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Combined effect of epigallocatechin gallate and triclosan on enoyl-ACP reductase of *Mycobacterium tuberculosis*

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Abstract

Among the various inhibitors known for enoyl-acyl carrier protein (ACP) reductases, triclosan and green tea catechins are two promising candidates. In the present study, we show, for the first time that epigallocatechin gallate (EGCG), a major component of green tea catechins, inhibits InhA, the enoyl-ACP reductase of *Mycobacterium tuberculosis* with an IC50 of 17.4 μM. EGCG interferes with the binding of NADH to InhA. We also demonstrate that EGCG increased the inhibitory activity of triclosan towards InhA and *vice-versa*. Direct binding assay using [^{3}H]EGCG and fluorescence titration assay support the spectrophotometric/kinetic inhibition data. The biochemical data has been explained by docking simulation studies.

Keywords: *Mycobacterium tuberculosis*; Tuberculosis; InhA; Enoyl reductase; FAS I; FAS II; Tea catechins; Epigallocatechin gallate; EGCG; Triclosan; Fluorescence titration; Scatchard plot

Tuberculosis (TB) is an infectious pulmonary disease caused by gram positive bacillus *Mycobacterium tuberculosis* (MTB), which causes two million deaths annually [1]. To combat the present multidrug-resistant strains (MDR) strains of MTB various pathways specific to the pathogen are being explored extensively and fatty acid biosynthesis pathway is one of them [2,3]. Fatty acids are indispensable to all living organisms. MTB is unique among bacteria because it contains both the types of fatty acid synthases (FAS) [2,3], type I or associative which is characteristic of eukaryotes [4] and type II or dissociative which is found in prokaryotes, plants, and certain protozoans [5,6]. MTB uses both the FAS systems to produce functional mycolic acids, which is exclusive to mycobacteria [2,3]. Mycolic acids are very long chain fatty acids comprising of a homologous series of C60–C90 α-alkyl β-hydroxy fatty acids and are main part of the mycobacterial cell wall. As mycolic acids are essential for mycobacterial survival, each enzyme of mycolic acid synthesis pathway can serve as a good target for the development of antimicrobacterial drugs [2,3].

The two types of FAS systems (FAS I and FAS II) differ significantly in their architecture and thus type II FAS has been validated as a good antibacterial target [5–7]. The fourth step of FAS II elongation cycle is catalyzed by a reductase known as enoyl-acyl carrier protein reductase (ENR or FabI or InhA in *M. tuberculosis*) [5–9]. It catalyzes the NADH dependent reduction of trans-2-enoyl fatty acyl chains to saturated fatty acyl chains [5–9]. Some of the potent inhibitors of this enzyme are isoniazid, triclosan, diazaborines, and green tea catechins [2,6–15]. Due to its importance in mycobacterial survival, InhA has been studied in great detail [2,9]. Isoniazid (INH), a front-line drug for treating TB, inhibits the type II FAS of MTB compromising the mycolic acid biosynthesis [2,3,9,10,14]. The target of INH still remains controversial. INH inhibits multiple components of mycobacterial metabolism [3,13]. Triclosan, a common additive in household products, spe-

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cifically inhibits enoyl-ACP reductase of type II FAS of bacteria [7–9] (including InhA [14]) and also *Plasmodium falciparum* [6,15].

In the past few years, due to its immense pharmacological potential, green tea catechins have gained enormous attention from the scientific world [16,17]. Green tea catechins are a group flavonoids mainly composed of (−) epigallocatechin gallate (EGCG), (−) epigallocatechin (EGC), (−) epicatechin gallate (ECG), (−) gallocatechin (GC), and (−) catechin gallate (CG) [16]. Earlier, it was reported that these catechins inhibit type II FAS of *Escherichia coli* [11]. Very recently, we have shown that green tea catechins and certain plant flavonoids are potent inhibitors of ENR from *Plasmodium falciparum* (PfENR) and also potentiate the binding of triclosan to PfENR [12].

