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Mimics of Diphosphate in ThDP to Improve Pharmacokinetics of Potential Drug Molecules

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ABSTRACT

It is well known that the diphosphate of ThDP is vital for the binding to the enzyme. However, this highly charged group is unsuitable for whole cell studies or pharmaceutical development, and compounds possessing it will suffer from poor bioavailability and cellular uptake. Various research groups have investigated the possibility to mimic the diphosphate with various neutral and more stable analogues without compromising too much binding affinity for the enzyme. This review discusses those efforts, which have been made to develop the identification of uncharged, chemically stable surrogates for the diphosphate linkage. Moreover, this field, is relevant not only to ThDP-dependent enzymes but also a wide range of other enzymes and proteins that bind diphosphate esters.

Keywords: ThDP, ThDP dependent enzymes, Diphosphate, Diphosphate mimics, Binding, Pharmacokinetics

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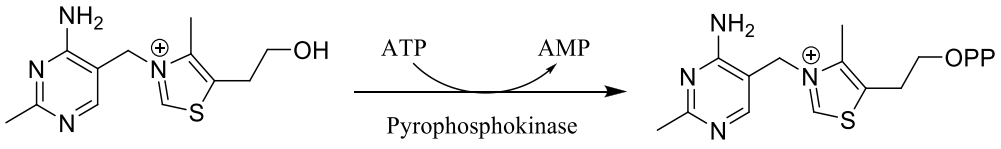
THIAMIN DIPHOSPHATE (ThDP)

The biologically active source of vitamin B1 is thiamin diphosphate (ThDP), an important cofactor and involved in a variety of metabolic pathways, including oxidative and non-oxidative decarboxylation of α-keto acids (pyruvate dehydrogenase, pyruvate decarboxylase), the formation of amino acid precursors (acetohydroxyacid synthase), electron-transfer reactions (pyruvate oxidase, pyruvate:ferredoxin oxidoreductase), and ketol transfer between sugars (transketolase) (Alfred Schellenberger, 1998). One common function of ThDP-dependent enzymes is to catalyze the cleavage and formation of carbon-adjacent bonds with ThDP acting as an electron sink during catalysis to stabilize what would otherwise be an acyl carbanion in the form of an intermediate enamine (Kluger, 1987). Since these ThDP-dependent enzymes are involved in numerous biological reactions which produce chiral compounds with high efficiency and high enantiomeric selectivity, they are of great interest to the pharmaceutical industry for use in the chemical synthesis of drugs(Müller, Gocke, & Pohl, 2009; Pohl, Lingen, & Müller, 2002).

STRUCTURE OF ThDP

Thiamin (vitamin B1) consists of two heterocyclic rings, a pyrimidine and a thiazolium ring, linked together by a methylene bridge. It is a vitamin because human cells cannot make thiamin and it therefore has to be in our diet. The enzyme thiamin pyrophosphokinase converts this thiamin into its diphosphate form by catalysing the transfer of a diphosphate group from ATP to thiamin (Bettendorff & Wins, 2009)



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Scheme 1: Pyrophosphorylation of thiamin by thiamin pyrophosphokinase

GENERAL CATALYTIC CYCLE

ThDP catalyses two type of reactions: non-oxidative, such as those of pyruvate decarboxylase (PDC) and benzoylformate decarboxylase (BFD) (Hasson *et al.*, 1998; Jordan *et al.*, 1998) producing acetaldehyde and benzaldehyde; and oxidative, such as those of pyruvate oxidases (POX), which use flavin as the oxidant to produce acetate (Bertagnolli & Hager, 1991) or acetyl phosphate (Muller & Schulz, 1993), the pyruvate dehydrogenase multienzyme complex (PDHc) and its family of enzymes which utilize lipoic acid as the oxidant to produce acetyl-CoA (Reed, 1974), and of pyruvate-ferredoxin oxidoreductases (PFOR) which use Fe4S4 cluster chemistry to produce acetyl-CoA (Chabrière *et al.*, 1999).

A generalised catalytic cycle for ThDP-dependent enzymes utilising pyruvate proposed by Breslow is depicted in fig 1.1 (Breslow, 1957, 1958). After formation of the ThDP ylide, the substrate carbonyl is attacked by the thiazolium C2 carbanion to form the tetrahedral pre-decarboxylation intermediate 2-(2-lactyl)-ThDP, LThDP. Binding of pyruvate in the covalent LThDP adduct introduces significant strain to the system, and the release of this strain is an important driving force in the decarboxylation reaction.

The presence of an electron sink in the form of + ve charging nitrogen is another significant aspect of this decarboxylation. Decarboxylation gives the post decarboxylation intermediate forms of carbanion /

enamine resonance. This intermediate represents a branching point of catalysis at which the non-oxidative and oxidative pathways diverge. As part of the oxidative pathways, for example, neighbouring redox-active cofactors such as flavins (POX), Fe4S4 clusters (pyruvate: ferredoxin oxidoreductase, PFOR), or lipoamide (PDHc) oxidise the enamine intermediate. Depending on the protein and the oxidizing cofactor, there may be transient formation of a radical ThDP intermediate, or simultaneous transfer of two electrons. The resulting 2-acetyl-ThDP intermediate can undergo enzyme-catalysed release of acetate or transfer of the acetate group to biological nucleophiles to produce, for example, acetyl phosphate, acetyl-CoA, or S8 acetyl-dihydrolipoamide. The enamine intermediate can also react non-oxidatively following protonation at C2α to give HEThDP, with subsequent elimination of acetaldehyde (as catalysed by PDC). Addition of the carbanion/enamine to the carbonyl of another keto acid (pyruvate, 2-ketobutyrate) leads to the formation of a conjugate resulting from both substrates and ThDP (acetohydroxyacid synthase, AHAS). Although ThDP-dependent enzymes catalyze a wide variety of reactions, there is a common pattern of analogous pathways that proceed via similar ThDP-derived covalent intermediates.

