate against TB.

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Introduction:

Tuberculosis (TB) according to the recent world health organization (WHO) report claimed about 1.3 million lives in 2012, with an overwhelming majority of these from developing portions of the world[1,2] Despite the effectiveness of WHO initiated directly observed treatment short course (DOTS) therapy with approximately 90% cure rate in human immunodeficiency virus (HIV)–negative drug-susceptible, active tuberculosis patients; the disease has however resurged in a more deadly drug resistant form causing substantial mortality and human suffering. Cofounding these issues is the association of TB with HIV, which compromises the host defence mechanism and renders individuals more susceptible to TB; necessitating further research into development of novel agents that lacks cross resistance mediated by mutation in bacterial target and shortens the duration of therapy.[3-8]

The current TB drug discovery pipeline has many promising leads at the various stages of clinical investigation including six new compounds specifically developed for tuberculosis.[8] The most promising among them is Moxifloxacin, a fluoroquinolone drug that acts through the inhibition of DNA gyrase subunit A (GyrA) and has demonstrated promising activity against both drug sensitive and drug resistant strains of *Mycobacterium tuberculosis* (*M.tb*). In *Mycobacterium tuberculosis*, DNA gyrase is the sole type II topoisomerase that ensures the regulation of DNA topology and has been genetically demonstrated to be a bactericidal drug target. It is a heterodimeric enzyme with A2B2 complex, the A subunit aids in breakage and reunion of the double stranded DNA, while the B subunit acts via ATPase activity, providing a sufficient amount of energy for the DNA supercoiling.[9-13] The GyrA subunit, the target of quinolone class of antibiotics has been clinically quite successful; however, the emergence of quinolone resistance strain and the occurrence of some serious side effects; calls for novel research in this field.[10, 14-17]

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Later, novel (non-fluoroquinolone) bacterial type II topoisomerase inhibitors (NBTIs) that displayed broad spectrum antibacterial potency and were structurally and mechanistically distinct from fluoroquinolones emerged as a probable replacement to quinolone class. However their translation into a future clinical candidate was hampered by their significant cardio toxic side effects, arising from their potent hERG inhibition and QTc prolongation in-vivo.[18,19]

Researchers now focus on the pharmaceutically unexploited subunit of DNA Gyrase; the GyrB subunit. Inhibitors of the GyrB subunit competitively block the ATPase activity conferred by the DNA GyrB subunit and thereby abolish the energy-dependent reactions catalyzed by DNA gyrase and hence offers tremendous opportunity to develop novel anti tubercular agents displaying broad spectra efficacy, evading existing bacterial resistance with minimal side effects.

In the light of these finding and in continuation of our group research efforts in designing novel anti tubercular drugs; we report the development of an improved class of GyrB inhibitor derived from a previously reported class[20]; that displays significant specificity towards the mycobacterial GyrB domain and exhibits considerable in-vitro enzyme efficacy and bacterial kill against *Mycobacterium tuberculosis* H_3Rv strain.

Results and Discussion:

The design strategy adopted here is based on the concept of molecular hybridisation through re-alignment and positioning of two or more pharmacophoric units previously demonstrated to have promising activity against the targeted protein / disease. The success of this strategy in developing new substances which are therapeutically attractive has observed a significant growth in distinct therapeutic classes, being amply used by the pharmaceutical industry to discover new analogues of therapeutic innovations commercially attractive and also as a tool

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useful in the molecular modification.[21-23] In the present protocol we integrated in one molecular platform, the pharmacophoric units of two previously reported DNA GyraseB inhibitors (**1**, **2** and **3** in **Figure 1**) to give the designed ligand **4** bearing a thiazole core on the left hand side that runs through a triazole linker to the right handed coumarin nucleus as shown in **Figure 1**.The left hand thiazole unit was derived from our previously reported GyrB inhibitor[21]; that displayed moderate GyrB inhibitory potency and mycobacterial MIC. Though N-aminopiperdine nucleus has served as the linking unit in many of the previously reported DNA Gyrase inhibitors, the significant cardiovascular safety risk associated with this class[18,19] encouraged us to explore a novel traizole core as the linking unit. Additionally, these 1,2,3-triazoles possess remarkable metabolic stability and prove to be amide surrogates in various bioactive compounds. The presence of the coumarin core in many of the previously reported GyrB inhibitor from natural source[9] made it the right hand choice. Thus a library of 36 hybrids analogues (**Table 1**) were designed and taken for synthesis as steps towards the derivation of structure-activity relationships (SAR) and lead optimization.

The synthetic pathway used to achieve the target compounds has been delineated in **Scheme1**. We utilised the Huisgen's (3+2) cycloaddition reaction as a useful strategy to generate the designed library. Azido coumarins, required to engineer the right hand core of the designed ligand were constructed as previously described by Sivakumar et al.[24] Synthesis of the left hand thiazole core began by condensing commercially available aryl (phenyl, 2-pyrdiyl, 3-pyrdiyl and 4-pyridyl) thioamides (**5a-d**) with diethyl bromo malonate in refluxing toluene as previously reported by Kerdesky et al[25], yielding the corresponding 4-hydroxy thiazole derivatives **6a-d**. The acetylene tail required to fuse the thaizole core to right hand nucleus was achieved by mistnobu reaction of hydroxyl group present in the thiazole moiety (**6a-d**) with propargyl alcohol, affording compounds **7a-d** in good yields**.** These analogues (**7a-d)** were further hydrolysed into their corresponding acid derivatives (**8a-d**) and later

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converted to their amide derivatives counterparts (**9a-d**) as well, in an effort to understand the ideal site for introducing chemical diversity. Finally Huisgen's (3+2) cycloaddition of the above obtained acetylenes (**7a-d, 8a-d, 9a-d)** with corresponding azido coumarins gave the titled compounds **(10-45** in **Table 1)** in excellent yields. An overview of the various modification/substituent attempted in synthesis has been outlined in **Figure 2**.

