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Flavin-dependent thymidylate synthase: A novel pathway towards thymine

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Abstract

For several decades only one chemical pathway was known for the *de novo* biosynthesis of the essential DNA nucleotide, thymidylate. This reaction catalyzed by *thyA* or *TYMS* encoded thymidylate synthases is the last committed step in the biosynthesis of thymidylate and proceeds *via* the reductive methylation of uridylate. However, many microorganisms have recently been shown to produce a novel, flavin-dependent thymidylate synthase encoded by the *thyX* gene. Preliminary structural and mechanistic studies have shown substantial differences between these deoxyuridylate-methylating enzymes. Recently, both the chemical and kinetic mechanisms of FDTs have provided further insight into the distinctions between *thyA* and *thyX* encoded thymidylate synthases. Since FDTs are found in several severe human pathogens their unusual mechanism offers a promising future for the development of antibiotic and antiviral drugs with little effect on human thymidylate biosynthesis.

Keywords

Thymidylate; Biosynthesis; Thymidylate synthase; Flavin; Enzyme mechanism

Introduction

Thymidylate is one of the four DNA building blocks and is crucial for survival of all organisms. This essential nucleotide is the product of the enzyme thymidylate synthase, which catalyzes the reductive methylation of 2'-deoxyuridine-5'-monophosphate (dUMP) to form thymidylate (2'-deoxythymidine-5'-monophosphate or dTMP). Until recently, it was thought that *thyA*-encoded (denoted *TYMS* in humans and other mammals) thymidylate synthases were the sole means to the *de novo* biosynthesis of thymidylate. These enzymes, called classical thymidylate synthases, have a well studied chemical and kinetic mechanism and have been the target of several chemotherapeutic and antimicrobial agents [1;2].

In 2002 the identification of several organisms that lacked the *thyA* and *Tdk* (the gene coding for thymidine kinase, a thymidine-scavenging enzyme) genes led to the discovery of an alternate flavin-dependent thymidylate synthase (FDTs) [3;4;5]. FDTs are encoded by the *thyX* gene, which is present in ~30% of all microorganisms including several severe human pathogens (see <http://www.cdc.gov>) [6]. Many organisms depend solely on FDTs as a source

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of thymidylate (e.g. all *Rickettsia*) and it has recently been suggested that dependence on the *thyX* gene limits chromosomal replication in these organisms [7;8;9].

FDTs share no structure or sequence homology with classical thymidylate synthases. The first crystal structure of FDTs obtained was from the organism *T. maritima* and is presented with the crystal structure of *E. coli* thymidylate synthase in Fig. 1 [10;11]. Classical thymidylate synthases are homodimers with one active site per subunit (Fig. 1A) [1]. This contrasts FDTs, which are homotetramers with four active sites, each located at the interface of three subunits (Fig. 1B). Catalytically important residues differ between these two enzymes, and an extended *thyX* motif has recently been characterized for FDTs enzymes [12]. Perhaps the most distinguishable feature of the FDTs structure is the short distance ($< 4\text{\AA}$) between the isoalloxazine moiety of the coenzyme flavin adenosine dinucleotide (FAD) and the substrate dUMP (Fig. 1B).

The overall chemical conversion catalyzed by thymidylate synthases is the net substitution of the C5 hydrogen of dUMP by a methyl group to form the product dTMP (Fig. 2). Although FDTs catalyze the conversion of dUMP to dTMP, early biochemical studies determined that the FDTs-catalyzed reaction differs from that seen in classical thymidylate synthases. Classical thymidylate synthases use $\text{N}^5, \text{N}^{10}$ -methylene-5,6,7,8-tetrahydrofolate ($\text{CH}_2\text{H}_4\text{folate}$) and dUMP to produce dihydrofolate (H_2folate) and dTMP (Fig. 2A). FDTs, on the other hand, consume dUMP, $\text{CH}_2\text{H}_4\text{folate}$ and reduced nicotinamide adenine dinucleotide phosphate (NADPH) to produce dTMP, tetrahydrofolate (H_4folate) and NADP^+ . The FDTs reaction is mediated by a flavin adenosine dinucleotide (FAD) coenzyme that cycles between reduced and oxidized forms ($\text{FAD} \rightleftharpoons \text{FADH}_2$) during a catalytic turnover (Fig. 2B). FDTs activity appears to take the place of both classical thymidylate synthase and dihydrofolate reductase (denoted DHFR, an enzyme that uses NADPH to reduce H_2folate to H_4folate).

