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SHORT COMMUNICATION

Differential inhibition of homotrimeric dUTPases by the 3′-azido derivative of dideoxy-UTP

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Abstract

The inhibitory effects of 3′-azido-2′,3′-dideoxyuridine-5′-triphosphate in complex with the Mg^{2+} ion (azido-ddUTP·Mg) on the dUTPases of the human, *E. coli*, and equine infectious anemia virus have been compared. Azido-ddUTP is analogous to drugs used in the treatment of HIV. Here it is shown to inhibit the bacterial dUTPase in a competitive manner ($K_i = 9.3$ μM), but to exhibit only marginal or no binding to the human and viral dUTPases, respectively. This is the first demonstration of an inhibitor with a strong preference for binding to a bacterial dUTPase over the human enzyme. The specific binding to the *E. coli* dUTPase is surprising in view of the close to identical substrate pockets among the three dUTPases tested. The results are discussed with reference to the possibility of designing active site directed inhibitors that bind to the homotrimeric dUTPase of a pathogen but not to the human form.

Keywords: *Azido-ddUTP; inhibition; specificity; homotrimeric dUTPases*

Abbreviations: dUTPase, deoxyuridine triphosphate nucleotidohydrolase; dUTP, deoxyuridine triphosphate; E. *coli*, *Escherichia coli*; azido-ddUTP, 3′-azido-2′,3′-dideoxyuridine-5′-triphosphate; dUMP, deoxyuridine monophosphate; dUDP, deoxyuridine diphosphate; TDP, thymidine diphosphate; EIAV, equine infectious anemia virus; IPTG, isopropyl-β-D-thiogalactopyranoside; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; DTT, 1,4-dithiothreitol; PMSF, phenylmethylsulfonyl fluoride.

Introduction

The ubiquitous enzyme deoxyuridine triphosphatase, or dUTPase, catalyzes the hydrolysis of dUTP into dUMP and pyrophosphate. Proliferating cells that lack dUTPase will accumulate dUTP and incorporate uracil instead of thymine in the growing DNA chain, which leads to fragmentation of nascent DNA and cell death[1–4]. The fact that dUTPase activity is essential to both prokaryotic and eukaryotic cells[5,6] makes the dUTPases attractive as targets for drugs against cancers and infectious diseases[7–11]. This also indicates that a drug intended to cure an infectious disease of the human by inhibiting the dUTPase of the pathogen, whether this is a virus, a bacterium, or a protozoan, must exhibit poor binding to the human dUTPase. However, the substrate pockets of the dUTPases of these and most other pathogens are closely homologous with that of the human enzyme[12], which makes the development of pathogen specific dUTPase inhibitors a difficult task.

To reduce the occurrence of resistance through spontaneous mutations, it is desirable that an inhibitor drug interacts primarily with conserved and functionally important groups of the target protein. With an enzyme as target, this is most easily accomplished by modifying the substrate itself. In the case of dUTP this would include removal of the phosphate moiety to enhance the cellular uptake. However, a drug based on deoxyuridine, and too small to reach out of the substrate pocket, may not achieve sufficient specificity and binding strength. To increase the chances for both tight and pathogen specific binding, it may become necessary to add groups to the deoxyuridine frame extensive enough to explore potential and less conserved binding sites outside the substrate pocket. Some progress along these lines toward an inhibitor of the *P. falciparum* dUTPase has recently been reported[13–15]. Also, triskelion shaped molecules carrying uracil at one or two of their flexible legs were recently shown to inhibit the human enzyme[16].

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This is not meant to say that pathogen specificity and tight binding has nothing to gain from more limited changes to the substrate. In the present work, we tested the reactivity of the substrate derivative 3′-azido-2′,3′-dideoxyuridine-5′-triphosphate in complex with the Mg^{2+} ion (azido-ddUTP·Mg) with the human, the *E. coli*, and the equine infectious anemia virus (EIAV) dUTPases. This derivative has been reported to inhibit the human immunodeficiency virus (HIV) reverse transcriptase[17]. It is analogous to the anti-HIV drug 3′-azido-dideoxythymidine (AZT; Zidovudine), which inhibits the HIV reverse transcriptase, as well as other enzymes involved in nucleotide metabolism[18].

Materials and methods

Materials

The dUTP was purchased from Jena Bioscience GmbH (Jena, Germany) and the azido-ddUTP from TriLink Bio Technologies (San Diego, USA). Other chemicals used (Sigma-Aldrich Sweden AB) were of analytical grade. Equipment for electrophoresis was from Bio-Rad (Bio-Rad Laboratories AB, Sweden), and for protein purification from GE Healthcare Bio-Sciences AB (Uppsala, Sweden).

