Inhibition of Thiamine Diphosphate (ThDP)-dependent Enzymes by Triazole-based Thiamine Analogues

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Abstract:

Thiamine (vitamin B$_1$) is metabolised into the coenzyme thiamine diphosphate (ThDP). Interrupting thiamine-utilisation leads to disease states and thiamine/ThDP analogues are commonly used as antagonists of thiamine-utilisation pathways to investigate the underlying pathophysiology. Oxythiamine, a thiamine analogue, uses the thiamine-utilising pathways for conversion into oxythiamine diphosphate (OxThDP), which binds to ThDP-dependent enzymes but lacks the catalytic activity of ThDP. Oxythiamine has been used to validate thiamine utilisation as an antimalarial drug target. However, high oxythiamine doses are often needed *in vivo* because 1) of its rapid clearance and 2) its potency decreases dramatically with thiamine levels. We report herein thiamine analogues possessing a triazole ring and a hydroxamate tail in place of the thiazolium ring and diphosphate group, respectively, of ThDP. We characterise their broad-spectrum inhibition of ThDP-dependent enzymes in biochemical and computational studies, and establish their ThDP-competitive action using *Plasmodium falciparum* as a model. We demonstrate how the cellular thiamine-utilisation pathway can be probed in mechanistic studies using our compounds and oxythiamine in parallel.

Introduction:

Thiamine **1** is essential for energy metabolism, and its deficiency leads to neurological disorders.[1-3] Thiamine being positively charged requires transport into the cytoplasm, where it is activated to thiamine diphosphate (ThDP) **2a** by thiamine pyrophosphokinase (TPK) (Figure 1A).[1-5] ThDP is a coenzyme of ThDP-dependent enzymes, a diverse class of proteins, including pyruvate dehydrogenase complex E1-subunit (PDHc E1), pyruvate decarboxylase (PDC), oxoglutarate dehydrogenase complex E1-subunit (OGDHc E1), pyruvate oxidase (PO) and transketolase (TK). Individual enzymes differ in substrate preferences and the biochemical reactions catalysed but they all require the coenzyme ThDP for activity. In mammalian tissues, ThDP is the major form of thiamine; the remaining forms are the non-coenzyme derivatives: free thiamine **1**, thiamine monophosphate, thiamine triphosphate **3** (ThTP) and adenosine thiamine triphosphate **4** (AThTP).[1-3] Since the discovery of **1**, research has focused on its coenzyme role in cellular metabolism,[4-5] but as more naturally occurring derivatives have been identified, increasing attention has also been directed to the non-coenzyme roles:[1-3] thiamine **1** is co-released with acetylcholine in acetylcholinergic neurons and seems to facilitate neurotransmission; ThTP **3** functions as a phosphate donor in a protein phosphorylation reaction; and AThTP **4** can inhibit poly-ADP-ribose polymerase 1, which is involved in DNA repair.[6] Even ThDP may have non-coenzyme roles: studies showed that elevated levels inhibit pyridoxal kinase and inhibit the binding of p53 to DNA. In bacteria, fungi and plants ThDP also down-regulates its own biosynthesis by binding to riboswitches in the mRNA coding for biosynthetic enzymes.[1-3] Collectively, the essentiality of thiamine is believed to be due to a combination of the coenzyme and non-coenzyme roles.

A common method to study the coenzyme role of thiamine is to use thiamine/ThDP analogues as ThDP-competitive inhibitors of ThDP-dependent enzymes.[3,7-21] Figure 1C summarises the three main types of thiamine/ThDP analogues that have been employed. The first type,[7,8] represented by triazole-ThDP **5**,[8] features a neutral central ring in place of the ThDP's positive thiazolium ring **2a**, which abolishes the catalytic activity. These ThDP analogues are potent ThDP-dependent enzyme inhibitors because they retain the ionic interaction between the diphosphate and the Mg^{2+} ion in the diphosphate pocket and the neutral central ring of **5** captures the strong stabilising interactions between the enzyme's hydrophobic region and the catalytically active high-energy ThDP-ylide **2b** (Figure 1B). These ThDP analogues are not selective towards individual ThDP-dependent enzymes, as they work by displacing the universally needed ThDP coenzyme, and they are not cell-permeable due to the polyanionic diphosphate.[20] The second type,[9-13] represented by furan **6**,[13] also features a neutral scaffold but bears a neutral tail replacing the diphosphate tail. This neutral tail compensates for the loss of the ionic interaction between the diphosphate and the Mg^{2+} ion by forming interactions with surrounding residues in the diphosphate pocket of PDHc E1. Since these interactions are specific to PDHc E1, **6** is a potent and PDHc-selective inhibitor. Also furan **6** can diffuse into cells so may be used to study the cellular roles of PDHc through selective inhibition. The third type,[14-21] represented by oxythiamine **7a**, is a thiamine analogue featuring a modified pyrimidine ring. **7a** is a prodrug: it enters cells, probably *via* thiamine transporters, and is activated by TPK to OxThDP **7b**.[1-3] OxThDP binds to ThDP-dependent enzymes, but its modified pyrimidine ring prevents the activation of the thiazolium ring to the catalytically active ylide (equivalent to **2b**); thus, OxThDP **7b** is an inhibitor of the ThDP-dependent enzyme family. ThDP analogues triazole-ThDP **5** and OxThDP **7b** are known ligands for ThDP riboswitches which recognise their diphosphate moiety.[22]

