Review

Structure-based studies on species-specific inhibition of thymidylate synthase

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Abstract

Thymidylate synthase (TS) is a well-recognized target for anticancer chemotherapy. Due to its key role in the sole de novo pathway for thymidylate synthesis and, hence, DNA synthesis, it is an essential enzyme in all life forms. As such, it has been recently recognized as a valuable new target against infectious diseases. There is also a pressing need for new antimicrobial agents that are able to target strains that are drug resistant toward currently used drugs. In this context, species specificity is of crucial importance to distinguish between the invading microorganism and the human host, yet thymidylate synthase is among the most highly conserved enzymes. We combine structure-based drug design with rapid synthetic techniques and mutagenesis, in an iterative fashion, to develop novel antifolates that are not derived from the substrate and cofactor, and to understand the molecular basis for the observed species specificity. The role of structural and computational studies in the discovery of nonanalog antifolate inhibitors of bacterial TS, naphthalein and dansyl derivatives, and in the understanding of their biological activity profile, are discussed. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Enzyme inhibition; Thymidylate synthase; Structure-based drug design; Specificity

1. Introduction

Folate-dependent enzymes have always been considered as attractive targets for hyperproliferative diseases, including cancer and infectious diseases [1,2]. Since they are usually involved in processes related to nucleic acid synthesis, their inhibitors act as antimetabolites, inhibiting cell division [3]. A problem with the current antimetabolite drugs is the development of resistance in human and pathogenic cells. Such resistance has arisen under intense and continuous chemotherapy, and can diminish or abrogate the therapeutic responses to these drugs [4]. The mechanisms of drug resistance can be seen both in multidrug resistant proteins that pump drug molecules out of the cell, and in mutations in the structural gene for the target itself that directly diminishes the affinity of the drug for the target enzyme. In recent years, with the advent of modern technologies in drug discovery, better understanding of the molecular basis of drug–target interactions have been reached and suggest new strategies to solve the above-mentioned problems [5].

Because of its essential role in DNA synthesis, thymidylate synthase (TS) is among the most studied of folate-dependent enzymes. We report the development of two novel classes of inhibitors of bacterial TS, naphthalein and dansyl derivatives, and in the understanding of their biological activity profile, are discussed. © 2002 Elsevier Science B.V. All rights reserved.

Abbreviations: hTS, human thymidylate synthase; LcTS, L. casei TS; EcTS, E. coli TS; dUMP, 2’-deoxyuridine-5’-monophosphate; FdUMP, 5-fluoro-2’-deoxyuridine-5’-monophosphate; RFC, reduced folate factor; DDT, didansyltyrosine; FPGS, folylpolyglutamyl synthetase; PTH, phenolphthalein

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phosphate (dTMP) [6]. As a regulatory protein, TS may also be involved in the synthesis of proteins that regulate the apoptotic process [7,8].

The sequences of the TS enzymes from more than 30 organisms have been determined. Among them, many are from pathogenic microorganisms that include the fungi Cryptococcus neoformans and Pneumocistis carinii; parasites, Trichinella spiralis, Leishmania major, Tripanosoma cruzi, Plasmodium malarias; and bacteria, Escherichia coli and Staphylococcus aureus. Sequence alignment and comparisons show that, of 26 residues involved in substrates binding, 16 are completely conserved, so that TS is one of the most conserved enzyme known [6]. Therefore, inhibitors against one species can be used as a starting point for development of species specific inhibitors, by taking advantage of the unique features of the active sites of each pathogenic species.

The inhibition of TS can be accomplished through the design of molecules that interfere with substrate binding. After 5-fluorouracil (the active form of which is 5-fluoro-2’-deoxyuridine-5’-monophosphate, FdUMP) the first mechanism-based inhibitor of TS introduced in anticancer chemotherapy, ZD1694 (Fig. 1), a folate-related compound, is effective in clinical therapy against colorectal cancer. Other molecules with folate-related structures have been synthesized, including CB3717, GW1843, AG337, ZD9331, LY21514 (Alimta) [9] (Fig. 1). These molecules have different metabolism according to how well they can be transported into the cells through the RFC system, or other transporter mechanisms. They can be polyglutamylated by folylpolyglutamyl synthetase (FPGS), which increases their intracellular concentration, and their affinity for the enzyme. Drug resistance mechanisms include changes in FPGS or in TS itself.