Enzyme expression, extraction, and purification

The respective plasmids containing the *E. coli*, the human, and the EIAV dUTPases coding gene pET3A/dut were used to transform competent cells *E. coli* BL21(DE3)pLysS (Novagen, UK) according to standard protocols[19]. A single colony, selected with chloramphenicol (34 μg/mL) and ampicillin (100 μg/mL), was used as the starting point for each culture. The cultures were grown to an OD_{600} (optical density at 600 nm) of 0.5–0.7, after which they were induced with IPTG (1 mM, final concentration) over 5 h at 37°C. The cells were harvested by centrifugation at 6650 × *g* (Beckman) for 45 min at 4°C. The supernatant was discarded and the pellets were stored at –20°C for extraction. The pellets were suspended in extraction buffer, Hepes (20 mM, pH 7.5), with the following additives: EDTA, EGTA, PMSF, and DTT, all at 1 mM, and glycerol to 10%. The extraction protocol involved three cycles of freezing and thawing, followed by three sonication cycles, each lasting for 4 min and performed on ice. The sonication was set to pulses of 2 s with a 60 mA pulse. The sonicated suspension was centrifuged at 6650 × *g* for 25 min at 4°C. The three dUTPases were isolated as described previously[20]. In short, the respective cleared extract was loaded on phosphocellulose (P11; Whatman International Ltd., England) packed in an XK26 column to a final bed volume of 16 mL. The proteins were eluted using a KCl gradient (0.04–1 M) in Hepes buffer (20 mM, pH 7.5). The fractions eluted were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)[21]. Fractions containing dUTPase were pooled. Each of the resulting enzyme solutions was estimated to be about 85% pure in dUTPase on a protein basis, as judged from the bands in the gel. The concentration of each enzyme was calculated based on this number, the 280 nm absorbance, and the associated extinction coefficient[22–24]. It is expressed throughout as the active site concentration.

Activity measurements

The hydrolysis of dUTP catalyzed by the three enzymes was monitored spectrophotometrically using the equipment and assay method described previously[25]. In addition to enzyme (0.2 μM) and substrate (2 μM), the reaction mixture contained $MgCl_2$ (5 mM), cresol red (50 μM), bicine (50 μM), and KCl to a final ionic strength of 0.1 M[25]. The inhibitory properties of azido-ddUTP were tested by including it at three concentrations in the 10–100 μM range in the standard reaction mixture. Each measurement was repeated three times or more. The azido-ddUTP·Mg was tested as a substrate for the three dUTPases by including it in the reaction mixture in place of dUTP.

Data evaluation

The differential equations describing the rate of change of the species of the Michaelis–Menten reaction were fitted numerically to the normalized data traces, using the program Dynafit[26]. The apparent V_{max} and K_M (maximum velocity and Michaelis constant) associated with each reaction were calculated based on the best fit rate constants.

Results

The azido-ddUTP·Mg substrate derivative was not hydrolyzed by any of the enzymes tested. Indicator absorbance progress curves reflecting the complete hydrolysis of the dUTP, in the absence and presence of azido-ddUTP, by the *E. coli*, human, and EIAV dUTPases were collected. The resulting traces were normalized to show product formation[25] and are displayed in Figure 1. A qualitative difference in the response to the presence of azido-ddUTP among the three dUTPases is obvious: the bacterial form is affected in a concentration dependent manner, whereas only a marginal or no effect at all is observed with the human and viral enzymes, respectively. A quantitative evaluation of the data obtained with the *E.coli* dUTPase in the absence and presence of azido-ddUTP, at 20, 40 and 60 μM, respectively, indicated that the inhibition is competitive, with an average K_i (inhibition constant) of 9.3 ± 1.2 μM (8.7, 8.9, 10.6 μM, at the respective inhibitor concentration tested) (Table 1). To illustrate the competitive nature of the inhibition and the amount of information contained in each complete reaction trace, the traces obtained with the *E. coli* dUTPase as a catalyst, without or with azido-ddUTP (60 μM), were converted to the corresponding Lineweaver–Burk data (Figure 2), as described in the legend to the figure. Simulations of the reactions catalyzed by the human and viral enzymes indicated that an effect of azido-ddUTP at the highest concentration tested (100 μM) should be clearly visible if the K_i is 1 mM or less, suggesting this number as the lower limit for the K_i of binding azido-ddUTP·Mg to the human and viral dUTPases.

