

Synthesis and Evaluation of α -Thymidine Analogues as Novel Antimalarials

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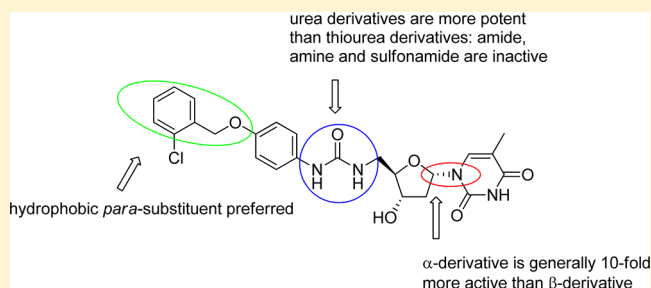
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Supporting Information

ABSTRACT: *Plasmodium falciparum* thymidylate kinase (*Pf*TMPK) is a key enzyme in pyrimidine nucleotide biosynthesis. 3-Trifluoromethyl-4-chloro-phenyl-urea- α -thymidine has been reported as an inhibitor of *Mycobacterium tuberculosis* TMPK (*Mt*TMPK). Starting from this point, we designed, synthesized and evaluated a number of thymidine analogues as antimalarials. Both 5'-urea- α - and β -thymidine derivatives were moderate inhibitors of *Pf*TMPK and furthermore showed moderate inhibition of parasite growth. The structure of several enzyme-inhibitor complexes provides a basis for improved inhibitor design. However, we found that certain 5'-urea- α -thymidine analogues had antimalarial activity where inhibition of *Pf*TMPK is not the major mode of action. Optimization of this series resulted in a compound with potent antimalarial activity ($EC_{50} = 28$ nM; $CC_{50} = 29$ μ M).



INTRODUCTION

There are approximately 300–500 million clinical cases of malaria each year.^{1–5} While there is significant progress in development of vaccines, they are still on trial and not available,³ so chemotherapy remains the mainstay for dealing with this enormous problem. Furthermore, owing to problems with resistance, there is a need for new drugs to treat the disease.

Analysis of the *Plasmodium falciparum* genome⁶ indicates that these parasites lack the enzymes required for pyrimidine salvage⁷ and are totally dependent on *de novo* pyrimidine nucleoside synthesis for DNA replication. In contrast, erythrocyte maturation is accompanied by the loss of the capacity to carry out *de novo* synthesis of pyrimidines⁸ reinforcing the importance of pyrimidine biosynthesis as a potential antimalarial drug target. Indeed, several enzymes in the pyrimidine metabolism pathway are validated antimalarial targets, such as dihydrofolate reductase⁹ and dihydroorotate dehydrogenase.¹⁰

Another enzyme involved in pyrimidine biosynthesis, *P. falciparum* thymidylate kinase (*Pf*TMPK), which catalyzes phosphorylation of thymidine monophosphate (TMP) to thymidine diphosphate (TDP), represents a potentially attractive drug target for malaria.^{11,12} TMPK has been characterized in several organisms including human,^{13,14} *Escherichia coli*,^{13,15} *Saccharomyces cerevisiae*,^{13,15} *Mycobacterium tuberculosis*,¹⁶ *Vaccinia virus*,¹⁷ and *P. falciparum*,^{5,12} and is

essential in most organisms. We have recently reported structural and kinetic studies of *Pf*TMPK, which indicate significant differences from the human homologue.¹²

TMPK is a homodimer with a subunit fold consisting of a five-stranded parallel β -sheet surrounded by 7–11 α -helices.¹² Two conserved sequence motifs (Figure 1) are found in the nucleotide substrate binding domain: the P-loop containing structural elements required for substrate recognition and catalysis and a LID domain that partially encloses the phosphate donor and is important for catalysis.¹²

Superposition of the structure of *Pf*TMPK-TMP-ADP with the equivalent complex of human TMPK (*h*TMPK) shows a highly conserved topology and mode of nucleotide binding, the TMP binding sites being essentially identical. However, several significant differences were observed in the P-loop and the LID domain.¹² Therefore, reported TMPK inhibitors containing the thymidine base, but an optimized 5'-OH motif, will potentially target the P-loop and LID domain, and could lead to selective inhibition of *Pf*TMPK compared to *h*TMPK.

TMPK has been investigated as a target against *M. tuberculosis*.¹⁶ Various series of inhibitors have been reported for *Mt*TMPK.^{16,19,20} An interesting class is a series of 5'-

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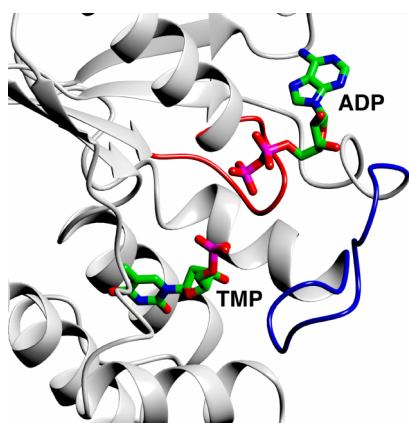


Figure 1. The crystal structure of the *Pf*TMPK TMP-ADP complex showing the P-loop (red) and LID domain (blue), PDB code 2wwf, Whittingham et al.¹² Figures 1 and 3 were made with CCP4mg.¹⁸

substituted α -thymidine derivatives (Figure 2), some of which showed sub-micromolar activity against *Mt*TMPK, and also good

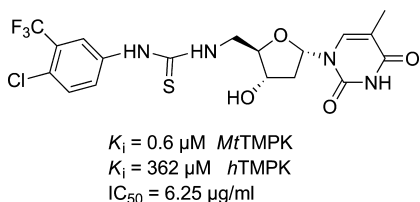


Figure 2. Example of a 5'-substituted α -thymidine inhibitor of *Mt*TMPK.¹⁶

selectivity compared to *h*TMPK (Figure 2).¹⁶ A number of *Mt*TMPK inhibitors were active against *M. tuberculosis* in cell culture, although others were inactive possibly due to issues