Due to the differences in structure and general reactivity of FDTs, from mammalian thymidylate synthase, it seems likely that inhibition of these enzymes is possible without disrupting human thymine biosynthesis. Rationally-designed compounds which could lead to selective inhibition of FDTs enzymes present a promising frontier for antibiotic and antiviral drug development. As a principal step in such a drug-development process, the distinctions between the catalytic mechanisms of classical thymidylate synthases and FDTs need to be clarified.

Chemical mechanisms of classical TS (EC 2.1.1.45) versus FDTs (EC 2.1.1.148)

In Fig. 3A, the proposed chemical mechanism [1;2] of classical thymidylate synthases is presented. A conserved active-site cysteine residue is required for catalysis and has been shown to covalently activate the substrate dUMP *via* Michael-addition to the C6 position of the uracil moiety (step 2, Fig. 3A). The resulting enolate anion can behave as a nucleophile and attack *via* Mannich condensation (step 3) the activated iminium form (produced in step 1) of $\text{CH}_2\text{H}_4\text{folate}$. H_4folate then undergoes Hofmann elimination to form a $\text{C}5=\text{C}7$ double bond resulting in a covalently bound exocyclic intermediate (step 4) [13]. The reaction is complete when a hydride from H_4folate is transferred to the C7 position (step 5), and both dTMP and H_2folate dissociate from the enzyme. Nucleophilic attack and covalent bonding of dUMP to the active site cysteine residue has been clearly demonstrated in the crystal structure of *EcTS* in complex with 5-fluoro-dUMP and $\text{CH}_2\text{H}_4\text{folate}$ (Protein Data Bank accession **1tts** [14]). This functionality is an important feature of the classical thymidylate synthase reaction and has been instrumental in inhibitor and chemotherapeutic drug development (e.g. 5-fluorodU).

Recent studies have indicated that the mechanism of FDTs differs substantially from that of classical thymidylate synthase enzymes. Since FDTs were identified in organisms that lacked the classical thymidylate biosynthesis genes (i.e. *thyA* and *folA* that codes for DHFR) and produce H₄folate instead of H₂folate (Fig. 2B), it was natural to suggest that these enzymes are bifunctional [15;16]. Bifunctional activity would be consistent with classical thymidylate synthase activity and flavin-dependent DHFR activity. The following observations have led to the rejection of this hypothesis: i) when conducting FDT reactions using (*R*)-6-³H-CH₂H₄folate [17] the tritium remains on the H₄folate [18]. This is in direct contrast to experiments with classical thymidylate synthase where this tritium always transfers to the C7 position of dTMP (step 5, Fig. 3A) [1;2;18;19]; ii) reactions preformed in D₂O result in deuterated dTMP, suggesting that the reduced flavin exchanges protons with the solvent prior to a hydride transfer directly to the nucleotide [18;20;21;22]; and iii) if tritiated NADPH is used to reduce the flavin, the tritium ends up in the water and not dTMP or H₄folate. These experiments support a mechanism where NADPH reduces FAD to FADH₂, and the pyrimidine moiety accepts a hydride equivalent from the FADH₂ to form dTMP (Fig. 2B). Notably, the FDT reaction differs from known mechanisms of bifunctional enzymes and classical thymidylate synthases [1;2;4;7;10;12;14;15;16;18;19;22;23;24;25;26;27;28;29;30].

Structural studies of FDT identified a conserved active-site serine that has been proposed to serve as an enzymatic nucleophile, in a manner analogous to the cysteine residue of classical thymidylate synthases. This suggestion was originally based on sequence alignments of *thyX* genes which exhibited no conserved cysteines, but did show a strictly conserved serine residue [3;31]. Crystal structures of FDTs from three different organisms indicated that this conserved serine residue is 4Å from the electrophilic C6 position of dUMP (Fig. 4) [10;23;32].

