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Inhibition of *M. tuberculosis* β-ketoacyl CoA reductase FabG4 (Rv0242c) by triazole linked polyphenol–aminobenzene hybrids: Comparison with the corresponding gallate counterparts

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ABSTRACT

Herein we report six novel triazole linked polyphenol–aminobenzene hybrids (**3**–**8**) as inhibitors of *Mycobacterium tuberculosis* FabG4 (Rv0242c), a less explored β-ketoacyl CoA reductase that has immense potential to be the future anti-tuberculosis drug target due to its possible involvement in drug resistance and latent infection. Novel triazole linked polyphenol–aminobenzene hybrids have been synthesized, characterized and evaluated for their inhibitory activity against FabG4. All of them inhibit FabG4 at low micromolar concentrations. In silico docking study has been carried out to explain the experimental findings. A comparative study of these new inhibitors with previously reported gallate counterparts leads to structure–activity relations (SAR) of substituent linked to N-1 of triazole ring.

Fatty acid synthesis (FAS) pathway, which functions to build lipid rich cell wall of *Mycobacterium tuberculosis* (*Mtb*), has remained as a key target for anti-tuberculosis research. Due to the presence of this lipid rich cell envelop, *Mtb* cells become virtually impenetrable and survive within the host cell. The FAS in *Mtb* has a complex biosynthetic mechanism consisting of two different pathways, namely FAS-I and FAS-II.[1] FAS-I which is involved in the de novo synthesis of short chain fatty acids, is a multi-domain enzyme. FAS-II is composed of discrete monofunctional enzymes and involved in the production of very long chain fatty acids which are finally converted to mycolic acid, the main constituent of cell wall. FAS carries four basic steps; condensation, reduction, dehydration and saturation. These steps are catalyzed by four major classes of enzymes all belonging to FAS namely, keto-acyl synthase (mtFabH/KasA/KasB), keto-acyl reductase (FabG1), hydroxyacyl dehydrase (HadAB/HadBC) and enoyl reductase (InhA). Naturally, these enzymes have been targeted by various researchers and many first-line anti-TB drugs (like Isoniazid)[2] target one of these four major classes of enzymes for their anti-TB action. But apart from these well-known enzymes, many new alternative enzymes have been reported recently. Apart from FAS I and FAS II, few bypass fatty acid metabolism pathways are hypothesized to be present in *Mtb* and implicated to its drug resistance behaviour.[3] Moreover, the inherent redundancy of polygenic *Mtb* network makes it impossible to shut down a cellular pathway by taking out a single target. Thus, the enzymes involved in bypass pathways are generally became attractive targets for alternate anti-TB drug discovery. One such less explored alternate target is FabG4 (Rv0242c) enzyme that belongs to a conserved putative fatty acid metabolizing operon.[4] FabG4 is a NADH-dependent β-ketoacyl CoA reductase and believed to involve in interlinked CoA dependent fatty acid metabolism pathway[5] instead of traditional FAS pathway. It is recently reported that beside FabG1, FabG4 is also an essential and functional gene for bacterial growth, survival and fatty acid synthesis.[3b,4,6] The FabG4 may also have a role in drug resistance of mycobacterial species as it is over-expressed in sub-inhibitory concentrations of streptomycin.[7] In 2014, it is reported that FabG4 is also over-expressed in pellicle or bio-film mode of growth which indicates its possible involvement in phenotypic resistance during latent infection.[8] Crystal structure of FabG4 shows that it is a high molecular weight ketoacyl reductase (HMwFabG) and contains two distinct domains, domain I and II.[9] Domain II is the typical 'ketoacyl CoA reductase (KAR) domain' containing conserved catalytic tetrad Ser347, Tyr360, Lys364 and Asn319. The active site of FabG4 is covered by two conserved

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loops, namely loop-I and loop-II, which play crucial roles for the activity. The structure of holo-FabG4 bound to hexanoyl-CoA (HXC) (PDB ID: 3V1U) shows that the active site of FabG4 could be accessed from two different sides—namely a major and a minor portal. The co-factor NADH binds at the relatively wide major portal, while CoA/ACP bound fatty acyl substrate accesses the active site via minor portal.[10] The NADH binding location comprise three subsites, namely the nicotinamide binding subsite (N-subsite), the adenosine binding subsite (A-subsite) and the pyrophosphate binding subsite (P-subsite). The basic structural features and function of FabG4 have been represented in Figure 1.

