Virtual Screening identification of 1H-imidazo[4,5-d]pyridazine derivatives as a new tool for the inhibition of Mycobacterium tuberculosis FAD-dependent Thymidylate Synthase.

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ABSTRACT. Thymidylate synthase-X (ThyX) represents an attractive target for tuberculosis drug discovery. Herein, we selected 19 compounds through a virtual screening approach. We solved the first X-ray crystal structure of *Thermatoga maritima* in complex with a non-substrate analogue inhibitor. Given the active site similarities between MtbThyX and Tm-ThyX, our crystal structure paves the way for a structure-based design of novel antimycobacterial compounds. The 1H-imidazo[4,5-d]pyridazine was identified as scaffold for the development of MtbThyX inhibitors.

INTRODUCTION

According to a study performed in 2006, a healthy human being has a 10% lifetime risk of developing active Tuberculosis (TB), and this risk increases to a 10% annual risk in already Human Immunodeficiency Virus (HIV) infected individuals.[1,2] The drugs available account for a reasonable success in treating the disease, but they have been in use for any years. Indeed, the introduction of the first-line, four-drug (isoniazid, rifampicin, pyrazinamide and ethambutol) combination therapy dates back to the 1960s. At that time, researchers thought that a combination therapy was preferable to a single drug therapy, because this would give less chances to the pathogen to mutate and re-invade the host's body. Additionally, the second-line treatments, including amikacin, capreomycin, ciprofloxacin, ethionamide, cycloserine and p-aminosalicylic acid, are almost 40 years old and show more adverse effects.[3] Furthermore, the spread of multi- and extensively drug resistant strains of *M. tuberculosis* have made the cure of the disease even more challenging to manage.[4]

The thymidylate synthase enzyme belongs to the family of transferases involved in the transfer of a one-carbon group (methyltransferases). They play an important role in the biosynthesis of the DNA base thymine, which is a necessary entity for the organism survival. The enzyme catalyzes the reductive methylation of the uracil moiety of 2'-deoxyuridine-5'-monophosphate (dUMP) turning it into 2'-deoxythymidine-5'-monophosphate (dTMP), which further serves as a precursor for 2'-deoxythymidine-5'-triphosphate, a building block of DNA. Two forms of thymidylate synthase are known, namely, the classical thymidylate synthase (TS or ThyA), which uses methylene tetrahydrofolate (CH_2H_4folate) as both the methylene group donor and the reducing agent, and the flavin-dependent thymidylate synthase (FDTS or ThyX) that requires NADPH and FAD as a reducing system while CH_2H_4folate serves only as the carbon donor (Figure 1). These forms of thymidylate synthases differ not only in their catalytic mechanisms but also in their amino acid composition and structural forms.[5, 6-9]

Mycobacterium tuberculosis is found to contain genes encoding for both enzymes, ThyA and ThyX. However, the biological significance of their presence is still not understood. Mutational studies performed on the organism showed that ThyX serves as an essential entity, confirming that it is a plausible drug target.[10] Compounds specifically designed to target *M. tuberculosis* ThyX were active towards this enzyme and inactive towards ThyA.[11]

ThyX from *M. tuberculosis* is a homotetrameric protein with a 222 symmetry and a central α/β domain flanked by two helical domains.[9] The FAD cofactor is bound in an extended conformation and provides a characteristic yellow color. The adenine rings are deeply buried in the interfacial pocket while the isoalloxazine rings make stacking interactions with the pyrimidine rings of dUMP substrates in the active site. Such a tailored binding explains the difficulties met in removing FAD from the enzyme.

