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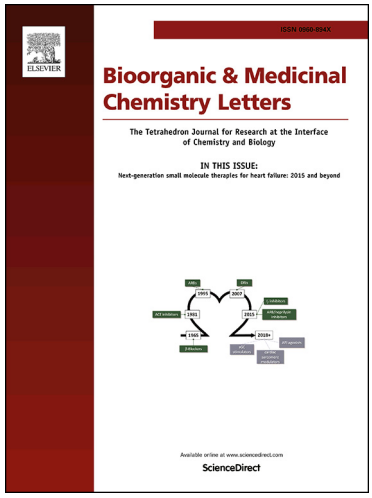

Hit Discovery of Mycobacterium tuberculosis Inosine 5′-Monophosphate Dehydrogenase, GuaB2, Inhibitors

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Hit Discovery of Mycobacterium tuberculosis Inosine 5′-Monophosphate Dehydrogenase, GuaB2, Inhibitors

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[b] *Drug Discovery and Development Centre (H3D), University of Cape Town, Rondebosch 7701, Cape Town, South Africa.*
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ABSTRACT

Tuberculosis remains a global concern. There is an urgent need of newer antitubercular drugs due to the development of resistant forms of *Mycobacterium tuberculosis* (*Mtb*). Inosine 5′-monophosphate dehydrogenase (IMPDH), *guaB2*, of *Mtb*, required for guanine nucleotide biosynthesis, is an attractive target for drug development. In this study, we screened a focused library of 73 drug-like molecules with desirable calculated/predicted physicochemical properties, for growth inhibitory activity against drug-sensitive *Mtb*H37Rv. The eight hits and mycophenolic acid, a prototype IMPDH inhibitor, were further evaluated for activity on purified *Mtb*-GuaB2 enzyme, target selectivity using a conditional knockdown mutant of *guaB2* in *Mtb*, followed by cross-resistance to IMPDH inhibitor-resistant SRMV2.6 strain of *Mtb*, and activity on human IMPDH2 isoform. One of the hits, **13**, a 5-amidophthalide derivative, has shown growth inhibitory potential and target specificity against the *Mtb*-GuaB2 enzyme. The hit, **13**, is a promising molecule with potential for further development as an antitubercular agent.

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Tuberculosis (TB), an infectious disease caused by the bacillus *Mycobacterium tuberculosis (Mtb)*, is the ninth leading cause of death worldwide and number one from a single infectious agent, ranking above HIV/AIDS.[1] The increased prevalence of infections caused by multidrug- resistant (MDR) and extensively drug-resistant (XDR) strains of *Mtb*, with limited treatment choices, is a wake-up call urging the development of more effective antitubercular agents, ideally with novel mechanism(s) of action. This is not the end of the story! Emergence of totally drug-resistant (TDR)-TB in which *Mtb* is resistant to all first- and second-line antitubercular drugs has terrified the healthcare professionals.[2]

Tuberculosis, mainly being the disease of the developing or underdeveloped nations, was a neglected disease in terms of drug discovery. Bedaquiline[3], a recently approved antitubercular drug, has shown promise in treating TB. Several drug repurposing campaigns of approved drugs[4,5] are likely to offer potential alternatives for the treatment of TB. In light of the fact that the strategies involving development of potent and target-selective enzyme inhibitors which arrested essential biochemical processes, failed miserably in whole-cell *Mtb* assays[6], newer molecules with novel mechanism(s) of action are essential to tackle TB menace. One such pathway is purine nucleotide biosynthesis. The enzyme inosine 5′-monophosphate dehydrogenase (IMPDH, EC 1.1.1.205) catalyzes a crucial step in the biosynthesis of guanine nucleotides leading to oxidation of inosine 5′-monophosphate (IMP) to xanthosine 5′-monophosphate (XMP) with concomitant reduction of nicotinamide adenine dinucleotide (NAD^+) to reduced nicotinamide adenine dinucleotide (NADH).[8] The XMP is further converted into guanosine 5´-monophosphate (GMP) by GMP synthase. Inhibition of IMPDH leads to depletion of guanine nucleotide pool, affecting cell division, and ultimately inhibition of cell proliferation.