Tea catechins inhibit MTB growth [18] but their inhibitory activities on InhA have not been examined. In the present report we have investigated the scope of tea catechins as antimicrobacterial agents targeting FAS II of MTB. Out of the five important catechins, we selected EGCG as a model compound because it was reported to have better activity over other catechins on PfENR [12]. Triclosan is a poor inhibitor of InhA [14]. Here we have also examined if the affinity of triclosan to InhA can be increased in the presence of EGCG and *vice-versa*. InhA from *M. tuberculosis* was cloned, expressed, and the inhibitory effects of EGCG and triclosan on purified InhA were examined. Radiolabeled EGCG was used to deduce the binding constant and binding site of EGCG on InhA. Using spectrophotometric and direct binding studies we show that EGCG inhibits InhA with low micromolar inhibition constant and binds reversibly to InhA at or near the cofactor (NADH) binding site. The results reported here present compelling evidence that EGCG enhances the affinity of triclosan for InhA and *vice-versa*. Docking simulation studies of EGCG alone and with triclosan were performed on InhA to support the biochemical data.

Materials and methods

Materials. EGCG and all the PAGE reagents were purchased from Sigma–Aldrich. Kanamycin and IPTG were obtained from Calbiochem. Trans-2-decenoic acid was bought from TCL chemicals. pET-28a(+) DNA, BL-21(DE3) cells were obtained from Novagen. Triclosan was a gift from Kumar Chemicals (Bangalore, India). The primers were obtained from Sigma.

Cloning and over-expression of InhA. InhA gene was amplified by PCR from the genomic DNA of MTB H37Rv strain using the following primers: 5′-GGAATTCCATATGACAGGACTGCTGGACGG-3′ (forward primer) and 5′-CGGGATCCCTAGAGCAATTGGGTGTGCGCG-3′ (reverse primer). The amplified gene was cloned in-frame in pET-28a(+) expression vector. The clones were primarily selected by restriction digestion and finally by gene sequencing.

InhA was over-expressed in BL-21(DE3) cells and purified by Ni-NTA affinity chromatography as described in the Supplementary material. The pure fractions were pooled and desalted in 30 mM PIPES buffer containing 150 mM NaCl, 2 mM EDTA, and 10% glycerol, pH 8.0. The concentration of purified InhA was determined spectrophotometrically using $\varepsilon_{280} = 37.3\ \mathrm{mM^{-1}\,cm^{-1}}$ [14,19]. Concentrated fractions of InhA were stored at −20 °C for further use.

Preparation of trans-2-decenoyl-CoA. trans-2-decenoyl-CoA was synthesized by mixed anhydride method as described earlier [19]. The product was further purified by HPLC on C-18 reverse phase column as mentioned earlier [19].

Inhibition of InhA by EGCG and triclosan. The inhibitory activities of EGCG and triclosan (Scheme 1) were checked, first separately and then in combination. The standard inhibition assay (100 μl) contained 50 nM of purified InhA, 250 μM of NADH, 25 μM of *trans*-2-decenoyl-CoA with various concentrations of inhibitors in 30 mM PIPES buffer, 150 mM NaCl, 10% glycerol (pH 8.0). The range of EGCG used for the inhibition study was from 100 nM to 20 μM and triclosan was used in the range of 50 nM to 5 μM. The progress of the reaction was monitored at 340 nm in Jobin–Yvon spectrophotometer. To examine the effect of triclosan on the inhibitory activity of EGCG, 0.5 μM of triclosan was pre-incubated in the reaction mixture with InhA for 10 min prior to addition of various concentrations of EGCG. Similarly, to check the effect of EGCG on the inhibitory activity of triclosan, 5 μM of EGCG was pre-incubated with InhA for 10 min before the addition of various concentrations of triclosan in the reaction mixture. In each case IC_{50} of the inhibitor was determined by analyzing the percent activity data *versus* log concentration of the inhibitor by non-linear regression method.