The catalytic mechanism of ThyX enzyme involves two semireactions: the first in which FAD is reduced to $FADH_2$ and NADPH is oxidized to $NADP^+$, and the second in which $FADH_2$ and CH_2H_4folate provide respectively an electron

and the methylene moiety, for the reductive methylation of dUMP to dTMP.[5, 12-14] Solution of the X-Ray Diffraction structure of the CH_2H_4folate-FAD-dUMP-ThyX complex (PDB-ID: 4GT9) showed stacking interactions involving rings of the three substrates and of His53 of the enzyme.[12] While no crystal with $NADP^+$ and FAD bound to ThyX has been obtained, docking studies[12] as well as steady-state kinetic analysis performed on Paramecium bursaria chlorella virus (PBCV-1) ThyX NADPH oxidase activity suggested that the binding sites of CH_2H_4folate and NADPH overlap.[15,16]

Noticeable structural and mechanistic differences among the ThyA and ThyX enzymes as well as the absence of the ThyX gene in eukaryotes (especially in humans) allow to selectively target the mycobacteria.[17]

Since dUMP binding to the active pocket of ThyX is necessary, not only for the second semireaction that directly involves it as a substrate, but also for the occurrence of the first one,[14] we aimed at identifying molecules that block such entry, thus eventually prevent the normal ThyX cycle and lead to 'thymineless' death of the pathogens.

In this work, we present the results of a virtual screening work on Mtb-ThyX, targeting the dUMP-substrate binding region. We have selected 19 molecules. Docking studies have shown that these compounds bind with an orientation similar to that of dUMP, making stacking interactions with the isoalloxazine rings of FAD. In order to confirm the binding mode of the compounds, we carried out X-ray crystallographic studies. No crystals of Mtb-ThyX in complex with our molecules was obtained but we solved the X-ray crystal structure *Termotoga maritima* ThyX (Tm-ThyX) in complex with compound **3**. Due to the similarities of Mtb-ThyX and Tm-ThyX active sites we could confirm the binding mode proposed with the docking studies. The library was also evaluated against human thymidylate synthase (hTS) in order to assess the selectivity of these class of compounds.

Herein, we provide the first X-ray crystal structure of Tm-ThyX in complex with a non-substrate (dUMP) analogue and we propose the 1H-imidazo[4,5-d]pyridazine moiety as a novel scaffold for the development of antimycobacterial compounds. We established a basis for a further structure-based design of molecules with improved binding affinities and thus more competitive versus the dUMP.

MATERIALS AND METHODS

Virtual screening towards Mtb-ThyX and molecular dynamics simulations.

The structure of Mtb-ThyX (PDB-ID: 2AF6) was retrieved from Protein data Bank. The structure contains the tetrameric form of ThyX along with the co-factor FAD and substrate analog BrdUMP.[9] BrdUMP substrate was extracted from the active site so that the enzyme only contains FAD in the active site. Virtual screening was then performed on ZINC database that contains approximately 8 million purchasable compounds and in a ready–to-dock format.[18] The database also supports its own screening tool, Dockblaster, which uses Dock version 3.6 software for docking compounds from ZINC database. The following files were given as inputs: (i) the protein file in the pdb format, (ii) pdb file containing the co-ordinates of three active site residues chosen in such a way that when joined with each other in the form of triangle, surrounds the target site: Arg199, Gln103', Arg172'. This screening resulted in the generation of a list containing 2000 molecules approximately. The list containing the coordinates of the filtered compound was downloaded and the compounds were then docked using Autodock version 4.2 software.[19] Following this second docking step, we selected 13 compounds (Table 1) that show similar binding pattern as that of the dUMP substrate. (Details on Autodock parameters used are reported in Supporting Information).

In order to see whether the compounds binding pose, generated from Autodock, is preserved, the compound-protein complexes were prepared and subjected to Molecular dynamics for time length of 1 ns. (See Supporting Information for details)

Compounds

13 computationally selected compounds, plus other 6 similar compounds were purchased from the respective vendors as reported in SI (Table S1). All reagents have been purchased at the highest purity level available from commercial sources (purity > 90%).

Protein purification

MTbThyX was purified following the protocol reported[11] TmThyX was produced as a C-terminal hexa-His tag fused protein in *E. coli* BL1(DE3) cells. Post culture, cells were spun down at 4200rpm and then re-suspended in ice cold lysis buffer (50mM Tris pH 8.5, 200mM NaCL, 1mM TCEP). They were lysed by sonication. The protein was purified on a 5mL Hi-flo Ni-ion exchange column. The wash buffer used was: Tris pH 8.5 50mM, NaCl 200mM, Imidazole 50mM, TCEP 0.5mM and FAD 10μM. For S191 the elution buffer was the same except using 1M imidazole and no FAD (this made the elution of the protein visible as it was yellow in colour). Concentrated protein was stored in Tris pH 8.5 50mM, NaCl 200mM.