Interrupting the thiamine-utilisation pathway can result in diseases such as diabetes and neurodegeneration and is also found in many cancers.[1-4] To understand these diseases, compounds causing thiamine deficiency within the cells can be used. Oxythiamine **7a** is the most widely applied tool for inducing thiamine deficiency in cells and *in vivo*.[1-3,14-20] For example, oxythiamine suppresses Ehrlich ascites tumour cell proliferation,[14] has a cytostatic effect against *Malassezia pachydermatis* (a yeast)[15] and inhibits the *in vitro* proliferation of *Plasmodium falciparum*.[17] However, oxythiamine **7a** has weaknesses as a probe: 1) its positively charged thiazolium ring can result in poor pharmacokinetic properties and degradation by thiaminases, so high oral doses are needed in *in vivo* studies;[17-19] 2) the diphosphate moiety of **7b** can be hydrolysed by ThDP-phosphatases;[23] 3) its similarity to ThDP could allow it to participate in non-coenzyme roles of ThDP **2** or (after appropriate modification of the diphosphate) of **3** and **4** (Figure 1A) and this makes assignment of any observed effects to the coenzyme role of ThDP uncertain;[1,2] and 4) the required intracellular activation of **7a** to **7b** makes its action highly sensitive to the levels of thiamine. As an example of this last weakness, oxythiamine inhibits *in vitro* proliferation of the 3D7 strain of *Plasmodium falciparum* with an IC_{50} value of 11 µM in the absence of extracellular thiamine, but the IC_{50} value increased 470-fold (to 5.2 mM) with added thiamine (2.97 µM).[17] We attribute the significant reduction of the anti-plasmodial effect with increasing thiamine levels to the competition with thiamine/ThDP at all stages of thiamine-utilisation, cell entry, pyrophosphorylation and enzyme binding.

In this paper we prepare a series of triazole-based thiamine analogues (Figure 1D), resulting in the discovery of thiamine analogues with inhibitory activity against multiple ThDP-dependent enzymes. The anti-plasmodial activity of the analogues are assessed and compared to that of oxythiamine. The analogues are uncharged under physiological conditions so they can passively diffuse into cells. They lack the diphosphate group of ThDP, which means that they are unlikely to be recognised by other ThDP-binding proteins or riboswitches. Also, they are not dependent on thiamine transporters and pyrophosphorylation by TPK, which reduces their sensitivity to the extracellular level of thiamine. We envision that these compounds will be useful tools to study the coenzyme roles of ThDP, complementing oxythiamine in inducing the effects of thiamine deficiency.
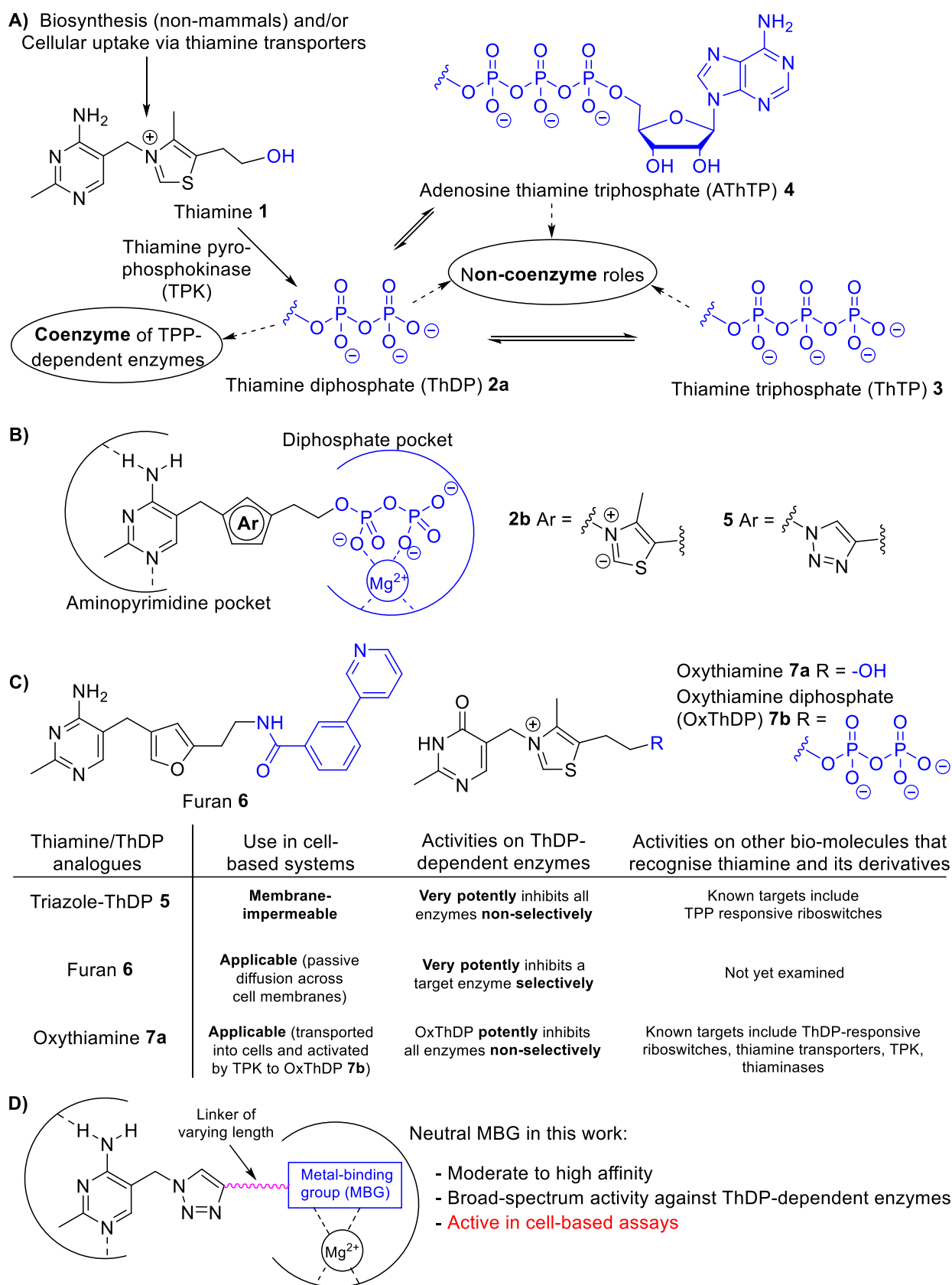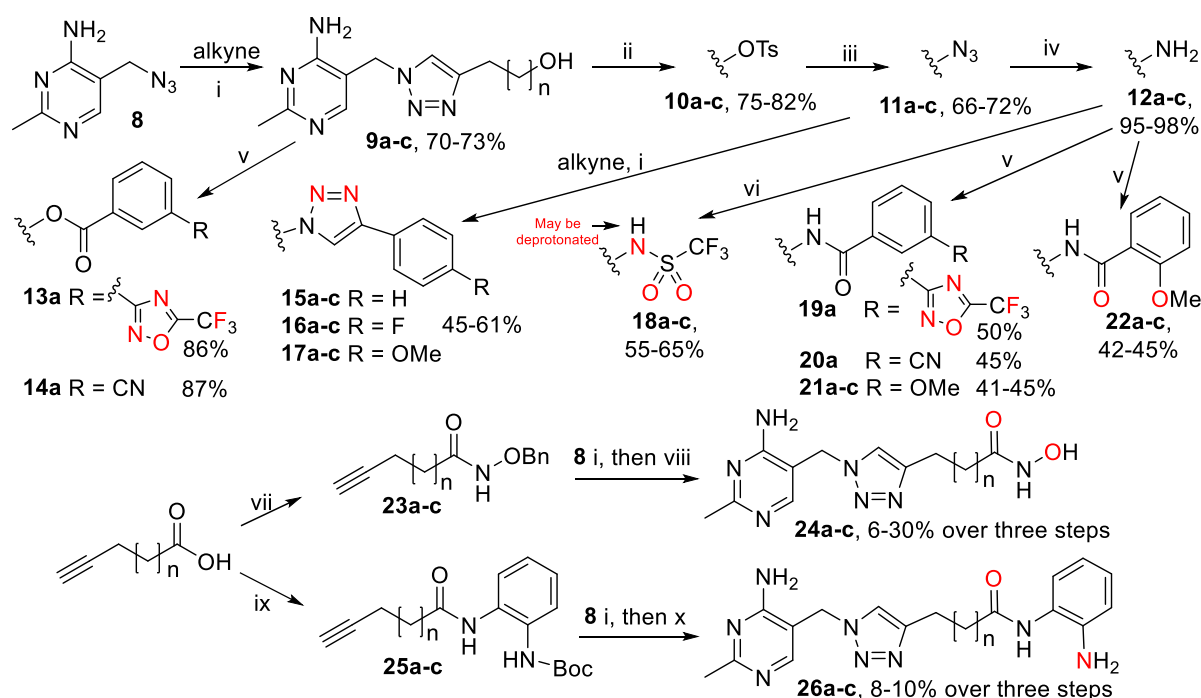
Figure 1. (**A**) Thiamine-utilisation pathways including the formation of thiamine derivatives from ThDP. (**B**) Binding mode of ylide **2b** and triazole diphosphate **5** in the ThDP pocket. (**C**) Comparison of the activities on the thiamine-utilisation pathways between thiamine/ThDP analogues. (**D**) Pharmacophore model of thiamine/ThDP analogues – ligand design strategy.