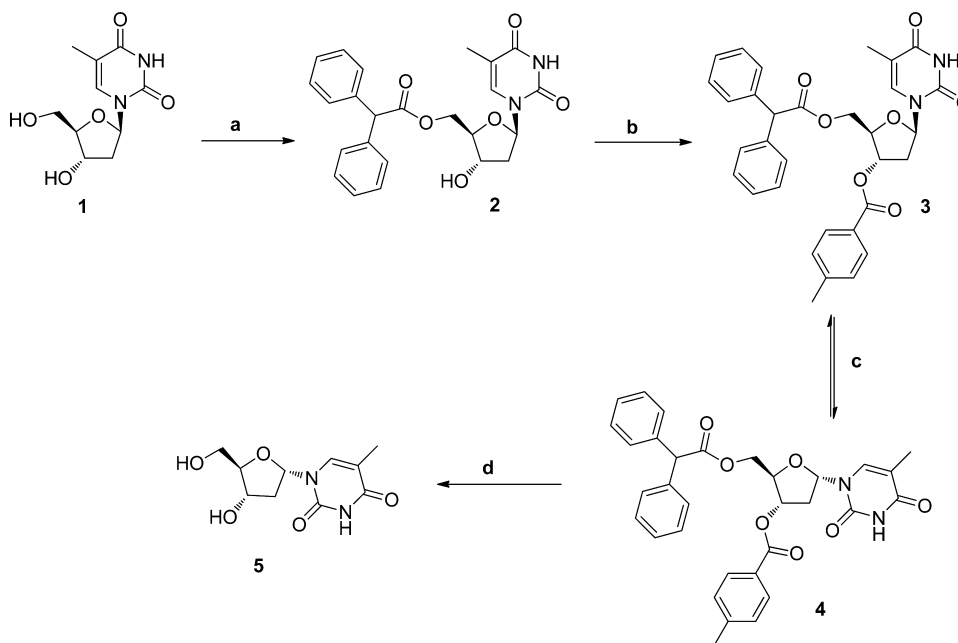
concerning chemical solubility and cell permeability. Superimposition of the structures of *h*TMPK and *Mt*TMPK revealed significant amino acid differences in the active site close to the 5'-position of the deoxyribose.²¹ Moreover, 5'-thiourea α -thymidine derivatives were completely inactive against human thymidine kinase 1 and only showed weak inhibition of human mitochondrial nucleoside kinase TK-2.^{22,23}

As a starting point we were interested to investigate if α -thymidine derivatives could act against *Pf*TMPK, as these have been shown to inhibit *Mt*TMPK, but not *h*TMPK.

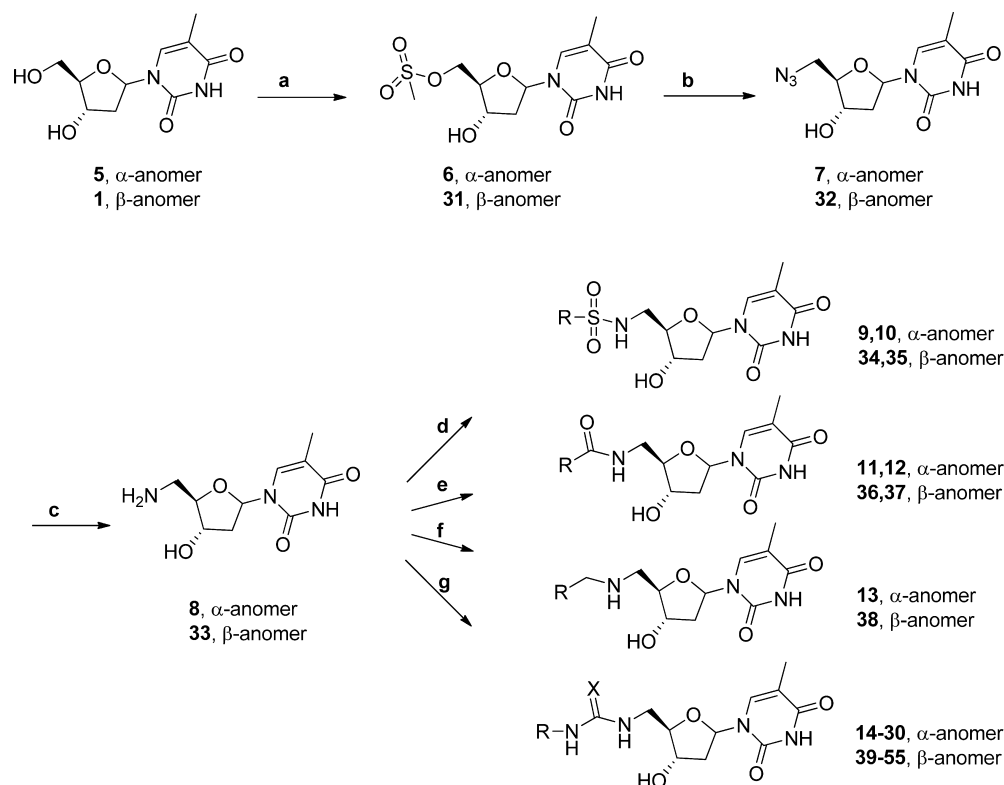
RESULTS AND DISCUSSION

Synthesis of α -Thymidine. The synthesis of α -thymidine was based on literature methods.^{24,25} The key step was epimerization of the base. Several methods are reported for epimerization of β -thymidine including the use of TMSOTf reported by Sato²⁵ and use of acetic anhydride/sulphuric acid as reported by Ward.²⁴ Initially the method by Sato was followed. First, β -thymidine was selectively protected with diphenylacetyl chloride on the primary position in 50% yield (Scheme 1). The 3'-position was then protected with *p*-toluoyl chloride to give compound 3, as this substituent was reported to be selective for generation of the α -analogue on epimerization of the anomeric center.²⁵ However, epimerization using TMSOTf was unsuccessful. It was decided to retain the 5' and 3' protecting groups suggested by Sato, since the compounds crystallized well, but epimerize the base using the acetic anhydride/sulphuric acid method of Ward.²⁴ The ratio of α/β analogues after epimerization was around 3/1. The 3',5'-*O*-diacylated α -thymidine derivative was readily separated by crystallization, giving the required product 4 in around 70% yield. Identification of the α/β -thymidine derivatives (3 and 4) was carried out by ¹H NMR (Supporting Information, Figure S4), in particular, by looking at the chemical shifts of H1' and H2', which were affected by the stereochemistry of C1'. The two acyl groups were

Scheme 1. Synthesis of α -Thymidine^a



^a(a) Diphenylacetyl chloride, pyridine, 0 °C, 50%; (b) *p*-toluoyl chloride, pyridine, RT, 56%; (c) acetic anhydride, H₂SO₄, CH₃CN, 51%; (d), NaOMe, MeOH, 90%.