One strategy to sidestep these problems is to design compounds that avoid the metabolic steps, do not need activation and bypass the FPGS transporter mechanisms. In fact, one of the most important mechanisms of resistance is overproduction of TS due to failure of the feedback regulation of TSmRNA transcription mediated by TS [10].

Fig. 1. Known folate analogs acting as TS inhibitors.
2. Experimental procedures

Docking calculations were performed with the computer program DOCK 3.5 using rigid body energy minimization (500 steps) [11]. Electrostatic and van der Waals interaction energies were calculated using a potential map calculated by CHEMGRID. For van der Waals terms, no upper maximum was set on the interaction energy (i.e., high-energy contacts were not truncated). For electrostatic terms, a distance-dependent dielectric constant of 4R was adopted. The standard AMBER-based parameter set was used [12]. Docking calculations used crystallographically determined structures of LcTS in complex with phenolphthalein [13], compounds a156 and MR20 (Fig. 2). The atoms of these inhibitors were used as “sphere centers” in these calculations [14].

Ligand structures were built using SYBYL (Tripos Associates, St. Louis) without minimization. Partial atomic charges were calculated using the method of Marsili and Gasteiger [15].

The hTS model used here was derived from the structure of E. coli TS [16]. The humanized E. coli TS crystal structure has been used very successfully as a model of the human enzyme in previous inhibitor design studies [17–19].

3. Structure-based design of non analog antifolate inhibitors

Thymidylate synthase remains a good target for structure-based drug design [20,21]. Over 90 X-ray crystal structures have been solved since 1987, when the first X-ray structure of the apoenzyme from Lactobacillus casei was determined in Stroud’s laboratory at UCSF in San Francisco. It was not until 1996 that the first structure of human TS was determined, initially for the apoenzyme [22,23]. Unfortunately, it was not suitable for typical antifolate drug design because the catalytic loop is twisted relative to the liganded structure [24,25]. In former years, to overcome the problem of not having the liganded hTS structure, scientists used a so-called humanized E.coli TS. This structure was a modification of the native EcTS in which some residues involved in ligand binding were substituted with the corresponding residues from hTS (i.e., Glu82 was truncated to alanine, Trp83 was modified to asparagine and Ile264 was modified to valine, according to EcTS numbering). Such a structure was suggested to have the correct sequence/folding for modelling hTS and it was used to design anticancer compounds [17]. Many leads and one drug have been discovered so far, using the humanized EcTS structure, such as the compounds developed at Agouron. Only recently have the ternary complex of hTS with dUMP and ZD1694 [24], and the complex with dUMP and LY21514, been obtained [25]. In principle, it is possible to rationally design compounds directed to a pathogenic TS whose X-ray structure is known, e.g., that for P. carinii [26], taking advantage of the sequence/structure differences with respect to human TS.

Efforts to discover species-specific inhibitors of TS often led to folate related compounds with classical nonspecific biological activity profiles. These molecules, structurally related to the folate cofactor, inhibit at the same level hTS and bacterial/fungal TS [27,28].

3.1. Phthalein derivatives discovery

In 1993, Shoichet et al. [13] published a pioneering structure-based inhibitor search based on application of docking algorithms to L. casei TS, and searching the available chemical database using the computational program DOCK [14]. For the X-ray ternary complex of LcTS–dUMP–CB3717, DOCK screened the FCD (Fine Chemical Directory) database, but notably excluding analogs of the substrates, i.e., dUMP and folate-related compounds. This led to discovery of phenolphthalein (PTH) as a low-micromolar inhibitor of LcTS. The biological activity profile of this molecule was nonspecific: $K_i$ vs. LcTS was 4.7 $\mu$M and $K_i$ vs. hTS was 1.3 $\mu$M. PTH is a dye and a well-known laxative agent, so it could not be considered a lead, but a hit. Nevertheless, it is an interesting molecule because it suggested that nonfolate related molecules could bind to the TS binding site with good affinity. The X-ray crystal structure revealed that this molecule binds competitively, close to the area where CB 3717 binds in the ligand binding cavity.