In the preliminary screening, synthesized compounds were evaluated for their in-vitro *Mycobacterium smegmatis* GyrB ATPase assay adapted to 96-well plate format as described previously [26,27] and detailed extensively in the experimental section. The use of *Mycobacterium smegmatis* GyrB protein as a surrogate for GyrB protein from *Mycobacterium tuberculosis* has been well demonstrated in literature [10,26-27] as the activity of GyrB protein from *Mycobacterium tuberculosis* was found be very low when compared to that of *Mycobacterium smegmatis*. Novobiocin which has been previously demonstrated to be a potent inhibitor of DNA GyrB was used as a positive control in the study. Negative controls (0% inhibition) did not contain any inhibitory compounds. Compounds were also tested in the presence of Brij-35, a non-anionic detergent to ascertain whether these inhibitions were an artifact due to sequestration of the enzyme by drug aggregates. The other artifacts like auto absorbance of the drug were also ruled out by nullifying its absorbance during the reaction.

In the GyrB assay, out of the 36 compounds tested, 34 compounds displayed GyrB inhibitory $IC_{50} < 50$ μM, out of which 13 compounds demonstrated $IC_{50} < 15$ μM, 9 compounds showed $IC_{50} < 5$ μM and 3 compounds were found to inhibit GyrB activity even at sub micromolar concentration (IC_{50} < less 1 μM). Compound **27** emerged as the most promising leads with and GyrB inhibitory IC_{50} of 0.44±1.1 μM.

With respect to structure-activity relationship study we explored various modifications at three different sites as shown in **Figure 2**. The site 1 of the thiazole core was derived from

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the previously reported GyrB inhibitor (**1**) and had phenyl, 2-pyridyl, 3-pyridyl, and 4-pyridyl substitutions. At site 2, we introduced a carboxylic acid and a carboxamide side chain in addition to the ethyl carboxylate chain present in the previous inhibitor/lead. The site 3 had a remarkable difference from the previous lead and had a coumarin nucleus with a hydroxyl and methoxy substitution attempted at the 7th position, in addition to the un-substituted analogue. Another notable difference is the newly introduced triazole linker for reasons mentioned earlier. In the in-vitro assay, out of the various explorations attempted in synthesis, the carboxamide analogues (**16**-**18**, **25**-**27**, **34**-**36**, **43**-**45**) turned out to be the most promising leads showing excellent GyrB inhibition even in the lower micromolar range. The most promising leads compound **27** also belonged to this class. A general trend derived from the GyrB assay has been summarized in **Figure 2**.

Information on the common properties of the binding groups is essential for resolving the type of inhibitor binding to the target protein. In order to analyse the interaction profile of the molecules reported from the assay, compounds were docked to the GyrB ATPase domain of *Mycobacterium smegmatis* retrieved from protein data bank (PDB ID–4B6C).[26] An initial validation of the active site pocket was performed by redocking the crystal ligand (6-(3,4-dimethylphenyl)-3-[[4-[3-(4-methylpiperazin-1-yl)propoxy]phenyl]amino]pyrazine-2-carboxamide), with the active site residues of the *Mycobacterium smegmatis* GyrB protein. Redocking results showed that the ligand exhibited similar interactions as that of the original crystal structure which was further confirmed with RMSD of 0.86A^{0}. Later compounds **10**-**45** were docked into the active site pocket using extra precision mode (XP) of Glide module.[28] All the molecules oriented nicely into the active pocket in the vicinity of the amino acids similar to the crystal ligand. The docking results further validated the in vitro GyrB findings with carboxamide derivatives (**16**-**18**, **25**-**27**, **34**-**36**, **43**-**45**) giving the best docking scores in the range of -7.87 to -6.02 kcal mol^{-1}. A closer look at the interaction profile of these

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molecules revealed the carboxamide –NH_2 at site 2 to be involved in an important hydrogen bonding interaction with Asp 79. This interaction is believed to be critical in improving the bioactivity. The carboxylic acid (**13-15**, **22-24**, **31-33**, **40-42**) and the ester analogues (**10-12**, **19-21**, **28-30**, **37-39**) however failed to retain this interaction thus accounting for their reduced activity. A cation-π interaction was also observed between the π-electron environment of core thiazole ring and the ammonium ion (NH_3^+) present in the guanidine group of Arg82 in the case of active hits. Effect of hydrophobicity on activity determination was also evident from the docking studies. The aryl/heteroaryl ring at site 1 was found to be stabilised by hydrophobic interaction with Ala59, Val128, Val50, Val49, Met100, Ile171, or Val199 amino acid residues and accounted for the good to moderate activity shown by these molecules. A 2D interaction profile of few active and inactive compounds has been provided in the supplementary information (**Figure S4-S9**) for a better understanding.

Furthermore, the binding affinity of the most potent analogue was evaluated by measuring the thermal stability of the protein–ligand complex using biophysical differential scanning fluorimetry experiments. by measuring the fluorescence of the native protein and protein-ligand complexes in presence of a fluorescent dye whose fluorescence increases when exposed to non polar residues of the protein and reach the maximum when the protein denature.[29] A higher or positive shift of melting temperature (T_M) of protein-ligand complex compared to the native protein T_M signify a better stabilization of the protein-ligand complex, which in turn would reflect on the inhibitor binding. The most potent analogue compound **27** displayed a T_m shift of 3.2°C compared with the native protein, a repercussion of strong binding of the ligand to the protein and highly correlated with its GyrB IC_{50} of 0.44±1.1 μM. The curves obtained are depicted in **Figure 4**.