Scheme 2. Synthesis of 5'-Substituted α - and β -Thymidine Derivatives^a

^a(a) Methanesulfonyl chloride, pyridine, -38°C , [α : 43%; β : 50%]; (b) sodium azide, DMF, 60°C , [α : 43%; β : 64%]; (c) 10% Pd/C, MeOH, [α : 95%; β : 95%]; (d) sulfonyl chloride, DMF, RT; (e) carbonyl chloride, DMF, RT; (f) aryl chloride, DMF, RT; (g) (thio)isocyanate, DMF, RT.

removed from compound 4 by sodium methoxide to give α -thymidine (5).

Preparation of 5' Substituted α - and β -Thymidine. Synthesis of 5'-amino substituted α -thymidine was carried out as shown in Scheme 2, by sulfonation, displacement with sodium azide and then hydrogenation to give the amine 8. Amine 8 was then coupled with thioisocyanate to give thioureas. The ureas, sulphonamides, amides and amines were also prepared to explore the SAR.

For comparison, some 5'-substituted β -thymidine analogues were prepared (Scheme 2) using similar conditions as for the preparation of 5'-substituted α -thymidine. The β -thymidine amine 33 was coupled to give the urea, sulfonamide, amide and amine.

PfTMPK Inhibition Assay. Compounds were screened against recombinant PfTMPK using a coupled assay with pyruvate kinase and lactate dehydrogenase (Figure S1).¹² Activity was measured spectrophotometrically by following the change in absorption at 340 nm due to the oxidation of NADH. The assay was carried out using TMP as substrate ($K_m = 11 \mu\text{M}$). Control spectrophotometric assays were performed to verify that the compounds were inhibitors of PfTMPK and not of the coupling enzymes, by using ADP as substrate and the two coupling enzymes pyruvate kinase and lactate dehydrogenase, but no PfTMPK or TMP.¹²

The 5'-urea α -thymidine derivatives (14–30) showed moderate inhibition of PfTMPK, with most compounds having K_i values between 80 and 200 μM (Table 1). The most active were the 3-trifluoromethyl-4-chlorophenyl derivative 28 ($K_i = 31 \mu\text{M}$) and the 4-nitrophenyl derivative 30 ($K_i = 11 \mu\text{M}$). There were no significant differences between urea and thio-urea

derivatives. 5'-(Thio)urea α -thymidine derivatives have been previously reported to be inhibitors of MtTMPK but are less active against hTMPK,¹⁶ human thymidine kinase²³ and human mitochondrial thymidine kinase.²³ They have also been shown to inhibit growth of *M. tuberculosis* and are selective compared to human cells.¹⁶

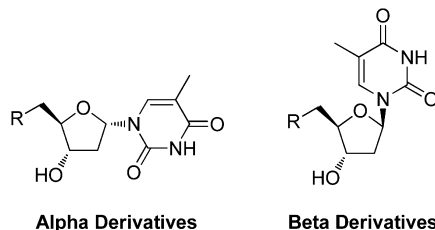
The 5'-urea β -thymidine derivatives (39–55) (Table 1) exhibited moderate inhibitory activity against PfTMPK in a similar fashion to the α -derivatives. Most of them inhibited in a similar range (50–100 μM), with again the 3-trifluoromethyl-4-chlorophenyl derivatives 53 ($K_i = 25 \mu\text{M}$) and 54 ($K_i = 27 \mu\text{M}$) and the 4-nitrophenyl derivative 55 ($K_i = 11 \mu\text{M}$) showing slightly higher potency.

There was no or very low inhibitory activity with the other 5' substituents in either the α - or the β - series: this includes the sulphonamides (9, 10, 34, 35), the amides (11, 12, 36, 37), and the amines (13, 38). Interestingly, some 5'-substituted β -thymidine derivatives, including 5'-sulfonamides, 5'-(thio) ureas and 5'-amides, have been reported as moderate inhibitors of *Bacillus anthracis* TMPK but do not inhibit bacterial growth.²⁶

Since the 5'-urea α -thymidine derivatives and the 5'-urea β -thymidine derivatives showed moderate inhibitory activity against PfTMPK, they were further investigated by cocrystallizing them with PfTMPK to assist in possible optimization.

CRYSTAL STRUCTURE OF LIGAND COMPLEXES

Co-crystallization with PfTMPK was successful with three of our active PfTMPK inhibitors (28, 30 and 53) (Figure 3). Crystallization conditions of the three inhibitors are listed in Supplementary Table S1, Supporting Information. Statistics for the X-ray data and refinement are summarized in Supplementary

Table 1. Evaluation of 5'- α and β -Thymidine Derivatives against *Pf*TMPK, *P. falciparum* and MRC5 Cells^c

R	alpha			beta			
	Cmpd	<i>Pf</i> TMPK K_i (μ M)	<i>P. fal.</i> EC ₅₀ (μ M)	MRC5 CC ₅₀ (μ M)	Cmpd	<i>Pf</i> TMPK K_i (μ M)	<i>P. fal.</i> EC ₅₀ (μ M)
	9	>1000	>100		34	>1000	>100
	10	>1000	>100		35	>1000	>100
	11	541	>100		36	>1000	>100
	12	541	>100		37	302	>100
	13	>1000	>100		38	742	>100
	14	>1000	>100		39	201	>100
	15	198	64		40	64	>200
	16	>1000	>100		41	222	>100
	17	166	24		42	60	>100
	18	205	36		43	52	>100
	19	106	4.8	>50	44	28	>100
	20 ^a	114	2.6	>50	45	14	>200

R	alpha				beta		
	Cmpd	<i>Pf</i> TMPK K_i (μ M)	<i>P. fal.</i> EC ₅₀ (μ M)	MRC5 CC ₅₀ (μ M)	Cmpd	<i>Pf</i> TMPK K_i (μ M)	<i>P. fal.</i> EC ₅₀ (μ M)
	21	90	13	>50	46	37	>200
	22	>1000	1.5	>50	47	15	30
	23	65	20		48	32	172
	24	138	44		49	107	>100
	25	159	56		50	69	>100
	26	132	2.5	>50	51	50	69
	27	167	2.9	>50	52	47	64
	28 ^b	31	2.3	>50	53	25	42
	29	88	9	>50	54	27	114
	30	11	31		55	11	69

^a*h*TMPK K_i > 1000 μ M. ^b*h*TMPK K_i = 362 μ M. ^cReference compound: chloroquine EC₅₀ = 0.007 μ M.