We have recently reported a novel class of triazole linked polyphenol–gallol hybrids (**1**–**2**) as potent inhibitors of *Mt*-FabG4 at low micromolar concentrations for the first time (Fig. 2).[11] A detailed computational, spectroscopic and thermodynamic analysis depicts that these inhibitors compete with co-factor NADH at major portal. Docking studies of these compounds with FabG4 suggest that they compete for all three subsites of NADH binding location. These findings prompted us to further explore the role of the gallol subunit by replacing with other electron rich aromatic rings, namely the aminobenzenes. Since an amino group is capable of forming hydrogen bonds both as a donor and an acceptor, it would be worth to replace the hydroxyl group(s) in the gallol moiety by one or more amino group(s) in an attempt to increase the potency of compounds.[12,13] This isosteric replacement (bioisosterism)[14] may also eliminate pharmacokinetic and toxicological limitations normally associated with phenols as presence of a large number of phenolic hydroxyl group causes instability due to oxidation by CYP 450 or O-methylation.[15] Based on the above rationale, we designed and subsequently synthesized a series of triazole linked polyphenol–amino benzene hybrids (**3**–**8**) and screened them for inhibition against *Mt*-FabG4. Molecular docking studies of these new hybrids have given an insight about the binding information which is different than previously reported compounds **1**–**2**. The results which are given here, provided a structure–activity relation (SAR) for substituents linked to N-1 of triazole moiety.

Synthesis: The methodology to obtain compounds **3**–**8** is similar to the one described in our previous work.[11] In brief, click reaction was performed between 3-*O*-propargyl tetrabenzyl epicatechin (**16**) or catechin (**17**) with respective azide counterparts (**9**, **12**, **15**). The benzyl ether was chosen as the protecting group of polyphenol because of its easy deprotection under neutral condition to provide the final compounds.

The key components for the click reaction, namely alkyne and azide counterparts, were synthesized prior to click procedure. The synthesis of 3-*O*-propargyl tetrabenzyl epicatechin (**16**) or catechin (**17**) was described previously.[11] The azide components were synthesized by following the procedure as depicted in Scheme 1A. Synthesis of 1-(azidomethyl)-4-nitrobenzene (**9**) was done *via* the attack of NaN_3 on commercially available 4-nitrobenzyl bromide. To obtain 1-(azidomethyl)-3-nitrobenzene (**12**), 3-nitrobenzaldehyde was reduced by $NaBH_4$ to get the corresponding alcohol. Successive S_N^2 displacements led to the azide **12**. The synthesis of the remaining azide component, 1-(azidomethyl)-3,5-dinitrobenzene (**15**), was carried out starting from 3,5-dinitrobenzoic acid via reducing the acid group by borane dimethyl sulfide complex in THF. The resulting benzyl alcohol **13** was converted to the azide **15** in a similar fashion.

The click reaction was performed in presence of Cu(I), made in situ by reduction of $CuSO_4$ with sodium ascorbate (Scheme 1B). Debenzylation and nitro group reduction were carried out in a single step using Pearlman catalyst in presence of high hydrogen pressure to furnish the final compounds (Scheme 1C). All final compounds were purified by repeated precipitation from methanol—ether and characterized by ^1H, ^{13}C NMR and mass spectra. Purity of these compounds was checked through reverse-phase analytical HPLC (traces are included in SI).

FabG4 inhibition assay: Screening of compound **3**–**8** was carried out to evaluate their FabG4 inhibition potencies. All six compounds inhibited FabG4 enzyme at micromolar concentrations (Table 1, dose–response plots are included in SI). Compounds **3**, **5** and **7** of epicatechin series have shown similarity in their inhibitory potencies (IC_{50} ~70 μM). Compounds belonging to catechin series (**4**, **6**, **8**) also have similar IC_{50} values (~60 μM). Compounds of catechin series have shown better inhibition of FabG4.