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Further the compounds were also subjected the DNA supercoiling assay[27] using DNA gyrase from *Mycobacterium tuberculosis* (Inspiralis, Norwich) to reconfirm their activity; as any inhibition of ATPase activity conferred by the DNA GyrB subunit should also inhibit the super coiling activity performed by GyrA domain. Moxifloxacin was used as a positive control in the study. In the supercoiling assay out of the 36 compounds tested, 33 compounds displayed supercoiling inhibitory IC_{50} < 25 μM, out of which 28 compounds demonstrated IC_{50} < 15 μM, 11 compounds showed IC_{50} < 5 μM and 8 compounds were found to inhibit supercoiling activity even at sub micromolar concentration (IC_{50} < less 1 μM). The most potent compound from the GyrB assay compound **27** with a GyrB IC_{50} of 0.44±1.1 μM showed an equally well correlating supercoiling IC_{50} of10.5±0.24 μM.

The compounds were also screened for their in-vitro antimycobacterial potency against *Mycobacterium tuberculosis* H_{37}Rv by the MABA assay.[30] In general; a good correlation was observed between the *Mycobacterium smegmatis* GyrB inhibitory IC_{50}, *Mycobacterium tuberculosis* DNA Gyrase supercoiling assay IC_{50} value and the in-vitro *Mycobacterium tuberculosis* minimum inhibitory concentration (MIC). Eleven compounds inhibited *Mycobacterium tuberculosis* with MIC less than 15 μM; Compounds **25**, and **27** were promising analogues, with MICs less than 10 μM. These compounds were found to be more active than the first-line anti-tubercular drug ethambutol (MIC = 15.31 μM) but were less active compared with isoniazid (MIC = 0.66 μM) and rifampicin (MIC = 0.23 μM)

The most potent compounds were further examined for hERG channel inhibition by assessing arrhythmogenic potential in a zebrafish model. Zebrafish (*Danio rerio*) is an attractive preliminary in-vivo model for screening compounds with potential pro-arrhythmic, hERG blockade & QT prolongation.[31] Compounds **18**, **25** and **27** were exposed from 1μM to 30μM concentration with 0.1% DMSO as a vehicle by using a previously reported protocol.[32-33]

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Terfenadine (20μM) was used as a positive control. All the compounds were analyzed for heart rate variations and AV ratio (**Figure 4**). The tested compounds though completely safe at lower concentrations tested showed mild ventricular bradycardia, as compared to control group at 30 μM concentration, which can be considered to be biologically insignificant from an overall perspective. Encouragingly, there was no effect on atrio-ventricular ratio in any concentration of the test compounds. This data is of significant importance considering the intrinsic challenges that some of the previously reported GyrB inhibitors (especially the NBTIs class) had in achieving a acceptable cardiovascular safety profile.

Finally the safety profile of these molecules were also evaluated by checking the in vitro cytotoxicity against RAW 264.7 celline (mouse macrophage) at 100 μM concentration by the (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[34] All the compounds tested were relatively safe and showed a selectivity profile.

A preliminary view of ADMET parameters predicted using QikProp3.5 module of Schrodinger software[28] depicted that all the hits preserved the basic criterion of drug-likeness showing characteristics (**Table S1** in supplementary information) of a promising drug leads which can be worked for rational drug design against *Mycobacterium tuberculosis* gyraseB from pharmaceutical point of view.

Conclusion:

In the present study, using the concept of molecular hybridization, a library of 36 molecules were designed and synthesized by fusing previously reported gyrase analogues through a newly introduced triazole linker, incorporated primarily to reduce the hERG toxicity that is believed to arise from the aminopiperidine nucleus - the linking unit in many of the previous gyrase inhibitors. The synthesized molecules displayed good GyrB inhibitory potency with

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equally well correlating supercoiling inhibitory activity. Compound **27** emerged as the most potent lead with a *Mycobacterium semgmatis* GyrB inhibitory IC_{50} of 0.44 ± 1.1 μM, *Mycobacterium tuberculosis* supercoiling IC_{50} of 0.5 ± 0.24 μM and *Mycobacterium tuberculosis* MIC of 6.5 μM. Structural handles critical for retaining the bioactivity were identified through in-silico investigations which revealed the interaction with Asp 79 along with the hydrophobic contacts that are the key attributes to activity. Compounds in the series demonstrated excellent in-vitro mycobacterial kill and showed no signs of cardiotoxicity unlike some of the previously reported gyrase analogues.

Experimental section:

Chemistry:

Representative experimental procedures

General procedure utilised for developing the designed analogues (10-45): To a mixture of the corresponding acetylene (1 equiv) and corresponding azide (1.1equiv) in THF:H_2O (1:1) system was added $CuSO_4.5H_2O$ (0.05 equiv), sodium ascorbate (0.1equiv) at 27^oC. The reaction was then stirred at 27^oC for 12-15 hours (monitored by TLC & LCMS for completion). The residue was further diluted with water (2 mL) and dichloromethane (4 mL) and the layers separated. The aqueous layer was re-extracted with dichloromethane (2 x 4 mL) and the combined organic layer was washed with brine (3 mL), dried over anhydrous sodium sulphate and evaporated under reduced pressure and the residue re-crystallized form diethyether/ethanol to afford the desired product in good yield as described below.

4-((1-(2-oxo-2H-chromen-3-yl)-1H-1,2,3-triazol-5-yl)methoxy)-2-phenylthiazole-5-carboxamide (16): The compound was synthesized according to the above general procedure using 4-(Prop-2-yn-1-yloxy)-2-phenylthiazole-5-carboxamide **(9a**, 0.1 g, 0.39 mmol), 3-azido-2H-chromen-2-one (0.08 g, 0.43 mmol), $CuSO_4.5H_2O$ (0.005 g, 0.02 mmol), sodium ascorbate (0.008 g, 0.04 mmol) to afford **16** (0.13 g, 75.6 %) as buff coloured solid. M.p: 190

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– 192°C. ^{1}H NMR ($CDCl_3$): $\delta_{H.}$ 5.79 (s, 2H), 6.96 – 8.79 (m, 11H). ^{13}C NMR ($CDCl_3$): δc.165.8, 163.3, 161.1, 153.4, 148.6, 144.1, 143.5, 141.1, 131, 129.3, 128.8, 128.5, 128.3, 127.8, 125.3, 123.5, 123.3, 122.2, 115.9, 63.4. ESI-MS *m/z* 446.3 $(M+H)^+$. Anal Calcd for $C_{22}H_{15}N_5O_4S$; C, 59.32; H, 3.39; N, 15.72; Found: C, 59.31; H, 3.4; N, 15.74.