The enzyme IMPDH, thus, is an interesting target for anticancer, antiviral, immunosuppressive and antimicrobial therapies.[9] Mycophenolic acid (MPA, **1**, Figure 1) is an IMPDH inhibitor widely used as immunosuppressant and antiviral drug.[10] Three genes encode IMPDH in *Mtb*, namely, *guaB1* (*Rv1843c*), *guaB2* (*Rv3411c*) and *guaB3* (*Rv3410c*). However, only *guaB2* has shown the IMPDH activity.[11,12]

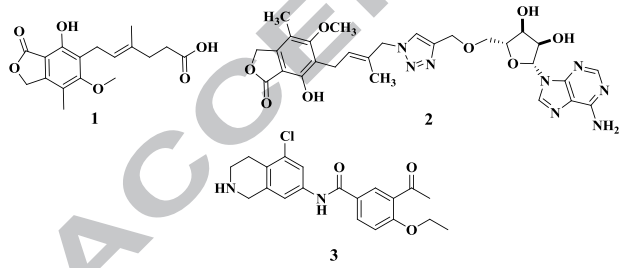


Figure 1. Mycophenolic acid (MPA) and earlier *Mtb*IMPDH inhibitors

Chen et. al. reported mycophenolic adenine dinucleotides (MAD1) as a *Mtb*IMPDH inhibitors, where the pyrophosphate linker in **NAD^+** was replaced with isosteric 1,2,3-triazole (**2**, K_i^{app}= 1.5 µM).[13] Recently, several *Mtb*-GuaB2 inhibitors have been identified in a target-based high-throughput resistance-based phenotypic screen.[14] The identified hits are represented by compound **3**. In similar studies, *Cryptosporidium purvum* IMPDH (*Cp*IMPDH) selective inhibitors belonging to five chemical series (**4**-**8**, Figure 2) were screened against *Mtb*IMPDH.[7] Few of these molecules were potent inhibitors (**9**, P series and **10**, Q series, Figure 3).[10] Another series, 1*H*-benzo[*d*]imidazole, of *Mtb*IMPDH was reported with submicromolar inhibition constants (**11**, Figure 3).[15]

Careful examination of the common structural features of the lead molecules (**3**, **9**, **10** and **11**, Figures 1 and 3), the authors adopted a pharmacophore-based design strategy for GuaB2 inhibitors - two aromatic moieties connected with a linker (Figure 4). Indeed, many human IMPDH2 inhibitors possessed similar features and the nature of the linker was shown to be crucial in modulating potency and selectivity, if any.[16,17] Learning from previous experience with IMPDH inhibitors, a subset of our in-house library (#60) matching the pharmacophore criteria along with few compounds similar to MPA (#12) were initially screened for *anti-Mtb* activity in drug-sensitive *Mtb* H37Rv strain, followed by evaluation of the hits (#8) on two derivatives of *Mtb H37Rv*: (i) for target selectivity - *guaB2-B3* Tet-OFF *attB*::*guaB3*, a conditional knockdown mutant (cKD, *guaB2* Tet-OFF) in which *Mtb*IMPDH levels are depleted by transcriptional silencing of the IMPDH-encoding gene, *guaB2* and (ii) for cross-resistance - *SRMV2.6*, this strain expresses the mutant *Mtb*IMPDH *Y487C*, which is resistant to an isoquinoline sulfonamide *Mtb*IMPDH2 inhibitor.[6] Here, we report the synthesis and biological testing of the hits discovered in whole cell-based and target (*Mtb*IMPDH, GuaB2)-specific assays.