Starting from PTH, the design of novel inhibitors of TS was further developed through a combination of X-ray structure analysis, database exploration and combinatorial studies. This led to new candidates that underwent synthetic optimization and biological evaluation in our hands. In such a structure-based approach, the knowledge of the 3D struc-
ture of the target in complex with the evolving compounds is key to improving the quality and reliability of successive iterations, as is the feedback from biochemical and biological screening. Applying these techniques, it was possible to develop a series of nonfolate analog TS inhibitors that included naphthalein and dansyl derivatives, as described in the following sections.

3.2. Naphthalein derivatives

Starting from the X-ray structure of the binary complex of LcTS with PTH [13], the structure of the ligand was further modified with other substituents. Small substituents on the phenolic ring, such as halogens, were introduced and their biological activity evaluated [29,30]. This led to the 2,3-naphthalein and the 1,8-naphthalein derivatives. With the latter, the results were most interesting: since introduction of the substituents on the naphthalene ring and, in particular, on the phenolic rings, influenced the biological activity profile, and the ‘affinity fingerprint’ [29]. Applications of docking algorithms using DOCK suggest that the naphthalein derivatives bind in the same site as PTH, with two families of orientations, both close to the classical folate recognition site. When the compounds were tested, a156 (Fig. 2) inhibited LcTS and EcTS with the same affinity ($K_i = 0.5$ μM) while it bound to hTS 40-fold less. This is, to our knowledge, the first species-specific TS inhibitor. By contrast, MR20 (Fig. 2) shows the same affinity for bacterial TS and hTS ($K_i = 1$ μM) [30]. The structural basis for the specificity of a156 was investigated by means of X-ray crystallography, mutagenesis and computation.

The X-ray crystal structure of the binary complex LcTS–a156 was determined [30]. Surprisingly, this molecule binds in a different binding site than the parent compound (PTH). It is shifted 5 Å towards the entrance of the active site with respect to the folate site. In Fig. 3, CB3717 is represented bound to its crystallographic binding site to show its relative position with respect to a156.

The X-ray structure of the LcTS–a156 complex differed from the DOCK prediction, in that the crystallographic binding mode placed the molecule close to the small domain, while DOCK placed the same molecule close to the folate recognition domain. It is well known that DOCK has such limitations, emphasizing the need for successive crystal structure determinations as the series progresses. Nevertheless, this case was not a simple one, since some experimental results suggested that these molecules could bind to more than one binding site. Mutagenesis studies were designed to better understand the mode of action of these compounds [30]. Residues that represented key interactions with the ligands involved were mutated: V316A, W82Y and E60D. The three residues are directly or indirectly involved in ligand interactions and direct or long range effects could be expected. The apparent $K_i$ values were measured and it was observed that some substitutions diminished the extent of inhibitor binding from 3- to 20-fold (V316A and E60D with a156). Several substitutions improved the extent of inhibitor binding up to 2.5-fold (E60D with PTH and MR20). These results were ascribed to multiple binding modes in which these molecules can bind to more than one binding site with different $K_i$ values. The crystallographic structure seems most definitive. The X-ray crystal structure was difficult to obtain and the occupancy (in terms of electron density of the ligand) of the crystallographic configuration was not complete, suggesting some disorder or multiple binding configurations [30]. Also,

Fig. 3. Superimposition of the folate analog CB3717 and the nonfolate analogs PTH and a156 in their crystallographic binding configuration in L. casei TS. The X-ray crystal structures of the three compounds with LcTS have been superimposed to show the different binding configurations.
the kinetics were difficult to interpret: since some compounds were almost competitive inhibitors with respect to the folate cofactor, some were ‘mixed’ type inhibitors, while others were noncompetitive [29].