Human Thymidylate synthase (hTS) was obtained as in literature reported.[20]

Enzyme catalytic assay

To determine the effect of the compounds on ThyX catalytic activity the tritium release assay was performed, essentially according to a previously published method.[21] The assay measures the formation of tritiated water resulting from the release of ^3H from the 5-carbon position of the dUMP pyrimidine ring, a prerequisite for the transfer of the methyl group from methylenetetrahydrofolate to the dUMP substrate.[22,23] Further details are reported in the Supporting Information.

hTS enzymatic activity inhibition was assessed spectrophotometrically as previously described.[24]

Protein Crystallization

ThyX (from Mtb and Tm) was crystallized using sitting drop method in a 96 well plate format. The ThyX (5-10 mg/mL)

protein was mixed with equal volumes of 1.6-2.0 M Ammonium sulphate, 50 mM tri-sodium citrate and 3-5% isopropanol and plates were left at 16 °C to yield diffraction quality crystals. Aiming to obtain the crystal structure of our compounds in complex with the enzyme, the protein crystals were soaked overnight in crystallization solution containing 10 mM of compound and cryo-protected with 20% ethylene glycol before freezing crystals in liquid nitrogen. Only the X-ray crystal complex of Tm-ThyX-compound **3** was obtained.

X-ray Data Collection and Structure Determination

X-ray diffraction data were collected at Diamond light source (Beamline: I03) and are shown in Table S2. The crystals diffracted to about 1.7Å. The crystal structure Tm-ThyX-**3** was determined using molecular replacement using Phaser[25] employing coordinates from PDB ID 3G4A as template with CCP4 package.[26]

RESULTS AND DISCUSSION

In-silico studies on Mtb-ThyX

ThyX is a homo-tetrameric protein with four substrate-binding cavities. X-ray crystallographic studies on Mtb-ThyX had shown that the binding region of dUMP analogues (FdUMP, BrdUMP) is formed by residues coming from two monomers (Figure 2A). The uridine ring makes a stacking interaction with the isoalloxazine ring of the FAD cofactor and hydrogen-bonds with the side-chains of Arg199 and Arg107' (the apostrophe after the sequence number indicates that the residues is part of a different monomer); moreover it forms other two hydrogen-bonds mediated by a crystallographic water molecule, with the side-chain of Glu92 and the backbone of Asp205. The 2'-deoxyribose moiety forms two hydrogen bonds with the side-chains of Arg95 and Gln103'; whereas the phosphate moiety forms hydrogen bonds with the side-chains of Arg87', Ser105' and Arg172' and with the backbones of Gln106' and Arg107', as well as making a water-mediated hydrogen bond with the side-chain of Ser88. The binding site is then formed by five other residues: His91, His203, Ala204, Ile94' and Tyr108'. The virtual screening protocol (Dockblaster screening followed by Autodock docking) generated 2000 compounds that could interact with the dUMP-binding residues. Since the binding of dUMP and its analogues is driven mainly by interactions with arginines, we searched for compounds able to interact with the arginines present in the active site and, at the same time, able to make stacking interactions with the isoalloxazine rings of FAD. Such filtration led to the recovery of 13 molecules (Table 1) showing binding patterns similar to that of dUMP.

The predicted binding modes showed that some compounds (**1**-**4**, **10**) span the whole binding site making several interactions with many residues of the binding pocket (Figure 2C). Others (**5**-**9**, **11**-**13**), having more planar geometries and being less functionalized, lie above the flavin rings and interact to residues that bind the uridine moiety of the substrate but are unable, due to structural limitations, to interact with the arginines and other residues that bind the phosphate moiety of the substrate (Figure 2D). MD experiments showed that all these compounds preserved their binding modes even if the system was dynamic, supporting our screening procedure.