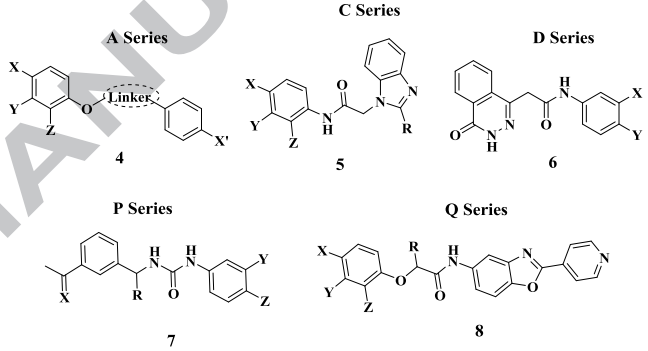


Figure 2. Chemotypes observed in *Cp* - and bacterial IMPDH inhibitors

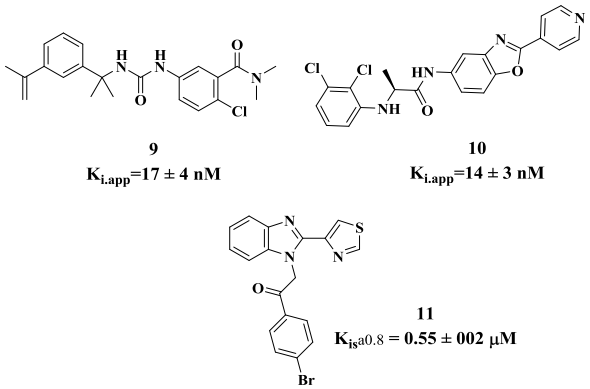


Figure 3. Few *Mtb*IMPDH (GuaB2) inhibitors

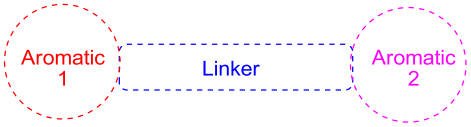


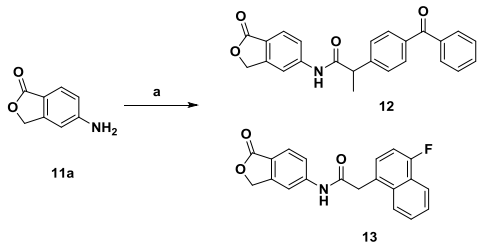
Figure 4. Pharmacophoric features for *Mtb*IMPDH (GuaB2) inhibitors

Methods

Compounds **12** and **13** were synthesized by reacting 5-aminophthalide (**11a**) and substituted acid chlorides, in presence of pyridine (Scheme 1). The synthesis of 1,2,3-triazole derivatives proceeded in two steps as described in Schemes 2, 3 and 4. First, intermediate **15** was synthesized using **14**, hydrazine hydrate and triethyl orthoacetate. Further **15**, α- and β-naphthols

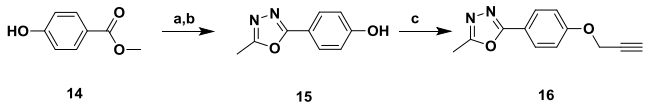
(**17a** and **17b**, respectively) were alkylated with propargyl chloride in presence of anhydrous K_2CO_3 and DMF to yield **16** and **18a-b** (Schemes 2-3). Substituted benzyl azides (**20a-e**, Scheme 3) prepared from corresponding benzyl bromides (**19a-e**), were further reacted with **16** and **18a-b**) in *tert*-butanol and water (3:1 mixture) along with $Cu(OAc)_2$ to yield title compounds **21a-d** and **22a-b** (Scheme 4) in 55-88% yield (Table 1). The synthetic procedures can be found in the Supporting Data section.

Scheme 1[a]. Synthesis of arylalkyl amides **12-13**



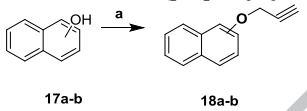
[a]*Reagents and conditions*. a. substituted acid chloride, pyridine, THF, 0 °C to RT, overnight.

