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Review

dUTP pyrophosphatase as a potential target for chemotherapeutic drug development*

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Aberrant dUTP metabolism plays a critical role in the molecular mechanism of cell killing induced by inhibitors of dihydrofolate reductase and thymidylate synthase. While considerable effort has been directed towards discovering new, more potent inhibitors of these two enzymes, little attention has been given dUTP pyrophosphatase (dUTPase) — the key modulator of cellular dUTP levels — as a potential target for chemotherapeutic drug development. Recent studies have provided evidence that dUTPase is vital for cellular and, in some cases, viral DNA replication. Furthermore, some retroviruses encode dUTPases — a fact which suggests that cellular dUTP metabolism may be more important than previously realized. Here, we briefly review current knowledge of cellular and viral dUTPases and discuss the potential of these enzymes as targets for cancer chemotherapeutic and anti-viral drug development.

In most, if not all cellular systems, uracil is not a normal component of DNA. It can arise in DNA, however, through the spontaneous deamination of cytosine, a process which occurs at low but biologically significant rates (Impellizzeri *et al.*, 1991; Lindahl, 1993). Alternatively, uracil can become incorporated into replicating DNA through the utilization of dUTP by DNA polymerases (see Williams, 1986). Under normal circumstances, the amount of uracil present in DNA is extremely low. This is due, in part, to the activity of uracil-DNA-glycosylase (uracil glycosylase) which catalyses the initial step in the excision repair process that specifically removes uracil residues from DNA (Lindahl, 1993). Additionally, the enzyme dUTP pyrophosphatase (dUTPase) hydrolyses dUTP to dUMP and PP_i and thereby reduces the availability of dUTP as a substrate for DNA polymerases (Fig. 1). Thus, uracil glycosylase and dUTPase are critical for the synthesis and maintenance of uracil-free DNA.

Although uracil has the same base-pairing and coding properties as thymine, its *extensive* incorporation into DNA is lethal in bac-

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Abbreviations: FIV, feline immunodeficiency virus; CAEV, caprine arthritis-encephalitis virus; EIAV, equine infectious anemia virus; dNTPs, deoxynucleoside-5′-triphosphates, FH_4, tetrahydrofolate; FH_2, dihydrofolate; 5-FU, 5-fluorouracil; FUdR, 5-fluoro-2′-deoxyuridine.

teria (El-Hajj *et al.*, 1988), yeast (Gadsden *et al.*, 1993) and likely all cellular systems. The reason for this is not completely known, however, the currently available data indicate that uracil glycosylase-mediated DNA repair is central to the process. For example, conditional mutations in the *E. coli* dUTPase gene (*dut*) are lethal at restrictive temperatures in the presence of a functional uracil glycosylase (*ung*) gene (Warner *et al.*, 1981). Deletion of the *ung* gene can suppress this lethality and lead to the stable incorporation of uracil into DNA (Warner *et al.*, 1981). Similarily, in yeast, DNA synthesis arrest caused by deletion of the dUTPase gene is dependent upon a functional uracil glycosylase gene (Gadsden *et al.*, 1993). Two related mechanisms may account for this effect. The first one involves the *ung*-dependent induction of extensive DNA strand breakage which leads to hyper-recombination, DNA fragmentation and cell death (Barclay *et al.*, 1982; Goulian *et al.*, 1980). According to this model, elevated dUTP levels lead to highly uracil-substituted DNA during replication. Uracil glycosylase then initiates excision repair at uracil-substituted sites. However, in the presence of persistently elevated dUTP levels, uracil becomes incorporated into the newly synthesized DNA repair patches. These substituted patches, in turn, become substrates for further rounds of excision repair such that the entire repair process is self-perpetuating. A consequence of this cyclical series of reactions is the generation of numerous single-strand breaks in the DNA at the sites of repair replication. In turn, these breaks serve as substrates for DNA recombination processes and, when excessive, lead to double-strand breaks, genome fragmentation and cell death. A second explanation is that the reiterative nature of this repair process simply delays the completion of cellular DNA replication such that cell division cannot be properly completed and cell death ensues. In either case, it is the combination of elevated dUTP levels during DNA replication, along with the activity of uracil glycosylase, that results in lethality. As outlined below, it is likely that these enzymes are central to the process of thymine-less death which can be induced by certain chemotherapeutic base and folate analogues. However, despite its potentially critical role in this process, little attention has been given to dUTPase as a potential target enzyme for the development of chemotherapeutic agents.