4-((1-(7-hydroxy-2-oxo-2H-chromen-3-yl)-1H-1,2,3-triazol-5-yl)methoxy)-2-phenylthiazole-5-carboxamide (17): The compound was synthesized according to the above general procedure using 4-(Prop-2-yn-1-yloxy)-2-phenylthiazole-5-carboxamide **(9a**, 0.1 g, 0.39 mmol), 3-azido-6-hydroxy-2H-chromen-2-one (0.087 g, 0.43 mmol), $CuSO_4.5H_2O$ (0.005 g, 0.02 mmol), sodium ascorbate (0.008 g, 0.04 mmol) to afford **17** (0.14 g, 78.2 %) as buff coloured solid. M.p: 262 - 264°C. ^{1}H NMR ($CDCl_3$): $\delta_{H.}$ 5.78 (s, 2H), 6.86 – 8.80 (m, 10H). ^{13}C NMR ($CDCl_3$): δc.165.4, 163.2, 161.3, 156.4, 154.2, 148.4, 143.8, 143.2, 141.3, 130.9, 130.2, 129.2, 128.4, 128.3, 123.1, 122.9, 114.6, 112.7, 102.6, 63.3. ESI-MS *m/z* 462.1 $(M+H)^+$. Anal Calcd for $C_{22}H_{15}N_5O_5S$; C, 57.26; H, 3.28; N, 15.18; Found: C, 57.24; H, 3.29; N, 15.2.

4-((1-(7-methoxy-2-oxo-2H-chromen-3-yl)-1H-1,2,3-triazol-5-yl)methoxy)-2-phenylthiazole-5-carboxamide (18): The compound was synthesized according to the above general procedure using 4-(Prop-2-yn-1-yloxy)-2-phenylthiazole-5-carboxamide **(9a**, 0.1 g, 0.39 mmol), 3-azido-6-methoxy-2H-chromen-2-one (0.094 g, 0.43 mmol), $CuSO_4.5H_2O$ (0.005 g, 0.02 mmol), sodium ascorbate (0.008 g, 0.04 mmol) to afford **18** (0.16 g, 88.9 %) as buff coloured solid. M.p: 240 - 242°C. ^{1}H NMR (DMSO-d_6): $\delta_{H.}$ 3.89 (s, 3H), 5.78 (s, 2H), 6.97 – 7.15 (m, 3H), 7.54 – 8.81 (m, 9H). ^{13}C NMR (DMSO-d_6): δc. 165.6, 163.4, 161.3, 159.4, 156.1, 154.5, 142.5, 135.7, 132.2, 131.3, 130.6, 129.3, 125.9, 120.1, 113.5, 111.4, 109, 100.6, 63.4, 56.1. ESI-MS *m/z* 476.1 $(M+H)^+$. Anal Calcd for $C_{23}H_{17}N_5O_5S$; C, 58.10; H, 3.60; N, 14.73; Found: C, 58.09; H, 3.59; N, 14.74.

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4-((1-(2-oxo-2H-chromen-3-yl)-1H-1,2,3-triazol-5-yl)methoxy)-2-(pyridin-2-yl)thiazole-5-carboxamide (25): The compound was synthesized according to the above general procedure using 4-(Prop-2-yn-1-yloxy)-2-(pyridin-2-yl)thiazole-5-carboxamide **(9b**, 0.1 g, 0.39 mmol), 3-azido-2H-chromen-2-one (0.08 g, 0.43 mmol), $CuSO_4.5H_2O$ (0.005 g, 0.02 mmol), sodium ascorbate (0.008 g, 0.04 mmol) to afford **25** (0.13 g, 75.6 %) as buff coloured solid. M.p: 184 – 186 ^oC. ^1H NMR ($CDCl_3$): $\delta_{H.}$ 5.77 (s, 2H), 7.01 - 8.82 (m, 10H), ^{13}C NMR ($CDCl_3$): δc. 164.2, 163, 160.7, 154.6, 152.9, 148.9, 147.6, 142.8, 141.2, 137.4, 128.7, 128.5, 127.9, 125.2, 123.9, 123.4, 123.1, 122.9, 122, 115.9, 63.2. ESI-MS *m*/*z* 447.1 $(M+H)^+$. Anal Calcd for $C_{21}H_{14}N_6O_4S$; C, 56.50; H, 3.16; N, 18.82; Found: C, 56.49; H, 3.14; N, 18.8.

4-((1-(7-hydroxy-2-oxo-2H-chromen-3-yl)-1H-1,2,3-triazol-5-yl)methoxy)-2-(pyridin-2-yl)thiazole-5-carboxamide (26): The compound was synthesized according to the above general procedure using 4-(Prop-2-yn-1-yloxy)-2-(pyridin-2-yl)thiazole-5-carboxamide **(9b**, 0.1 g, 0.39 mmol), 3-azido-6-hydroxy-2H-chromen-2-one (0.08 g, 0.43 mmol), $CuSO_4.5H_2O$ (0.005 g, 0.02 mmol), sodium ascorbate (0.008 g, 0.04 mmol) to afford **26** (0.15 g, 84.3 %) as buff coloured solid. M.p: 237 – 239 ^oC. ^1H NMR ($CDCl_3$): $\delta_{H.}$ 5.75 (s, 2H), 6.89 – 8.81 (m, 9H). ^{13}C NMR ($CDCl_3$): δc. 164, 162.8, 160.5, 156.1, 154.6, 154.3, 148.7, 147.6, 143, 141.1, 137.2, 130.2, 128.5, 124, 123.5, 123.3, 123.1, 114.6, 112.6, 102.4, 63. ESI-MS *m*/*z* 463.2 $(M+H)^+$. Anal Calcd for $C_{21}H_{14}N_6O_5S$; C, 54.54; H, 3.05; N, 18.17; Found: C, 54.52; H, 3.06; N, 18.14.