Table S2, and data for the three complexes have been deposited in the PDB with codes, 2YOG (28), 2YOH (30) and 2YOF (53). Compounds 28 and 53 are pairs of α - and β -thymidine derivatives (3-trifluoromethyl-4-chlorophenyl) that allow for comparison. The structures of all three complexes show that the enzyme is well ordered, with an essentially identical fold to that reported for the complexes with nucleotides and nucleotide derivatives.¹² There was clear electron density for most of the ligands in all three complexes. A summary of some key features of the structural data on the complexes is given in Supporting Information Table S3. One substantial change in the protein is that the extended loop, residues 141–152, is disordered with no visible density in all three ligand structures, with the exception of Chain C in the complex with 53 where it packs against one of the two alternate conformations of the ligand.

The thymidine ring of the synthetic ligands mimics that of the natural TMP substrate (Figure 3B). The deoxyribose ring in contrast shows its typical variation in pucker and orientation relative to the thymidine allowing the end of the synthetic ligands

to take up optimum interactions with the enzyme. The thymidine and deoxyribose are buried deep within the pocket, which also accommodates two water molecules, W27 and W303, that form bridges between ligand and protein (Figure 3C). Two additional waters form bridging H-bonds between ligand and protein. The H-bonds between the ligand and the protein and surrounding ordered water molecules (red spheres) are shown. The aromatic ring with its fluoro substituents extends out of the pocket and packs against the protein surface.

Chain C of the 53 complex displays the least well-ordered ligand (Figure 3D), and we have modeled this in two conformations. There is reasonable density for the thymidine and deoxyribose, though the deoxyribose is already showing disorder over two alternative puckers. There is essentially no density for the aromatic ring, and its positions are solely based on density for the fluorine groups. This ligand is well ordered in Chain B. A summary of the experimental electron density for the ligands is given in Supporting Table S3.

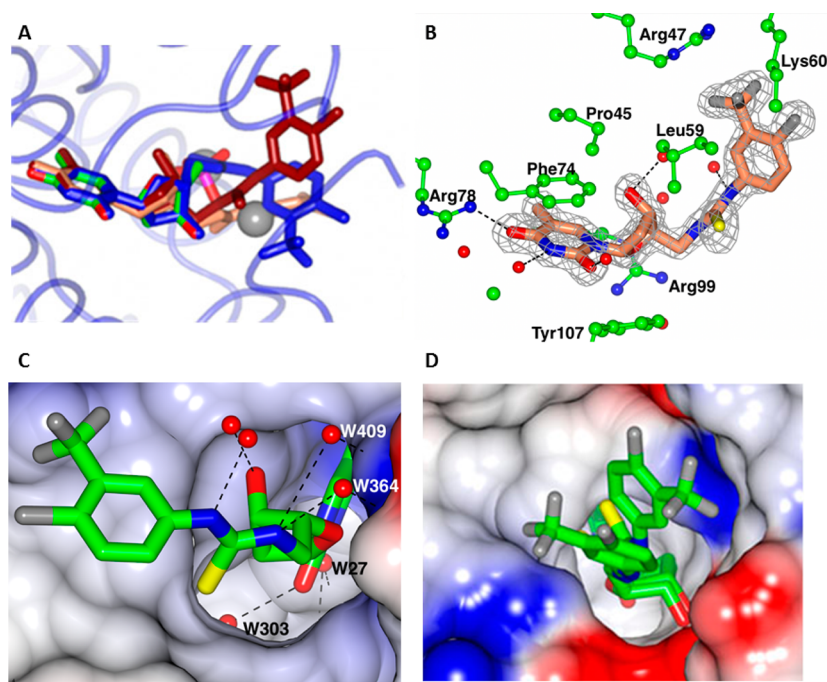


Figure 3. (A) Superposition of three ligand complexes **53** (blue), **30** (coral) and **28** (tan) on the TMP–ADP complex (colored by atom type) determined previously (pdb: 1wwf). The chain was selected in which the ligand was best ordered. The two sodium atoms in the TMP–ADP complex are shown as spheres. The peptide backbone is shown for the **53** complex (pale blue). (B) The binding pocket for **28**, showing interactions with the protein. (C) The binding pocket for **28**. The surface of the protein is colored by electrostatic potential. The four waters that form H-bond bridges to the protein are numbered. (D) The two conformations for **53** in Chain C, the least well-ordered ligand site.

■ ANTI-MALARIAL ACTIVITY

All of the compounds were evaluated for growth inhibition activity against *P. falciparum* using a SYBR green assay as reported in the literature.²⁷ Most compounds from both series with K_i values for *Pf*TMPK below 50 μM inhibited parasite growth. A number of compounds gave low micromolar inhibition of growth, the actives belonging almost exclusively to the α -thymidine analogue series (Table 1). The most potent compounds were **20**, **26**, **27** and **28**, which had EC_{50} values of approximately 2 μM . They were all 4'-substituted phenyl compounds, which was the most active subset of this series of inhibitors in producing inhibition of parasite growth. All of the tested inhibitors showed good selectivity between malaria parasites and human MRC5 cells (Table 1). The β -thymidine derivatives had very low or no activity against the parasite (Table 1), and we therefore focused our attention on the α -derivatives.

There was only a low correlation between inhibition of *Pf*TMPK and inhibition of the growth of *P. falciparum*. Most compounds were more potent inhibitors of parasite growth than inhibitors of the enzyme. For example, compound **17** (Figure 4) has a K_i of 166 μM against *Pf*TMPK, but an EC_{50} of 23 μM against the parasite suggesting that the principal mode of action is not through inhibition of *Pf*TMPK. These compounds showed good selectivity between the malaria parasite and human MRC5

cells. For instance, compound **26** has an EC_{50} of 2.5 μM , but a CC_{50} > 50 μM . Additional experiments were performed to probe the antimalarial activity of these derivatives.