Binding studies of [^{3}H]EGCG to InhA in the presence of triclosan. Direct binding of [^{3}H]EGCG, alone and in presence of triclosan to InhA, was studied by filter binding assay as described earlier for PfENR [12]. In brief, 1 μM of InhA was incubated with various concentrations of [^{3}H]EGCG (500 nM to 10 μM) in 50 μl of reaction volume in PIPES buffer (30 mM PIPES with 150 mM NaCl, pH 8.0) for 10 min and then the reaction mixture was spotted on activated PVDF membrane and washed twice with 100 μl PIPES buffer. The membrane was dried completely and the counts of bound [^{3}H]EGCG determined in scintillation counter. To check the binding in the presence of triclosan in all the above reactions, 10 μM triclosan was incubated with InhA for 10 min before addition of [^{3}H]EGCG. The data was analyzed by saturation binding kinetics and Scatchard plot as mentioned earlier [12] using following equations [20].

$$[S]_b = \frac{[S]_{b\max} \times [S]_f}{K_d + [S]_f} \tag{1a}$$

$$\frac{[S]_b}{[S]_f} = -\frac{1}{K_d[S]_b} + \frac{n[E]_t}{K_d} \tag{1b}$$

Where $[S]_b$ is the bound, $[S]_f$ is free and $[S]_{bmax}$ is the maximum binding of the ligand being analyzed and K_d is the dissociation constant. Association constant (K_a) = $1/K_d$.

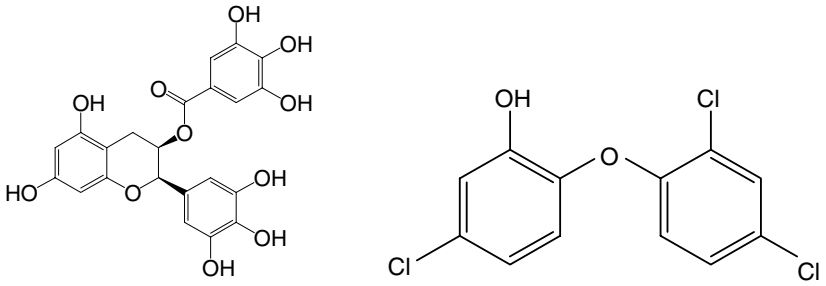
Direct binding studies of EGCG and triclosan with InhA by fluorescence titration studies. Direct binding studies of EGCG and triclosan to InhA were performed by fluorescence titrations on a Jobin–Yvon Horiba fluorimeter. The monochromator slit widths for excitation and emission were 3 and 5 nm, respectively. InhA samples (1 μM) were excited at 295 nm and emission intensity was recorded at 336 nm. In separate experiments, InhA was titrated with different concentrations of EGCG and triclosan to determine their individual dissociation constants. To analyze the effect of triclosan on EGCG binding to InhA and *vice-versa*, InhA was incubated with 5 μM of triclosan or 10 μM of EGCG for 10 min and then titrated with various concentrations of EGCG or triclosan, respectively. In all the four cases the decrease in fluorescence (F_o–F) upon addition of the inhibitors alone or in conjugation was fitted to Eq. (2) to obtain the values of K_i for each condition [21].

$$F_o - F = \frac{\Delta F_{\max}}{(1 + \frac{K_i}{[I]})} \tag{2}$$

Inner filter effect associated with the system was corrected using the following equation [22]

$$F_c = Fanti \log\left[\frac{A_{ex} + A_{em}}{2}\right] \tag{3}$$

Where, F_0, F_c and F are the fluorescence of native protein, corrected and measured fluorescence intensities, A_{ex} and A_{em} are the absorbance at excitation wavelength and emission wavelength, respectively. K_i is the dissociation constant of the inhibitor for InhA.



EGCG **Triclosan**

Scheme 1.

Docking of inhibitors with InhA. All the docking simulations were performed using AutoDock 3.05 [23] and MOE (Molecular Operating Environment) [24].

Preparation of the receptor and ligand molecules. The crystal structure of InhA submitted by He et al. [25] to PDB (www.rcsb.org) was used for docking studies. The structure co-ordinates were converted into mol2 format with MMFF94 charges assigned using the MOE suite of programs [24]. The molto2pdbqs utility (provided with AutoDock program) was used to prepare the input receptor file containing fragmental volume and solvation parameters. Inhibitors were also built using MOE and energy minimized with MMFF94 charges.

Docking simulations. Grid maps for docking simulations were generated and docking was performed as described earlier [12]. For each inhibitor hundred independent runs were conducted.