dUTP METABOLISM AND THYMINELESS DEATH

In addition to its role in eliminating dUTP as a substrate for DNA replication, dUTPase supplies a major source of cellular dUMP, the nucleotide substrate of thymidylate synthase (Fig. 1). This enzyme catalyses the

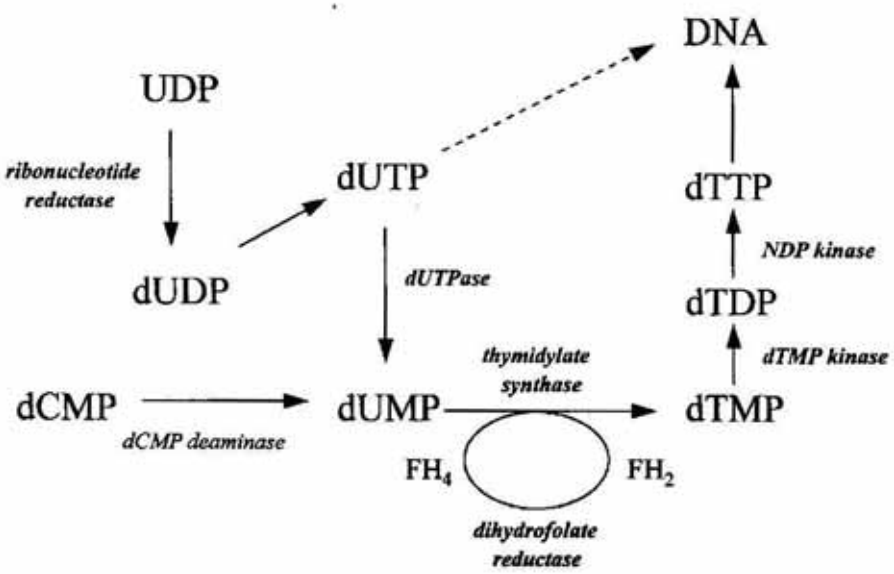
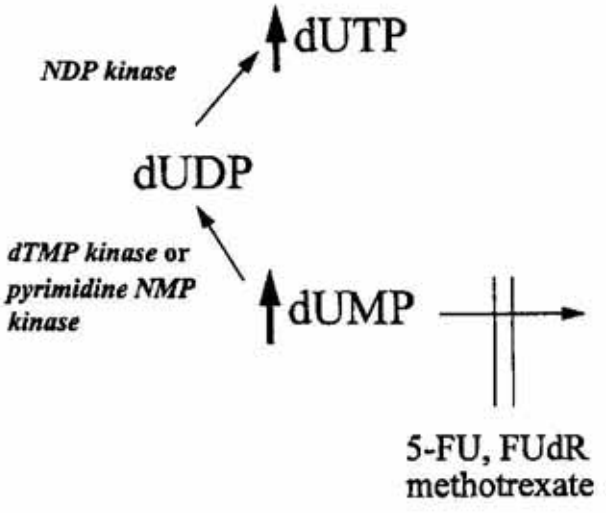


Figure 1. Enzymes involved in *de novo* pyrimidine dNTP synthesis.