4. Docking analysis of naphthalein binding

Computational analysis, based on the three X-ray structures of PTH, a156, MR20 with LcTS, were applied to define the basis of species specificity. Rigid-body energy minimization and molecular docking calculations were performed to investigate how well the compounds fit into each others’ binding sites, and the analogous sites in a humanized (see Section 2) E. coli EcTS structure [33]. The results derived from a comparison with the humanized EcTS showed how the small domain is especially important in terms of specificity, since this region is much shorter in the human enzyme.

4.1. Binding analysis of phthalein derivatives to hTS

When compound a156 is fitted into the human TS structure, in the same binding site as that of LcTS, it appears to be poorly accommodated, physically intersecting several residues. In this orientation in hTS, a156 has a high energy of interaction due to these steric conflicts. On rigid-body minimization, which allows the ligand to move as a rigid body but keeps the receptor rigid, the ligand moved by 4.2 Å from its analogous position in hTS, achieving a final energy that was higher (worse) than when minimized from its LcTS orientation (Tables 1 and 2). Molecular docking, which typically allows for further relaxation, could not fit compound a156 into hTS in a similar orientation as it adopts in LcTS (data not shown).

Although the complex between LcTS and MR20 involved several residues that differed between the human and L. casei enzymes, MR20 lacked the steric conflicts with the humanized E. coli model that compound a156 had (Table 1). Moreover, compound MR20 had few interactions with the “small domain” of LcTS. Both observations are consistent with the low specificity of compound MR20 for the bacterial vs. the human enzyme. Similar conclusions can be drawn for PTH, which can be well accommodated in the humanized EcTS binding site (Table 1).

4.2. Binding of inhibitors to other inhibitors site

In the calculations for LcTS, the question was whether compounds for which enzyme complex structures were available could fit well into the binding modes defined by the other two inhibitors. For instance, how well does PTH fit into the sites occupied by compounds a156 and MR20, and how well does compound a156 fit into the site occupied by compound MR20. The compounds were fitted into the three different orientations to match equivalent atoms. High-energy contacts were relaxed through rigid-body minimization keeping the conformation of the enzyme constant.

PTH and a156 fitted best in their own crystal structures of TS, while MR20 fitted equally well in all three sites (Table 2). Compound a156 showed the highest specificity for its site, both energetically and in terms of displacement on rigid body minimization, and compound MR20 showed the lowest specificity.

4.3. Molecular docking

To investigate the problem more thoroughly, we docked the individual molecules into crystal structures of LcTS using the molecular docking program DOCK 3.5 (Table 3) [11]. DOCK considers multiple orientations of a ligand in a site and is able to explore possibilities that might be missed by rigid-body energy minimizations. Docking led to orientations whose geometry corresponded more closely to the crystallographic result than did the rigid-body minimization calculations, but did not find geometries that were energetically more favorable unless we allowed for large deviations from the canonical binding modes. Considering both energy and RMS deviation, PTH and compound MR20 showed little specificity for their own binding sites, whereas compound a156 could only be fitted into its own binding sites; it could not be fitted into the PTH or compound MR20 sites unless substantial deviation was allowed.

In conclusion, modeling suggests that the crystallographic structure of each molecule represents a unique

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Site of</th>
<th>RMS (Å)</th>
<th>Site of</th>
<th>RMS (Å)</th>
<th>Site of</th>
<th>RMS (Å)</th>
</tr>
</thead>
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<tr>
<td>PTH</td>
<td>−25.4</td>
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<td>−20.9</td>
<td>0.25</td>
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<td>−26.7</td>
<td>0.13</td>
<td>−14.1</td>
<td>1.68</td>
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<tr>
<td>MR20</td>
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<td>0.9</td>
<td>−17.2</td>
<td>1.95</td>
<td>−17.4</td>
<td>1.60</td>
</tr>
</tbody>
</table>

* RMS deviation from X-ray crystallographic configuration, or best fit onto that configuration using common atoms.