Mtb-ThyX enzyme kinetics inhibition studies

All compounds have been tested against the recombinant Mtb-ThyX; the percentages of inhibition (%I) were measured using a concentration of 100 μM for each compound. Moreover the compounds were tested against hTS but none was shown to be active (data not shown). The Mtb-ThyX kinetics inhibition studies were based on a radioactive-ligand competition assay where dUMP was used as the competing substrate. We used 5-fluordeoxyurdine-5'monophosphate (5-FdUMP) as reference compound, which showed a percentage of inhibition equal to 97% at 100 μM concentration value. This result is consistent with the data reported in literature.[21]

Among the 19 compounds studied, compound **7**, bearing a 1H-imidazo[4,5-d]pyridazine moiety, shows the best inhibition activity with %I of 29%, while compounds **1**, **6**, **11**, **12**, **14**, **15** and **18** inhibit the Mtb-ThyX activity by about 10%-15%. All the other compounds (**2**, **3**, **4**, **5**, **8**, **9**, **10**, **13**, **16**, **17**, **19**) do not significantly inhibit Mtb-ThyX (% I: < 10%). Among all chemical structures reported here, the imidazo-pyridazine (compounds **6**, **7**, **12** and **18**) and benzo-pyridazine (compounds **14** and **15**) scaffolds showed the best activity.

X-ray Crystallographic studies: X-ray crystal complex Tm-ThyX-FAD-3

In order to confirm the binding modes proposed by the docking studies, we tried to crystallize the compounds with Mtb-ThyX protein but no X-ray crystal complex could be obtained. Therefore, we performed an X-ray crystallographic screening on Tm-ThyX. The sequence identity between the Mtb-ThyX and Tm-ThyX is only 28%, but the superimposition of their active sites reveals that many of the key residues responsible for substrate binding and activity are retained (Table S3 in the Supporting Information).[27] As shown in Figure 2A and 2B, in both enzymes the dUMP binding site is deeply buried and the access is controlled by the flavin part of the FAD. In our X-ray crystallographic screening towards Tm-ThyX, we solved the crystal complex Tm-ThyX-FAD-**3**.

The 1,2,4-triazine ring of compound **3** is involved in a stacking interaction with FAD (Figure 3A) and H-bonds with Tm-Arg174, Tm-Ser88' and Tm-Arg90', and water-mediated H-bonds with Tm-Gln75, Tm-Gln180 and FAD. The carboxylate group forms H-bonds with Tm-Gln75, Tm-Ser88', Tm-Gly89', and Tm-Arg147'(Figure 3B) (for the sake of clarity in the paper we use always the Mtb-ThyX residue sequence numbering; when we need refer to Tm-thyX residue sequence numbering, this is indicated by "Tm-" added before the residue indication).

The availability of the X-ray crystal complex allowed us to validate the *in silico* studies: the analysis of the structure revealed that compound **3** binds in the same way as predicted by docking studies and confirmed the binding pocket similarity between Tm-ThyX and Mtb-ThyX (Figure 3B and 3C).

The conformation adopted by compound **3** closely resembles that of dUMP: the triazine ring overlaps the uracil ring of dUMP and the carboxylate group occupies a position similar to that of the phosphate group in dUMP. Since the substrate-binding site is conserved between Mtb-ThyX and Tm-ThyX, compound **3** might bind into Mtb-ThyX in a way similar to that found in Tm-ThyX. However, compound **3** showed a modest inhibitory activity towards Mtb-ThyX, being among the weakest inhibitors of the identified compound set. The phosphate group of dUMP serves as an anchor and drives the binding to all the arginine residues, while the carboxylate group of compound **3** does not establish H-bonds with Tm-Arg147 and Tm-Arg90 (corresponding to Arg107 and Arg172 in Mtb-ThyX).

CONCLUSION

We here present the first X-ray crystal structure of Tm-ThyX in complex with a non-substrate analogue. Due to the similarities between Mtb-Thyx and Tm-ThyX active sites, the Tm-ThyX-FAD-**3** can represent a starting point for further structure-based approaches to drug discovery against Tuberculosis. Among the tested library, compound **7**, bearing a 1H-imidazo[4,5-d]pyridazine moiety, showed the highest inhibitory activity towards Mtb-ThyX, making it a promising scaffold for structure-based drug design aimed at identifying novel antimycobacterial agents. Our work suggests that we should design novel compounds linking the selected scaffold to chemical moieties able to establish H-bonds with all the arginines constituting the binding site, in order to increase the potency towards Mtb-ThyX.