dUTPase occupies a central position in this pathway and serves two roles; (i) to supply dUMP for thymidylate synthase, and (ii) to eliminate dUTP as a possible substrate for DNA polymerases (dashed arrow). The methyl group donor in the thymidylate synthase reaction is N^5N^{10}-methylene tetrahydrofolate which is synthesized from tetrahydrofolate (FH_4) and serine by serine hydroxymethyl transferase (not shown). FH_2, dihydrofolate.

reductive methylation of dUMP to yield dTMP which, in turn, generates dTTP for DNA replication. This reaction is of considerable therapeutic importance for two reasons. First, thymidylate synthase is essential for cell viability in the absence of exogenous thymine or thymidine. Inhibitors of the enzyme, such as the cancer chemotherapeutic agents 5-fluorouracil and FUdR, are lethal to dividing cells (the active metabolite of these analogues is 5-fluoro-2'-deoxyuridylate which forms a tight-binding ternary complex with thymidylate synthase). A second important aspect of this reaction is that it constitutes a major drain of reduced folates within a cell. Therefore, inhibition of folate synthesis also blocks the ability of thymidylate synthase to convert dUMP to dTMP (Fig. 1). This is important pharmacologically and is principally achieved using inhibitors of dihydrofolate reductase (DHFR) such as methotrexate, a widely used cancer chemotherapeutic agent, trimethoprim, which inhibits bacterial DHFRs, or pyrimethamine, an effective anti-malarial agent. Additionally, sulphonamide antibiotics block *de novo* folate synthesis and also inhibit this reaction.

Because of the proven clinical utility of thymidylate synthase and DHFR as therapeutic targets, considerable effort continues to be directed toward developing new, more effective inhibitors of these enzymes (Schweitzer *et al.*, 1990; Shoichet *et al.*, 1993). Unfortunately, less attention has been given to the molecular mechanism of cell-killing induced by these agents. Many researchers have assumed that cell death caused by inhibition of the thymidylate synthase reaction simply results from DNA synthesis arrest secondary to limitation of dTTP synthesis. Indeed, the process has been termed "thymineless death" to reflect this notion. However, considerable evidence points to aberrant dUTP metabolism and uracil glycosylase-mediated DNA damage as the underlying the mechanism of thymineless death. For example, inhibition of thymidylate synthase and DHFR through various means has been shown to induce DNA strand breakage which correlates with increased cell death in bacteria (Hochhauser & Weiss, 1978; Taylor, 1982; Tye *et al.*, 1977), yeast (Barclay *et al.*, 1982; Kunz *et al.*, 1980, 1994) and mammalian cells (Curtin *et al.*, 1991; Goulian *et al.*, 1980a; Goulian *et al.*, 1980b; Ingraham *et al.*, 1986; Richards *et al.*, 1986; Sedwick *et al.*, 1981). In bacteria, thymineless death can be suppressed by deletion of uracil glycosylase activity (Makino & Munakata, 1978), a finding which strongly implicates uracil-substituted DNA and excision repair in this process. Treatment of yeast with thymidylate synthase inhibitors, sulphonamide antifolates or DHFR inhibitors such as methotrexate, also induces extensive mitotic recombination, DNA strand breakage and cell killing (Barclay *et al.*, 1982; Kunz *et al.*, 1980, 1994). Studies with a variety of mammalian cell lines have shown that inhibitors of thymidylate synthase (FUdR, CB3717) and DHFR (methotrexate, metoprine) induce largely elevated intracellular dUTP:dTTP ratios and also promote the incorporation of uracil into DNA, all of which



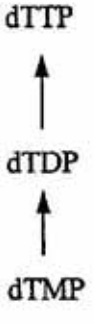


Figure 2. The possible route to elevated dUTP levels in response to inhibition of thymidylate synthase or DHFR.

dUMP accumulates behind the metabolic block imposed by methotrexate or fluoropyrimidines and its phosphorylated to dUTP *via* pyrimidine nucleoside monophosphate kinase, or thymidylate kinase, and nucleoside diphosphate kinase (NDP kinase). Since dTMP, and consequently dTTP synthesis is reduced, this results in a largely increased dUTP:dTTP ratio and favors uracil incorporation into DNA.