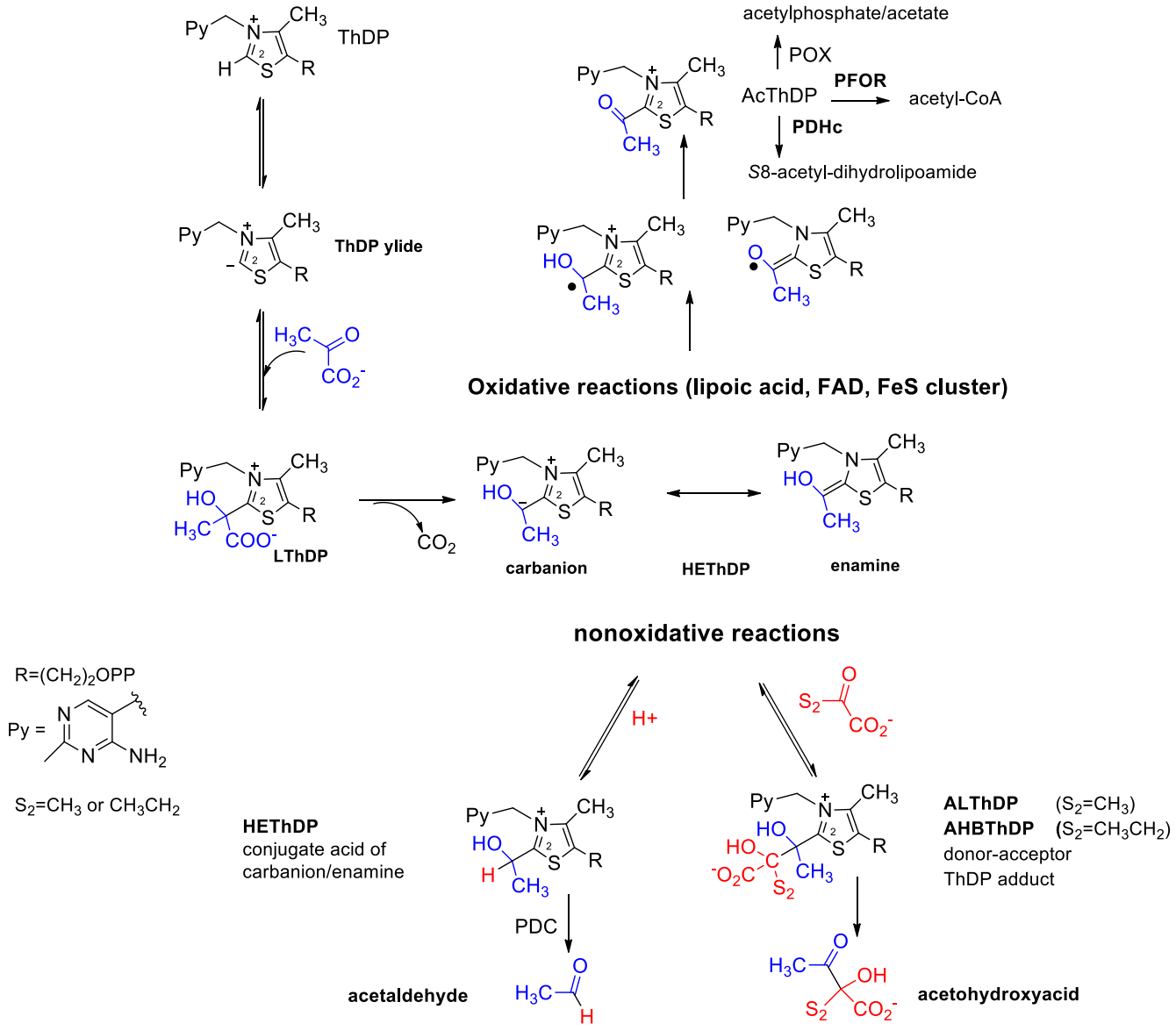


Figure 1. Catalytic cycle of ThDP (Kluger & Tittmann, 2008)

ROLE OF THE DIPHOSPHATE GROUP (BINDING OF ThDP IN ACTIVE SITE)

It has been shown that inorganic diphosphate on its own will compete with the coenzyme for its active site. Common features among ThDP enzymes are (i) consistency of ThDP binding site across the enzyme family and (ii) their quaternary structure architecture, which always consists of two or four subunits, giving a dimer or a tetramer (dimer of dimers), respectively. This assembly is in fact stabilised by the binding of ThDP, which occurs at the interface between two subunits, where the diphosphate is anchored to one subunit and the pyrimidine binds in a cleft formed by two different subunits. The binding site is buried inside the enzyme, at the bottom of a rather narrow entrance funnel (Muller *et al.*, 1993), and is generally quite hydrophobic. Without its diphosphate group thiamin itself shows little if any

binding affinity in recombination experiments with apoPDC suggesting that the binding of ThDP is very much dependent on the interaction between the diphosphate-ion complex and the enzyme. The rest of the molecule contributes additional binding interactions, including key hydrogen bonding interactions between the nitrogen atoms of the