ASSOCIATED CONTENT

Supporting Information.

AUTHOR INFORMATION

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Author Contributions

SS carried out the structural studies and TLB oversaw the analyses and reviewed the manuscript.

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ABBREVATIONS USED

dUMP, deoxyuridine-5'-monophosphate; dTMP, 2'-deoxythymidine-5'-monophosphate; TS, thymidylate synthase; CH_2H_4folate, methylene tetrahydrofolate; ThyX flavin-dependent thymidylate synthase; *Mtb*-ThyX, *Mycobacterium tuberculosis* flavin-dependent thymidylate synthase; *Tm*-ThyX, *Termotoga maritima* flavin-dependent thymidylate synthase.

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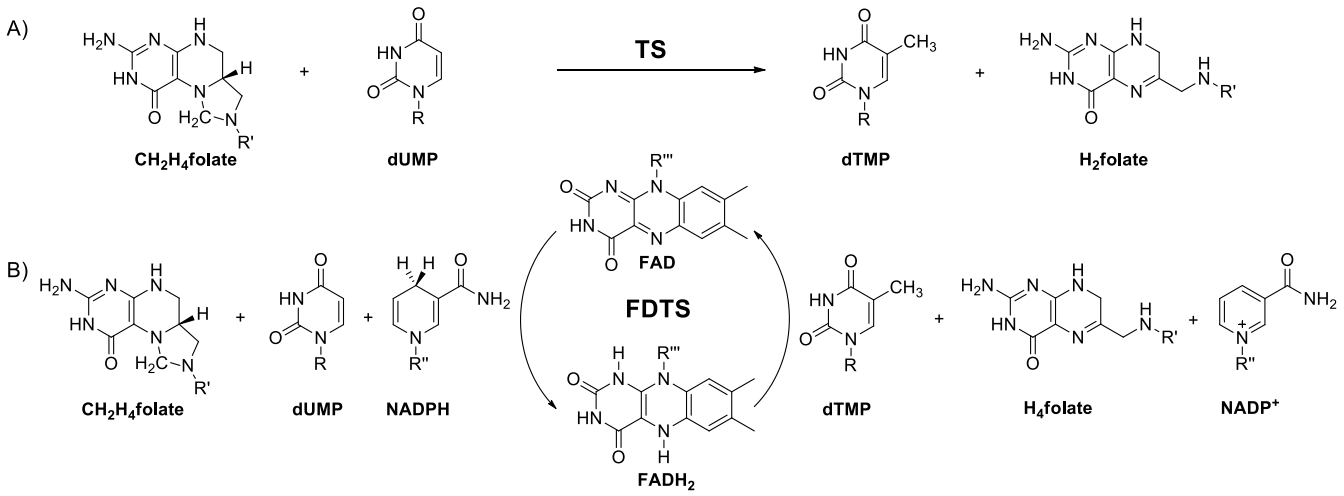
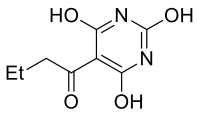
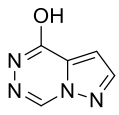
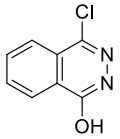
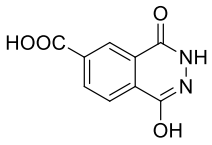
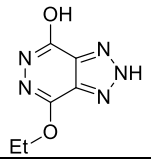
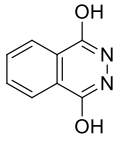
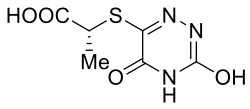
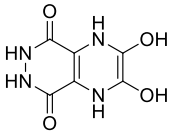
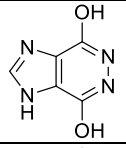
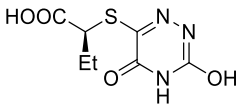
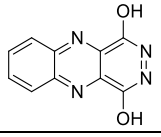
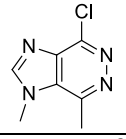
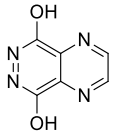
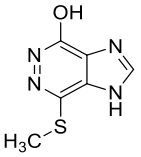
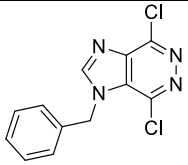
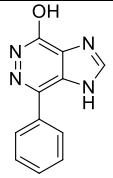
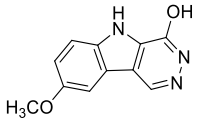
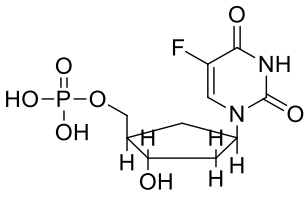
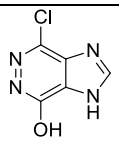
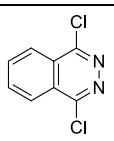