correlates with DNA strand breakage and cell death (Curtin *et al.*, 1991; Goulian *et al.*, 1980a; Goulian *et al.*, 1980b; Ingraham *et al.*, 1986; Richards *et al.*, 1986; Sedwick *et al.*, 1981). Similarly, starvation of proliferating bone marrow cells for folate or vitamin B_{12} (required for reduced folate synthesis) also promote uracil incorporation into DNA (Wickramasinghe & Fida, 1994).

How can inhibition of thymidylate synthesis cause such effects? One important, but often overlooked consequence of blocking thymidylate synthase is a drastic elevation of the dUTP:dTTP ratio within a cell (Goulian *et al.*, 1980a). The most dramatic effect reported is a $> 10^4$ increase in this ratio in human lymphoblasts treated with methotrexate (Goulian *et al.*, 1980a). This likely results from the massive build-up of dUMP behind the metabolic block and its subsequent phosphorylation to dUTP *via* nucleotide kinases as outlined in Fig. 2. dTTP levels are reduced because of the impaired *de novo* synthesis of dTMP, and dUTP levels increase presumably owing to supersaturation of dUTPase. The resulting increase in the dUTP:dTTP ratio facilitates the synthesis of uracil-substituted DNA which is then subject to attack by uracil glycosylase as outlined above.

More recently, Canman *et al.* (1993) provided further evidence for the role of dUTP metabolism in thymineless death. They found that resistance to FUdR-induced DNA damage and cytotoxicity in a human colorectal tumor cell line (SW620) correlated with elevated endogenous dUTPase activity relative to a control cell line (HT29). SW620 cells were found to have 5-fold more dUTPase activity than HT29 cells and accumulated far less dUTP and DNA strand breaks than HT29 cells treated with the same dose of FUdR. In a subsequent and complementary study, it was found that transfection of HT29 cells with a vector expressing the *E. coli* dUTPase gene conferred resistance to FUdR -induced DNA strand breakage and cell killing (Canman *et al.*, 1994). These studies have provided the first direct link between dUTPase activity and thymineless death induced by fluoropyrimidines.

Collectively, the findings described above provide strong evidence that dUTP metabolism is important in thymineless death. This indicates that dUTPase is generally a vital enzyme — a notion consistent with the estab-

Table 1. Identified dUTPase genes from various sources

Cellular	Retroviruses
Escherichia coli	equine infectious anemia virus (EIAV)
Haemophilus influenzae	feline immunodeficiency virus (FIV)
Coxiella burnetti	visna virus
Saccharomyces cerevisiae	ovine lentivirus
Candida albicans	sheep pulmonary adenomatosis virus (Jaagsiekte sheep retrovirus)
Tomato	mouse mammary tumor virus (MMTV)
Rat	simian retrovirus 1, 2 (SRV-1, SRV-2)
Human	human endogenous retrovirus K (HERV-K)
	human endogenous retrovirus L (HERV-L)
Herpesviruses	mouse, hamster intracisternal A particles
	squirrel monkey retrovirus
Epstein–Barr virus	
Varicella zoster virus	**non-herpes DNA viruses**
Herpes simplex virus 1 (HSV-1)	vaccinia virus
Bovine herpesvirus 1	African swine fever virus
Channel catfish virus	orf virus
Equine herpesvirus 1	avian adenovirus
Herpesvirus samiri	bacteriophage T4
	bacteriophage rlt

dUTPase genes that have been cloned or identified by DNA sequence analysis are listed. The herpesvirus enzymes have an atypical arrangement of the 5 conserved amino-acid sequence motifs and are functional as monomers. The channel catfish virus is unique among this group in that its motifs are arranged the same as that of the non-herpesvirus dUTPases which are all presumed to be active as homotrimers.