Modeling of the ternary complex. First InhA was docked with EGCG to get the binary complex and then the InhA–EGCG complex was docked with triclosan to generate the complex of InhA–EGCG–TCL as mentioned elsewhere [12]. The ligand–receptor interactions were calculated using LPC/CSU Server (http://ligin.weizmann.ac.il/cgi-bin/lpccsu/LpcCsu.cgi).

Results and discussion

InhA is one of the important targets for the development of anti-tuberculosis drugs. Triclosan scores over INH in not being a prodrug [26]. Triclosan could be treated as an ideal candidate for the development of antitubercular drug provided the affinity of triclosan could be increased for InhA [8,26]. Several derivatives of triclosan have been synthesized and their antitubercular activities have been examined and efforts are underway to synthesize more potent derivatives of triclosan on the basis of structure–function relationship [9,26]. In our recent report with PfENR, we showed that certain tea catechins could increase the inhibitory activity of triclosan to an appreciable extent [12]. Here, we determined the inhibitory activity of EGCG and also investigated the possibility of potentiating the inhibitory activity of triclosan by EGCG.

Inhibition of InhA by EGCG and triclosan

InhA was over-expressed and purified to near homogeneity as inferred from the single band of 28.5 kDa on SDS–PAGE (Supplementary Fig. 1). EGCG inhibited InhA activity in a concentration dependent manner with an IC_{50} of 17.4 ± 2.3 μM (Fig. 1A). When 0.5 μM of triclosan was added to the reaction the inhibitory activity of EGCG was enhanced and the IC_{50} of EGCG came down to 1.5 ± 0.3 μM (Fig. 1A). IC_{50} value of EGCG for EcFabI and PfENR is 15 μM and 250 nM, respectively [11,12]. The results indicate that EGCG is a poor inhibitor of InhA as compared to PfENR. Triclosan also showed concentration dependent inactivation of InhA with IC_{50} of 2.8 ± 0.2 μM (Fig. 1B). In comparison to EcFabI [12] and PfENR [19],

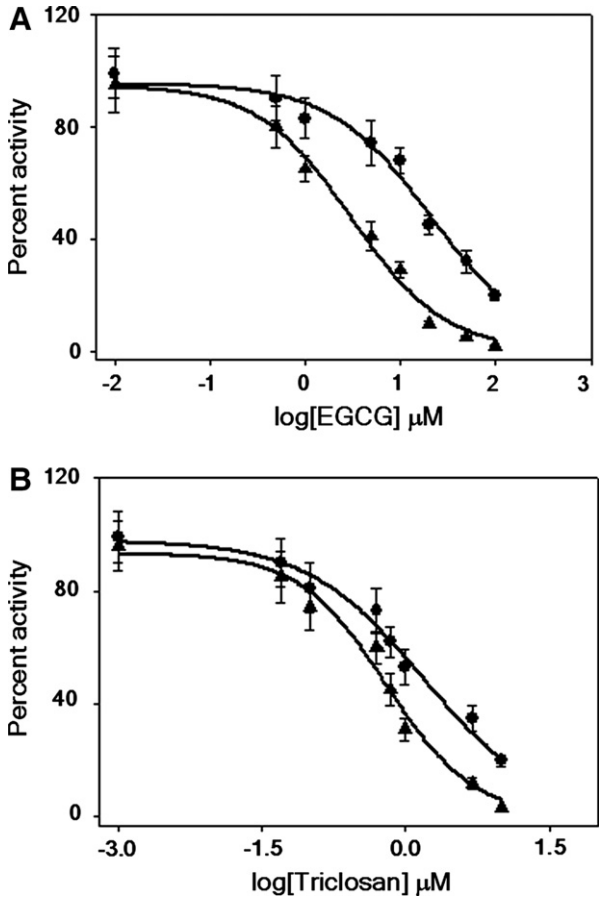


Fig. 1. Inhibitory effect of EGCG and triclosan against InhA. The data was analyzed by non-linear regression method and the best fit gave sigmoidal curves. (A) EGCG alone (●) and in the presence of 0.5 μM triclosan (▲). (B) Triclosan alone (●) and with 5 μM EGCG preincubation (▲). Each data point in the plot represents mean of three experiments. The error bars represent the standard deviation of the data.