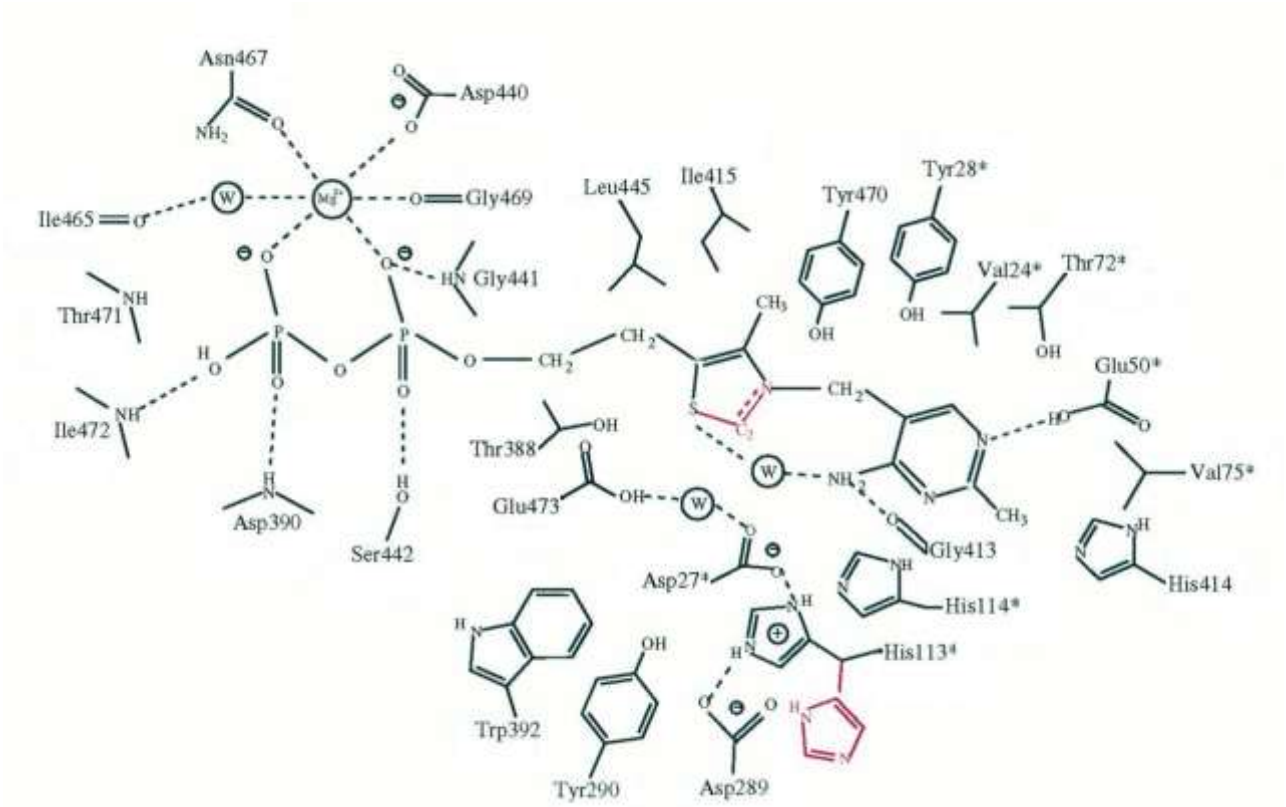


Figure 2. ThDP binding in PDC from Z. mobilis (ZmPDC). (Dobritzsch, König, Schneider, & Lu, 1998)

aminopyrimidine and the enzyme (also confirmed by X-ray analysis). To assess the contribution of each part of the molecule to its overall binding affinity, the binding capacities of a number of analogues were compared (Table 1) (Bertagnolli & Hager, 1991).

Table 1. The binding capacity of different parts of ThDP

Binding capacity	%
Thiamin diphosphate (ThDP)	100
Thiamin	0
Diphosphate	27
Thiazolium Diphosphate	41
Methyl-thiazolium diphosphate	57
Benzyl-thiazolium Diphosphate	73
4'-Hydroxy-ThDP	>99

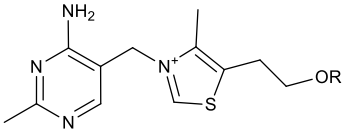
The diphosphate group of ThDP forms the most important binding interactions with the enzyme by coordinating to Mg2+ (A Schellenberger, 1967). It is necessary for thiamine diphosphate to interact with its target protein, and therefore for its biological activity. In order to synthesize potential drug molecules with good ThDP-based pharmacokinetic profiles, mimics of the diphosphate group will be necessary, which are not as highly charged but retain good affinity to the enzyme. The high charge of the diphosphate group can lead to poor uptake and bioavailability due to its difficulty in crossing the lipid membranes of cells. With this in mind, efforts have been made to develop the identification of uncharged, chemically stable surrogates for the diphosphate linkage.

DIPHOSPHATE MIMICRY

Diphosphate is an extremely central and important group, involved in the biochemistry of all organisms e.g. nucleotides, carbohydrates and vitamins. Strangely, there are not many papers published in the area of pyrophosphate analogues. However, since many biomolecules are dependent on the diphosphate group to interact with enzymes and receptors in the cell, the group might also be important in drug development. For example, in antiviral therapy, where nucleoside analogues are used as drugs and the active form is the triphosphate, a diphosphate analogue would give the ability to by-pass some of the naturally occurring phosphorylation steps and thus provide a much more stable and reliable drug delivery (Boyle, 2006; Eummer, Gibbs, Zahn, Sebolt-Leopold, & Gibbs, 1999; JohnáJennings, 1992; Klein, Nghiêm, Valleix, Mioskowski, & Lebeau, 2002; G. Wang *et al.*, 2004). This field of research, to find novel analogues of pyrophosphate, is therefore of great general interest.
Diphosphate bonds are inherently unstable linkages, susceptible to both chemical and enzymatic hydrolysis.

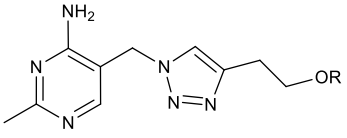
Therefore, molecules containing a diphosphate bond have a short half-life within the body. A wide variety of diphosphate modifications have been made in an attempt to increase stability of such molecules, and numerous potential bioisosteres have also been investigated.