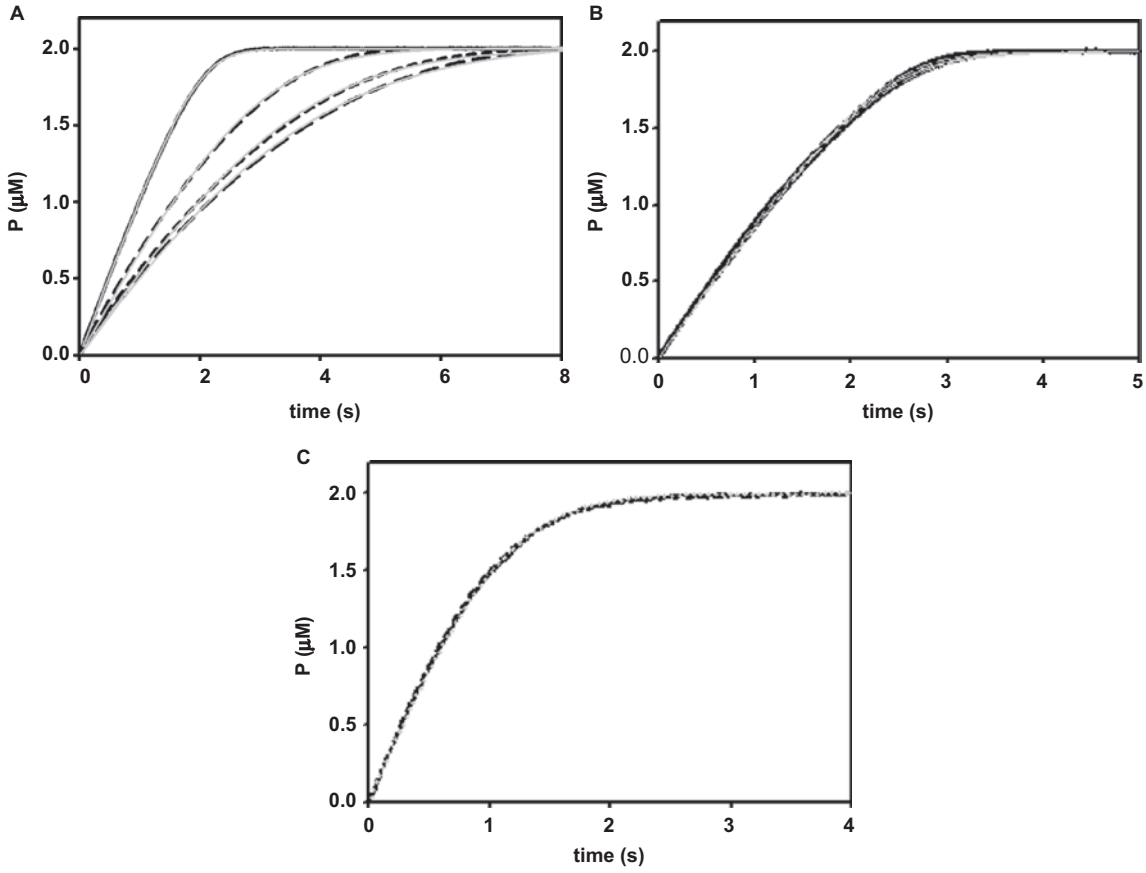

Figure 1. Inhibition of homotrimeric dUTPases by 3′-azido-ddUTP·Mg. Reaction traces showing the effects of azido-ddUTP·Mg on the reactions catalyzed by (A) the *E. coli*, (B) the human, and (C) the EIAV dUTPases. The traces were obtained by reacting the enzymes (0.2 μM) with substrate (2 μM) in the presence of Mg^{2+} (5 mM) before (solid black lines) and after including azido-ddUTP (10–100 μM) (dashed black lines) in the reaction mixtures. The gray lines were obtained by simulating the reactions using the associated best fit parameters, as estimated by Dynafit.

Table 1. Binding of dUTP·Mg and azido-ddUTP·Mg to the *E. coli*, human, and EIAV dUTPases. Binding parameters (K_M and K_i) were calculated based on the best fit parameters obtained for the data presented in Figure 1.