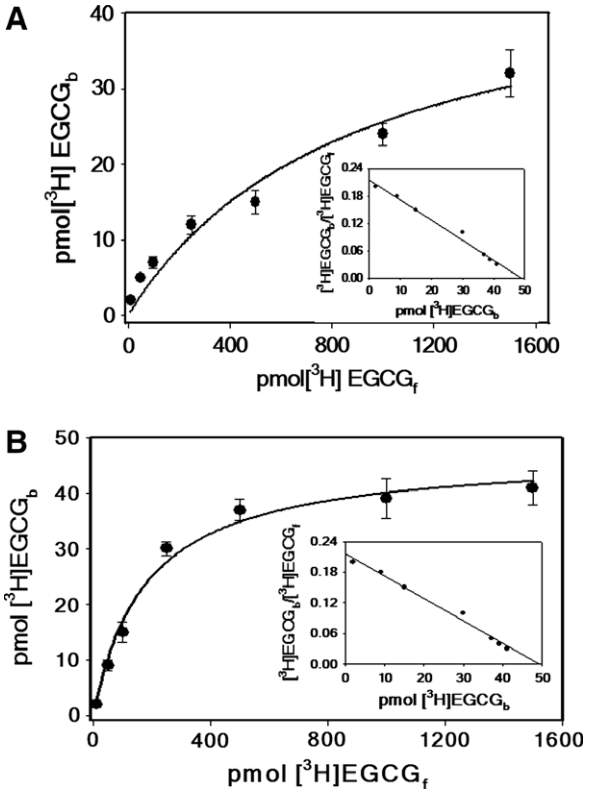


Fig. 2. Binding of [^{3}H]EGCG to InhA. (A) Direct binding of [^{3}H]EGCG alone with InhA showed saturation binding and (B) in the presence of 10 μM of triclosan. The inset in both the figures shows the linear treatment of the same data by Scatchard plot. Each experiment was done in triplicate and the mean values were used to generate the plots. The error bars are indicative of the standard deviation associated with the data.

triclosan showed reduced activity against InhA which is consistent with the earlier report [18]. The reason for the reduced affinity of triclosan for InhA has been investigated in detail and it is mainly attributed to the difference in the substrate binding loops of these otherwise overall similar reductases [27]. When EGCG was preincubated with InhA, considerable increase (eight times) in triclosan activity ($IC_{50} = 0.35 \pm 0.04$ μM) was observed. The data indicate that there is an enhancement of inhibitory activity of EGCG in the presence of triclosan and *vice-versa* for InhA.

Binding of [^{3}H]EGCG to InhA

The binding of [^{3}H]EGCG with InhA followed saturation kinetics with an association constant (K_a) of 1.25 μM^{-1} (Fig. 2A). In the presence of 10 μM of triclosan, the binding of [^{3}H]EGCG increased for InhA with K_a of 5.2 ± 0.8 μM^{-1} (Fig. 2B). The binding data were also analyzed by Scatchard plot using Eq. (1b) and are presented as inset in the respective saturation plots. From Scatchard plot, K_a of EGCG alone and in the presence of triclosan was calculated to be 1.53 μM^{-1} and 5.9 μM^{-1}, respectively. [^{3}H]EGCG binding experiment proves that triclosan is able to potentiate the binding of EGCG to InhA. Similar mechanism was observed in the case of PfENR where presence of saturating concentration of triclosan increased the affinity of EGCG more than five times for the enzyme [12]. Further, by competitive binding assay we found that NADH replaced the bound [^{3}H]EGCG from [^{3}H]EGCG–InhA binary complex in a concentration dependent manner with EC_{50} of 24.0 μM (Supplementary Fig. 2). As shown for