Erixon, Dabalos, and Leeper (2007) synthesised six analogues of the coenzyme ThDP, containing different diphosphate mimicking moieties (3a-f) (fig 3). When tested against PDC, compounds (3a-b), in which the bridging oxygen of the diphosphate group was replaced by a CH2 or a CF2 group, were found to be quite potent inhibitors, with Ki values of 1.2 and 0.95 nM, respectively. (The apparent KM value for ThDP itself is 1.28 μM) (Bringer-Meyer, Schimz, & Sahm, 1986). The phosphoramidic analogue (3c) exhibited a weaker inhibitory effect, with a Ki of 0.14 μM. Whereas for carbamate (3d), malonate (3e) and iminodiacetate (3f) analogues, no binding was observed. These results illustrate the importance of the charge on the diphosphate group to biological activity. A marked decrease in inhibition is seen going from analogues (3-b) (charge = -3) to analogue (3c) (charge = -2) to analogues (3d-f) (charge = -1). It is known that the diphosphate is of great importance in the binding of ThDP to enzymes, where it is coordinated to a magnesium ion (or sometimes Ca2+ ion) in the active site. Therefore, it makes sense that the more negatively charged analogues will bind best.



(4) R = H, vitamin B_1

(**5**) R = $P_2O_6^{3-}$, TPP (Km = 1.28 μM)



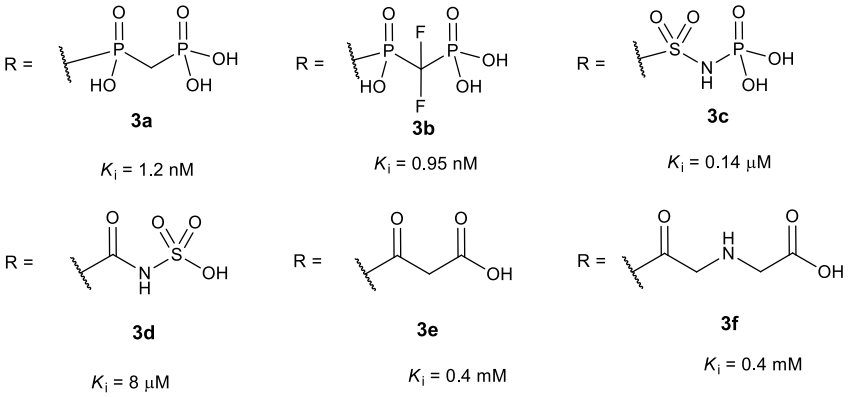


Figure 3. Thiamine diphosphate analogues

The key reason to synthesize less charged diphosphate mimics is to improve the analog's cell permeability so that it can be used in whole cell experiments. Another solution is to use the required alcohol (e.g. 4) and hope that it will either migrate through the cell wall or be carried by thiamine transporters. Furthermore, such approach would also be dependent on the analogue being a substrate for TPPK, so that it could be converted to the corresponding pyrophosphate inside the cell. In this project, masking of the methylenediphosphonate ester with acyloxymethyl (AM) ester groups was attempted instead (Niemi, Vepsäläinen, Taipale, & Järvinen, 1999; Troutman, Chehade, Kiegiel, Andres, & Spielmann, 2004; Vepsäläinen, 1999). Although attempts to synthesis the acetyloxymethyl ester were unsuccessful, a pivaloyloxymethyl (POM) ester 6 was prepared (figure 4). The concept with POM ester 6 is that the hydrophobic compound will enter the cell membrane and, once within the cell, different esterases would cleave their labile ester groups to release the charged species. Unfortunately, assays on E. coli did not show any effects on the cell proliferation (by means of OD600) even at high concentrations of 6 (>0.5 mM). Nonetheless, in a study on human umbilical vein endothelial cells (HUVECs), 6 showed an IC50 of 40-50 μM, which is, however, still higher than expected given the KI value (1.2 ± 0.1 nM) of the corresponding methylene analogue. In conclusion, it appears like this strategy, to protect the pyrophosphate moiety, might only be useful in studies on mammalian cells, which is also indicated in the literature by the lack of studies using AM ester derivatives on bacterial cells.

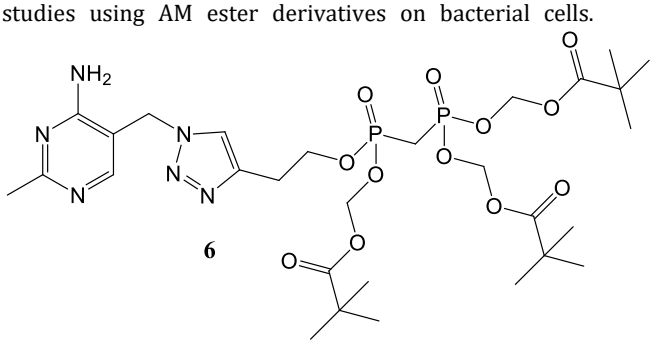
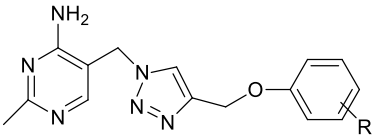


Figure 4: Pivaloyloxymehtyl ester 6.

Using docking simulations, four novel compounds (7a-d) with high inhibitory activity against PDHc-E1 from E. coli were designed by (Ren *et al.*, 2011). The mechanism of interaction between the active site of PDHc-E1 and its inhibitors was further explored by the combined use of molecular dynamics (MD) simulation and fragment molecular orbital (FMO) methods. The consistency between the docking results and the results of MD simulation shows that the residues Glu571, Met194, Val192 and Phe602 are well preserved and may act in aminopyrimidine stabilization on compound (7a). Furthermore, the nitro group on compound (7a) can not only form a hydrogen-bond with residue Asn260 but also coordinate to the Mg2+, which is very important for stabilizing the complex. The four compounds with high inhibitory activities against PDHc-E1 from E. coli were validated by determining their IC50 values. which lent partial credit to the quality of molecular modelling strategy presented in this study. Experimental results suggested that the nitro group on compound (7a) significantly affects its inhibitory activity again PDHc-E1.