Results

Rational Ligand Design and Efficient Synthesis of Triazole-based Thiamine Analogues

Our ligand design came from analysis of the binding mode of triazole-ThDP **5** (one of the most potent inhibitors with K_I for PDC from *Zymomonas mobilis* of 30 pM[8]) in the ThDP pocket (Figure 1B).[24] Based on this, our pharmacophore model of thiamine/ThDP analogues (Figure 1D) consists of the aminopyrimidine-CH_2-triazole moiety joined to a neutral metal-binding group (MBG) by a linker of variable length. Seven different MBGs were tested in this study, adapted from other reported metalloenzyme inhibitors (highlighted in Scheme 1).[25] Most MBGs were attached by linkers of three different lengths to find the optimum positioning of the MBG relative to the aminopyrimidine-CH_2-triazole moiety. All ligands were efficiently synthesised in 2-5 steps from a common precursor azide **8**, in turn obtained in a single-step reaction[8] from thiamine hydrochloride (Scheme 1). OxThDP was synthesised using reported procedures.[20]

Scheme 1. Synthesis of triazole-based thiamine analogues from a common precursor, azide 8. *Reagents and conditions*: i) $CuSO_4.5H_2O$, sodium ascorbate, *t*-BuOH, H_2O, RT; ii) TsCl, pyridine, RT; iii) NaN_3, DMF, RT; iv) $H_2(g)$, 10% Pd/C, MeOH, RT; v) carboxylic acid, DCC, DMAP, DMF, RT; vi) trifluoromethanesulfonic anhydride, pyridine, 35 °C; vii) $NH_2OBn \cdot HCl$, CDI, DMF, THF, RT; viii) method a: BCl_3, DCM, RT; or method b: $H_2(g)$, 10% Pd/C, MeOH, RT; ix) *N*-Boc-1,2-phenylenediamine, DCC, DMAP, DMF, 40 °C; x) TFA, DCM, RT. For all compounds: **a** n=1, **b** n=2, **c** n=3. The potential metal-binding atoms of each metal binding group (MBG) are highlighted in red.
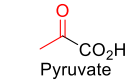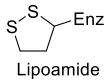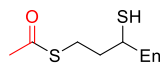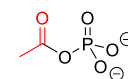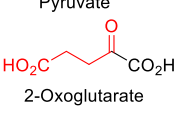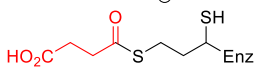
Compound Screening to Identify Broad-spectrum Inhibitors of ThDP-dependent Enzymes

To demonstrate widespread inhibition of ThDP-dependent enzymes, we chose four enzymes across three kingdoms (animals, fungi and bacteria) – porcine PDHc E1, *Saccharomyces cerevisiae* PDC, *E. coli* OGDHc E1, and *Aerococcus viridans* PO. The different reactions catalysed, preferred substrates (Table 1) and primary sequences reflect the structurally and functionally diverse members of the family.