Compound **17** is a representative of the 5'-phenyl urea α -thymidine derivatives (Figure 4). Compound **17** can be divided into three components: the α -anomeric thymidine base, the deoxyribose ring and the substituted phenyl urea. The initial SAR showed that a substitution of the 5'-phenyl urea gave a significant increase in the activity against the parasite. For example, a *para*-methoxy group on the phenyl increased potency against the parasite 10-fold (compound **26**, Figure 4). Work was carried out to optimize the phenyl group in the 5'-position, and the corresponding β -thymidine analogues were also evaluated for comparison purposes. Compounds were prepared as shown in Scheme 2 and tested against the parasite (Table 2).

Since the 4-benzyloxy derivatives appeared to give very potent activity (compound **60**), this series was expanded by preparing 4-benzyloxy phenyl isocyanates using the conditions reported by Knaggs et al.²⁸ with triphosgene. The isocyanates were rapidly passed through a column for purification and then reacted with α - or β -thymidine amine to give the final compounds **84–90** (Scheme 3 and Table 3).

■ ANTI-MALARIAL STRUCTURE–ACTIVITY RELATIONSHIP

Data for the antiparasite assays are given in Tables 1–3. Compounds **57**, **60**, **63**, **66**, **84**, **85**, **86**, **87** and **89** showed sub-micromolar activity. Most notably, compound **84** had an EC_{50} of 28 nM, which represents an increase in inhibition of approximately 1000-fold compared to the starting compound **17** (EC_{50} = 23 μM). We can summarize the key trends as follows:

- α -Anomers are much more potent than β -anomers. Five of the most active pairs of derivatives are compared in Table

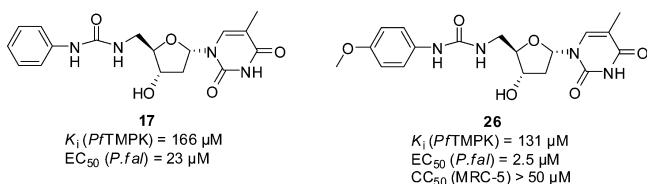
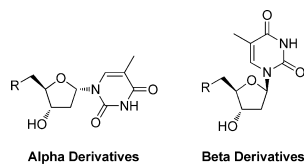
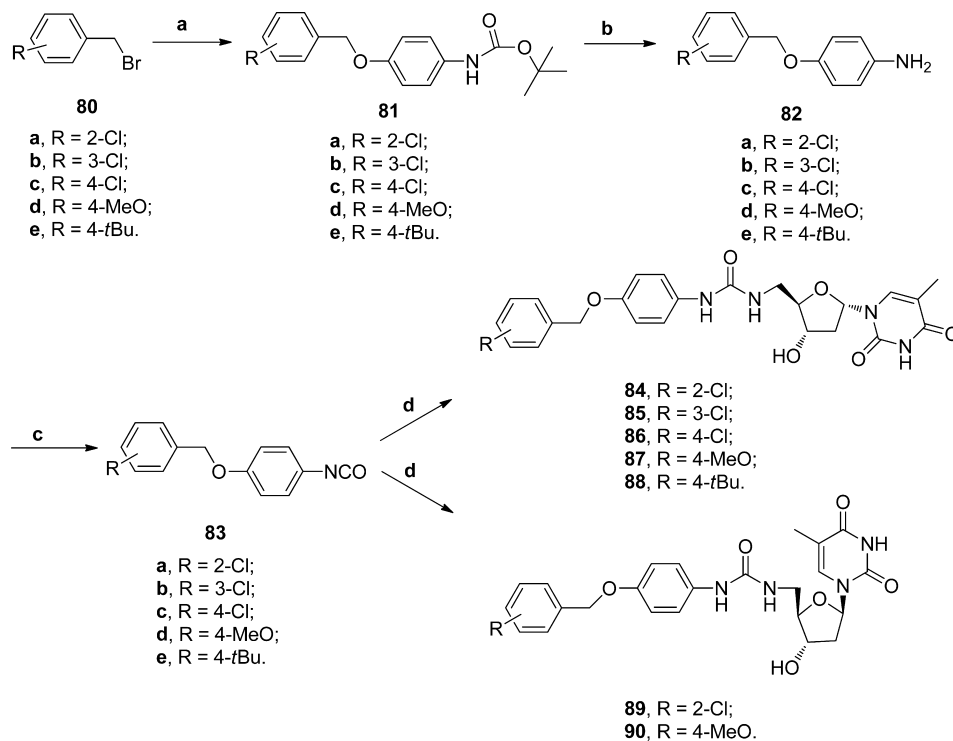


Figure 4. Two 5'-phenyl urea α -thymidine derivatives.

Table 2. Evaluation of 5'-Phenyl (thio)urea α - and β -Thymidine Derivatives against *P. falciparum* and MRC5 Cells^a

R	alpha			beta		
	Cmpd	<i>P. fal.</i> EC ₅₀ (μM)	MRC5 CC ₅₀ (μM)	Cmpd	<i>P. fal.</i> EC ₅₀ (μM)	MRC5 CC ₅₀ (μM)
	56	96		70	253	
	57	0.29	>50	71	3.3	>50
				72	>100	
	58	1.8	>50	73	148	
	59	7.2	>50	74	48	
	60	0.24	>50	75	4.0	>50
	61	9.5	>50			
	62	1.3	>50	76	33	
	63	0.34	>50	77	4.8	>50
	64	29		78	26	
	65	4.6	>50	79	>200	
	66	0.24	44			
	67	35				
	68	1.5	>50			
	69	1.4	>50			

^aReference compound: chloroquine EC₅₀ = 0.007 μM.

Scheme 3. Preparation of 4-Benzoyloxy-phenyl Urea α - and β -Thymidine Derivatives^a

^a(a) NaH, *N*-Boc protected 4-aminophenol, DMF; (b) TFA, DCM; (c) Et₃N, triphosgene, EtOAc; (d) DMF, RT.