Scheme 2[a]. Synthesis of 1,2,3-triazole analogs



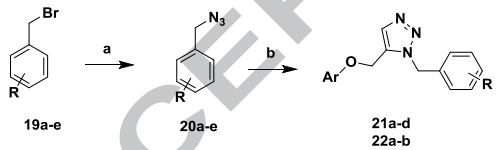
[a]*Reagents and conditions*. a. $NH_2NH_2.H_2O$, EtOH, Reflux; b. triethyl orthoacetate; c. propagyl chloride, K_2CO_3, DMF, 12 hrs

Scheme 3[a]. Synthesis of 1- and 2-(prop-2-ynyloxy)naph-thalene



[a]*Reagents and conditions*. a. Propargyl chloride, K_2CO_3, DMF, 12 hrs

Scheme 4[a]. Synthesis of title compounds



[a]Reagents and conditions
a. NaN_3, *i*-PrOH:H_2O (4:1); b. **16**/**18a**/**18b**, $Cu(OAc)_2$, *t*-BuOH:H_2O (3:1), RT, 8 hrs

Drug Susceptibility Testing

Unless indicated otherwise, minimum inhibitory concentration (MIC) testing was carried out by broth microdilution using the AlamarBlue (AB, Invitrogen) assay.[6] For pairwise combination (checkerboard) assays, a two-dimensional array of serial dilutions of test compound and anyhydrotetracycline (ATc) was prepared in 96-well plates, as previously described.[6,18] The results are shown in Table 1 and Figure 5.

The hits were evaluated for antibacterial activity against five bacterial strains at 60 to 146 μM (32 μg/mL) concentration. The bacterial strains included one Gram-positive (*Staphylococcus aureus*, MRSA) and four Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*).[19] The results are summarized in Table 3S (*Supplementary Data)*.

Biochemical assay

All the hits were further tested in our laboratory for human IMPDH2 inhibition according to protocols reported previously.[16,17] The results are shown in Figure 6 and Table 3S (*Supplementary Data*). Inhibition of tested compounds on recombinant *Mtb*-GuaB2 enzymatic activity was assayed as previously reported protocol.[6]

Results and Discussion

From our in-house library generated for the human and *Staphylococcus aureus* IMPDH (*Sa*IMPDH) inhibitor programs, we selected 60 molecules based on their pharmacophoric features for further screening against *Mtb*. Mycophenolic acid (**1**), a prototypical IMPDH inhibitor, was included in this set since *Mtb*IMPDH inhibitors based on MPA structure (**2**, Figure 1) are known.[13] We also included 12 molecules (alkanoic acids) structurally similar to MPA in the screening set. The molecular property ranges for these molecules are shown in Table 2S (*Supplementary Data*). The average logP for the screening set was 2.899 (Min. 0.2022, Max. 5.325). The MPA analogs exhibited lower logP values due to alkanoic acid functionality. None of these molecules exhibited appreciable anti-*Mtb* activity against *Mtb* H37Rv strain (MIC_{90} > 100 μM) (Table 1S). This was not surprising owing to the higher lipophilicity required for crossing *Mtb* cell wall. The screening results for the hits ($MIC_{90} \leq$ 100 μM) are summarized in Table 1.

Of the nine hits, out of 73 molecules screened (Table 1), **21a**, **21c** and **22b** (logP between 3.5 and 4.5) were two-fold more potent than others (**13**, **21d**: logP between 2.5 and 3.5; and **21b**: logP >4.5) except **1** (logP 2.679). Compound **12** (logP 3.669) exhibited MIC_{90} of 100 μM. This could be due to its lower predicted logS (-0.228). Presence of a polar substituent (-OMe, -Cl) at 3rd position on the arylalkyl moiety attached to triazole N (**21a**, **21c** and **22b**) could contribute to higher potency in the 1,2,3-triazole series. Moving this substituent to the 4th position led to complete loss of activity (MIC_{90}> 100 μM) (Table 1S). A planar [mono- or bicyclic (hetero)aromatic] substituent on the left side of the linker was tolerated for anti-*Mtb* activity in the hits (**12**, **21c** and **22b**, Table 1). Replacing this aromatic ring with alicyclic/spirocyclic ring(s) abolished the anti-*Mtb* activity (Table 1S).