The 32 compounds shown in Table 1 were subjected to preliminary screening for inhibition of the four enzymes. Free alcohols **9a-c**, which lack the MBG, were included as reference compounds as they reflect the binding affinity of the aminopyridine-CH_2-triazole moiety alone. OxThDP **7b** was used as a positive control. The compounds showed a wide range of percentage inhibition on PDHc E1, PDC and OGDHc E1; they were mostly more potent than the reference set **9a-c**, indicating that the modified tail can contribute to binding. Surprisingly, most ligands were inactive on PO, with only **7b**, **15a**, **18c**, **24b**

and **24c** inhibiting weakly. Five inhibitors particularly attracted our attention: bis-triazole **15c**, **16c** and **17c** and hydroxamates **24b** and **24c**. The two hydroxamates (**24b** and **24c**) showed inhibitory activities comparable to OxThDP **7b** and markedly greater than that of **9a-c** in all four enzyme assays, thus they were considered as truly multi-targeting ligands, warranting further characterisation. On the other hand, **15-17c** were very selective, showing potent inhibition of PDHc E1 and no inhibition of the other three enzymes. The remaining compounds were neither potent enough against individual enzymes nor sufficiently multi-targeting to warrant further biological testing. However, some docking studies were performed to explain the observed inhibitory effects (Figures S1-3).

Table 1. Comparison of assay enzymes and preliminary screening data.

	Inhibition (%)[a]			
Compounds	**PDHc E1[b]**	**PDC[c]**	**OGDHc E1[b]**	**PO[b]**
OxThDP 7b	> 90	81 ± 3	62 ± 4	48 ± 4
9a	55 ± 2	37 ± 3	57 ± 4	< 20
9b	52 ± 3	35 ± 2	50 ± 3	< 20
9c	57 ± 2	33 ± 4	51 ± 2	< 20
13a	72 ± 2	Insoluble	< 20	< 20
14a	71 ± 4	Insoluble	< 20	< 20
15a	45 ± 3	Insoluble	23 ± 3	22 ± 2
15b	65 ± 4	Insoluble	< 20	< 20
15c	> 90	Insoluble	< 20	< 20
16a	42 ± 2	Insoluble	25 ± 4	< 20
16b	66 ± 4	Insoluble	< 20	< 20
16c	> 90	Insoluble	< 20	< 20
17a	46 ± 4	Insoluble	21 ± 3	< 20
17b	71 ± 2	Insoluble	< 20	< 20
17c	> 90	Insoluble	< 20	< 20
18a	50 ± 2	< 20	48 ± 2	< 20
18b	55 ± 2	37 ± 4	42 ± 3	< 20
18c	71 ± 3	Insoluble	58 ± 4	22 ± 4
19a	48 ± 3	Insoluble	< 20	< 20
20a	46 ± 4	< 20	< 20	< 20
21a	67 ± 4	48 ± 2	63 ± 3	< 20
21b	61 ± 2	38 ± 5	58 ± 5	< 20
21c	60 ± 5	Insoluble	25 ± 3	< 20
22a	83 ± 4	61 ± 4	80 ± 4	< 20
22b	58 ± 3	Insoluble	56 ± 3	< 20
22c	58 ± 2	Insoluble	35 ± 3	< 20
24a	77 ± 3	41 ± 3	73 ± 2	< 20
24b	> 90	65 ± 2	> 90	35 ± 5
24c	> 90	63 ± 4	> 90	42 ± 5
26a	75 ± 5	< 20	63 ± 2	< 20
26b	59 ± 2	28 ± 3	52 ± 2	< 20
26c	64 ± 4	< 20	30 ± 2	< 20

[a] Data are the means of measurements in three technical replicates. Data for compounds with the greatest % inhibition for each enzyme are highlighted in red. [b] Inhibition (%) determined for compounds at 250 μM with [ThDP] = 50 μM. [c] Inhibition (%) determined for compounds at 1.5 mM with [ThDP] = 0.3 mM; compounds insufficiently soluble under the assay conditions were excluded. The K_M of yeast PDC for ThDP was found to be 11-23 μM so a high [ThDP] was needed to fully saturate the enzyme and so to keep the [inhibitor]:[ThDP] ratio constant, a higher [inhibitor] was needed.

Biochemical and Computational Characterisation of Enzyme Inhibition

The ThDP-competitive relationship of the inhibitors was validated by repeating the assays at increased [ThDP]:[compound] ratio (Table 2). As expected, the percentage inhibition by **7b, 15-17** and **24b** and **c** all decreased, consistent with competitive inhibition. These assays also showed that **17c** is the strongest binder among the bis-triazole series **15-17** and selective to PDHc.

In silico docking studies into the ThDP-binding pocket of human PDHc E1 (Figures 2 and S4-7) suggested **7b** and **24b** and **c** overlay well with ThDP, with the same V-shaped conformation of the aminopyridine-CH_2-thiazole moiety. Hydroxamates **24b** and **c** showed a non-ionic, bidentate metal-binding pose (Figure 2C). For bis-triazole **17c**, docking studies suggested that two of the triazole nitrogen atoms interact with the Mg^{2+} (Figure 2D). **17c**, with the *p*-OMe group, seemed to be a better inhibitor than **15c** or **16c** because the O of the OMe group hydrogen bonds to the side chain of a glutamine residue. The *in silico* studies also suggested that the selectivity of **15-17** for PDHc E1 is because the longer side-chains are too bulky to be accommodated in the smaller diphosphate pockets in OGDHc E1, PDC and PO (Figure S7).

Full IC_{50} determinations were conducted for bis-triazole **17c** and hydroxamates **24b** and **c** and showed that they inhibited ThDP-dependent enzymes in a dose-dependent manner (Figure 3A-D). As ThDP-competitive ligands, their K_I value, affinity relative to ThDP and ligand efficiency (L.E., binding energy in kcal mol^{-1} per heavy atom)[26] are shown in Figure 3E. Bis-triazole **17c** showed the best inhibition of PDHc E1, with a K_I of 30 nM.