lished role of the enzyme in bacteria and yeast — and therefore a potentially useful target for chemotherapeutic drug development. The possible advantages of using dUTPase as a target are discussed later in this review.

dUTPases FROM VARIOUS SOURCES

dUTPase was initially characterized by Bertani *et al.* in 1961, and first purified from *E. coli* by Shlomai & Kornberg in 1978. In recent years, there has been an increased interest in this enzyme and dUTPase genes have been cloned and characterized from a diverse array of sources ranging from bacteria, fungi and plants to humans (Table 1). dUTPase genes have also been characterized from a broad spectrum of viruses including the medically important herpesviruses such as HSV-1, Epstein-Barr and varicella zoster virus (Table 1). Comparison of these enzymes reveals five well conserved amino-acid sequence motifs that may be regarded as signature sequences typical of dUTPases (McGeoch, 1990). The crystal structures of the *E. coli* and human dUTPases complexed with deoxyuridine nucleotides have been recently resolved to 2 Å resolution (Larsson *et al.*, 1996; Mol *et al.*, 1996). In both cases, the holoenzyme is found to be a homotrimer containing three active sites, each comprised of segments from the three subunits. A homotrimeric configuration has also been found for the EIAV enzyme (Bergman *et al.*, 1995) and it is likely that this will be true of most other dUTPases with the exception of the herpesvirus-encoded enzymes (described below). The 5 conserved motifs contain residues

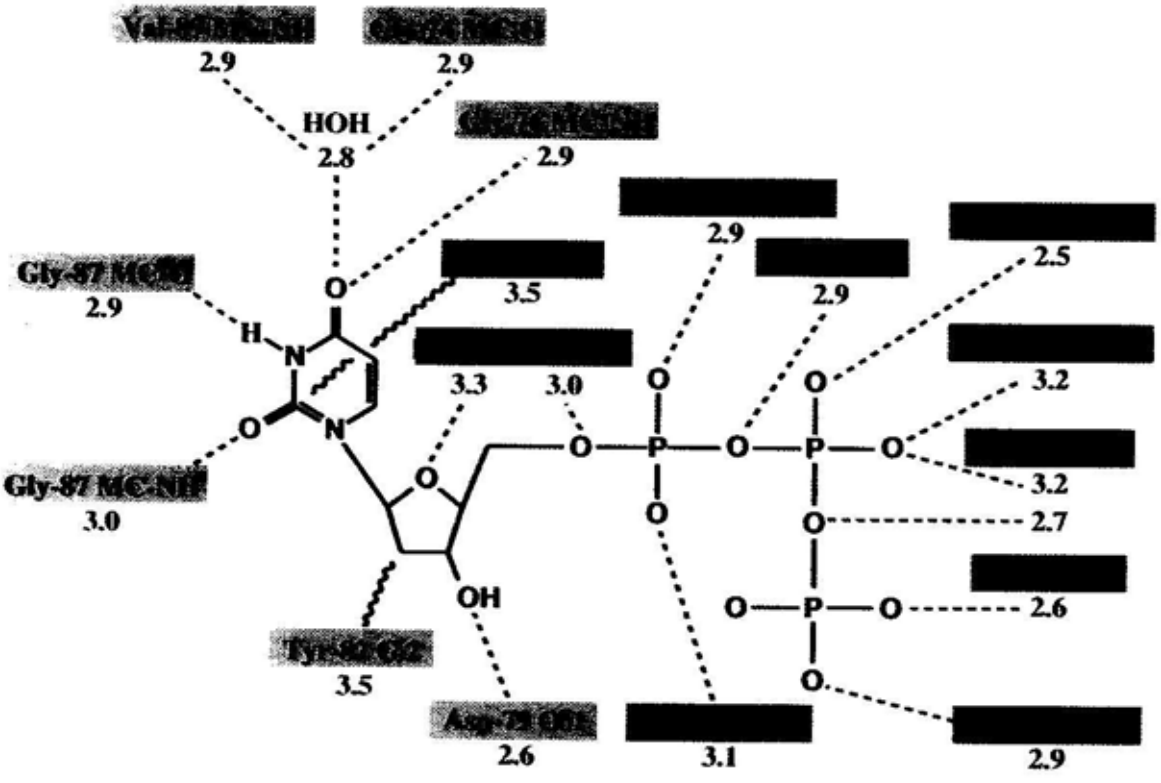


Figure 3. Substrate interactions with conserved amino-acid residues in the human dUTPase.