Structure–activity relations (SAR) study: We have previously reported that hybrid with catechin unit (**2**) has been better inhibitor than the hybrid with epicatechin unit (**1**).[11] This previous finding is also supported by the present study, as the catechin linked hybrids (**4**, **6**, **8**) have shown better inhibitory activities than the respective epicatechin linked analogues (**3**, **5**, **7**). This finding indicates that the stereochemistry in the polyphenolic part of the scaffold plays an important role in case of inhibitory capabilities. On the other hand, replacement of galloyl fragment by aminobenzyl unit has resulted in 1.5 to 2-fold decrease in inhibition potencies. Thus, the aminobenzyl units (in **3**–**8**) are comparatively inferior bioisostere than galloyl unit (as in **1**–**2**). The decrease in inhibition by the aminobenzene hybrids may be due to their poor binding as supported by docking studies. Interestingly, the binding is not affected by the number or positions of the amino groups as

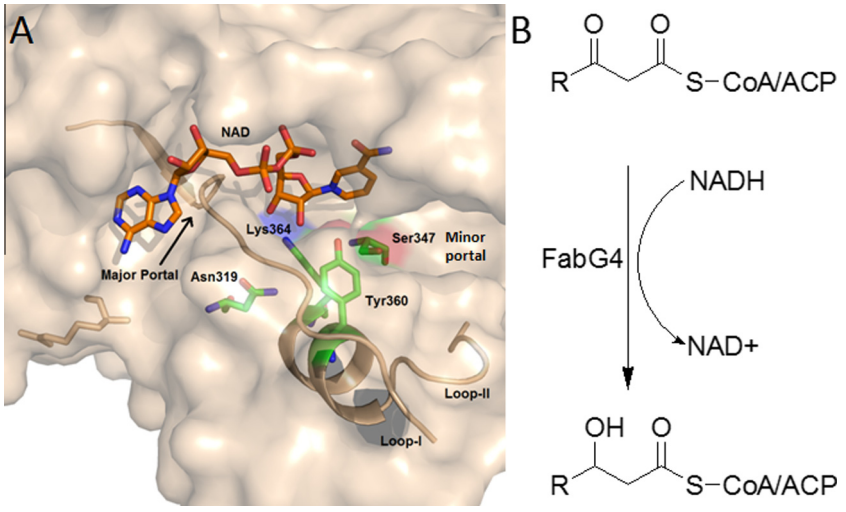


Figure 1. (A) Major portal of FabG4 with bound NAD. Catalytic tetrad and loops are shown. (B) Basic function of FabG4.

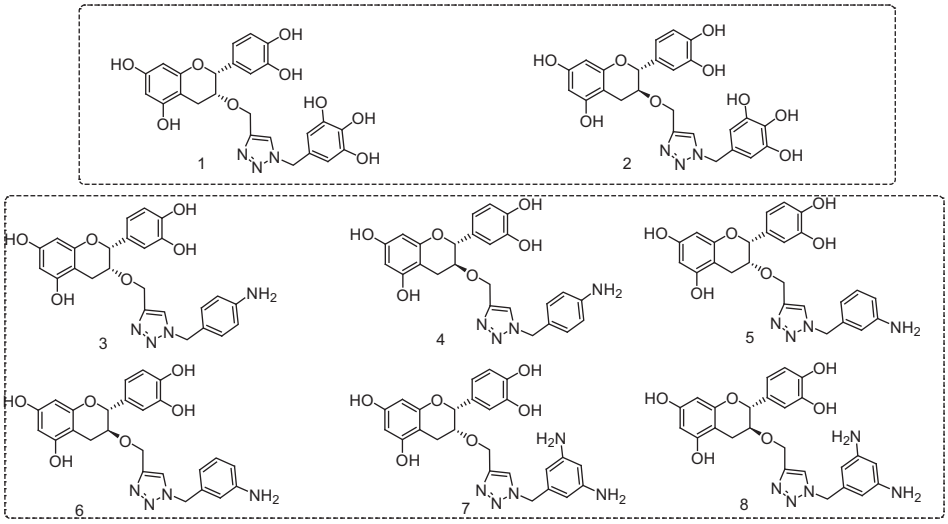
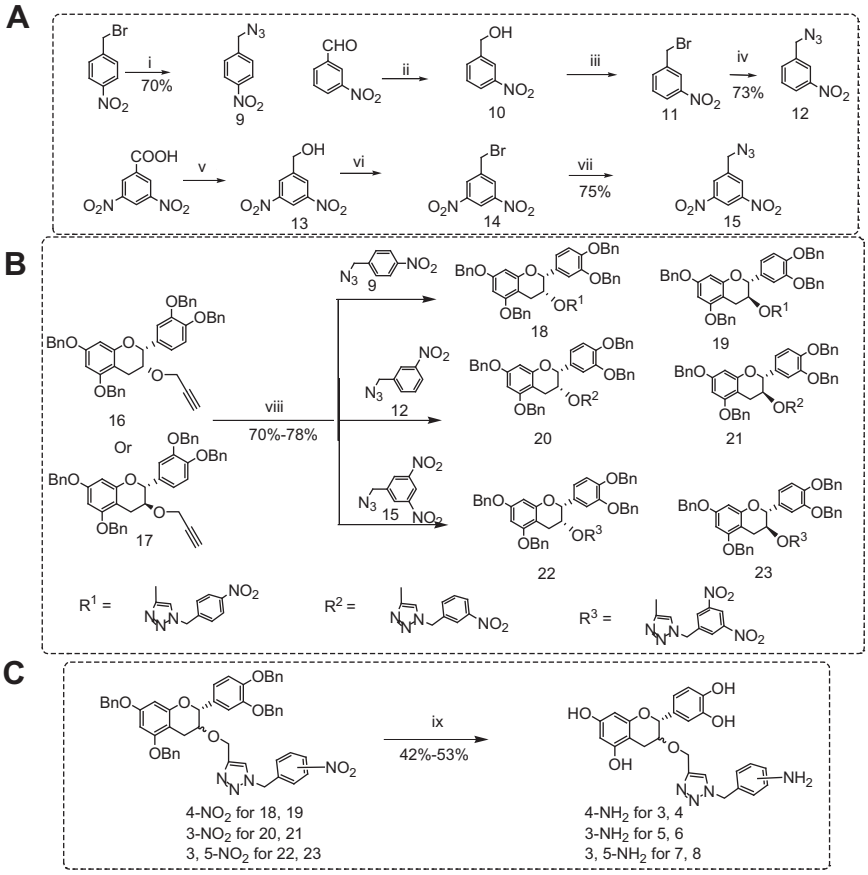