The hits were further taken up to test the activity against GuaB2 in target-specific whole-cell *Mtb* assays. The data of the checkerboard assay (Anhydrotetracycline, ATc, vs compounds) against *guaB2* Tet-OFF are presented in Figure 5 (**1**, **12**, **13** and **21a**) and Figures 1S (**21b**, **21c** and **21d)** and 2S (**22a** and **22b**) (*Supplementary Data*). The *guaB2* Tet-OFF strain was a *guaB2* cKD mutant in which *guaB3* expression was unaffected by ATc (i.e., upon ATc treatment only *guaB2* expression was down regulated).[6] The data clearly demonstrated that the transcriptional silencing of *guaB2* (upon ATc addition) in *Mtb* confers hypersensitivity (shift of MICs from left to right) to the compounds (more profound with **13**, MIC_{90} reduced from 100 μM in WT strain to 12.5 μM upon depletion of *guaB2* – both at 0.15 and 0.31 ng/mL ATc). Compounds **1**, **12** and **13** definitely were active against *guaB2*, whereas the activity of other compounds at *guaB2* could not be ascertained from the data obtained from checkerboard assays.

The hits (Table 1) were tested against *SRMV2.6* strain (nsSNP in *guaB2*, *Y487C*)[18] which showed resistance to earlier lead compound VCC234718, an isoquinoline sulfonamide. Here, no significant deviation in the MICs was observed (Table 1), suggesting the unique binding of the hits to the GuaB2 compared to VCC234718. Further these hits were screened for *Mtb*-GuaB2 inhibition assay, where eight molecules inhibited *Mtb*-GuaB2 at 50 μM. Molecules exhibiting more than 50% inhibition were

further subjected for IC_{50} determination, out of which **1**, **12** and **13** showed promising activity 27.2, 6.2 and 3.2 μM, respectively.

The hits were further tested for *h*IMPDH2 inhibition. As seen in Figure 6, compound **1** exhibited the highest *h*IMPDH2 inhibition at 10 μM. In addition, **12**, **22a** and **22b** showed appreciable inhibition (23, 14.75 and 24.93%, respectively, Table 3S, *Supplementary Data*), compared to other hits (*h*IMPDH2 inhibition ≤ 0.1 % at 10 μM). The authors did not pursue these 'nonselective' compounds (**12**, **22a** and **22b**) further and focused the attention on the remaining hits. Compound **13**, due to its potency at GuaB2 (IC_{50} = 3.04 ± 0.03 μM) and selectivity (over *h*IMPDH2) was studied further. The data (Table 1 and Table 3S), thus, affirms the selectivity of **13** for GuaB2 over *h*IMPDH2.

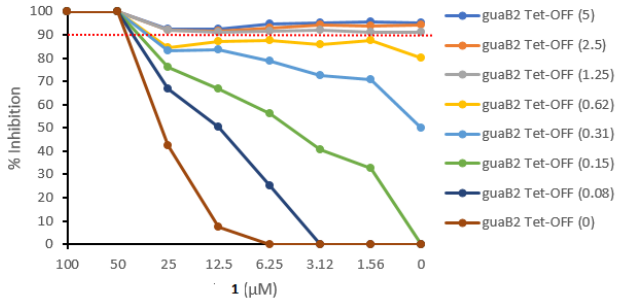
None of the hits showed any antibacterial and antifungal activity at 60 to 146 μM (32 μg/mL) concentration.[19] No further studies were carried out for evaluating target activity against *Sa*IMPDH since the hits were inactive.

Overall, our efforts directed towards discovering hit molecules targeting *Mtb*-GuaB2 led to fruition. We have successfully identified hits belonging to 5-amidophthalide (**13**, Table 1) series which were selective for GuaB2 over *h*IMPDH2. Although compounds from 1,2,3-triazole series showed appreciable anti-*Mtb* activity (**21a**, **21c** and **22b**), their activity at GuaB2 remains to be proved. Earlier, Shaikh et al. reported antitubercular activity of 1,2,3-triazole derivatives against *Mtb H37Rv* strain (MIC 5.8 – 29.9 μg/mL).[20] Similarly, Stanley et al. identified novel *Mtb* growth inhibitors belonging to 1,2,3-triazole series.[21] Interestingly, the nitrotriazole compound was shown to inhibit DprE1 (decaprenyl-phosphoryl-β-d-ribose-2′-epimerase) enzyme required for cell wall biosynthesis in *Mtb*. Future work in this direction is likely to yield desirable results.