The affinities of hydroxamates **24b** and **c** and OxThDP **7b** for PDHc E1 are all comparable to that of ThDP (Table 2 and Figure 3), which is in line with the findings of others on OxThDP.[2-3] The molecular properties of hydroxamates **24b** and **c** (Table S1) are predicted to be in the range preferred for drugs;[26,27] so they should be active on intact live cells.

Table 2. Inhibitory data of bis-triazoles 15-17 and hydroxamates 24b,c under increased ThDP levels.

| Compound | Inhibition (%)[a] | | | |
	PDHc E1[b]	PDC[c]	OGDHc E1[b]	PO[b]
OxThDP 7b	75 ± 3	63 ± 3	41 ± 4	32 ± 5
15a	< 20	Insoluble	< 20	< 20
15b	40 ± 4	Insoluble	< 20	< 20
15c	75 ± 4	Insoluble	< 20	< 20
16a	< 20	Insoluble	< 20	< 20
16b	43 ± 2	Insoluble	< 20	< 20
16c	79 ± 2	Insoluble	< 20	< 20
17a	< 20	Insoluble	< 20	< 20
17b	45 ± 3	Insoluble	< 20	< 20
17c	82 ± 4	Insoluble	< 20	< 20
24b	70 ± 3	42 ± 4	78 ± 3	27 ± 4
24c	73 ± 4	37 ± 4	82 ± 2	24 ± 4

[a] Data are the means of measurements in three technical replicates. Compared to the assay conditions for Table 1, the assays here were conducted with an increase in [ThDP]:[inhibitor] from 1/5 to 1/2. [b] Inhibition (%) determined for compounds at 250 μM with [ThDP] = 125 μM. [c] Inhibition (%) determined for compounds at 1500 μM with [ThDP] = 750 μM; compounds not dissolved under the assay conditions were excluded.
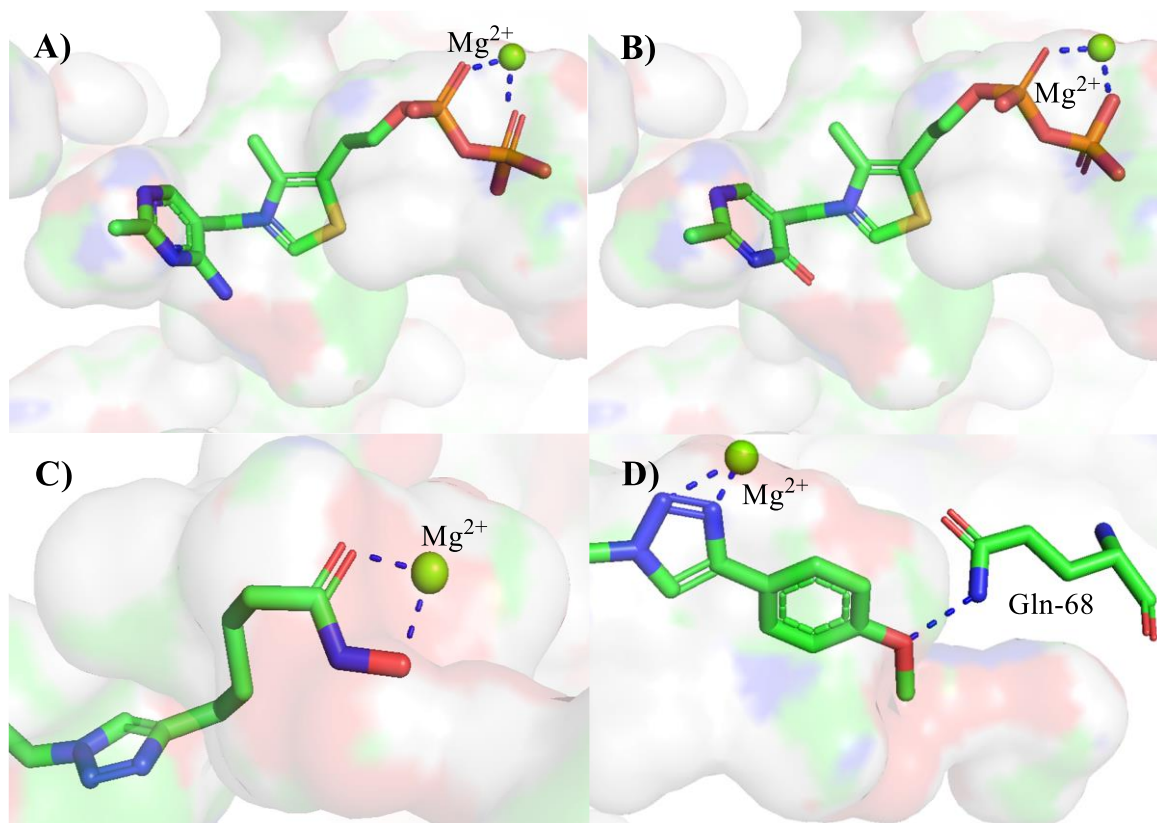
Figure 2. *In silico* **prediction of metal-binding interactions in the human PDHc E1 ThDP pocket.** (**A**) ThDP **2a**. (**B**) OxThDP **7b**. (**C**) hydroxamate **24c**. (**D**) bis-triazole **17c**.
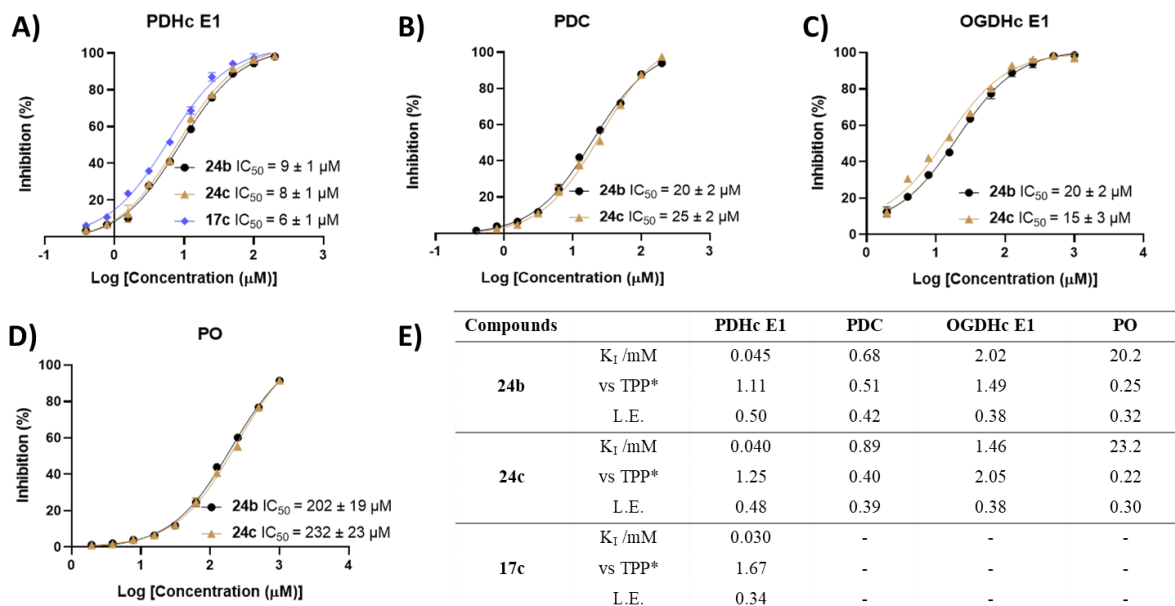