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a, R=2-Cl, 4-NO_2
b, R=4-NO_2
c, R=H
d, R=4-CO_2Et

Figure 5. Inhibitors of PDH-E1

MONOSACCHARIDES AS DIPHOSPHATE MIMICS

Huang and co-workers (Huang, Chopra, Verdine, & Harrison, 1998) determined the crystal structure of a covalently trapped catalytic complex of deoxythymidine triphosphate (dTTP) with a DNA template:primer in the active site of human immunodeficiency virus-type 1 (HIV-1) reverse transcriptase (Figure 6).

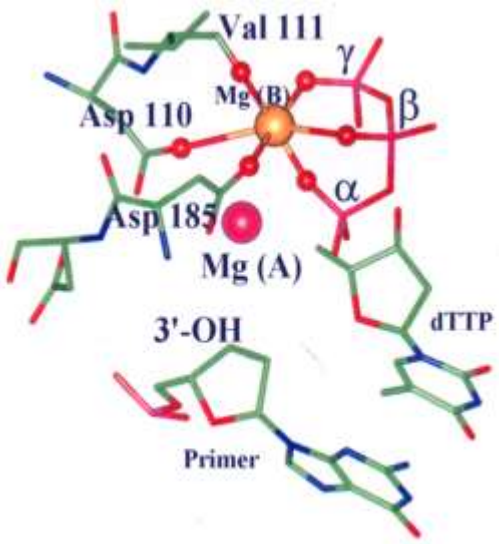


Figure 6. The catalytic complex of HIV-1 reverse transcriptase (PDB code = 1RTD)(Huang *et al.*, 1998)

This was achieved by inserting a dideoxynucleotide at the 3'-primer terminus, poised for attack on dTTP, but unable to due to the absence of a 3'-hydroxyl group. Analysis proved that the presence of two Mg2+ ions within this catalytic complex is essential for correct substrate binding and catalysis. One of the Mg2+ ions, Mg-(A), resides within the active site at all times, whereas the other, Mg-(B), enters the active site as a chelate with the triphosphate chain of the NTP substrate. Mg-(B) also coordinates to two aspartate residues on the protein, and a backbone carbonyl group on a valine residue, and thus is essential for the substrate to bind in the correct conformation. Mg-(B) is also necessary for the catalysis itself, as it activates the β,γ-pyrophosphate leaving group on the NTP.

When dTTP is bound in the active site, a six-membered chelate ring is formed between Mg-(B) and the β,γ-pyrophosphate moiety of the NTP (fig 7a). This ring closely resembles the 4C1-chair conformation expected of a nucleoside monophospho-α-glycoside (NMP sugar) (fig 7b)

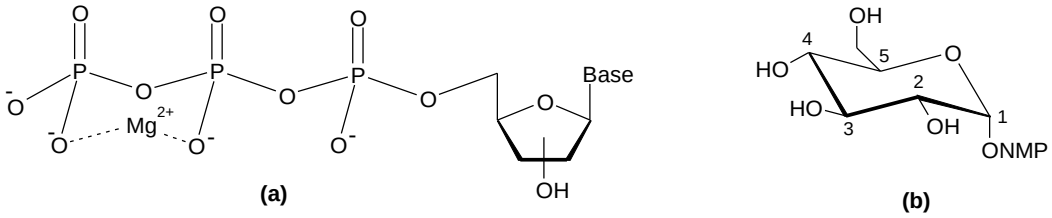


Figure 7. NTP-Mg chelate ring (a) and an NMP sugar (b)

It is therefore logical to suggest that the terminal β, γ-pyrophosphate moiety of the NTP could perhaps be replaced with a monosaccharide unit and still lead to favourable enzyme binding, but result in inhibition. Figure 8 shows the crystal structure of α-D-glucose superimposed over the crystal structure of the polymerase-bound NTP such that C-1 and C-4 overlay Pβ and Pγ of the triphosphate chain, respectively. It illustrates how the hydroxyl groups on C-2 and C-3 of the monosaccharide sit in close proximity to the position occupied by Mg-(B) in the HIV-reverse transcriptase catalytic complex. It can therefore be reasonably suggested that the ionic interaction that occurs between Mg-(B) and the charged aspartate residues in the enzyme active site could be replaced by hydrogen bonding interactions between the aspartate residues and one or both of these hydroxyl groups.

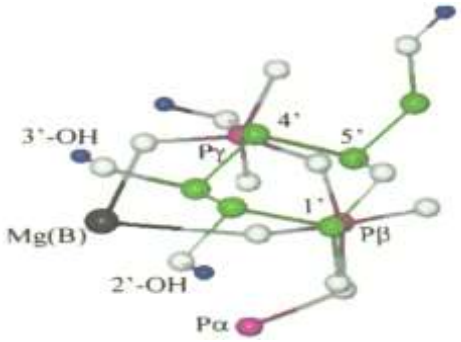
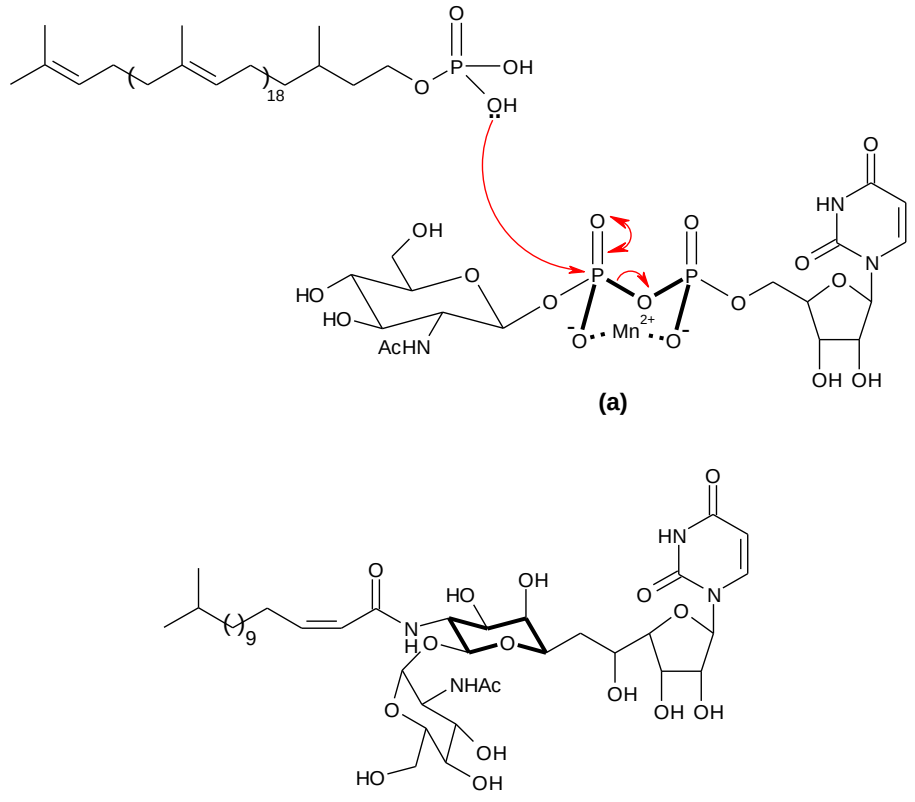