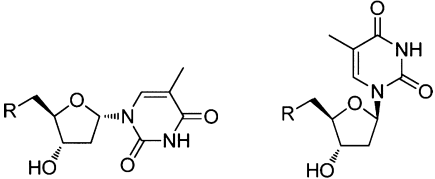
- 4 which shows that α - had at least 10 times more activity than β -derivatives, presumably due to the α -anomer constraining the base in a much more favorable orientation. Interestingly compound **84**, which has an *ortho* substitution in the benzyl group, gave the best antimalarial activity with an EC₅₀ of 28 nM, and the related β -derivative **89** also gave the best inhibition activity of the β -derivatives, albeit with a 20-fold drop in activity.
- A *para*-substitution gives enhanced activity. The phenyl urea is optimally substituted in the *para*-position rather than the *ortho* or *meta* positions for both the α - and β -anomers. For example, **56** (2-phenyl, EC₅₀ = 96 μ M) is much less active than **57** (4-phenyl, EC₅₀ = 0.29 μ M) (Table 2). Most of the active compounds in this study are *para*-substituted derivatives, for both α - and β - derivatives of the same pairs.
 - Ureas are more potent than thiourea. In the α -derivatives, thiourea and urea derivatives were investigated for their contribution to the inhibitory activity. Four pairs of inhibitors were chosen (Table 5). Urea derivatives are more active than thioureas except for the pair containing the 3-trifluoromethyl-4-chloro substituent, which is slightly more active for the thio-urea (**28** and **29**).
 - The *para*-substituent of the phenyl urea should be preferably a hydrophobic group. For the α -derivatives, improved activity is observed when the *para*-substituents are hydrophobic groups. This is evident in comparisons of **66** (4-phenyloxy, EC₅₀ = 0.24 μ M) versus **62** (4-tetrahydropyran-oxy, EC₅₀ = 1.3 μ M); **63** (4-piperidine, EC₅₀ = 0.34 μ M) versus **67** (4-morpholino, EC₅₀ = 35 μ M) or **64** (4-(4-methyl-piperazine), EC₅₀ = 29 μ M).

DMPK Studies. To assess the further developability of the compounds, we carried out *in vitro* DMPK studies on five key compounds (Table 6).²⁹ All showed reasonable microsomal stability ($C_{Li} < 5$ mL/min/g) except for compound **84**, which exhibited rapid turnover. Plasma protein binding was less than 95% in most cases, with compound **63** showing a particularly large unbound fraction. Unfortunately the most potent compound **84** was the most unstable when incubated with hepatic microsomes. However, related compounds showed good *in vitro* DMPK properties (**57**, **60**, **63**, **66**), suggesting that there is nothing inherently problematic associated with the scaffold in terms of microsomal stability and protein binding.

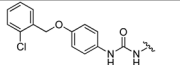
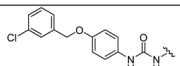
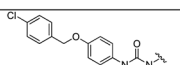
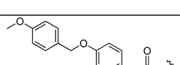
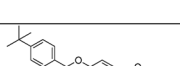
CONCLUSION

In this study, we optimized a series of 5'-*para* substituted phenyl urea α -thymidine derivatives to produce compounds with improved antimalarial activity. Initially different series of compounds were designed as inhibitors of PfTMPK. While inhibition of the enzyme and parasite growth was moderate, the elucidation here of the crystal structure of several enzyme-inhibitor complexes provides the basis for a future structure-based drug discovery program.

Interestingly, while poorly active against the enzyme, the α -thymidine derivatives showed promising antimalarial activity and optimization was performed in order to increase potency. Several features were found to contribute to antiplasmodial action. The α -thymidine derivatives with *para* substituted phenyl groups (preferably hydrophobic substituents) and ureas (better than thiourea) exhibited increased growth inhibition. Testing of the inhibitors gave activities in the nanomolar range and compounds showed a good selectivity between *P. falciparum* and human

Table 3. Biological Evaluation of 4-Benzyloxy-phenyl Urea α - and β -Thymidine Derivatives^a


The image shows the chemical structures of Alpha and Beta Derivatives. The Alpha Derivative has a 4-benzyloxyphenyl group attached to the nitrogen of a thymidine derivative. The Beta Derivative has a 2-benzyloxyphenyl group attached to the nitrogen of a thymidine derivative.

R	alpha			beta		
	Cmpd	EC ₅₀ (μM)	CC ₅₀ (μM)	Cmpd	EC ₅₀ (μM)	CC ₅₀ (μM)
	84	0.028	29	89	0.41	>50
	85	0.24	>50			
	86	0.88	>50			
	87	0.21	>50	90	3.8	>50
	88	1.8	>50			

^aReference compound: chloroquine EC₅₀ = 0.007 μM.

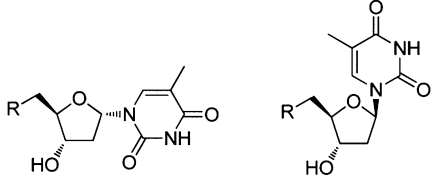
MRC5 cells. The most potent inhibitor from this series is compound **84** with an EC₅₀ of 28 nM and CC₅₀ of 29 μM, an increase in potency of nearly 1000 times compared to the starting compound **17** (EC₅₀ = 23 μM). Furthermore some of the most active compounds have reasonable microsomal stability and free fractions. The resulting SAR information obtained for this series of inhibitors is shown in Figure 5.

EXPERIMENTAL SECTION

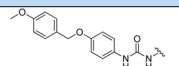
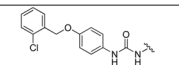
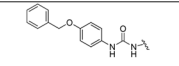
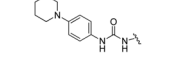
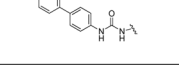
Chemistry. General. Chemicals and solvents were purchased from the Sigma-Aldrich Chemical Co., Fluka, VWR, Acros, Fisher Chemicals and Alfa Aesar. ¹H NMR and ¹³C NMR were recorded on a Bruker Avance DPX 500 spectrometer (¹H at 500.1 MHz and ¹³C at 125.8 MHz). Chemical shifts (δ) are expressed in ppm. Signal splitting patterns are described as singlet (s), double (d), double doublet (dd), triplet (t), quarter (qt), multiplet (m). Low resolution electrospray (ES) mass spectra were recorded either on an Agilent Technologies 1200 series HPLC connected to an Agilent Technologies 6130 quadrupole spectrometer and to an Agilent diode array detector or on a Bruker MicroTof mass spectrometer, run in a positive ion mode, using either methanol, methanol/water (95:5), or water/acetonitrile (1:1) + 0.2% formic acid as the mobile phase. High resolution electrospray measurements were performed on a Bruker Daltonics MicroTOF mass spectrometer. Column chromatography was carried out using silica gel 60 from Fluka. Thin layer chromatography (TLC) was carried out on Merck silica gel 60 F254 plates using UV light or PMA for visualization.

Purity was determined using both LCMS and NMR spectroscopy. Compounds had a purity of >95%.