4-((1-(7-methoxy-2-oxo-2H-chromen-3-yl)-1H-1,2,3-triazol-5-yl)methoxy)-2-(pyridin-2-yl)thiazole-5-carboxamide (27): The compound was synthesized according to the above general procedure using 4-(Prop-2-yn-1-yloxy)-2-(pyridin-2-yl)thiazole-5-carboxamide **(9b**, 0.1 g, 0.39 mmol), 3-azido-6-methoxy-2H-chromen-2-one (0.08 g, 0.43 mmol), $CuSO_4.5H_2O$ (0.005 g, 0.02 mmol), sodium ascorbate (0.008 g, 0.04 mmol) to afford **27** (0.16 g, 87 %) as buff coloured solid. M.p: 224 – 226 ^oC. ^1H NMR (DMSO-d_6): $\delta_{H.}$ 3.89 (s, 3H), 5.78 (s, 2H),

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6.94 – 8.84 (m, 9H). ^{13}C NMR (DMSO-d_6): δc. 164.3, 163.1, 160.6, 159.9, 154.6, 153.9, 149, 147.8, 143.1, 141.2, 137.4, 130, 128.6, 124, 123.7, 123.4, 123.2, 114.8, 110.9, 100.5, 63.1, 56.2. ESI-MS *m/z* 477.1 $(M+H)^+$. Anal Calcd for $C_{22}H_{16}N_6O_5S$; C, 55.46; H, 3.38; N, 17.64; Found: C, 55.43; H, 3.35; N, 17.62. For details regarding the experimental section of other compounds synthesised eg. 6a-d, 7a-d, 8a-d, 9a-d, 10-15, 19-24 and 28-45 refer ESI.

Biological evaluation

MS GyrB ATPase assay:

Being the gyrase enzyme catalytic site the gyraseB domain performs the ATPase assay with the sole GyrB subunit. The assay was performed in 30 µL reaction volume for 120 min at 25°C in reaction buffer containing 60 mM HEPES-KOH pH 7.7, 250 mM potassium glutamate, 200 mM KCl, 2 mM magnesium chloride, 1 mM DTT, 2% Glycerol, 4% DMSO, 0.001% BriJ, 0.65 mM ATP, 40 nM GyrB as previously published method (Pravin etal, 2012). All the test compounds were diluted in 4% DMSO to about eight concentrations for the determination of IC_{50}. ATPase assay was performed in V-shaped 96-well plates (Polystyrene untreated). Initially 15 µL of 2x assay buffer containing purified GyrB enzyme and substrate mix were placed in the assay well followed by 1 µL of test compound, subsequently the enzyme reaction was initiated by adding 14 µL of $MgCl_2$ solution, as metal ion triggers the enzyme. The reaction was allowed to proceed for 120 min at room temperature. At the end, 20µL malachite green reagent (Bioassay systems) was added to quench the reaction and incubated for 20 min to determine the inorganic phosphates (Pi) released when measured at 635 nm wavelength against the blank absorbance. In this assay, novobiocin was considered as positive control and moxifloxacin as the negative control.

In-vitro *Mycobacterium tuberculosis* (*M.tb*) MABA assay:

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The compounds were further screened for their *in vitro* antimycobacterial activity against *M. tuberculosis* H37Rv by microplate Alamar blue assay method[S7]. Briefly, the inoculum was prepared from fresh LJ medium re-suspended in 7H9-S medium (7H9 broth, 0.1% casitone, 0.5% glycerol, supplemented oleic acid, albumin, dextrose, and catalase [OADC]), adjusted to a McFarland tube No. 1, and diluted 1:20; 100 µl was used as inoculum. Each drug stock solution was thawed and diluted in 7H9-S at four-fold the final highest concentration tested. Serial two-fold dilutions of each drug were prepared directly in a sterile 96-well microtiter plate using 100 µl 7H9-S. A growth control containing no antibiotic and a sterile control were also prepared on each plate. Sterile water was added to all perimetre wells to avoid evaporation during the incubation. The plate was covered, sealed in plastic bags and incubated at 37^{o}C in normal atmosphere. After 7 days incubation, 30 ml of alamar blue solution was added to each well, and the plate was re-incubated overnight. A change in colour from blue (oxidised state) to pink (reduced) indicated the growth of bacteria, and the MIC was defined as the lowest concentration of drug that prevented this change in colour. For details regarding the protocol utilised for cloning and purification of protien, supercoiling assay, docking, toxicity evaluation, DSF experiments and ADMET calculations refer ESI.