Mutagenesis studies of FDT from *H. pylori* and *M. tuberculosis* (*HpFDT* and *MtbFDT*, respectively) have been used to support the putative role of serine as a nucleophile [12;31]. The conserved serine (Ser84) in the *HpFDT* enzyme was mutated to alanine and this mutant enzyme retained activity. However, it was reasoned that a neighboring serine (Ser85) was able to rescue the activity of the S84A mutant enzyme. This hypothesis was supported by a double mutant S84A/S85A which had no observed activity. The conserved serine (Ser105) of *MtbFDT* was mutated to glutamic acid, which resulted in an enzyme that failed to complement in thymidine deficient media. This S105E mutant was shown to slowly oxidize NADPH, however, this rate was less than 1/100 of the rate of oxidation observed for wild-type [12]. These results were taken as a support to the conclusion that without serine in the active site, FDT catalysis cannot take place.

Additionally, a mutation of Ser84 in the *HpFDT* and Ser105 in the *MtbFDT* to cysteine resulted in an active enzymes [12;31]. These observed activities suggest that cysteine can take the place of the serine nucleophile. Further studies using MALDI-TOF mass spectrometry of the *HpFDT*-S84C mutant indicated a covalent adduct between the enzymatic cysteine and the product dTMP [31]. This S84C-dTMP covalent complex, in addition to the lack of observable synthase activity for both the *HpFDT*-S84A/S85A double mutant and the *MtbFDT*-S105E mutant were taken by the authors as further support to the role of serine as an active site nucleophile.

Taking the suggestion that serine is the active site nucleophile, together with the evidence ruling out a bifunctional enzyme, led to the proposed chemical mechanism for FDT enzymes presented in Fig. 3B [18]. This mechanism parallels the classical thymidylate synthase mechanism, and differs from it in that a serine residue serves as the enzymatic nucleophile (Fig. 3B, step 2) and the reduced flavin cofactor provides the reductive hydride to terminate the reaction (step 4).

We approached the idea that serine was acting as an enzymatic nucleophile with some doubt due to a lack of structural evidence and the ambiguity that may arise from the study of a double mutant *Hp*FDTs enzyme. For serine to act as a nucleophile it must be activated by a basic system in the enzyme's active site (e.g. the catalytic triad present in hydrolytic enzymes). Upon evaluating the available crystal structures of FDTs, we were unable to identify such a basic system required to deprotonate serine (Fig. 4) [10;22;32]. This observation suggests that the conserved serine of FDTs is much less reactive than its classical cysteine counterpart, thus diminishing the likelihood of its reactivity towards dUMP.

To directly determine serine's role in the FDTs reaction, we performed mutation studies using *T. Maritima* FDTs. The active site of *Tm*FDTs contains only the conserved serine (Ser88) and no other strictly conserved nucleophilic residues [10;23;32]. Mutation of Ser88 to alanine (PDB **3g4a**) resulted in an enzyme which surprisingly retained activity [22], an anomaly that could not be explained easily based on the mechanism proposed in Fig. 3B.

When Ser88 was mutated to cysteine the resulting enzyme had 1/20 the activity of S88A and 1/400 the activity of the wild-type *Tm*FDTs. The crystal structure of S88C (PDB **3g4c**) in complex with dUMP did not show covalent binding to the C6 position of the uracil moiety. Nevertheless, MALDI-TOF mass spectrometry analysis of the *Tm*FDTs-S88C mutant indicated an apparent covalent adduct with the substrate dUMP, consistent with the previous studies on the *Hp*FDTs-S84C mutant. However, even in solution, cysteine has been shown to covalently bind to pyrimidine moieties. This fact, along with the low activity of the cysteine mutants, suggests that covalent binding of cysteine to the nucleotide may represent a dead-end complex which is not part of the catalytic pathway [22].

The conserved residues, Ser83 and Tyr91, are the only other potential enzymatic nucleophiles besides Ser88 (Fig. 4) within *Tm*FDTs. Tyr91 is an unlikely nucleophile, because its mutation to Phe in *Hp*FDTs results in an enzyme that is 50% more active than the wild type [31]. The Ser83 residue is 17.7 Å from C6 of dUMP and is hydrogen-bonded to the adenine ring of FAD at the core of the tetramer. For Ser83 to activate dUMP, FAD would need to dissociate, which is inconsistent with both our steady-state results [18;30] and crystal structures with NADP⁺ bound instead of FAD (PDB **2GQ2**), which show Ser83 still H-bonded to the adenine moiety [33]. These points diminish the possibility Ser83 or Tyr91 acting as nucleophiles during the FDTs reaction.