	E. coli	Human	EIAV
$K_{M,dUTP}$ (μM)	0.22 ± 0.03	0.17 ± 0.02	0.96 ± 0.1
K_i (μM)	9.3 ± 1.2	>1000	>>1000

Discussion

This is the first demonstration of an inhibitor with a strong preference for binding to a bacterial dUTPase over the human form of the enzyme. To probe for a potential position of the azido group of azido-ddUTP·Mg, we modeled a 3′-azido nucleotide into the respective active site of the three enzymes. This was done by aligning the dideoxy-thymidine part of 3′-azido-dideoxy-TDP·Mg (AZT diphosphate), as it appears in complex with nucleoside diphosphate kinase (PDB (Protein Data Bank) ID: 1lwx), with matching nuclei of the α,β-imido-dUTP·Mg bound to the human and bacterial enzymes, or of the dUDP·Sr (PDB ID: 1duc) bound to the viral enzyme. This suggested that, in all cases, a substrate-like binding of the azido-ddUTP·Mg would require an adjustment of its position relative to the bound substrate analog,

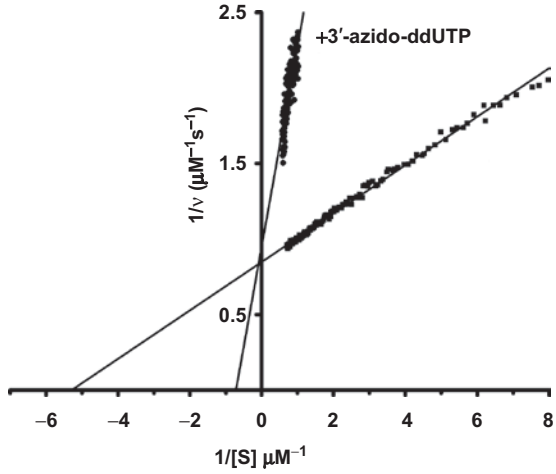

Figure 2. Lineweaver–Burk representation of complete reaction traces. The slope of the tangent to the progress curves obtained with the *E. coli* dUTPase before and after inclusion of azido-ddUTP·Mg (40 μM) was obtained pointwise along the curves as the local first derivative. The slopes were used as the reaction rates (v) resulting at the corresponding concentrations of residual free substrate [S].

or a twist of its sugar moiety, to avoid a steric clash with the strictly conserved tyrosine (Tyr93 in the *E. coli* dUTPase), as shown in Figure 3. Either change would place the azido

substituent closer to the strictly conserved and catalytically important aspartate (Asp90 in the *E. coli* dUTPase), where it may interfere with, or even displace, the substrate water molecule. This could explain why the derivative is not hydrolyzed by the dUTPase to which it binds. It serves to mention that 2′,3′-dideoxy-UTP·Mg is a good substrate for the human[27], the *E. coli*, and the EIAV dUTPases (unpublished data).

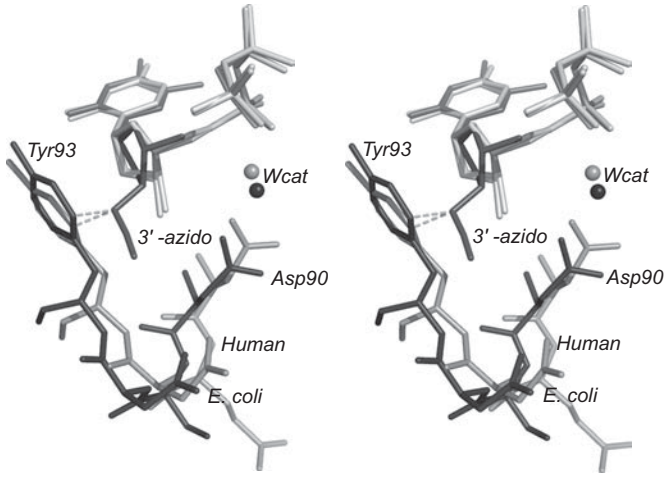

Figure 3. Stereo image of a model of azido-ddUTP·Mg binding to the human and *E. coli* dUTPases. Parts of the bacterial dUTPase in complex with the substrate analog α,β-imido-dUTP.Mg (PDB ID: 1rn8) were structurally aligned with the corresponding parts of the human dUTPase, also in complex with the imido-analog (2hqu). The selected parts carry the groups that may interfere directly with an azido-group at the 3′-position of the ribose moiety of a bound dUTP. The 3′-azido-dideoxythymidine part (AZT) of the 3′-azido-dideoxy-TDP·Mg, as it appears in complex with nucleoside diphosphate kinase (1lwx), was superimposed on the imido-analog bound to the human enzyme, using matching atoms of the two nucleotides. The AZT is shown in a darker nuance. The dashed lines mark the distances (~1.7 Å) from the N3′ of the modeled azido group to the closest ring carbon of the strictly conserved tyrosine, in the respective enzyme. The position of the putative substrate water molecule (Wcat) in the respective complex is indicated. Residues are numbered according to their position in the bacterial enzyme. (Figure generated with PyMol™, Copyright © 2006 DeLano Scientific LC.)

