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Design, synthesis and evaluation of potent thymidylate synthase X inhibitors

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ABSTRACT

Three synthesized series of compounds based on a thiazolidine core allowed identification of potent inhibitors of thymidylate synthase X. The evaluation of the catalytic activity of the enzyme in the presence of these molecules revealed two distinct classes of compounds that inhibit ThyX with submicromolar concentrations, which could lead, after optimization, to effective inhibitors with potential biomedical interest.

Infectious diseases caused by bacteria and fungi affect millions of people worldwide. The widespread bacterial resistance has increased the interest in identifying new targets for the development of effective antibacterial agents. The recently discovered thymidylate synthase X [ThyX (EC 2.1.1.148)] constitutes a promising target, since this protein is present in many pathogenic bacteria and dsDNA viruses but is absent from human and most eukaryotes.[1,2]

DNA polymerase catalyzes DNA synthesis using four different nucleoside triphosphates as substrates. Thymidylate synthases participate in the biosynthesis of DNA by producing thymidylate (dTMP) that is phosphorylated to the corresponding triphosphate (dTTP). There are two different classes of thymidylate synthases, with no structural[3] or sequence[4] similarities: the well-known homodimeric ThyA[5] (EC 2.1.1.145) and the homotetrameric ThyX.[6,7] The canonical thymidylate synthase ThyA, found in humans, catalyses the reductive methylation reaction where methylenetetrahydrofolate (CH_2H_4folate) functions both as the carbon source and reductant. On the other hand, ThyX proteins use a FAD/NAPDH couple to mediate hydride transfer during catalysis. Note that in some species, thymidylate can also be salvaged by phosphorylation of thymidine.

Whereas a large number of ThyA inhibitors has been synthesized, only a few ThyX inhibitors are available. In this study, we sought to identify inhibitors of ThyX proteins by developing a library of compounds around an ethyl thiazolidine-4-carboxylate ring that allows the introduction of a wide range of diversity in few transformations. In addition, this structure can improve protein binding properties by decreasing the degrees of conformational freedom.[8] Using this specific structure, we developed two kinds of transformations. The first one involves the modification of the secondary amine in order to mimic the natural substrate. The second one introduces diversity via various transformation of the ethyl carboxylate (Fig. 1). Two compound families composed

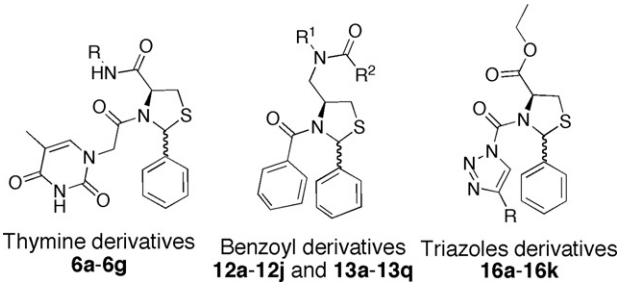

Figure 1. Thiazolidine families synthesized.