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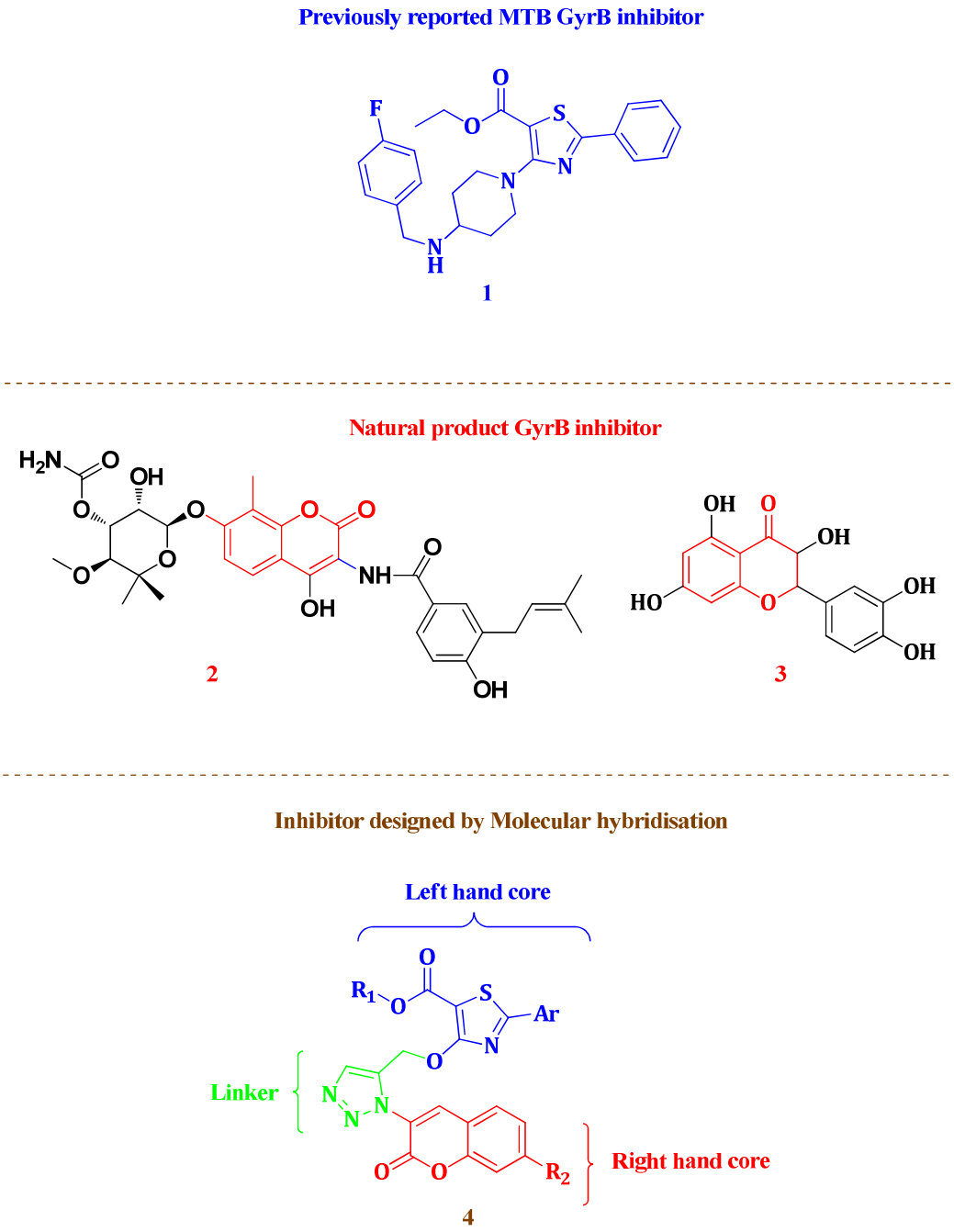

Figure 1: Chemical structure of previously reported synthetic inhibitors of DNA GyrB bearing thiazole nucleus (**1**), natural product inhibitor novobiocin (**2**) and quercetin (**3**) bearing coumarin nucleus and the inhibitor designed through molecular hybridisation (**4**).

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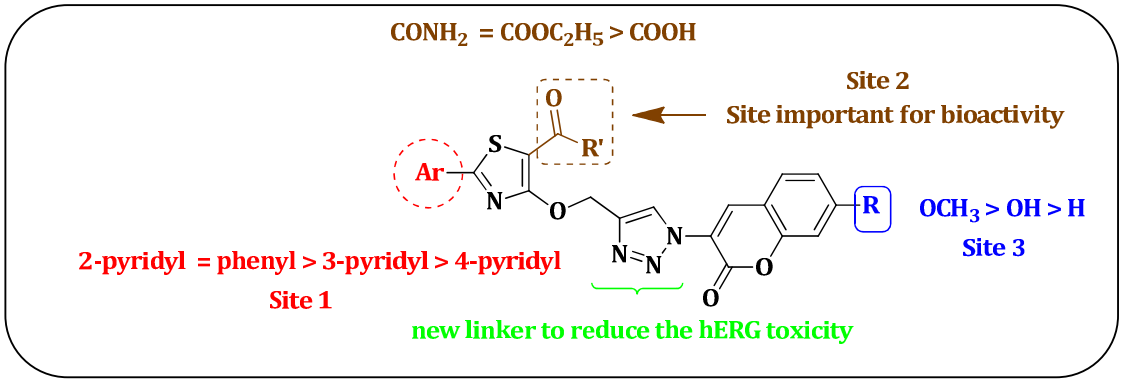
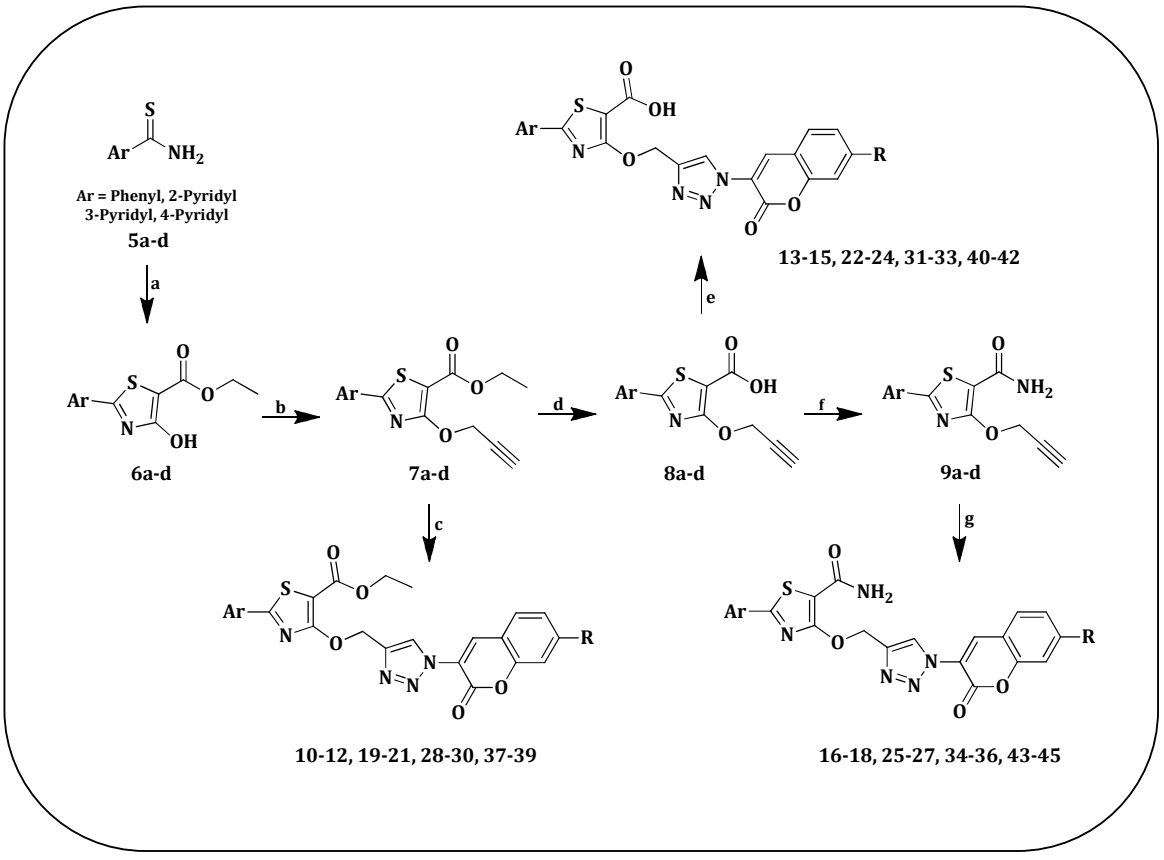