Figure 1.Thymidylate synthase reactions. A) The reaction catalyzed by classical thymidylate synthase (TS or ThyA). B) The reaction catalyzed by flavin-dependent thymidylate synthase (FDTS or ThyX).
R = 2'-deoxyribose-5'-phosphate; R'= (*p*-aminobenzoyl)-glutamate; R'' = adenosine 5'-pyrophospho ribosyl; R''' = adenosine-5'-pyroposphate-ribityl.

Table 1. Percentage of inhibition of the 19 compounds towards Mtb-ThyX. All compounds were tested at 100 μM. The reference compound is 5-FdUMP.

code	Compounds Structure	% Inh. ThyX	code	Compounds Structure	% Inh. ThyX	code	Compounds Structure	% Inh. ThyX
1		11.0 ± 1.0	**8**		4.9 ± 0.5	**15**		11.5 ± 3.0
2		3.9 ± 0.2	**9**		3.6 ± 0.2	**16**		1.0 ± 0.1
3		6.1 ± 0.7	**10**		4.3 ± 0.4	**17**		7.1 ± 2.0
4		5.7 ± 0.4	**11**		10.0 ± 2.0	**18**		14.0 ± 3.0
5		5.3 ± 0.3	**12**		10.0 ± 2.0	**19**		2.1 ± 0.5
6		15.0 ± 2.0	**13**		7.0 ± 1.0	**5FdUMP**		97.3 ± 3.0
7		29.0 ± 5.0	**14**		10.3 ± 2.0			