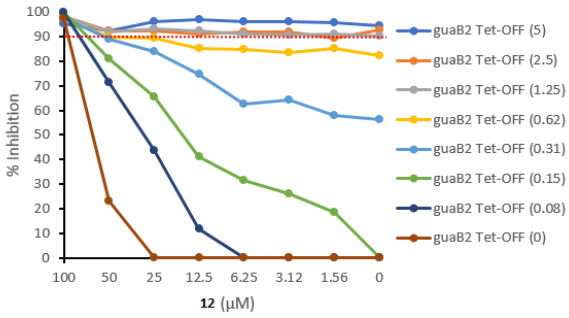
Conclusion

The systematic selection and biological screening of in-house library of potential IMPDH inhibitors led to the identification of novel and selective inhibitors of *Mtb*IMPDH encoded by *guaB2* over *h*IMPDH2. The hits belonged to 5-amidophthalide and 1,2,3-triazole series. Further screening of the hits in target-specific assays confirmed significant activity of **13** on *Mtb*-GuaB2. The triazole hits, despite more potency, failed to establish clear connection with GuaB2 inhibition and anti-*Mtb* activity. The hits did not exhibit significant inhibition against drug-resistant *SRMV2.6* strain, pointing to unique mode of GuaB2 inhibition. The hits can be further exploited in a typical medicinal chemistry program to enhance potency and target selectivity.

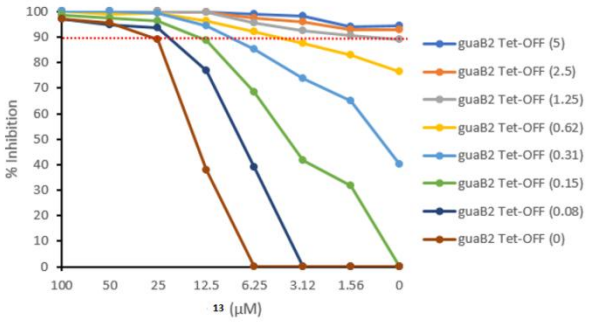
a.



b.



c.



d.

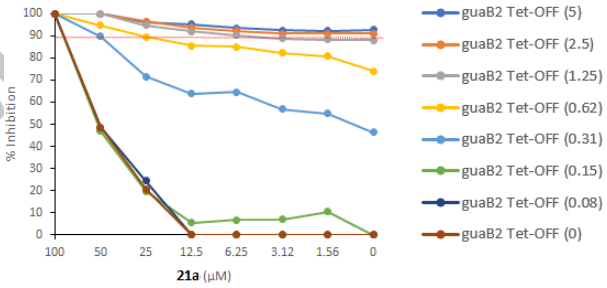


Figure 5. Effects of *guaB2* silencing on susceptibility of *Mtb* to a. **1**; b. **12**; c. **13** and d. **21a**. Values in the parentheses represent ATc concentration (ng/mL). The red-dashed lines represent MIC_{90} values.