To further investigate why azido-ddUTP·Mg is excluded from the pockets of the human and viral dUTPases, or at least binds more weakly than to the bacterial form, the human and viral enzymes were aligned individually with the *E. coli* dUTPase, as demonstrated for the bacterial and human forms in Figure 3. Guiding the alignment by the atoms of the analogs only, resulted in an almost perfect match of the ligands, as shown at the top of Figure 3, and a slightly different orientation of the bound nucleotides relative to the respective protein. However, this difference was not observed in the corresponding alignment of the bacterial and viral dUTPases. We conclude that neither the individual models, nor the structural alignments, reveal an obvious explanation as to why the azido-ddUTP·Mg binds to the *E. coli* dUTPase but not to the other forms. Whatever the cause, our data show that the bacterial enzyme can adapt to the bulky azido substituent, whereas the energetic costs of the corresponding adaptations in the human and viral dUTPases are too high to allow significant binding even at a concentration of the derivative as high as 100 μM.

The binding of azido-ddUTP·Mg to the *E. coli* dUTPase is weaker than anticipated for a typical drug, but substitutions at other positions in the azido-dideoxyuridine frame may increase the binding strength and at the same time retain or improve the specificity. A sequence alignment (Figure 4) shows that whereas the human dUTPase exhibits a low homology (~30%) with that of the *E. coli*, the putative dUTPases of some pathogenic enterobacteria display close to identical sequences. This strongly suggests that the azido-ddUTP·Mg will bind also to these enzymes. However that may be, the final design of specific drugs must be engineered on an individual pathogen basis. Besides finding a potential lead for drug design, the virtue of the present study is of principal nature: it demonstrates that an analog of the substrate shows a strong preference for binding to one form of a homotrimeric dUTPase, despite the close to identical substrate pockets, and suggests that substitutions at the 3′-carbon of the deoxyribose moiety may prove fruitful in achieving specificity.

Figure 4. Alignment of the sequences of isolated and putative dUTPases. The alignment was made using the Clustal W program[28] and compares the sequence of the *E. coli* dUTPase with that of the putative dUTPases of some pathogenic enterobacteria, as well as that of the human dUTPase. Lowercase gray residue symbols on a white background are used for positions non-homologous to the *E. coli* dUTPase. The human dUTPase, which shows less than 30% sequence homology with the *E. coli* form, nevertheless folds and assembles into a structure closely overlapping that of the *E. coli* enzyme, as well as other homotrimeric dUTPases.