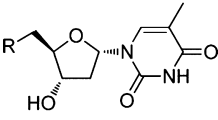
General Procedure for Compounds 84–90. For the synthesis of compounds **84–90**, amine **8** or **33** (1 equiv) was dissolved in DMF at 0 °C. The coupling reagents (1.1 equiv) were added, and the reaction

Table 4. Comparison in Parallel of the α - and β -Urea Derivatives^a


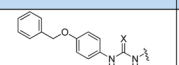
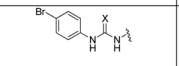
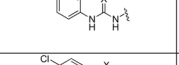
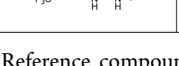
The image shows the chemical structures of Alpha and Beta Derivatives. The Alpha Derivative has a 4-benzyloxyphenyl group attached to the nitrogen of a thymidine derivative. The Beta Derivative has a 2-benzyloxyphenyl group attached to the nitrogen of a thymidine derivative.

R	α derivatives		β derivatives	
	Cmpd	EC ₅₀ (μM)	Cmpd	EC ₅₀ (μM)
	87	0.21	90	3.8
	84	0.028	89	0.41
	60	0.24	75	4.0
	63	0.34	77	4.8
	57	0.29	71	3.3

^aReference compound: chloroquine EC₅₀ = 0.007 μM.

Table 5. Comparison in Parallel of the Thiourea and Urea Derivatives^a


The image shows the chemical structure of Alpha Derivatives, which are thymidine derivatives with a 4-benzyloxyphenyl group attached to the nitrogen.

R	Urea		Thiourea	
	X=O			
	Cmpd	EC ₅₀ (μM)	Cmpd	EC ₅₀ (μM)
	60	0.24	61	9.5
	22	1.4	23	20
	20	2.6	21	13
	29	9.0	28	2.3

^aReference compound: chloroquine EC₅₀ = 0.007 μM.

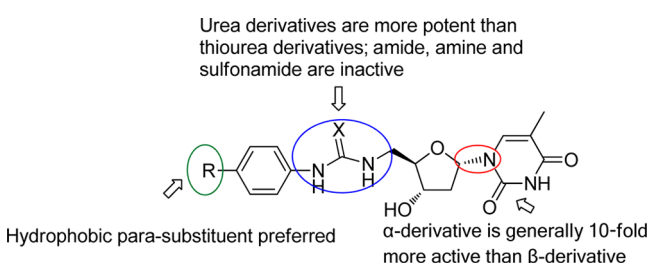
mixture was allowed to stir at room temperature for 3 h. After the completion of the reaction, the reaction mixture was evaporated to dry (ethanol and toluene were used to coevaporate), and the residue was purified by column chromatography to yield the compounds **84–90** as a solid with the yields ranging from 67% to 83%.

N-(5'-Deoxy- α -thymidin-5'-yl)-*N'*-(4-(2-chlorobenzyloxy)phenyl)-urea **84**. 4-(2-Chlorobenzyloxy)phenyl isocyanate reacted with amine **8** to yield compound **84** as a solid; ¹H NMR (500 MHz, DMSO): δ 11.28

Table 6. The Stability and Plasma Protein Binding Data of Five Selected Compounds^a

compd	microsomal stability (mL/min/g)	plasma protein binding (% bound)	EC ₅₀ (μM)	CC ₅₀ (μM)
84	6	94.1	0.028	29
57	1.9	92.0	0.29	>50
60	3	81.2	0.24	>50
63	0.5	52.3	0.34	>50
66	1.8	90.5	0.24	43.6

^aReference compound: chloroquine EC₅₀ = 0.007 μM.

**Figure 5.** Summary of the SAR data for the thymidine-derived inhibitors.

(s, 1H, NH), 8.40 (s, 1H, NH), 7.78 (d, *J* = 1.20 Hz, 1H, H₆), 7.57–7.60, 7.50–7.53, 7.37–7.40, 7.29–7.31, 6.90–6.94 (m, 8H, *H*-Ph), 6.16–6.19 (m, 2H, H₁' and NH), 5.45 (d, *J* = 3.20 Hz, 1H, OH), 5.09 (s, 2H, CH₂), 4.18–4.21 (m, 2H, H₃' and H₄'), 3.21–3.28, 3.09–3.13 (m, 2H, H₅'), 2.53–2.60, 1.91–1.97 (m, 2H, H₂'), 1.78 (s, 3H, CH₃); ¹³C NMR (125 MHz, DMSO): δ 163.8 (C₄), 155.4 (CO), 150.5 (C₂), 136.9 (C₆), 152.8, 134.6, 132.5, 130.0, 129.7, 129.3, 127.3, 119.3, 114.9 (C-Ph), 108.8 (C₅), 86.9 (C₁'), 84.7 (C₄'), 70.8 (C₃'), 67.0 (CH₂), 55.0 (C₅'), 41.0 (C₅'), 39.0 (C₂'), 12.3 (CH₃); LCMS (ES⁺): *m/z* (%) 501 (100) [M + H]⁺; HRMS (ES⁺): calcd for C₂₄H₂₆Cl₁N₄O₆ [M + H]⁺ 501.1535 *m/z*, found 501.1533 *m/z* (0.54 ppm).

N-(5'-Deoxy-α-thymidin-5'-yl)-*N'*-(4-(3-chlorobenzoyloxy)phenyl)-urea **85**. 4-(3-Chlorobenzoyloxy)phenyl isocyanate reacted with amine **8** to yield compound **85** as a solid; ¹H NMR (500 MHz, DMSO): δ 11.28 (s, 1H, NH), 8.38 (s, 1H, NH), 7.78 (d, *J* = 1.20 Hz, 1H, H₆), 7.37–7.42, 7.28–7.30, 6.89–6.91 (m, 8H, *H*-Ph), 6.16–6.19 (m, 2H, H₁' and NH), 5.45 (d, *J* = 3.25 Hz, 1H, OH), 5.06 (s, 2H, CH₂), 4.16–4.20 (m, 2H, H₃' and H₄'), 3.12–3.22 (m, 1H, H₅'), 2.57 (m, 1H, H₂'), 1.92–1.95 (m, 1H, H₂'), 1.78 (s, 3H, CH₃); ¹³C NMR (125 MHz, DMSO): δ 163.8 (C₄), 155.3 (CO), 150.5 (C₂), 136.9 (C₆), 152.7, 136.9, 133.9, 133.0, 130.0, 127.6, 127.2, 126.1, 119.3, 115.0 (C-Ph), 108.8 (C₅), 86.9 (C₁'), 84.7 (C₄'), 70.8 (C₃'), 68.4 (CH₂), 41.0 (C₅'), 39.0 (C₂'), 12.3 (CH₃); LCMS (ES⁺): *m/z* (%) 501 (100) [M + H]⁺; HRMS (ES⁺): calcd for C₂₄H₂₆Cl₁N₄O₆ [M + H]⁺ 501.1535 *m/z*, found 501.1533 *m/z* (0.54 ppm).