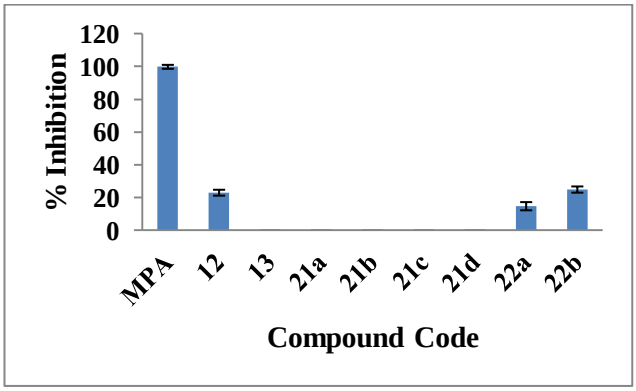


Figure 6. % *h*IMPDH2 inhibition by the hits at 10 μM concentration

Acknowledgment

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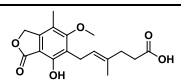
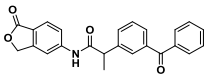
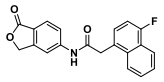
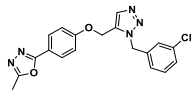
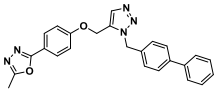
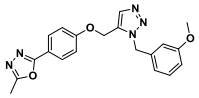
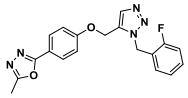
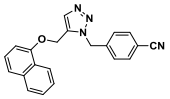
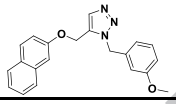
Assistant Professor with SVKM's NMIMS, SPP School of Pharmacy and Technology Management, Mumbai, India and Mr. Chetan Shah, PhD student in PK's group during *h*IMPDH2 inhibition studies. The South African Medical Research Council (SAMRC) is acknowledged for funding. PK thank Optibrium Ltd., USA, for providing one-month demo license of StarDrop™.

Table 1. Anti-*Mtb* activity of the hits

Compound No.	Structure	MIC_{90} (μM)		GuaB2	
		H37Rv	SRMV2.6	**% Inhibiton** (50 μM)	IC_{50} (μM)
1		50	50	83.0 ± 5.7	27.2 ± 0.1
12		100	100	95.2 ± 0.4	6.2 ± 0.1
13		100	100	99.9 ± 0.6	3.04 ± 0.03
21a		50	>100	ni	nd
21b		100	100	72.6 ± 8.4	nd
21c		50	100	35.0 ± 6.4	nd
21d		100	>100	71.0 ± 0.6	nd
22a		100	>100	43.5 ± 3.1	174.7 ± 0.1
22b		50	>100	52.4 ± 8.0	nd

*ni- no inhibition, nd- not determined

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References and notes

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Supplementary Data

The synthetic procedures along with the summary of physicochemical and molecular properties of the screening set, % *h*IMPDH2 inhibition,antibacterial and antifungal activity results, checkerboard assay results for compounds **21b-d** and **22a-b**, and the data from ^{1}H-NMR, mass and HPLC analyses for the hits are included.

Notes

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Graphical Abstract

Hit Discovery of Mycobacterium tuberculosis Inosine 5′-Monophosphate Dehydrogenase, GuaB2, Inhibitors

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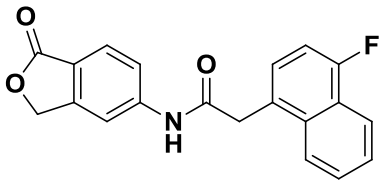
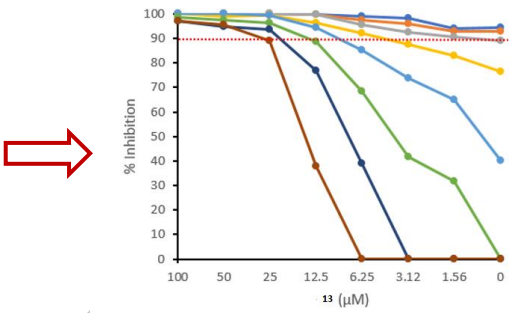
Niteshkumar U. Sahu[a], Vinayak Singh[b,c], Davide M. Ferraris[d], Menico Rizzi[d], and Prashant S. Kharkar[a,*]



13



GuaB2 Tet-OFF

Highlights

- Novel *Mycobacterium tuberculosis* GuaB2 inhibitor (Compound **13**) reported
- Moderately potent biochemical inhibition of *Mtb* GuaB2 by **13**
- Compound **13** is highly selective for Mtb enzyme over its human counterpart (**h**IMPDH2)
- The hit molecule may potentially serve as a lead for further antitubercular drug development