Table 1

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Energy in E. coli site (kcal/mol)</th>
<th>RMS (Å)</th>
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<tr>
<td>PTH</td>
<td>−17</td>
<td>2.4</td>
</tr>
<tr>
<td>a156</td>
<td>−7.6</td>
<td>4.2</td>
</tr>
<tr>
<td>MR20</td>
<td>−17</td>
<td>2.2</td>
</tr>
</tbody>
</table>

* The structures of the LcTS complexes were RMS-fit on the “humanized” E. coli structure to orient compounds PTH, a156 and MR20 in the humanized E. coli structure. The positions of the inhibitors were then allowed to relax as rigid bodies to minimize their interaction energies with the protein.

* The RMS distance from the initial fit positions to the final energy minimized positions.
low energy binding orientation. Alternatively this family of molecules might bind to LcTS in several low energy modes, of which the primary ones are represented by the three crystal structures. The theory of multiple binding modes is complex and cannot yet be completely interpreted by energy calculations, since the inherent errors are large. Therefore modeling studies have to be considered in light of mutagenesis and enzymology.

5. Dansyl derivatives discovery

In 1999, a new series of nonfolate analog inhibitors of LcTS were discovered through a combined approach of structure-based drug design and in parallel synthetic chemistry [31,32]. This approach has the potential to discover new hits and new scaffolds rapidly for further chemical elaboration toward a specific biological profile [32]. DOCK 3.5 was applied to screen the ACD (Available Chemicals Database) database for compounds that might bind to the binding site from the ternary complex LcTS–dUMP–CB3717. The aim was to identify nonfolate chemical structures, and particularly those suitable for further rapid elaboration by parallel synthetic chemistry and solid phase methods [33].

Table 3

Molecular docking energies of inhibitors in three different binding modes

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Site of PTH RMS (Å)</th>
<th>Site of a156 RMS (Å)</th>
<th>Site of MR20 RMS (Å)</th>
</tr>
</thead>
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<td>a156</td>
<td>2.5 1.00</td>
<td>−26.7 0.10</td>
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<tr>
<td>MR20</td>
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<td>−7.0 1.35</td>
<td>−11.5 0.85</td>
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</table>

* RMS deviation from X-ray crystallographic configuration, or best fit onto that configuration using common atoms.

Fig. 4. Evolution of the molecular structure of the dansyl derivatives. The fragments in black show the increasing molecular complexity that is related to the improved affinity for bacterial TS and improved specificity with respect to human TS.
Four hundred molecules with the highest score were gleaned by this method and scored on the basis of the electrostatic and van der Waals (VdW) interaction energy with the enzyme, after correcting for ligand solvation [12,34]. On the basis of the DOCK score, and the number of specific interactions with the protein, five hits (IC$_{50}$ between 300 μM and 2 mM) were tested for their ability to inhibit LcTS. Among the selected molecules, dansyl hydrazine (IC$_{50}$ 439 μM; $K_i$ = 176 μM) (Fig. 4) was a good starting point for synthetic elaboration.

The predicted binding mode for dansyl hydrazine suggested that bulkier fragments attached to the dansyl scaffold could be accommodated in the enzyme pocket. Among the more interesting was dansyl tyrosine, with a somewhat better $K_i$ (=65 μM) than dansyl hydrazine (Fig. 4). The molecule bears both an ‘anchor site,’ a functional group for linking the scaffold to the resin, and a diversity-derivatizable group (the carboxyl group and the amino group, respectively). Molecular diversity was introduced in the dansyl tyrosine molecule by synthesizing a series of analogs that carry different substituents on the amino group. A library of 34 molecules was created using in-parallel solid phase synthesis. The best of the synthesized analogs, DDT, had a $K_i$ of 1.4 μM towards LcTS, while several others had $K_i$ values below 10 μM. These analogs (Fig. 4) are structurally dissimilar to either of the substrates but bind competitively with them.