Besides the enzyme residues Ser83, Tyr91 and Ser88, the absence of any other possible enzymatic nucleophile is further supported by experiments using halogenated dUMP analogs. Perhaps the most convincing evidence for Michael-addition in classical thymidylate synthases is the covalent complex of 5F-dUMP with CH₂H₄folate (PDB **1tfs**). Similar complexes have not been identified for FDTs as detected by MALDI-TOF mass spectrometry [22] and X-ray crystallography (PDB **1o28**) [10;22]. Furthermore, FDTs does not catalyze the dehalogenation of 5Br-dUMP a common assay for enzymatic Michael-addition in the classical thymidylate synthase reaction [22;29]. In light of the fact that S88A is an active enzyme, and no other experimental evidence suggests activation of the substrate by an enzyme residue, we have concluded that the FDTs-catalyzed reaction does not rely on an enzymatic nucleophile. This feature fundamentally distinguishes the FDTs reaction from that of classical thymidylate synthases [22].

In the absence of an enzymatic nucleophile, the mechanism proposed in Fig. 3B does not explain the reaction catalyzed by FDTs. To further elucidate the FDTs reaction we followed the flow of hydrogens from the reduced flavin coenzyme by a range of analytical techniques. Previous experiments used ESI-MS to identify a mono-deuterated dTMP as a product of FDTs reactions in D₂O [18]. We recently revisited these experiments, performing reactions in D₂O

at both 37 °C and 65 °C and analyzing the product by ^1H and ^2H NMR. At 65 °C (close to the physiological temperature of *T. maritima*) the product observed was 7- ^2H -dTMP. However, the product of the 37 °C reactions showed a significant percentage (up to 60%) of 6- ^2H -dTMP, which is consistent with hydrogen transfer directly to the uracil moiety (Fig. 5). Both the lack of enzymatic nucleophile and the observation of deuterium at C6 of dTMP for reactions in D_2O , suggest a strikingly different mechanism for FDTS than for classical TS, or, for that matter, any other known biological methylation.

Besides an enzymatic residue, a hydroxide ion or the flavin coenzyme could activate dUMP. A hydroxide nucleophile is unlikely due to the lack of a basic system in the FDTS active sites required to produce a hydroxide ion by deprotonation of a water molecule. Additional tests with the flavin derivative, 5-carba-deaza-FAD, resulted in catalytic activity suggesting that the N5 of the flavin is not the source of nucleophile. These observations, along with the finding that the reduced flavin can transfer a hydrogen to the C6 position of dUMP, are consistent with reduction of the uracil moiety by hydride transfer from the reduced flavin to the dUMP substrate [22].

As a consequence, we have recently proposed a mechanism that is consistent with all currently published data regarding the chemical mechanism of FDTS enzymes [10;18;25;26;31;32]. Inspired by known flavo-protein reactions [34;35], we suggested a mechanism wherein a hydride is transferred to the uracil moiety from FADH_2 (see Fig. 3C, step 1). Such a mechanism would result in a noncovalently bound enolate anion, which could act as a nucleophile and attack the activated iminium form of $\text{CH}_2\text{H}_4\text{folate}$ (step 2). Following elimination of H_4folate (step 3), an isomer of thymine is then formed, which can undergo rearrangement to form the product dTMP (step 4) [22]. Since we have no direct evidence regarding the methylene transfer, the steps in this newly proposed mechanism are presented as the simplest case, and other more complex mechanisms can be envisioned.

The putative exocyclic methylene intermediate formed in step 3, of Fig. 3C is an isomer of thymine and has been shown to be stable in solution (Koehn and Kohen unpublished data and ref [36]). The product dTMP is thermodynamically favored over this isomer, and the enzyme could catalyze its isomerization either by an addition-elimination mechanism or by a sigmatropic 1,3-hydrogen rearrangement [37]. Both these isomerization mechanisms have been investigated by following the flow of hydrogens during the FDTS reaction (Fig. 5B). The observation that 6- ^2H -dTMP is formed when enzyme reactions are preformed in D_2O at reduced temperatures can be explained by reduced stereoselectivity and normal kinetic isotope effect on that step. If there was a loss of stereoselectivity, and the H-transfer is faster than D-transfer during an addition-elimination mechanism, it should be possible to observe 6,7- $^2\text{H}_2$ -dTMP (Fig. 5B, AEM). This product would be formed upon addition of $^2\text{H}^+$ to the C7 position and elimination of H^+ from the C6, resulting in a di-deuterated product. Mass spectrometric and NMR analysis of the reaction products has always indicated a mono-deuterated dTMP product, suggesting a rearrangement of the hydrogen between the C6 and C7 positions consistent with a 1,3-hydrogen shift (Fig. 5B, 1,3-H-shift) [22]. Such an isomerization reaction could be catalyzed by the oxidized flavin in the active site via oxidation of the C6 and reduction of C7.