Figure 2: Structure-activity profiling.



Scheme 1: Reagents and conditions. a. diethylbromomalonate, pyridine, toluene, reflux; b. propargyl alscohol, DEAD, triphenylphosphine, THF,0^{o}C to rt; c. Azido coumarin, $CuSO_4$, sodium ascorbate, THF:H_2O, rt; d. LiOH, THF:CH_3OH::H_2O, rt; e. azido coumarin, $CuSO_4$, sodium ascorbate, THF:H_2O, rt; f. (i) CO_2Cl_2, DCM, DMF (cat), (ii) NH_4OH; g. azido coumarin, $CuSO_4$, Sodiuma ascorbate, THF:H_2O.

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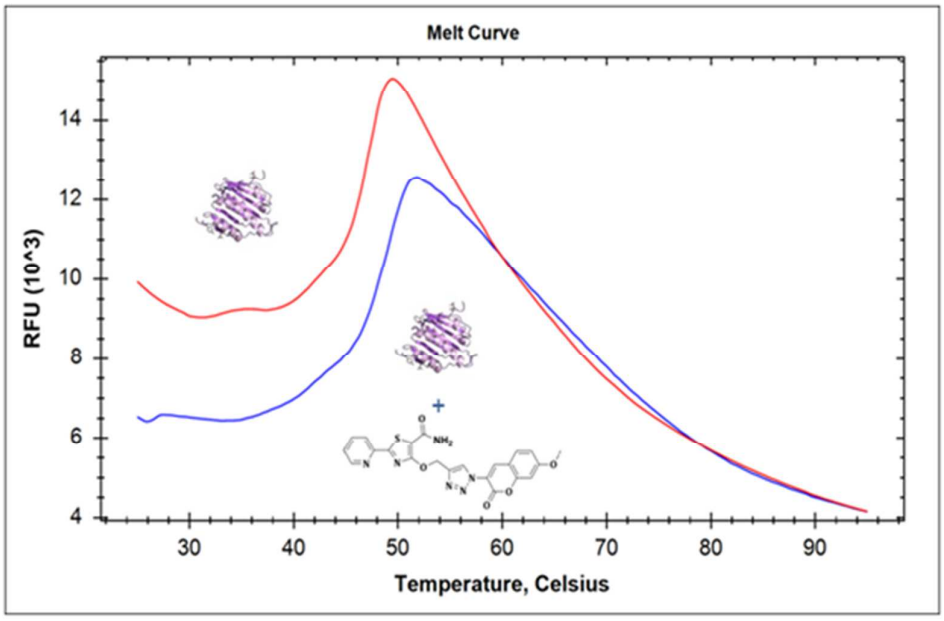
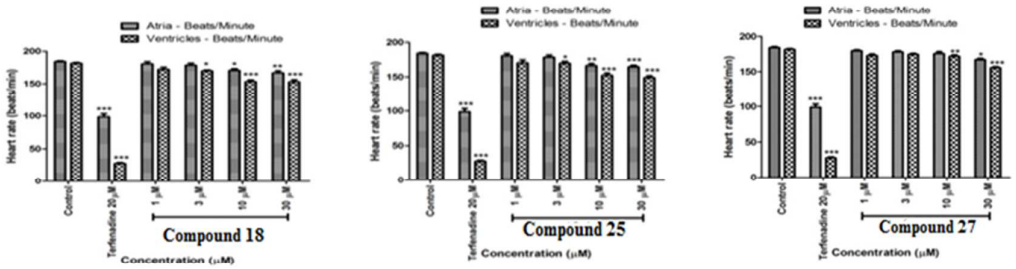


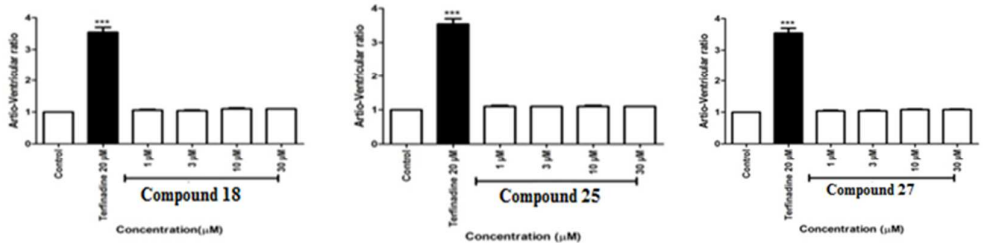
Figure 3: DSF curve for compound **27** a T_M shift of 3.2°C showing an increase in thermal stability between the native protein G24 protein (red) and DNA gyraseB protein-compound complex (blue).