N-(5'-Deoxy-α-thymidin-5'-yl)-*N'*-(4-(4-chlorobenzoyloxy)phenyl)-urea **86**. 4-(4-Chlorobenzoyloxy)phenyl isocyanate reacted with amine **8** to yield compound **86** as a solid; ¹H NMR (500 MHz, DMSO): δ 11.28 (s, 1H, NH), 8.37 (s, 1H, NH), 7.77 (d, *J* = 1.20 Hz, 1H, H₆), 7.44–7.45, 7.27–7.29, 6.88–6.90 (m, 8H, *H*-Ph), 6.16–6.19 (m, 2H, H₁' and NH), 5.45 (d, *J* = 3.30 Hz, 1H, OH), 5.03 (s, 2H, CH₂), 4.16–4.18 (m, 2H, H₃' and H₄'), 3.75 (s, 3H, CH₃), 3.22–3.32 (m, 1H, H₅'), 3.15–3.27 (m, 1H, H₅'), 2.57 (m, 1H, H₂'), 1.92–1.94 (m, 1H, H₂'), 1.78 (s, 3H, CH₃); ¹³C NMR (125 MHz, DMSO): δ 163.8 (C₄), 155.3 (CO), 150.5 (C₂), 136.9 (C₆), 152.7, 136.4, 133.8, 132.2, 129.4, 128.4, 119.2, 115.0 (C-Ph), 108.8 (C₅), 86.9 (C₁'), 84.6 (C₄'), 70.8 (C₃'), 68.9 (CH₂), 41.0 (C₅'), 39.0 (C₂'), 12.3 (CH₃); LCMS (ES⁺): *m/z* (%) 501 (100) [M + H]⁺; HRMS (ES⁺): calcd for C₂₄H₂₆Cl₁N₄O₆ [M + H]⁺ 501.1535 *m/z*, found 501.1519 *m/z* (3.33 ppm).

N-(5'-Deoxy-α-thymidin-5'-yl)-*N'*-(4-(4-methoxybenzoyloxy)phenyl)-urea **87**. 4-(4-Methoxybenzoyloxy)phenyl isocyanate reacted with amine **8** to yield compound **87** as a solid; ¹H NMR (500 MHz, DMSO): δ 11.28 (s, 1H, NH), 8.37 (s, 1H, NH), 7.78 (d, *J* = 1.20 Hz, 1H, H₆), 7.27–7.31, 6.99–7.00, 6.87–6.90 (m, 8H, *H*-Ph), 6.16–6.19

(m, 2H, H₁' and NH), 5.45 (d, *J* = 3.25 Hz, 1H, OH), 5.01 (s, 2H, CH₂), 4.16–4.18 (m, 2H, H₃' and H₄'), 3.75 (s, 3H, CH₃), 3.22–3.32 (m, 1H, H₅'), 3.15–3.27 (m, 1H, H₅'), 2.57 (m, 1H, H₂'), 1.94 (m, 1H, H₂'), 1.78 (s, 3H, CH₃); ¹³C NMR (125 MHz, DMSO): δ 163.8 (C₄), 155.4 (CO), 150.5 (C₂), 136.9 (C₆), 159.3, 152.9, 138.9, 129.5, 119.2, 114.9, 113.1, 113.0 (C-Ph), 108.8 (C₅), 87.0 (C₁'), 84.6 (C₄'), 70.8 (C₃'), 69.2 (CH₂), 55.0 (CH₃), 41.0 (C₅'), 39.0 (C₂'), 12.3 (CH₃); LCMS (ES⁺): *m/z* (%) 497 (100) [M + H]⁺; HRMS (ES⁺): calcd for C₂₅H₂₉N₄O₇ [M + H]⁺ 497.2031 *m/z*, found 497.2031 *m/z* (−0.12 ppm).

N-(5'-Deoxy-α-thymidin-5'-yl)-*N'*-(4-(4-*tert*-butylbenzoyloxy)phenyl)-urea **88**. 4-(4-*tert*-Butylbenzoyloxy)phenyl isocyanate reacted with amine **8** to yield compound **88** as a solid; ¹H NMR (500 MHz, DMSO): δ 11.23 (s, 1H, NH), 8.37 (s, 1H, NH), 7.77 (d, *J* = 1.20 Hz, 1H, H₆), 7.67–7.76, 7.26–7.41, 6.77–6.89 (m, 8H, *H*-Ph), 6.16–6.19 (m, 2H, H₁' and NH), 5.45 (d, *J* = 3.20 Hz, 1H, OH), 4.99 (s, 2H, CH₂), 4.18–4.21 (m, 2H, H₃' and H₄'), 3.21–3.28, 3.09–3.13 (m, 2H, H₅'), 2.52–2.59, 1.91–1.98 (m, 2H, H₂'), 1.78 (s, 3H, CH₃), 1.28 (s, 9H, CH₃); LCMS (ES⁺): *m/z* (%) 523 (100) [M + H]⁺; HRMS (ES⁺): calcd for C₂₈H₃₅N₄O₆ [M + H]⁺ 523.2551 *m/z*, found 523.2554 *m/z* (−0.57 ppm).

■ ASSOCIATED CONTENT

Supporting Information

Methodology for enzyme assays, parasite assays, crystallography, DMPK and chemistry. Stereo views of several protein–ligand complexes. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

CC₅₀, concentration required to reduce growth of MRC5 cells by 50%; C_{Li}, intrinsic clearance; TDP, thymidine diphosphate; TMP, thymidine monophosphate; hTMPK, human thymidylate kinase; Pf, *Plasmodium falciparum*; Mt, *Mycobacterium tuberculosis*; TMPK, thymidylate kinase; TMSOTf, trimethylsilyl trifluoromethanesulfonate

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