The suggestion that FDTS does not use an enzymatic nucleophile, and that the reduced flavin transfers a hydride to the pyrimidine ring during the reaction, leads to a strikingly different chemical cascade than the classical thymidylate synthase enzymes. This presents significant opportunities for rational design of selective inhibitors for FDTs. Since the FDTS reaction appears to follow a reaction path leading to noncovalently bound intermediates, analogs which can mimic these intermediate species or transition states for their formation may bind tightly to FDTS and have little effect on classical thymidylate synthases. Such tight-binding inhibitors

of FDTS may also consequently facilitate crystallographic studies, and thus lay the foundations for rational drug design.

Kinetic mechanism of FDTS

Along with efforts to establish the chemical mechanism of the reaction catalyzed by FDTS, the binding and release features of the substrates and products have been investigated. Recently many works have explored the order of substrate binding, product release and their associated binding constants [23;25;27;30;38;39;40]. Overall, it appears that FDTS follows a sequential order of binding for all its substrates; however, little information is available regarding the order of product release. Measurement of substrate binding constants has proved to be challenging due to the ability of FDTS to function as an oxidase (catalyzing the conversion of O_2 to H_2O_2) and the ability for the substrate dUMP to function as an activator of the enzyme's oxidative half reaction [25;30;38].

Early studies of the substrate binding order offered conflicting results with regard to the kinetic mechanism of FDTs. Myllykallio and co-workers determined that the rate of oxidation of NADPH was increased in the presence of dUMP [24]. Their results contrasted the mechanism suggested by McClarty and co-workers, wherein NADPH and CH_2H_4 folate bind and $NADP^+$ and H_4 folate are released from the enzyme prior to dUMP binding. This latter mechanism proposed that an Arg residue serves as a vehicle for the methylene; however, subsequent studies [23;25;30;39] have not been able to confirm methylene transfer to the enzyme experimentally. Furthermore, several recent studies have observed sequential binding between CH_2H_4 folate and dUMP during the FDTS reaction, excluding the possibility for the release of H_4 folate prior to dUMP binding [23;30;38;39].

Recent investigations of the oxidase activity of FDTS have provided insight into the activation kinetics previously observed for the dUMP-FADH₂-FDTS complex [40] and moreover, have provided the means to monitor the binding features of the FDTS synthase reaction [30]. FDTS can catalyze the reduction of O_2 to H_2O_2 , and displays normal Michaelis-Menton kinetics for molecular oxygen with a relatively small K_M . This is unusual for many flavo-proteins that function as oxidases, and may imply that FDTS has a binding site for O_2 , or more likely, that some other step becomes rate-limiting under saturating concentrations of oxygen [41].

We have shown that CH_2H_4 folate and O_2 compete for the same activated form of the enzyme, namely the dUMP-FADH₂- $NADP^+$ -FDTS complex [30;38]. Furthermore, we found that the rate of FAD reduction is independent of dUMP concentration. However, oxidation of FADH₂ has a strong dependence on dUMP (with a functional activation constant, $K_f \sim 2\mu M$) [25]. This observation supports a mechanism where dUMP is not required to bind prior to NADPH, as previously suggested [23;24;40]. Additionally, a sequential mechanism between NADPH and O_2 , along with high affinity of $NADP^+$ for the dUMP-FADH₂-FDTS, suggests that $NADP^+$ does not leave the reactive complex prior to CH_2H_4 folate binding as previously suggested [18;23;27]. Overall, the oxidase activity has proved to be a useful tool to probe the substrate-binding features of FDTS synthase activity, and also emphasizes the importance of studying these enzymes under anaerobic conditions in order to avoid artifacts produced by this side reaction.