Figure 3. Dose-dependent inhibition of ThDP-dependent enzymes by PDHc E1-selective bis-triazole 17c and multi-targeting hydroxamates 24b,c. (**A-D**) IC_{50} values determined at [ThDP] = 10 µM (PDHc E1 and bacterial PDC), 30 µM (OGDHc E1) and 50 µM (PO). Data are the means of measurements in three technical replicates for all four enzymes. Where error bars are not visible they are smaller than the symbol used. The PDC used here was bacterial (from *Zymomonas mobilis*) due to the better assay conditions that avoid solubility issues. (**E**) Summary of the affinity (K_I) and ligand efficiency (L.E.). K_I values are determined according to the K_M of the respective enzymes for ThDP (refer to Methods). *Affinity of the inhibitor versus that of ThDP (*i.e.* [ThDP]/IC_{50} or $K_{M(ThDP)}/K_I$).

Mechanistic Investigation of the ThDP-competitive Thiamine Analogues in Cell-based Studies

Next the effect of the thiamine/ThDP analogues on *in vitro* proliferation of the 3D7 strain of *P. falciparum* was determined. Infected erythrocytes were treated with eight selected compounds, bis-triazoles **16a-c** and **17a-c** and hydroxamates **24b** and **c**. Parasite proliferation was measured by SYBR-Safe assay of parasite DNA.[11,17] The IC_{50} (concentration at which the compound suppresses parasite proliferation by 50%) was determined at various extracellular thiamine levels (Table 3) and is compared to our previously reported data on oxythiamine **7a**.[17]

Most of the thiamine analogues tested inhibited proliferation of the parasite (Table 3) in a dose-dependent manner (Figures 4A and S8). In thiamine-free culture medium, the PDHc-selective **16** and **17** ($IC_{50} \geq 44$ µM) were considerably weaker than the multi-targeting hydroxamates **24b** and **c** (IC_{50} = 0.9-3 µM) and oxythiamine **7a** (IC_{50} = 11 µM). With 2.97 µM thiamine in the culture medium, the IC_{50} of the better performing hydroxamate **24b** changed from 0.9 ± 0.1 µM to 1.3 ± 0.2 µM ($n=3$; $P=0.0188$; T-test), and the IC_{50} of the best-performing bis-triazole **17c** changed from 44 ± 2 µM to 62 ± 7 µM ($n=3$; $P=0.0136$; T-test) (Figure 4A). Increasing the thiamine concentration in the culture medium 100-fold to 297 µM resulted in a statistically insignificant further change in the anti-plasmodial potency of all compounds (including **7a**) (Table 3).

Some of the compounds were also tested on Human Foreskin Fibroblast (HFF). Most of the compounds exhibited dose-dependent cytotoxicity (Table 3, and Figures 4 and S9). In the bis-triazole series, the longer derivatives (**16c** and **17c**) showed higher potencies (than their shorter counterparts) on both *P. falciparum* and human cells. Hydroxamate **24b** was the strongest inhibitor of proliferation of HFF cells, consistent with its potent inhibition of multiple ThDP-dependent enzymes. Although **7a** was not tested on HFF cells in this study, numerous *in vitro* and *in vivo* studies have confirmed that **7a** is toxic;[14-19] for instance, our previous study showed that, relative to the controls, the average body weight of mice declined progressively with each daily dose of **7a** over a three-day course.[17]

Table 3. IC_{50} values (µM) for the anti-plasmodial activity and cytotoxicity of selected compounds.

Compound	IC_{50} of Suppression of *P. falciparum* proliferation (µM)			HFF Cytotoxicity
	Thiamine-free	[Thiamine] = 2.97 µM	[Thiamine] = 297 µM	
7a*	11 ± 4	5200 ± 300	5500 ± 500	ND
16a	>200	>200	>200	ND
16b	>200	>200	>200	>200
16c	67 ± 5	70 ± 8	89 ± 19	48 ± 5
17a	129 ± 9	156 ± 13	169 ± 11	>200
17b	51 ± 3	78 ± 8	82 ± 11	90 ± 5
17c	44 ± 2	62 ± 7	69 ± 9	46 ± 7
24b	0.9 ± 0.1	1.3 ± 0.2	1.4 ± 0.4	15 ± 3
24c	3.0 ± 1.2	3.8 ± 2	4.0 ± 1.8	44 ± 4

ND, not determined. Refer to Figures S8 and S9 for details. *Ref. 17.