5.1. Docking of didansyl tyrosine to L. casei TS

Further calculations were performed to understand the molecular basis for DDT affinity: 500 multiple conformations were generated by rotating all single, nonterminal bonds in increments of 120° [35]. Each conformer was independently docked and scored into the LcTS binding site for the active site.

![Fig. 5. Superimposition of the X-ray ternary complex EcTS–dUMP–DDT (green yellow) and the DOCK model of DDT (brown blue) with LcTS. The two molecules are reported in the same frame of reference to show the difference in the respective binding modes: DDT from the X-ray crystal structure is in the folded conformation and DDT from the DOCK model is oriented towards the entrance to the active site.](image-url)
site. In the best scoring orientation, the anchor dansyl ring forms nonpolar charge transfer interactions with the pyrimidine ring of dUMP.

Increasing the molecular complexity of the dansyl derivatives, the predicted binding mode is conserved, demonstrating the value of the incremental approach to design new hits. By adding fragments under computational control, a sensible gain in binding energy can be observed (from dansyl hydrazine to DDT, the $K_i$ is improved by 500-fold). The biological activity profile of the dansyl derivatives shows the same trend observed in the computational studies in that increasing the molecular diversity led to rapid improvement of selectivity (Table 4).

5.2. X-ray crystal structure of EcTS–DDT–dUMP

The crystal structure of the ternary complex DDT–dUMP–EcTS was subsequently solved at 2.0 Å resolution [36] (Fig. 5). The O-dansyl ring of DDT binds also to the site normally occupied by the quinazoline group of folate analogues, as predicted by our model for LcTS, though in a different orientation. It interacts more deeply in the binding site due to specific protein rearrangements (i.e., Arg23, Ala315, dUMP). These deep rearrangements avoid bad clashes with the inhibitor DDT and could not be predicted in our present calculations since the receptor was kept rigid. This underlines the failure of the rigid receptor hypothesis in drug design.

In the EcTS X-ray structure, the tyrosine ring is oriented close to Phe228 and Leu224, in the area normally occupied by the PABA moiety. However, this region is now occupied by the combination of the phenyl ring and N-dansyl group of DDT. The phenyl ring and the N-dansyl group form favourable aromatic stacking interactions with each other and are sandwiched between the hydrophobic residues Leu172 and Ile79. Consequently, we encounter several protein rearrangements in this site, so that the enzyme could accommodate such a complex moiety (Leu224, Phe228, Ile81, Leu56, Lys259 are the residues most involved in protein rearrangements).

The X-ray structure shows that DDT binds to EcTS in a conformation not predicted by molecular docking studies and one substantially different from other TS inhibitors. Binding of DDT is accompanied by large rearrangements of the protein that involve areas both near and far from the active site. Not surprisingly, the predicted model for DDT in LcTS and the crystallographic orientation of DDT in EcTS differ considerably from each other (Fig. 5). Moreover, the protein plasticity results in novel interactions of DDT with residues conserved in bacterial TS but not humanTS and which are hypothesized to account for DDT’s species specificity.

The comparison between the binding mode that DDT displays with the two enzymes, LcTS and EcTS, underlies the fact that binding of a ligand by a protein is highly specific, often relying on subtle changes between the structures of even closely related species of a protein, and in this case, closely related species of one of the most conserved of all enzymes, TS.

6. Conclusions

Structure-based drug design is a powerful technique, especially when combined with mutagenesis and species specific competition assays. Starting from early hits, it is possible to discover new scaffolds placing the immediate computational products alongside the structural analysis of the crystal/model of the complexes. In the present cases, two series of compounds were discovered and elaborated to develop a ~100-fold selective inhibitor for bacterial TSs vs. human TS. The naphthalene derivatives, after two cycles of design, synthesis and evaluation, have reached an advanced stage. The dansyl derivatives are being attractive probes for structural/functional studies and more efforts are directed to improve their biological activity profiles.

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