Figure 8. Superimposed crystal structures of α-D-glucose and a polymerase-bound NTP

There is precedent in the literature for the use of monosaccharides as diphosphate mimics. This primarily stems from the discovery of the naturally occurring nucleoside antibiotic tunicamycin, first discovered and isolated from Streptomyces lysosuperificus by Takatsuki *et al.* in 1971 (Takatsuki, Arima, & Tamura, 1971). Tunicamycin is actually a mixture of homologous antibiotics which differ in the chain length of their fatty acid components, the most important of which is tunicamycin V (Scheme 2b) (Sarabia, Martín-Ortiz, & López-Herrera, 2003).The antibacterial action of tunicamycin is due to the inhibition of translocase MraY (or translocase I), an enzyme essential for bacterial cell wall peptidoglycan biosynthesis (Schouten *et al.*, 2006). However, in eukaryotic cells it also inhibits N-linked protein glycosylation, an important process for the folding of some proteins, by inhibiting UDP-GlcNAc:dolichyl-phosphate GlcNAc-1-phosphate transferase, the enzyme responsible for catalysing the transfer of GlcNAc-1-phosphate from UDP-GlcNAc to dolichol phosphate. This non-specific activity occurs because tunicamycin is a donor-acceptor bisubstrate mimetic which contains a monosaccharide unit that mimics the diphosphate-Mn2+ complex of the natural donor substrate UDP-GlcNAc (Scheme 2a) (Heifetz, Keenan, & Elbein, 1979). Due to this mechanism of action, tunicamycin exhibits very high toxicity in mammals, and so is unsuitable for use in humans (Sarabia *et al.*, 2003).



Scheme 2: a) Natural donor & acceptor substrates of GlcNAc-1-phosphate transferase b) Donor-acceptor bisubstrate mimetic tunicamycin V (Pyrophosphate-metal chelate and mimicking monosaccharide unit are in bold)

Mimicry of the diphosphate group was the strategy of Wang and co-workers in the development of inhibitors of β-1,4-galactosyltransferase (β-1,4-GalT), an enzyme that catalyses the transfer of a galactose unit from UDP-galactose onto an acceptor sugar. The diphosphate moiety of the natural substrate UDP-galactose (UDP-Gal) forms a

six membered chelate ring with a Mn2+ ion (fig 9a). Taking inspiration from tunicamycin, it was decided to replace this pyrophosphate with a glucose residue, resulting in 5'-O-β-lactosyl-uridine (fig 9b). This molecule was found to be a competitive inhibitor of the β-1,4-GalT in L1210 leukaemia ascites fluid, with a Ki of 120 μM (R. Wang *et al.*, 1997). This is very similar to the KM of the natural substrate, 127 μM, indicating that the inhibitor is able to effectively compete with the natural substrate for the active site of the enzyme. In this particular case, this implies that glucose is a good bioisostere of the diphosphate-metal chelate.

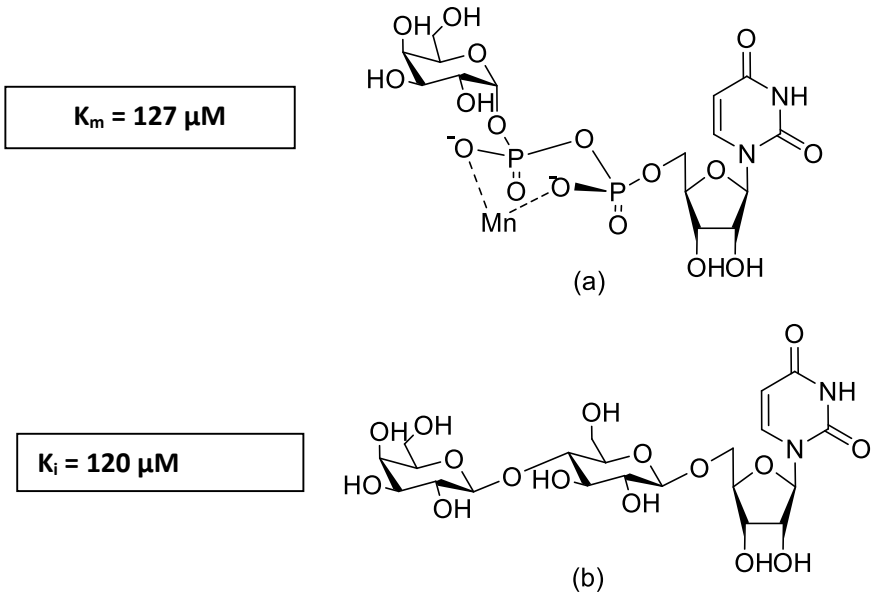


Figure 9. Natural substrate UDP-Gal (a) and competitive inhibitor (b) of β-1,4-GalT from L1210 leukaemia ascites fluid

Yuasa and co-workers synthesised 2,4-diaminopentopyranosides as hinge molecules that change conformation from $_4C^1$ to $_1C^4$, like a hinge, upon the chelation of metal ions. It was envisaged that these compounds would serve as diphosphate mimics because they can chelate Mn2+ through the hinge motion and that the pyranose structure would be accommodated into the diphosphate cleft of GTs. The hinge-like motion potentially endows the inhibitors with induced-fit abilities (Mitsuhashi & Yuasa, 2009).