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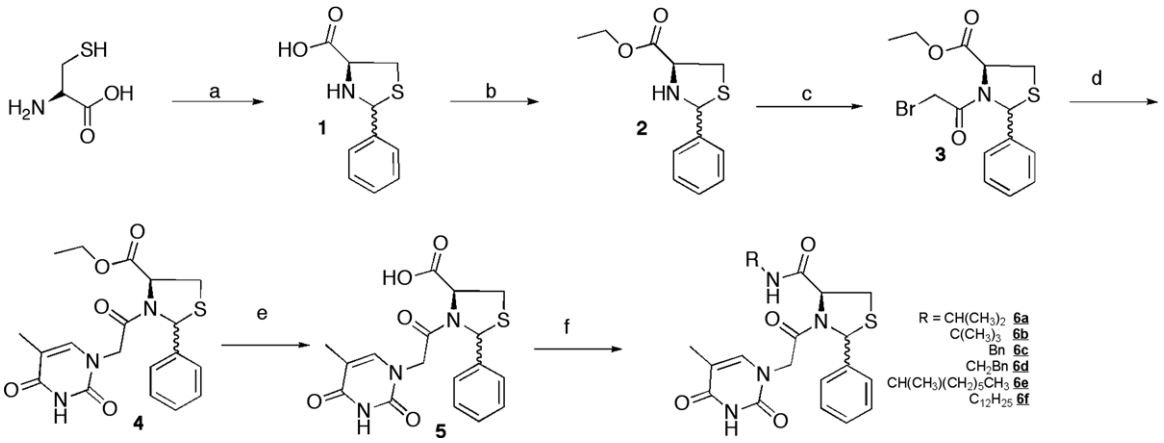
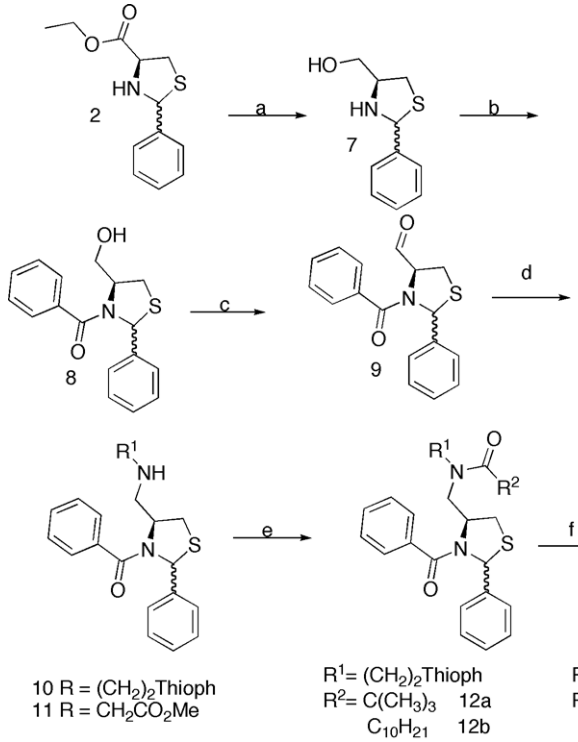


Scheme 1. Synthesis of thymine derivatives. Reagents and conditions: (a) benzaldehyde, EtOH/H_2O, 3 h at rt; (b) $SOCl_2$, anhydrous EtOH, 1 h at 0 °C, then 18 h at rt; (c) bromoacetyl bromide, pyridine, anhydrous CH_2Cl_2; (d) thymine, K_2CO_3, anhydrous DMF; (e) RNH_2, EDC, HOBt.



R^1 = $(CH_2)_2$Thioph	R^2 =	
	$C(CH_3)_3$	12a
	$C_{10}H_{21}$	12b
	Bz	12c
	p-F-Bz	12d
	p-CN-Bz	12e
	p-NO_2-Bz	12f
	o-I-Bz	12g
	m-CF_3-Bz	12h
	C_6F_5	12i
	C_6H_5-C_6H_5	12j

R^1 = CH_2COOMe	R^2 =	
	$C(CH_3)_3$	13a
	C_6H_{13}	13b
	$C_{10}H_{21}$	13c
	Bz	13d
	p-F-Bz	13e
	p-CN-Bz	13f
	p-NO_2-Bz	13g
	o-I-Bz	13h
	m-CF_3-Bz	13i
	C_6F_5	13j
	C_6H_5-C_6H_5	13k

R^1 =	
$C(CH_3)_3$	14a
C_6H_{13}	14b
p-CN-Bz	14c
p-NO_2-Bz	14d
C_6F_5	14e
C_6H_5-C_6H_5	14j

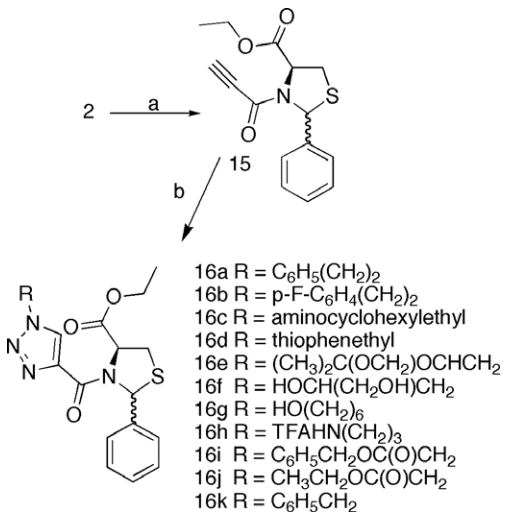
Scheme 2. Introduction of diversity. Reagents and conditions: (a) 1—$CaCl_2$/$NaBH_4$ in EtOH at −10 °C; 2—addition at 10 °C, 2 h at 10 °C; (b) benzoyl chloride 2 h at −20 °C; (c) DMSO, pyridine/SO_3; (d) R^1NH_2, $NaBH_3CN$ in MeOH; (e) 1—$R^2C(O)Cl$, pyridine in anhydrous CH_2Cl_2; 2—A-15.

of related molecules where synthesized in solution using efficient multistep syntheses. Obtained molecules were then purified and biologically evaluated. The synthesis of thymine derivatives was carried out as depicted in Scheme 1.