Figure 2. Previously reported triazole linked polyphenol–gallol hybrids as FabG4 inhibitors (at top side, compounds **1**–**2**) and current triazole linked polyphenol–aminobenzene hybrids (compounds **3**–**8**).

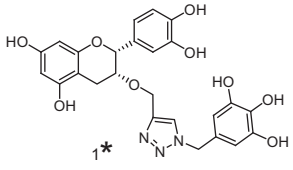
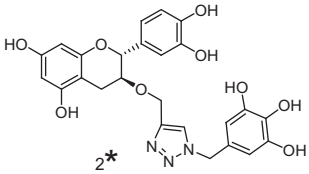
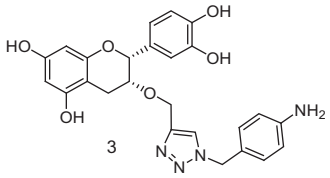
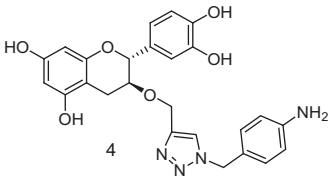
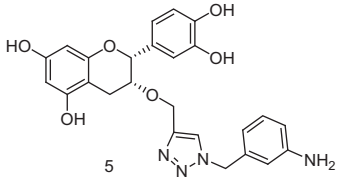
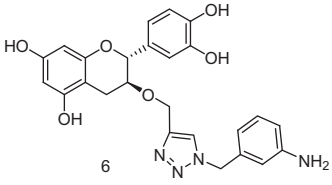
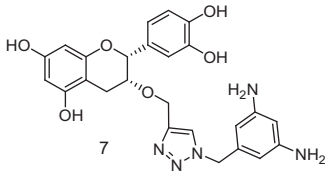
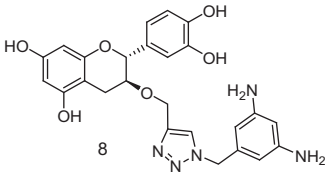


Scheme 1. (A–C) Synthesis of triazole–aminobenzene hybrids (**3**–**8**). Reagents and conditions: (i) NaN_3, dry DMF(s), rt, 12 h; (ii) $NaBH_4$, methanol (s), 2 h; (iii) PBr_3, dry DCM (s), 0 °C, 15 min; (iv) NaN_3, dry DMF(s), rt, 12 h; (v) borane dimethyl sulfide, dry THF (s), 0 °C to rt, 3 h; (vi) PBr_3, dry DCM (s), 0 °C, 20 min; (vii) NaN_3, dry DMF(s), rt, 12 h; (viii) $CuSO_4$–sodium ascorbate, MeCN–H_2O (1:1), 36 h–48 h; (ix) 20% $Pd(OH)_2$–C, THF–MeOH–H_2O (20:1:0.5), 5–7 h.

compounds of catechin series or of epicatechin series have very similar IC_{50} values.