Dashed lines indicate hydrogen bonds with the donor-acceptor atom distance shown underneath each protein atom label. Wavy lines indicate hydrophobic interactions. The active site is formed by contributions from each of the three subunits. The 3 different colour codes correspond to residues from each individual subunit (this Figure is reproduced from Mol *et al.*, 1996, with kind permission of the authors).

that line the active sites in both the human and *E. coli* enzymes and contribute to substrate binding and catalysis. The amino-acid residues from each subunit that contribute to substrate binding by the human enzyme are shown in Fig. 3 (Mol *et al.*, 1996). A similar pattern of substrate binding occurs in the *E. coli* enzyme (Larsson *et al.*, 1996). This information has already proved to be useful in the design of initial inhibitory substrate analogues (described below). Crystallographic analysis of the *Candida albicans* dUTPase is currently in progress and comparison of this structure, particularly with the human enzyme, may be useful in the development of anti-fungal compounds (McIntosh *et al.*, 1994).

While most dUTPases characterized to date are similar in size and amino-acid sequence composition to the human and *E. coli* enzymes, surprisingly, this is not true of the herpesvirus dUTPases. These enzymes are distinct in three respects. First, they are considerably larger than the cellular or other

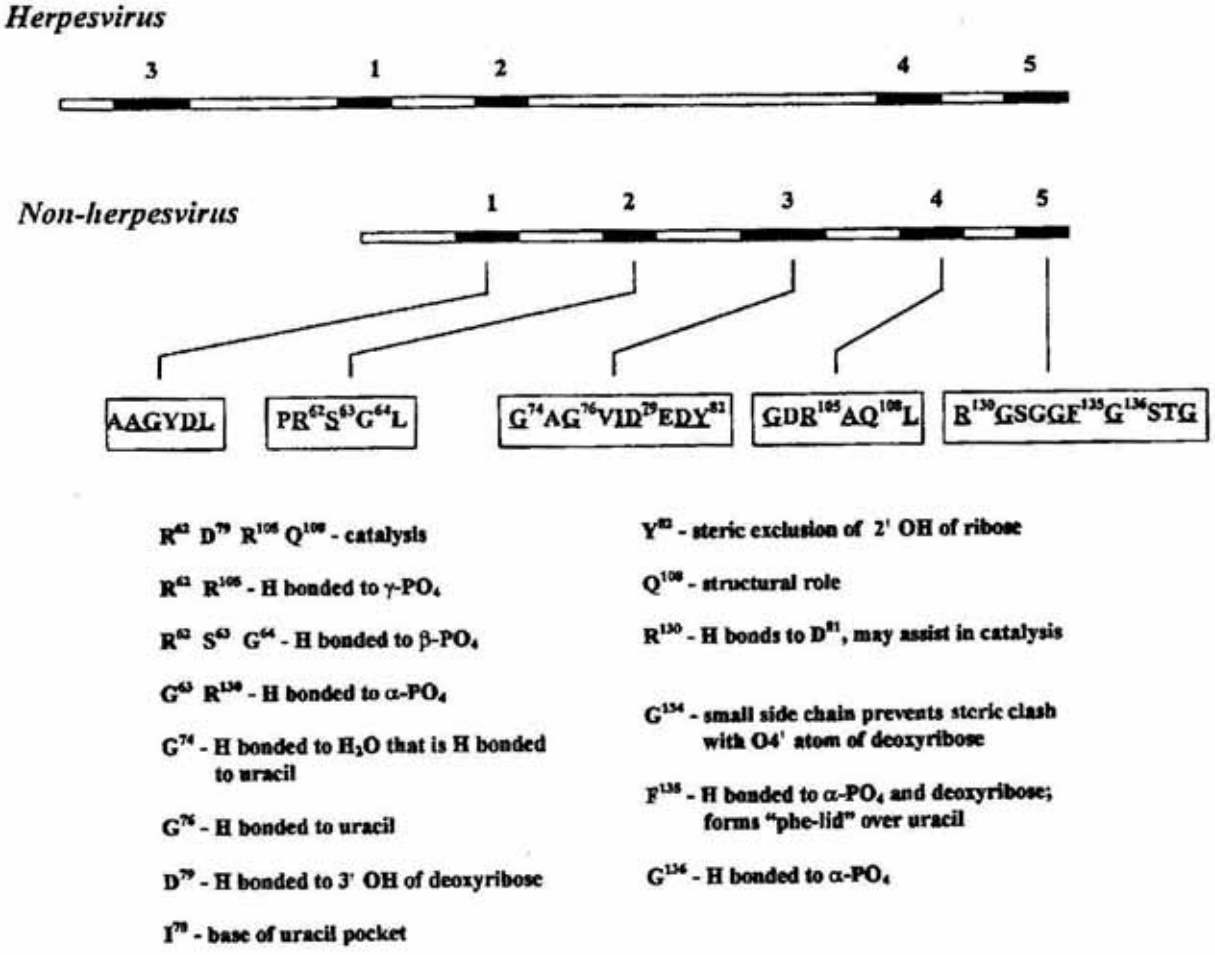


Figure 4. Schematic representation of herpesvirus and non-herpesvirus dUTPases.

The filled-in boxes indicate the positions of the 5 conserved amino-acid sequence motifs common among all currently known dUTPases. The linear order of these motifs is characteristically altered in the larger primary structures of the herpesvirus dUTPases (3-1-2-4-5) which are functional as monomers with the exception of the channel catfish dUTPase. The amino-acid sequences of the 5 human motifs are shown and the roles of residues within these motifs that are indicated numerically are summarized below (for more detail, see Strahler *et al.