Acknowledgements

***Declaration of interest*:** The authors report no conflicts of interest.

References

1. Tye BK, Nyman PO, Lehman IR, Hochhauser S, Weiss B. Transient accumulation of Okazaki fragments as a result of uracil incorporation into nascent DNA. *Proc Natl Acad Sci USA* 1977;74:154–7.
2. Tye BK, Chien J, Lehman IR, Duncan BK, Warner HR. Uracil incorporation: a source of pulse-labeled DNA fragments in the replication of the Escherichia coli chromosome. *Proc Natl Acad Sci USA* 1978;75:233–7.
3. Lehman IR, Tye BK, Nyman PO. Excision-repair of uracil in DNA: its implications for the discontinuous replication of DNA in vivo and in vitro. *Cold Spring Harb Symp Quant Biol* 1979;43:221–30.
4. Richards RG, Sowers LC, Laszlo J, Sedwick WD. The occurrence and consequences of deoxyuridine in DNA. *Adv Enzyme Regul* 1984;22:157–85.
5. el-Hajj HH, Zhang H, Weiss B. Lethality of a dut (deoxyuridine triphosphatase) mutation in Escherichia coli. *J Bacteriol* 1988;170:1069–75.
6. Gadsden MH, McIntosh EM, Game JC, Wilson PJ, Haynes RH. dUTP pyrophosphatase is an essential enzyme in Saccharomyces cerevisiae. *EMBO J* 1993;12:4425–31.
7. Zalud P, Wachs WO, Nyman PO, Zeppezauer MM. Inhibition of the proliferation of human cancer cells in-vitro by substrate-analogous inhibitors of dUTPase. *Adv Exp Med Biol* 1994;370:135–8.
8. McIntosh EM, Haynes RH. dUTP pyrophosphatase as a potential target for chemotherapeutic drug development. *Acta Biochim Pol* 1997;44:159–71.
9. Grässer FA, Romeike BF, Niedobitek G, Nicholls J, Kremmer E. dUTPase in human neoplastic cells as a potential target for therapeutic intervention. *Curr Protein Pept Sci* 2001;2:349–60.
10. Ladner RD. The role of dUTPase and uracil-DNA repair in cancer chemotherapy. *Curr Protein Pept Sci* 2001;2:361–70.
11. Studebaker AW, Balendiran GK, Williams MV. The herpesvirus encoded dUTPase as a potential chemotherapeutic target. *Curr Protein Pept Sci* 2001;2:371–9.
12. McGeoch DJ. Protein sequence comparisons show that the pseudoproteases encoded by poxviruses and certain retroviruses belong to the deoxyuridine triphosphatase family. *Nucleic Acids Res* 1990;18:4105–10.
13. Whittingham JL, Leal I, Nguyen C, Kasinathan G, Bell E, Jones AF, et al. dUTPase as a platform for antimalarial drug design: structural basis for the selectivity of a class of nucleoside inhibitors. *Structure* 2005;13:329–38.
14. Nguyen C, Ruda GF, Schipani A, Kasinathan G, Leal I, Musso-Buendia A, et al. Acyclic nucleoside analogues as inhibitors of Plasmodium falciparum dUTPase. *J Med Chem* 2006;49:4183–95.
15. McCarthy O, Musso-Buendia A, Kaiser M, Brun R, Ruiz-Perez LM, Johansson NG, et al. Design, synthesis and evaluation of novel uracil acetamide derivatives as potential inhibitors of Plasmodium falciparum dUTP nucleotidohydrolase. *Eur J Med Chem* 2009;44:678–88.
16. Jiang YL, Chung S, Krosky DJ, Stivers JT. Synthesis and high-throughput evaluation of triskelion uracil libraries for inhibition of human dUTPase and UNG2. *Bioorg Med Chem* 2006;14:5666–72.
17. Eriksson BF, Chu CK, Schinazi RF. Phosphorylation of 3′-azido-2′,3′-dideoxyuridine and preferential inhibition of human and simian immunodeficiency virus reverse transcriptases by its 5′-triphosphate. *Antimicrob Agents Chemother* 1989;33:1729–34.
18. Huang P, Farquhar D, Plunkett W. Selective action of 3′-azido-3′-deoxythymidine 5′-triphosphate on viral reverse transcriptases and human DNA polymerases. *J Biol Chem* 1990;265:11914–18.
19. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989.
20. Persson R, Nord J, Roth R, Nyman PO. dUTPase from Escherichia coli; high-level expression and one-step purification. *Prep Biochem Biotechnol* 2002;32:157–72.
21. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–5.
22. Hoffmann I, Widström J, Zeppezauer M, Nyman PO. Overproduction and large-scale preparation of deoxyuridine triphosphate nucleotidohydrolase from Escherichia coli. *Eur J Biochem* 1987;164:45–51.
23. Nord J. Nucleotide binding to trimeric dUTPase: studies on recombinant enzymes using kinetic and spectroscopic methods. Doctoral Thesis, Department of Biochemistry, Center for Chemistry and Chemical Engineering, Lund University, Sweden, 2000.
24. Bergman AC, Björnberg O, Nord J, Rosengren AM, Nyman PO. dUTPase from the retrovirus equine infectious anemia virus: high-level expression in Escherichia coli and purification. *Protein Expr Purif* 1995;6:379–87.
25. Larsson G, Nyman PO, Kvassman JO. Kinetic characterization of dUTPase from *E.coli*. *J Biol Chem* 1996;271:24010–16.
26. Kuzmic P. Program DYNAFIT for the analysis of enzyme kinetic data: application to HIV proteinase. *Anal Biochem* 1996;237:260–73.
27. Ingraham HA, Goulian M. Deoxyuridine triphosphatase: a potential site of interaction with pyrimidine nucleotide analogues. *Biochem Biophys Res Commun* 1982;109:746–52.
28. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;22:4673–80.