Using the information obtained during studies of the oxidase reaction [25;30], along with previous steady state measurements on the synthase reaction of FDTS [23;27;38;39], a kinetic mechanism for the FDTS-catalyzed reaction has been proposed. This mechanism is sequential with respect to all substrates, and can be divided into two half reactions as depicted in Fig. 6 [25;30;38]. In the reductive half reaction it is proposed that NADPH binds first to reduce FAD to FADH₂, and then dUMP can bind to form the dUMP-FADH₂- $NADP^+$ -FDTS complex. Both

molecular oxygen and $\text{CH}_2\text{H}_4\text{folate}$ have been shown to compete for this quaternary enzyme complex. Binding of $\text{CH}_2\text{H}_4\text{folate}$ (top path, Fig. 5) or reaction of FADH_2 with O_2 (bottom path, Fig. 5) begins the oxidative half reaction where these species are converted to H_4folate and H_2O_2 , respectively. Since there is no evidence of the order of product release, the dissociation of the enzyme complex after oxidation of the flavin is proposed to proceed by the 'first come, last to leave' principal, and more rigorous experimental study is needed to resolve the question of binding and release order.

Mechanism-based inhibition of FDTs

The biosynthetic pathways for thymidylate present good targets for antibacterial and antiviral drugs, as nucleotides are essential for cellular reproduction. This, together with the presence of *thyX* in the genome of human pathogens, biowarfare agents and many other microbes makes FDTs attractive for selective inhibition without affecting human thymidylate biosynthesis. Such inhibitors would need to exploit the differences between these enzymes in order to selectively disrupt FDTs function, and not that of classical thymidylate synthases.

A striking difference between FDTs and classical thymidylate synthases is the lack of an enzymatic nucleophile. One of the most effective classical thymidylate synthase inhibitors, 5F-dUMP, has been shown to covalently bind the active-site cysteine in these enzymes. FDTs however, lack this functionality and consequently do not bind 5F-dUMP covalently. The intermediate proposed after step 3, in Fig. 3C, is a noncovalent species that may tightly bind to the enzyme [6;22]. Analogues that mimic this species or the transition state for its formation are likely to have a high affinity for FDTs, but not for classical thymidylate synthases. Compounds that take advantage of covalent versus noncovalent binding modes could comprise one avenue for rational inhibitors of FDTs.

A notable characteristic of FDTs enzymes is the activation of the enzyme by dUMP, and the high affinity of $\text{CH}_2\text{H}_4\text{folate}$ to the dUMP-FADH₂-NADP⁺-FDTs complex. To date, the only mechanistically relevant complex for which crystal structures have been solved are with dUMP or its halogenated derivatives bound to the oxidized enzyme (e.g. (5F or 5Br)-dUMP-FAD-FDTs complex). Efforts to crystallize complexes with folate moieties have not been successful, and the only complex with the nicotinamide cofactor indicates that NADP⁺ has substituted for the FAD [33], and is unlikely to be part of the catalytic pathway. Our recent findings suggest that under strictly anaerobic conditions the possibility to crystallize the reduced form of FDTs bound to folate moieties may be greatly increased. Studies of the activated dUMP-FADH₂-NADP⁺-FDTs complex may provide valuable structural data, such as the location of the binding site for $\text{CH}_2\text{H}_4\text{folate}$, which could result in the identification of previously unrecognized mechanism-based compounds or inhibitors.

To date there are few inhibitors of FDTs activity [42], and none that show high specificity for FDTs over classical thymidylate synthase. As mentioned above, the chemical and kinetic mechanisms of FDTs have recently been greatly clarified; however, even with current knowledge of these enzymes, the scope of rationally designed inhibitors is limited. Preliminary suggestions of rational inhibitors have included bridged or hybrid ligands, such as linked adenosine moieties [32;38], which could effectively bind more than one active site, thus inhibiting the enzyme. Also, the linking of dUMP analogs to the pterin ring of NADPH or the isoalloxazine moiety of the FAD may produce bridged ligands that will bind tightly and specifically to FDTs. These conceptually simple inhibitors may give rise to alternate ligands for crystallization, providing access to new structural conformations.