Computational study: As already mentioned, FabG4 is a NADH-dependent enzyme in which the active site can be accessed from two different directions: narrow minor portal and wide major portal. The compounds (**3**–**8**) are expected to access the NAD-binding active site via the major portal. The inhibitory power of the ligand will depend on the extent of interaction with the subsites. A ligand that covers more subsites should have stronger interaction and expected to be a better inhibitor. As reported previously, compounds **1**–**2** span the entire major portal of FabG4 protein and competitively bind at all three NADH-subsites, with the gallol

Table 1
Inhibition of FabG4 by new polyphenol–aminobenzene hybrids (**3**–**8**) in terms of their IC_{50} values and SAR relation table; all experiments were repeated thrice and their variation was added in error values

Epicatechin series	IC_{50} in μM	Catechin series	IC_{50} in μM
 1*	44.7	 2*	34.9
 3	73.2 ± 1.5	 4	59.3 ± 1.0
 5	79.1 ± 0.8	 6	58.7 ± 1.2
 7	73.5 ± 1.3	 8	61.6 ± 0.5

* = previously reported compounds.

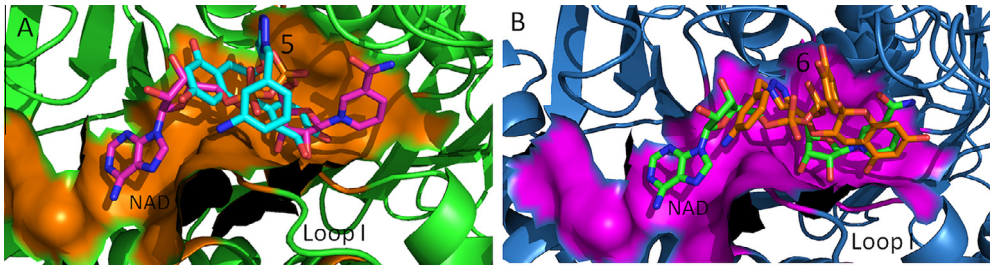


Figure 3. (A) Docked conformations of **5** of epicatechin series (cyan) is overlaid on co-crystallized NAD (magenta) for comparison which depicts that **5** will compete with pyrophosphate fragment of NADH for same binding site (P-subsite) over loop I. (B) Docked conformations of **6** of catechin series (orange) is overlaid on co-crystallized NAD (green) for comparison which depicts that **6** will compete with both pyrophosphate and nicotinamide fragments of NADH for same binding sites (P-subsite and N-subsite).

fragment at N-1 interacting with the N-subsite.[11] In case of the polyphenol–aminobenzene hybrids (**3**–**8**), molecular docking studies showed that the aminobenzyl unit does not interact with N-subsite or the neighbouring catalytic site of FabG4. Instead, the unit interacts in opposite direction with the residues present in loop-I thus preventing the molecules to cover the entire major portal, that is, NADH binding area. The inability to make strong interaction with catalytic residues because of less area covering at NADH binding site may be the reason behind lower activity of compounds **3**–**8** as compared to **1**–**2**.

Bindings of epicatechin and catechin series compounds with FabG4 depicts that epicatechin series compounds can only block P-subsite; whereas, catechin series compounds block P- subsite and N-subsite of NADH binding site (Fig. 3). Thus compared to epicatechin series, the compounds in catechin series show stronger interactions with FabG4 and cover more area at major portal that supports their slightly better inhibitory activities. Details interactions are given in Supporting information.

Thus we have presented six novel triazole linked polyphenol–aminobenzene hybrids as FabG4 inhibitors. These new compounds have been synthesized, characterized and evaluated for their inhibitory activity, against FabG4 at micromolar concentrations. Compounds with catechin sub-unit have shown marginally better inhibition of FabG4. Computational study has supported the experimental findings and given crucial insight binding information. Comparison of the inhibitory activities between previously reported and the present triazole linked polyphenol–amino benzene hybrids emphasised the importance of the gallol moiety. Any future design will have to focus on the hybrids containing gallol surrogates like phloroglucinol.

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Supplementary data

Supplementary data (Chemistry experimental, procedures of inhibition kinetics and docking study, dose–response plots, selected NMR spectra, mass spectra, HPLC traces and additional docking data are included in supporting information.) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.01.014.

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