Fig 4a : depicts the results of the heart rates of atria and ventricles



Mean (±S.E.M.) of the heart rates of atria and ventricles of (Compound 18, 25 and 27) treatment groups. (*p<0.05, **p<0.01 and ***p<0.001). Statistical significance was analyzed with respect to the control group.

Fig 4b: depicts the atrio-venticular beat ratio



Mean (±S.E.M.) score of atrio ventricular ratio of (Compound 18, 25 and 27) treatment groups. (*p<0.05, **p<0.01 and ***p<0.001). Statistical significance was analyzed comparing control group Vs all groups.

Figure 4: Evaluation of cardiotoxicity of the compounds **18**, **25** and **27** in zebra fish model.

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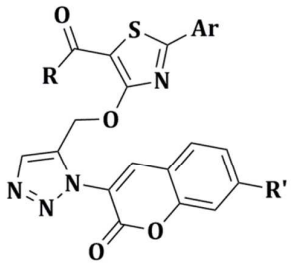
Table 1: In-vitro inhibitory potency of synthesised analogues **10 - 45**.



10 - 45

Cmpd.	Ar	R	R'	GyrB assay (IC_{50})[b]	Supercoiling assay (IC_{50})[a]	MIC µM[c]	Cytotoxicity (% inhib:)[c]
10	Phenyl	OC_2H_5	H	19.1±1.3	7.3±0.62	26.34	23.57
11	Phenyl	OC_2H_5	OH	16.13±0.17	11.31±0.13	25.49	24.23
12	Phenyl	OC_2H_5	OCH_3	18.38±0.76	13.5±0.81	24.79	25.88
13	Phenyl	OH	H	>50	>25	56.0	27.31
14	Phenyl	OH	OH	21.8±0.82	17.4±0.66	27.03	36.06
15	Phenyl	OH	OCH_3	20.36±1.3	14.9±0.42	26.23	27.99
16	Phenyl	NH_2	H	1.2±0.48	0.62±0.30	14.03	26.31
17	Phenyl	NH_2	OH	1.8±0.66	0.71±0.16	27.08	30.17
18	Phenyl	NH_2	OCH_3	0.92±0.28	0.63±0.28	13.15	21.86
19	2-Pyridyl	OC_2H_5	H	21.45±0.97	10.2±0.71	26.29	14.58
20	2-Pyridyl	OC_2H_5	OH	14.66±0.33	11.2±0.64	12.72	29.35
21	2-Pyridyl	OC_2H_5	OCH_3	5.7±0.18	5.5±0.24	12.72	24.69
22	2-Pyridyl	OH	H	>50	>25	111.75	39.16
23	2-Pyridyl	OH	OH	16.7±1.2	15.8±0.63	26.97	13.96
24	2-Pyridyl	OH	OCH_3	18.93±0.77	15.6±0.31	26.18	31.93
25	2-Pyridyl	NH_2	H	0.71±0.72	0.44±0.17	7.00	23.61
26	2-Pyridyl	NH_2	OH	1.6±0.72	0.8±0.32	13.5	29.42
27	2-Pyridyl	NH_2	OCH_3	0.44±1.1	0.5 ±0.55	6.5	26.72
28	3-Pyridyl	OC_2H_5	H	15.8±1.5	11.3±0.92	26.29	11.32
29	3-Pyridyl	OC_2H_5	OH	14.3±0.91	10.6±1.12	25.43	22.22
30	3-Pyridyl	OC_2H_5	OCH_3	2.24±0.22	0.5±0.22	12.36	11.52
31	3-Pyridyl	OH	H	23.6±0.82	16.1±0.41	27.93	27.57
32	3-Pyridyl	OH	OH	27.54±1.1	18.2±0.76	53.95	24.78
33	3-Pyridyl	OH	OCH_3	22.7±2.1	12.4±0.27	26.18	20.67
34	3-Pyridyl	NH_2	H	2.896±0.14	1.7±0.61	13.99	31.89
35	3-Pyridyl	NH_2	OH	1.354±0.25	0.75±0.21	27.03	24.91
36	3-Pyridyl	NH_2	OCH_3	7.3±0.21	8.5±0.92	13.12	13.80
37	4-Pyridyl	OC_2H_5	H	18.91±0.8	9.1±0.51	26.28	35.5
38	4-Pyridyl	OC_2H_5	OH	15.25±0.53	9.7±0.13	25.43	56.5
39	4-Pyridyl	OC_2H_5	OCH_3	19.8±0.24	8.3±0.31	24.72	41.5
40	4-Pyridyl	OH	H	32.1±0.46	>25	111.75	15.8
41	4-Pyridyl	OH	OH	17.38±0.75	14.9±0.48	26.97	18.64
42	4-Pyridyl	OH	OCH_3	14.98±0.44	11.4±0.91	26.18	34.49

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43	4-Pyridyl	NH_2	H	17.27±1.6	11.8±0.31	27.99	30.41
44	4-Pyridyl	NH_2	OH	7.79±0.43	1.56±0.89	13.51	26.88
45	4-Pyridyl	NH_2	OCH_3	16.28±0.18	13.3±0.24	26.23	29.69
	Novobiocin			46±10 nM	180±3.9 nM	nd	81.67
	Isoniazid			nd	nd	0.66	nd
	Rifampicin			nd	nd	0.23	nd
	Ofloxacin			nd	nd	2.16	nd

[a]*Mycobacterium smegmatis* GyrB ATPase activity, [b] *Mycobacterium tuberculosis* DNA Gyrase supercoiling activity, [c]in vitro *Mycobacterium tuberculosis* activity, [d]At 100μM against RAW 264.7 cells, nd: indicates not determined

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