Acetalation of L-cysteine with benzaldehyde efficiently gave the thiazolidine **1**.[9] Esterification of the carboxylic acid function by treatment with thionyl chloride in ethanol yielded the ethyl ester **2**. Acylation of the endocyclic nitrogen with bromoacetyl bromide introduced an halide that can be substituted by thymine in the presence of K_2CO_3 and 18-crown-6 to afford **4**.[10,11] Finally, saponification of the ethyl ester in basic medium provided **5**, which was converted into compounds **6a** to **6f** by a peptidic coupling with commercial amines in the presence of EDC.

Compounds of the second family, where a benzoyl group replaces thymine, are synthesized by a five-step procedure as shown in Scheme 2.

Reduction of the thiazolidine **2** with sodium borohydride yielded the diol **7**. Exposure of **7** to benzoyl chloride at low temperature selectively afforded the amide **8**. Oxidation of the primary alcohol conducted in the presence of DMSO and SO_3/pyridine complex provided the aldehyde **9** as a solid. Then the reductive amination of **9** with glycine methyl ester and thiophenylamine yielded **10** and **11**. The amines **10** and **11** were acylated with various acyl chlorides to yield the amides **12a**–**12j** and **13a**–**13k**, respectively. Finally, acids **14a**–**14f** were synthesized by removal of the ethyl group.



Scheme 3. Synthesis of triazoles derivatives. Reagents and conditions: (a) DCC, propiolic acid in anhydrous CH_2Cl_2 16 h in rt; (b) R-N_3, 5% Cu(I), DIPEA in CH_2Cl_2, 5 h, rt.

By synthesizing compounds **16a**–**16k**, we developed a family of *N*-acetyled triazoles that was prepared in two steps (Scheme 3). First propiolic acid was reacted with thiazolidine **2** to yield **15**. Then alkyne condensation with a series of azides using a catalytic amount of Cu(I) afforded the triazole ring by a [2+3] Huisgen cycloaddition.[12–14] The azides was freshly synthesized from corresponding alcohols by a preliminary mesylation followed by a nucleophilic substitution of the mesylate moiety with NaN_3.

The synthesized compounds were screened using a spectrophotometric method designed to detect ThyX activity.[6,15] This test is based on the capability of ThyX proteins to oxidize NADPH that is used as substrate. As NADPH has a maximum of absorption at 340 nm, the ThyX catalysis results in decrease of absorption at 340 nm (Fig. 2, positive control). This analysis revealed four triazole derivatives and one *N*-benzoylated thiazolidine that inhibited ThyX catalytic activity (Fig. 3). Among these five compounds **14b**[16] (IC_{50} = 0.13 μM) and **16h**[17] (IC_{50} = 0.057 μM) showed inhibitory activity at submicromolar concentrations. As these compounds could constitute a promising starting point for optimized inhibitors, we performed additional tests in the presence of a surfactant Triton X-100 (to exclude aggregation effects) and bovine serum albumin (to exclude non-specific protein binding) (Fig. 2). Neither one of these conditions diminished the inhibition effect of the compounds, thus indicating that the observed inhibitory effect is caused by a specific interaction between the chemicals and the ThyX protein (data not shown).

Monitoring the changes in the OD of the reaction medium at different dUMP concentrations revealed the different behavior for the compounds **14b** and **16h**, suggesting that their inhibitory mechanisms are not identical (Fig. 4). In particular, inhibitory activity of class I inhibitors such as **14b** can be reversed by addition of molecular excess of dUMP whereas class II inhibitors such as **16h** are insensitive to addition of dUMP.