To further support the proposed mode of action of the thiamine analogues, we tested **17b** and **24c** on a parasite line that expresses extra copies of TPK with a GFP-tag.[11] As a negative control, we also tested the compounds on another parasite line transfected with an empty plasmid. Our previous studies showed that in the thiamine-free culture medium, the parasite that over-expresses TPK was 70-fold more sensitive to oxythiamine **7a** than the parasite that bears an empty plasmid.[17] This is because the extra TPK copies significantly improved the conversion ratio of extracellular **7a** to intracellular **7b**; the increased OxThDP levels resulted in stronger antagonism on the parasite's thiamine-utilisation pathway. By contrast, in the thiamine-free culture medium, the sensitivity of the two parasite lines to thiamine analogues **17b** and **24c** was almost identical (Figures 4C and S10).
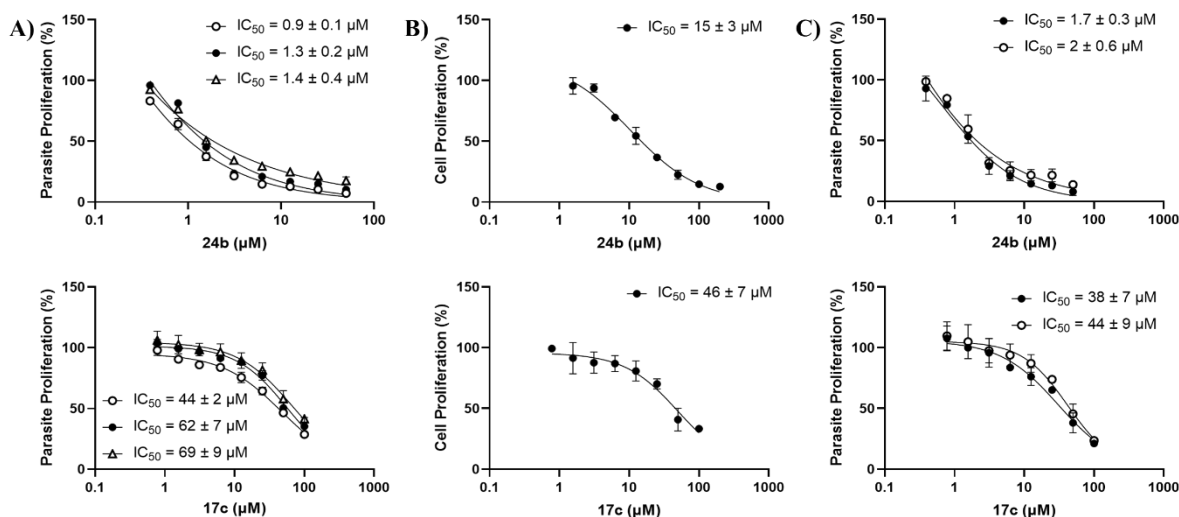
Figure 4. Anti-plasmodial activities of hydroxamate 24b and bis-triazole 17c. (**A**) Anti-plasmodial activities against *P. falciparum* 3D7 parasites in thiamine-free medium (○), medium with 2.97 μM thiamine (●), and medium with 297 μM thiamine (△). (**B**) Cytotoxicities on HFF cells. (**C**) Anti-plasmodial activities against *P. falciparum* 3D7 parasites transfected with an empty plasmid (●), and 3D7 parasites expressing *Pf*TPK-GFP (○) in thiamine-free medium. Data are the means of measurements from three independent experiments, each performed in two technical replicates.

Discussion

Our ligand design strategy came from retaining the aminopyrimidine-CH_2-triazole moiety of the potent inhibitor of ThDP-dependent enzymes, triazole-ThDP **5**[8] for affinity, but changing the diphosphate to a neutral metal-binding group for membrane-permeability.[28] All the compounds had short synthetic routes starting from inexpensive thiamine hydrochloride. Among the 31 such compounds tested, hydroxamates **24b** and **c** stand out as inhibiting all the ThDP-dependent enzymes tested, in a dose-dependent and ThDP-competitive manner, with affinities comparable to those of ThDP **2a** and OxThDP **7b** (Figure 3). Ligand efficiency (L.E.), measuring the binding energy of a ligand to its target (in kcal mol^{-1}) per heavy atom of the ligand, is a widely applied metric in medicinal chemistry.[26] Drug discovery efforts often aim to develop clinical candidates with L.E. > 0.3.[26,27] Thus, both bis-triazoles, **24b** (L.E. = 0.50) and **24c** (L.E. = 0.48), are extremely efficient inhibitors of PDHc and very good inhibitors of the other three enzymes too.

In the cell-based studies in thiamine-free medium, the comparable enzyme inhibition by **24b** and **c** and **7b** translated into similar low-micromolar anti-plasmodial activities of **24b** and **c** and oxythiamine **7a**, though **24b** is 3-fold more active than **24c** and 11-fold more active than **7a**. This suggests that the passive diffusion of **24b** and **c** results in a greater concentration in the parasites than the presumed active transport and pyrophosphorylation of **7a**. The thiamine-antagonistic effect of **24b** was demonstrated in cell-based assays: its dose-dependent activity decreased with added thiamine in the medium (Figure 4), presumably because this leads to higher levels of ThDP within the parasites. Interestingly adding thiamine (2.97 μM) to the medium lowered the inhibitory effect of **24b** much less than it did for oxythiamine.[17] This suggests that the lowered inhibitory effect of oxythiamine is more due to competition with thiamine for transport into the cell and/or pyrophosphorylation by TPK (resulting in much less OxThDP being formed) than due to competition of OxThDP with ThDP for binding to the enzymes. Raising the thiamine concentration in the medium still further (to 297 μM) had no further effect on the IC$_{50}$ values of either **24b** or oxythiamine.[17] Probably one or more parasite's thiamine-utilisation steps become saturated even at 2.97 μM thiamine, so adding more extracellular thiamine no longer significantly increases the intracellular thiamine/ThDP levels.[17]