FDTs represent an under characterized family of enzymes that is critical for the survival of many human pathogens. With the recent advances in the understanding of both the chemical and kinetic mechanism of these enzymes, future antibiotic drug design looks promising.

Advancement towards this goal hinges on the discovery of novel inhibitors selective to FDTSS with little impact upon human thymine biosynthesis and consequently, low toxicity.

Conclusion

Studies of FDTSS are in their early stages, and both the chemical and kinetic mechanisms of these enzymes have not thoroughly been established. However, it has been shown that the mechanism of the FDTs-catalyzed reaction differs greatly from that of the classical thymidylate synthases. Perhaps the most notable distinction between these pathways toward thymidylate, is the role of an enzymatic nucleophile. Classical thymidylate synthases absolutely require an active site nucleophile to covalently activate the substrate, dUMP. FDTSS, on the other hand, lack such functionality leading to a chemical cascade where the dUMP and intermediates along the reaction path, do not covalently bind the enzyme. Instead, the FDTs-catalyzed reaction relies on the reduced flavin cofactor, which has been suggested to reduce the uracil moiety by hydride transfer.

The *thyX* gene is present in many microbes, including several pathogens that threaten human life. FDTSS represent an attractive target for antimicrobial drugs due to the structural differences between *thyA* and *thyX* encoded enzymes, and the unusual chemical mechanism observed for FDTSS. Currently, there are several known potent inhibitors of classical thymidylate synthases, which are broadly used clinically, but have little effect on FDTSS. This suggests that it is possible to develop compounds selective to FDTSS. Future research of FDTSS will include crystallization attempts with folate moieties, investigation of the chemical properties and binding modes of noncovalent intermediates, elucidation of the relative timing of events during the FDTs-catalyzed reaction, and foremost, design and combinatorial efforts to find specific inhibitors.

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Figure 1. Thymidylate synthase structures

A) Classical thymidylate synthase dimer from *E. coli* (PDB entry 2KCE). The substrate dUMP is red and the cofactor analogue (Zd1694, Ralitrexed) is magenta and are highlighted as space filling shapes. B) *T. maritima* FDTs tetramer (PDB entry 1O26). FAD (blue) and dUMP (red) are highlighted as space filling shapes.

Figure 2. Thymidylate synthase reactions

A) The reaction catalyzed by classical thymidylate synthases (TSs). B) The reaction catalyzed by FDTs enzymes. R = 2'-deoxyribose-5'-phosphate; R' = (*p*-aminobenzoyl)-glutamate; R'' = adenosine-5'-pyrophosphate-ribityl.

Figure 3. Thymidylate synthase mechanisms

A) The chemical mechanism for the classical thymidylate synthase catalyzed reaction. **B)** The chemical mechanism for the FDTs which depends on an enzymatic nucleophile. **C)** A mechanism for the FDTs that does not rely on an enzymatic nucleophile. The conserved enzymatic nucleophile is orange, the methylene is purple, the reducing hydride from H_4folate is green, and the hydride from FADH_2 is red. $\text{R} = 2'$ -deoxyribose-5'-phosphate; $\text{R}' = (p\text{-aminobenzoyl})\text{-glutamate}$; $\text{R}'' = \text{adenosine-5'-pyrophosphate-ribose}$. Reproduced with permission from ref [22].

Figure 4. Crystal structure of *Tm* FDTS-dUMP-FAD complex

The three possible conserved nucleophiles Y91, S88 and S83 are shown as sticks. The oxygen of the S88 and the N5 of FAD are 4Å and 3.5Å from the electrophilic C6 of dUMP, respectively. Reproduced with permission from ref [22].

Figure 5. FDTS deuterium labeling experiment

A) An illustration of the observed products of the FTDS-catalyzed reaction when performed in D_2O at both 65 °C and 37 °C. **B)** Labeling outcomes of isomerization in D_2O by an addition-elimination mechanism (AEM, top path) or a 1,3-hydrogen rearrangement (1,3-H-shift, bottom path). This figure has been modified and reproduced with permission from ref [22].

Figure 6. Kinetic mechanism of FDTs

A proposed binding and release order for substrates and products of the FDTs catalyzed reaction. E_{ox} and E_{red} correspond to the oxidized (FAD-FDTS) and reduced (FADH₂-FDTS) enzyme complexes, respectively. Reproduced with permission from ref [30].