In summary, a library based on a thiazolidine scaffold has been prepared using various chemistry including aminative reduction and click chemistry. Evaluation of their inhibiting properties was performed against ThyX, a relatively recently discovered protein with few identified inhibitors (FdUMP and BrdUMP). Using a screening method adapted to a rapid identification of potent hits,

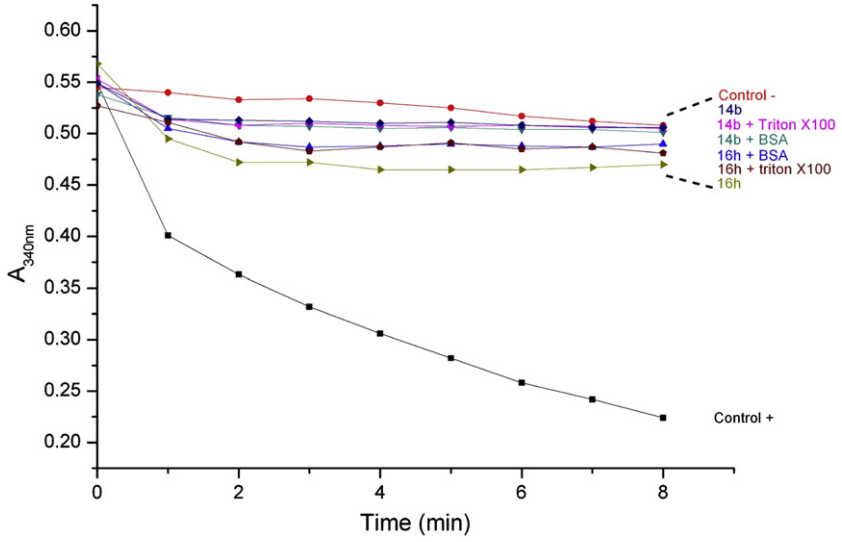


Figure 2. Compounds inhibiting ThyX's catalytic activity.

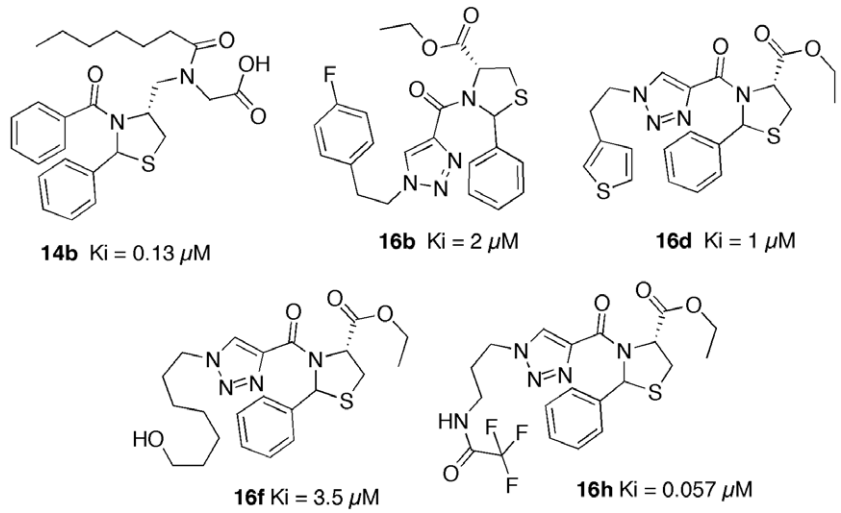


Figure 3. ThyX activity was measured by monitoring decrease in A_{340} as the function of time (see Materials and methods). Molecules **14b** and **16h** were used at 20 μM. Where indicated, BSA (240 μg/ml) or Triton X-100 (0.1% v/v) were included in reaction mixtures.