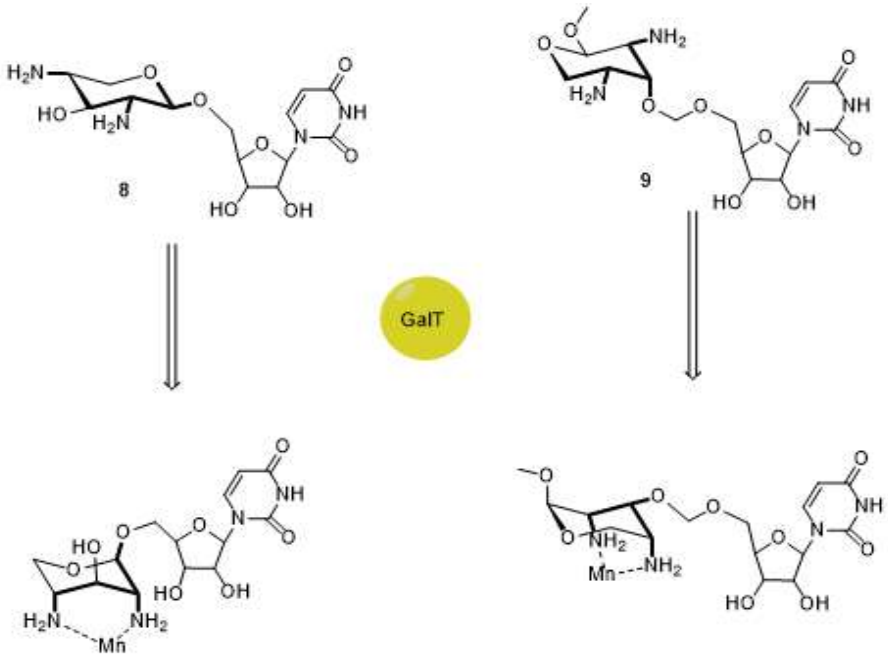


Figure 10. Proposed mechanism for GalT inhibition

Similarly Hamilton and his group (Rowan *et al.*, 2009) synthesised a library of nucleoside triphoshate mimetics where the Mg2+ chelated triphosphate side chain was replaced by an uncharged methylene-triazole linked monosaccharide side chain.

The compounds were evaluated as Bacillus anthracis pantothenate kinase inhibitors and a competitive inhibitor was detected with a Ki 3 times lower than ATP's KM value.

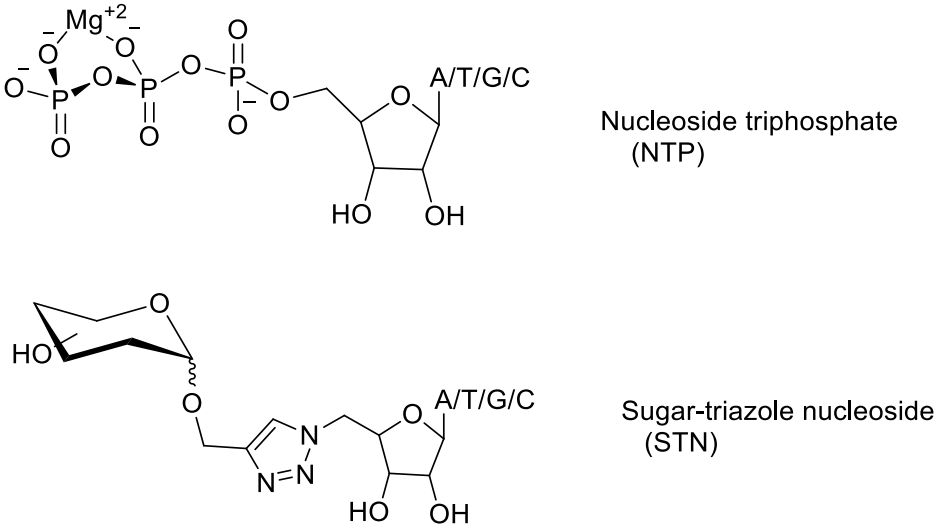
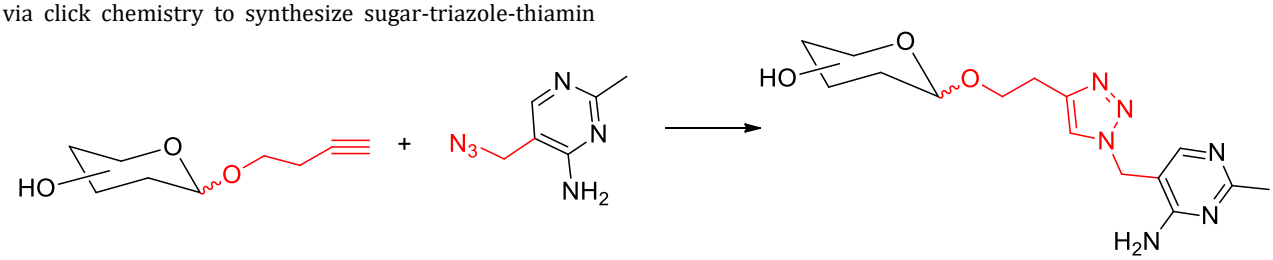