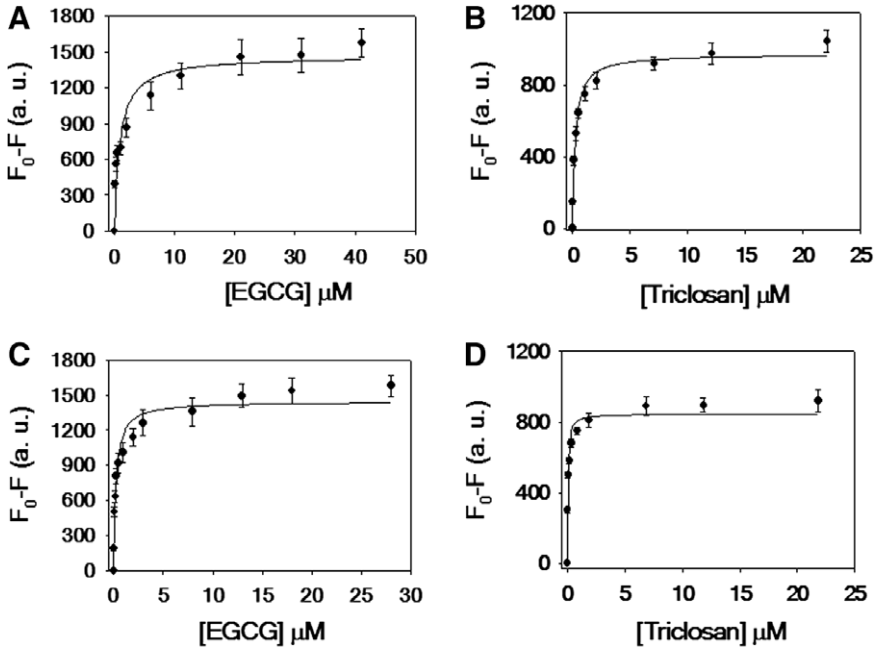


Fig. 3. Fluorescence titration of EGCG and triclosan with InhA. K_i values were determined from the fluorescence quenching data using Eq. (2). In all the four cases the data fitted well and followed saturation kinetics. (A) EGCG alone. (B) In the presence of triclosan. (C) Triclosan alone. (D) In the presence of EGCG. The data used to plot the curve are mean values from three experiments and the error bars show the standard deviation of the data.

EcFabI [11] and PfENR [12], the results presented here clearly indicate that in InhA also EGCG binds reversibly at or somewhere near to the NADH binding site.

Direct binding studies of EGCG and triclosan to InhA by fluorescence spectroscopy

The titration of InhA with EGCG or triclosan alone and in the presence of each other followed saturation kinetics and the data fitted well to Eq. (2) (Fig. 3A–D). K_i of EGCG was calculated to be 876.0 ± 21.0 nM (Fig. 3A) whereas for triclosan it was 251.4 ± 8.7 nM (Fig. 3B). When triclosan was pre-incubated with InhA, K_i of EGCG became 138.0 ± 7.0 nM (Fig. 3C). Similarly, pre-incubation of EGCG with InhA reduced the K_i of triclosan to 21.4 ± 2.7 nM (Fig. 3D). These results thus show that EGCG was able to improve the affinity of triclosan for InhA and *vice versa*.