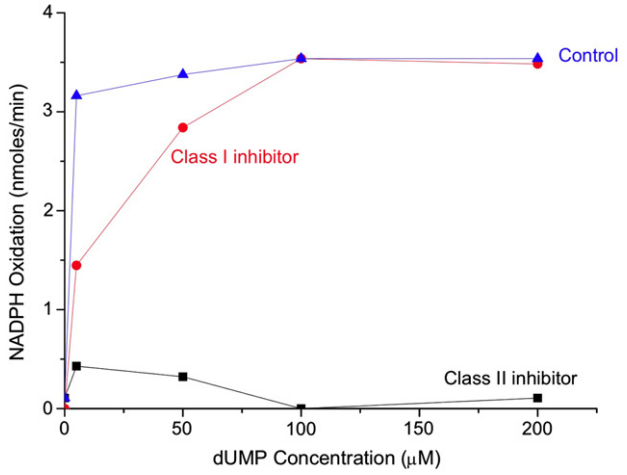


Figure 4. NADPH oxidation activity (nmol/min) of PBCV-1 ThyX as the function of the added dUMP. Experiment was performed in the absence and presence of inhibitors (20 μM) **14b** (class I) and **16h** (class II).

we detected a benzoyl and a triazole derivative that demonstrated inhibition of the catalytic activity. Optimization of these leads by further structure–activity relationship (SAR) analyses and screening of the library against additional methyltransferases are under investigation and will be the subject of future communications.

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15. Assay reactions, with a final volume of 200 μl, consisted of 200 μM NADPH, 5 μM CH_2H_4 folate, 5 μM dUMP, 1 mM $MgCl_2$, 1% glycerol, 62.5 μM FAD and 12.5 μM of viral PBCV-1 ThyX. Note that active site configurations of this viral enzyme is very similar to that of ThyX proteins from pathogenic species (not shown). The reactions were initiated by injection of NADPH to each well of the Microtiter 96-well clear flat-bottom plate, followed by rapid shaking of the microplate. ThyX activity was determined by following the decrease of A_{340} (due to oxidation of NADPH) that was measured with a reading interval of 30 s for duration of 15 min. All assays used a kinetic mode of a multilabel microplate reader [CHAMELEON II (Hidex, Finland)] with an injector. All screening reactions were performed in triplicates. The primary screen was performed with the small molecules dissolved in DMSO, including DMSO alone as low-activity control. The concentration of screened compounds was 20 μM unless otherwise indicated. Dose–response curves of the chosen compounds were determined using a wide range of compounds.
16. *2-(N-{[(4R)-3-benzoyl-2-phenyl-1,3-thiazolidine-4-yl]methyl}hexanamido)acetic acid* (**14b**). ^{1}H NMR ($CDCl_3$) δ 0.87–1.60 (m, 7H, H-19 à H-21), 1.68–2.25 (m, 4H, H-17+H-18), 3.91–4.27 (m, 4H, H-6+H-8), 4.69–4.98 (m, 3H, H-14+H-7) 6.05 (sl, 1H, H-5), 7.38–7.81 (m, 10H, H-arom).^{13}C NMR ($CDCl_3$) δ 14.0 (C-21), 22.4 (C-20), 26.0 (C-19), 31.5 (C-20), 31.8 (C-17), 36.2 (C-6), 50.6 (C-14), 53.3 (C-16), 58.8 (C-7), 65.8 (C-5), 127.0, 128.3, 128.8, 129.5, 134.2, 138.3 (C-arom). LC–MS Calcd for $C_{25}H_{30}N_2O_4S$ 454.59$^-$ *m/z* 472 (M+Na)$^+$. Purity by ELSD 99%.
17. *Ethyl (4R)-2-phenyl-3-({(1-[3-2,2,2-trifluoroacetamido)propyl]-1H-1,2,3- triazol-4-yl}carbonyl)-1,3-thiazolidine-4-carboxylate* (**16h**). ^{1}H NMR ($CDCl_3$) δ 1.27–1.37 (m, 3H, H-1), 1.93–2.20 (m, 2H, H-17), 3.14–3.56 (m, 4H, H-5+H-18), 4.21–4.43 (m, 4H, H-2+H-16), 5.04–5.16 (m,1H, H-4), 6.04–6.18 (m, 0.6H, H-6), 6.49 (s, 0.4H, H-6), 7.12–8.31 (m, 6H, H-arom+H-15). ^{13}C NMR ($CDCl_3$) δ 14.1 (C-1), 29.1 (C-17), 31.3 (C-5), 34.8 (C-5), 36.9 (C-18), 47.7 (C-16), 61.9 (C-2), 65.0 (C-4), 65.7 (C-4), 67.1 (C-6), 68.6 (C-6), 127.1, 128.3, 129.4, 130.0 (C-arom). LC–MS: *m/z* 486 (M+H)$^+$, 508 (M+Na)$^+$. Purity by ELSD 99%.