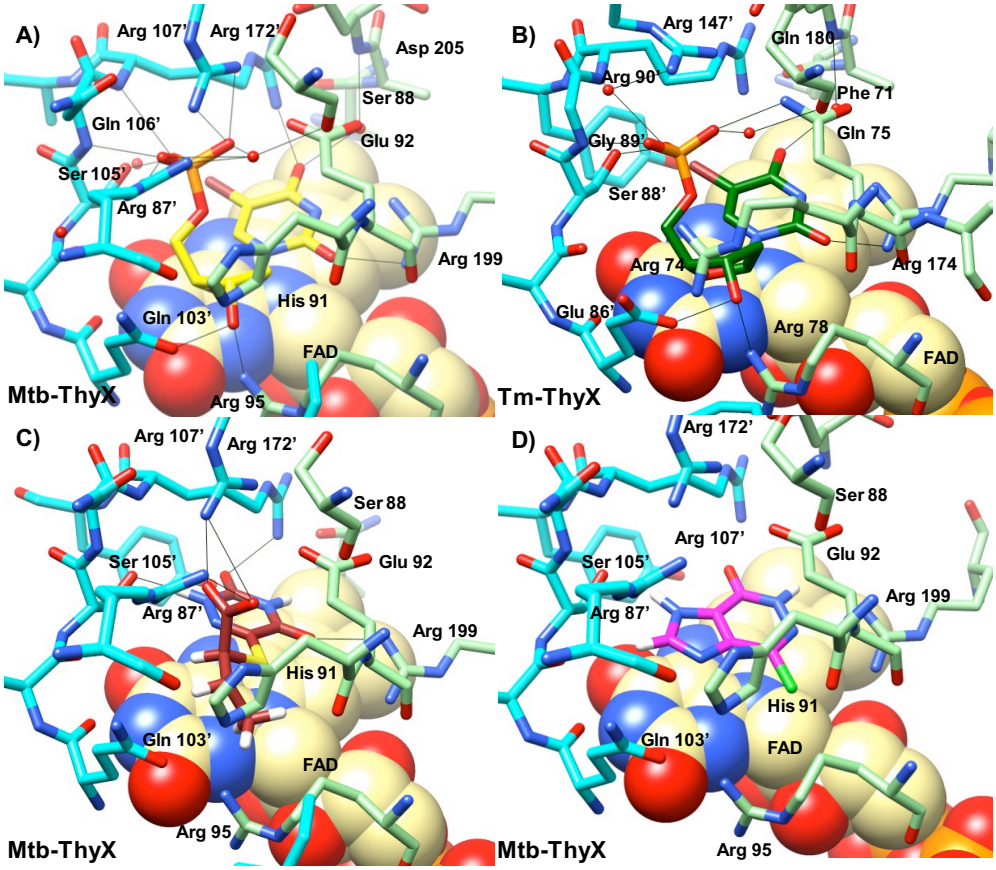


Figure 2. A) BrdUMP interactions with Mtb-ThyX; from PDB ID 2AF6. BrdUMP is colored in yellow and FAD in khaki. **B)** BrdUMP interactions with Tm-ThyX; from PDB ID IO27. BrdUMP is colored in dark green and FAD in khaki. **C)** Docking of compound 4 into Mtb-ThyX. Compound 4 is colored in brown and FAD in khaki. **D)** Docking of compound 7 into Mtb-ThyX. Compound 7 is colored in magenta and FAD in khaki.
C atoms from one monomer are colored in light green, while C atoms from the other monomer are colored in cyan. H-bonds are shown as black lines.

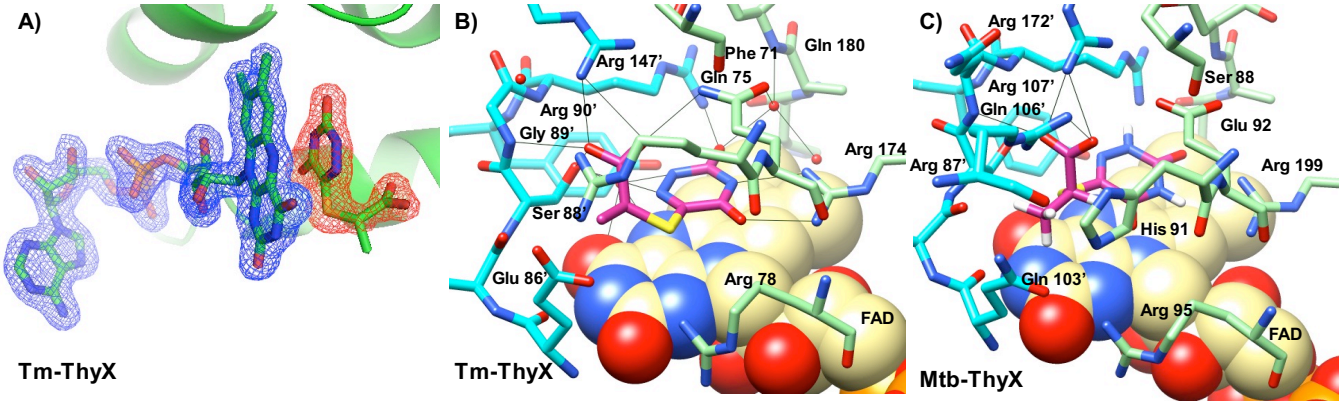


Figure 3. A) Stacking interaction between FAD and compound **3** in the Tm-ThyX active site; from PDB ID 5CHP. **B)** X-ray crystallographic complex Tm-ThyX-FAD-compound **3**; from PDB ID 5CHP. **C)** Docking of compound 3 into Mtb-ThyX.
C atoms from one protomer of the dimer are colored in light green, while C atoms from the other protomer are colored in cyan. Compound **3** is colored in magenta; FAD in khaki. The H-bonds are shown as black lines.

TOC