Our biochemical, computational and cell-based studies collectively suggest that our hydroxamate **24b** is as competent as oxythiamine to cause thiamine deficiency in cells. However, with the triazole scaffold replacing the thiazolium ring, **24b** is uncharged under physiological conditions so it is more drug-

like[19,26,27] and unlikely to be degraded by thiaminases.[18] Moreover, without the diphosphate moiety, **24b** would likely retain activity on oxythiamine-resistant organisms that over-express the SLC19A1 transporter[16] (which removes OxThDP from the cell) or ThDP-hydrolases[23] (which hydrolyse OxThDP to OxTMP). Also, the neutral triazole ring of **24b** is expected to improve the selectivity (relative to OxThDP) to the coenzyme role of ThDP over the non-coenzyme roles. ThDP riboswitches are known to prefer a charged central ring[22] and the other (unknown) non-coenzyme targets probably do as well, as they are unlikely to be optimised for binding the neutral ThDP ylide **2b**, as the enzymes are. Furthermore, it most unlikely that **24b**, lacking the diphosphate, would be converted into derivatives equivalent to ThTP **3** or AThTP **4**, whereas OxThDP may well be.

Although the structural and mechanistic differences may make hydroxamate **24b** superior to oxythiamine **7a** in some ways, its utility in understanding the roles of the thiamine-utilisation pathway in disease states is maximised when applied alongside **7a**, as we have done in this study. For instance, the activity of an inhibitor such as **24b** will either be unchanged when the thiamine-utilisation pathway is up-regulated or drop (as a result of increased intracellular ThDP levels and/or ThDP-dependent enzymes being over-expressed). For **7a**, however, there is a dramatic increase in activity (in thiamine-free medium) if TPK is over-expressed because **7a** needs TPK for activation.[11,17] By contrast, the fact that the anti-plasmodial activity of **24b** is insensitive towards TPK over-expression not only confirms that **24b** does not require the action of TPK for enzyme binding, but also indicates that TPK is not the target. Using our studies on *P. falciparum* as an example: we have previously attributed the anti-plasmodial activities of oxythiamine to the inhibition of multiple ThDP-dependent enzymes in the parasite, but it is possible that its action on some non-coenzyme role of thiamine may have also contributed. In this study, however, hydroxamate **24b**, almost certainly lacking any non-coenzyme activity, has exhibited comparable activities to oxythiamine **7a** (in thiamine-free culture medium), which suggests that inhibition of ThDP-dependent enzymes is indeed the main target of oxythiamine.

Apart from the multi-targeting hydroxamates **24b,c**, we discovered a series of bis-triazoles **15-17** as selective inhibitors of PDHc E1, with **17c** being the best-performing thiamine analogue. As PDHc plays a vital role in bioenergetic processes by linking glycolysis with the Krebs cycle,[4] the relative activities on cells (**17c**>**b**>**a**, Table 3) is likely to be a function of the PDHc E1 inhibition (**17c**>**b**>**a**, Table 2). It is, however, possible that the higher hydrophobicity of the longest derivative **17c** led to better cellular uptake. Since a narrow-spectrum coenzyme antagonist, such as **17c**, was not the main focus of this work, further cell-based characterisation was not pursued. However, recent studies have shown that selective PDHc E1 inhibitors have potential against certain cancers,[29-32] so **17c** may be of interest in this field. The ligand efficiency of **17c** is good (0.34), so it could be a good starting point for the development of anti-cancer drugs.

Although **17c**, **24b** and **24c** are all very similar in their inhibition of PDHc E1 (Figure 3A) (with **17c** marginally most potent), **24b** is almost 50-fold more potent than **17c** at inhibiting proliferation of the parasites (Figure 4A). This suggests that some other ThDP-dependent enzyme(s) may be more critical than PDHc for proliferation. Possibilities include transketolase, which is required for the synthesis of the ribose 5 phosphate, needed for *de novo* RNA and DNA synthesis, by the non-oxidative pentose phosphate pathway and 1-deoxyxylulose 5-phosphate synthase, the first and rate-determining enzyme of terpene biosynthesis *via* the non-mevalonate pathway. Previous studies have shown that inhibition of the non-mevalonate pathway in *P. falciparum* does have an antiproliferative effect.[33] The greater activity on the parasites than on the HFF cells would be consistent with this, as human cells do not use the non-mevalonate pathway. The low anti-proliferative effect of **17c** might also be because PDHc and OGDHc enzymes can, to some extent, accept each other's substrates, and so OGDHc could compensate for the lack of activity of the inhibited PDHc.[17]

In summary, the current study has demonstrated hydroxamate **24b** is an antagonist of thiamine through inhibiting the coenzyme role of ThDP. Its utility is broad: active on both *P. falciparum* and human cells, and capable of inhibiting different ThDP-dependent enzymes from various species. We have also discussed its use alongside oxythiamine **7a** in inducing thiamine deficiency in cell-based systems. This can help to avoid misinterpretation of the role of these multi-targeting coenzyme antagonists and indicate whether coenzyme or non-coenzyme roles of thiamine are the main target.

Author Contributions

AHYC conceived the project, designed and synthesised most compounds and performed the molecular property calculations and some enzyme assays. TCSH performed most enzyme assays and the computational dockings. RP synthesised some compounds. FJL supervised the work of AHYC, TCSH and RP. IF performed cell assays under the supervision of KJS. AHYC wrote the first draft with input from TCSH and IF. All authors contributed to and approved the final version of the paper.

Conflicts of Interest

The authors declare no competing financial interest.

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