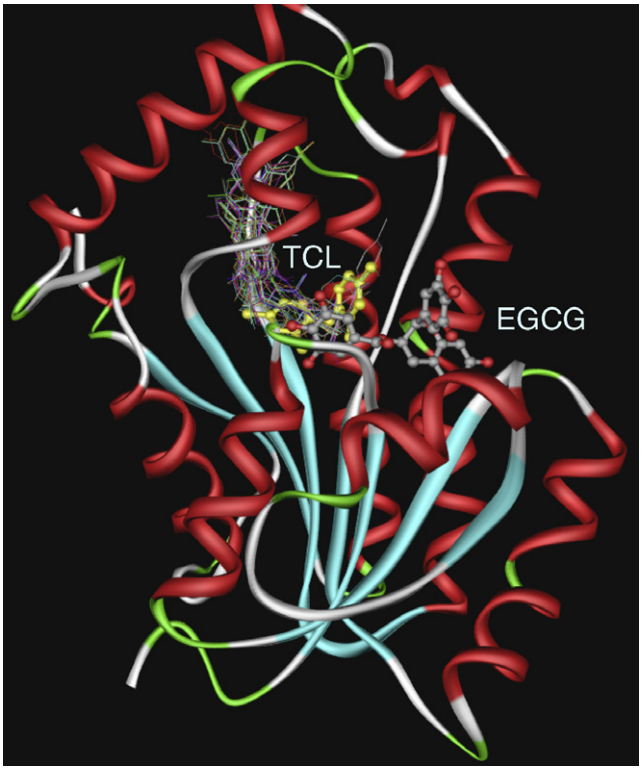


Fig. 4. Ensemble of triclosan conformations docked with InhA-EGCG binary complex. EGCG is represented in balls and sticks and triclosan conformations in lines, the best energy conformation of the biggest cluster is shown in yellow balls and sticks. EGCG is also shown in balls and sticks (Atom colors; C in grey, O in red). InhA is shown in ribbons. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Docking of EGCG with InhA

Docking studies were performed to find out the remarkable difference in the affinity of EGCG for InhA as compared to PfENR. Docking studies with AutoDock show that EGCG occupies NAD^+ binding site on InhA (Fig. 4). The mean docked energy of InhA–EGCG complex was -14.71 kcal/mol ($IC_{50} = 17.4$ μM) while that of PfENR–EGCG and EcENR–EGCG complexes was -18.00 kcal/mol ($IC_{50} = 0.25$ μM) [12] and -15.80 kcal/mol ($IC_{50} = 15.0$ μM) [11], respectively. Cluster analysis of the docked conformations from 100 independent docking simulations was performed with 1 Å rmsd. The highest energy cluster had 10 conformations and they occupied the space where adenine moiety of NAD^+ binds. The galloyl moiety plays a major role in the affinity of EGCG with ENRs [11,12]. In case of InhA the galloyl moiety makes T-shaped aromatic interactions with Phe93 and EGCG binds in a different orientation as compared to PfENR. Overall, the favorable interactions between EGCG and InhA are less, and unfavorable contacts significantly more, as compared to PfENR (Table 1) explaining the lower affinity of EGCG for InhA as compared to PfENR.

Docking of Triclosan with the EGCG–InhA complex

Triclosan was docked with the binary complex of InhA and EGCG to achieve the ternary complex and compared with the ternary complex of PfENR–EGCG and triclosan modeled by us earlier [12]. Cluster analysis was carried out for the docked conformations of triclosan from the 100 independent runs. The clusters with highest number of conformations lie in the pocket where nicotinamide moiety of NAD^+ would otherwise bind. In case of InhA the biggest cluster had only 22 conformations while there were 51 conformations in the biggest cluster for PfENR with few smaller clusters. The various clusters span through the InhA active site, which is much bigger than that of PfENR for the obvious reason of accommodating longer fatty acids. Stacking of the 3-chloro-phenol ring of triclosan and benzene-1, 2, 3-triol moiety of EGCG contributes enormously in the formation of a strong PfENR–EGCG–triclosan ternary complex [12]. Such kind of stacking interactions were not found in case of InhA, instead the OH of triclosan forms hydrogen bond with oxygen of the galloyl

Table 1
Comparison of all the stabilizing and destabilizing contacts made by EGCG in binary complexes with PfENR and InhA

Contacts made in binary complexes	Hydrogen bonds	Hydrophobic contacts	Aromatic–Aromatic contacts	Hydrophilic–Hydrophobic contacts (destabilizing)	Acceptor–Acceptor (destabilizing)
ECGC–PfENR	27	21	10	19	2
ECGC–InhA	22	26	13	28	4

moiety of EGCG making weaker ternary complex compared to PfENR–EGCG–triclosan.

Conclusions

In the present study, using direct inhibition assay, [^{3}H]EGCG binding assay and fluorescence titration assay we show that EGCG is indeed an inhibitor of InhA and binds reversibly at or near the binding site of NADH. Further, we also demonstrate that the affinity of triclosan for InhA is increased more than eight times in the presence of EGCG. The affinity of EGCG for InhA is also increased by five times in the presence of triclosan. The biochemical data is well supported and explained by docking studies. Our study opens up a platform for the development of tea catechins and triclosan based drugs for treating tuberculosis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.10.191.

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