Figure 11. Proposed NTP mimics

Iqbal *et al.* (2012) investigated the possibility to mimic the diphosphate with various neutral and more stable sugar analogues without compromising too much binding affinity for the enzyme. For this purpose, variety of anomerically pure (where possible) butynyl glycosides of monosaccharides such as glucose, N-acetyl glucosamine, galactose, mannose and xylose were synthesized. Finally, these glycosides were linked with aminopyrimidine azide via click chemistry to synthesize sugar-triazole-thiamin (STT) compounds. It is well known that triazole rings can be easily formed by the use of 'click' chemistry in just one step. One of the most recognised reactions in terms of 'click' chemistry is the 1,3-dipolar cycloaddition of a terminal alkyne and an azide (Rostovtsev, Green, Fokin, & Sharpless, 2002). The synthetic scheme for the synthesis of sugar-triazole-thiamin derivatives is (scheme 3) is:



Scheme 3: Proposed scheme for the synthesis of STT

During the investigation of binding behaviour of these sugar analogues with PDC, it turned out that all sugar analogues bind weakly and were displaced by ThDP when the enzyme activity assay is started (ThDP is added in a large excess) and consequently, any binding that might have occurred will not be seen. All ligands were docked in ZmPDC using Glide (Rostovtsev *et al.*, 2002).

Both of these figures show that similar to the binding mode of crystal triazole ThDP in active site of ZmPDC , these sugar based analogues are also bound in a pocket of PDC with the 'V' conformation. Furthermore, sugar moieties can occupy the binding pocket of PDC and side chain –CH2OH at position 5 is not restricting their entry.

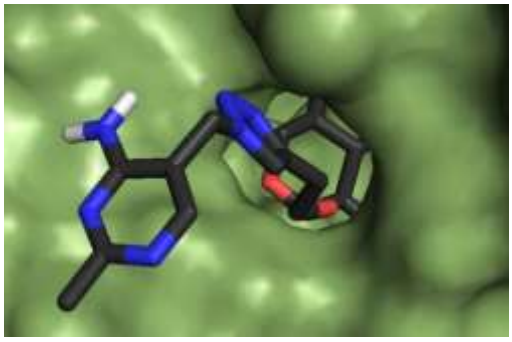


Figure 12. Entry of xylose sugar into the binding pocket of PDC

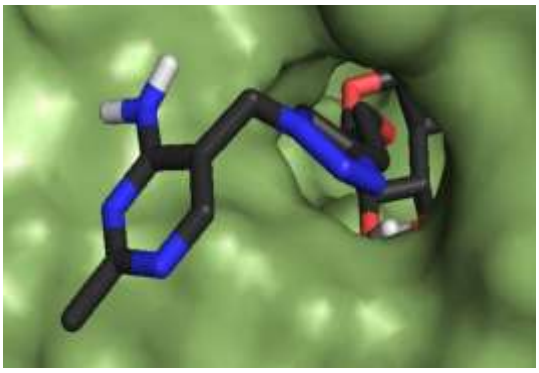


Figure 13. Entry of glucose sugar (used as representative of other sugars with side chain at position-5) into binding pocket of PDC

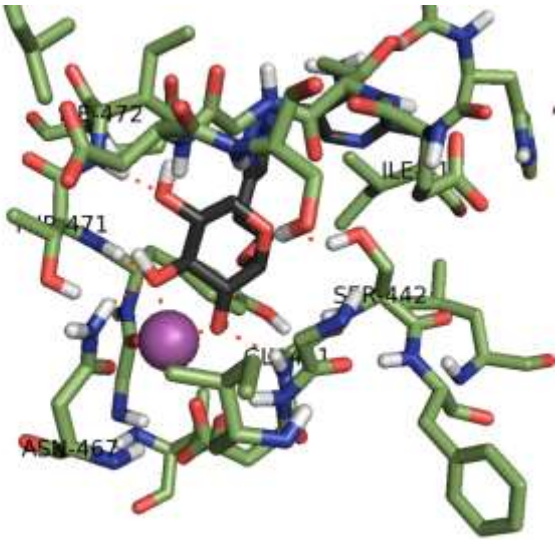


Figure 14. Binding interactions between xylose sugar and amino acid residues in the active site of PDC after docking

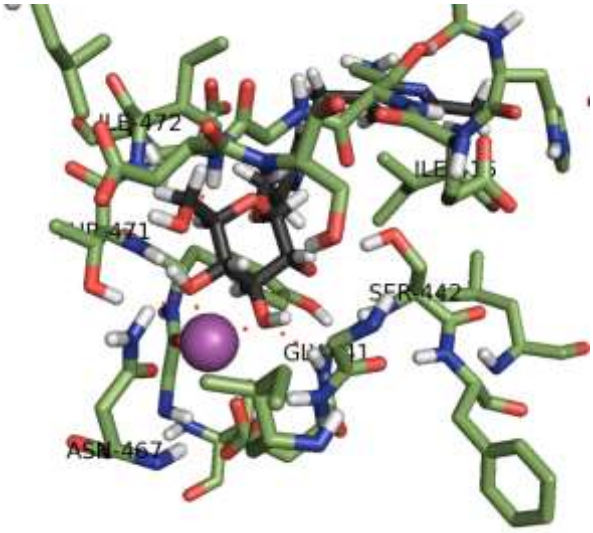


Figure 15. Binding interactions between glucose and amino acid residues in the active site of PDC after docking

Binding interaction of both xylose and glucose with binding pocket residues is almost identical. Our docking results show that –OH group on sugar ring not only form hydrogen bonds with amino acid residues, but also co-ordinate with the Mg+2 in the active site. So, at this point, it is not clear that why xylose show some inhibition during inhibition study as compared to other sugar analogues.

CONCLUSION

Being a highly charged group, diphosphate is not suitable for pharmaceutical development and compounds possessing it will suffer from poor bioavailability and cellular uptake. With this in mind, the possibilities of mimicking the diphosphate with various neutral and more stable surrogates has been investigated by various research groups.

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