*, 1993). Residues that are well conserved between these motifs are underlined. Note that the numbering of amino-acid residues corresponds to that reported in references (McIntosh *et al.*, 1992; Strahler *et al.*, 1993), as opposed to the longer sequences of the nuclear and mitochondrial isoforms (Ladner *et al.*, 1996).

viral enzymes. Second, they display a disordered arrangement of the 5 conserved motifs common to dUTPases (Fig. 4). Third, the herpesvirus dUTPases are active as monomers as opposed to homotrimers (Caradonna *et al.*, 1984; McGeoch, 1990). Thus, the tertiary structure of these enzymes is considerably different from that of the non-herpesvirus dUTPases. Curiously, although dUTPase sequences have been reported for members of the β-herpesvirus group (cytomegalovirus, HHSV-6, HHSV-7), these putative dUTPases do not contain any of the 5 conserved amino-acids sequence motifs. At least in the case of CMV, the assignment of the dUTPase gene was based on its location in comparison with other herpesvirus genomes and enzyme activity has not been demonstrated for this particular gene. Thus, the designation of these genes as dUTPases may be incorrect and β-herpesviruses may characteristically lack such genes.

Interest in the herpesvirus enzymes stems from the suggestion that inhibitors specific for these dUTPases may be clinically useful (Williams, 1986, 1988). Consistent with this notion is the finding that mutants of HSV-1 defective in dUTPase display largely reduced virulence, neuroinvasiveness and reactivation from latency in a mouse model (Pyles *et al.*, 1992). As described below, some substrate analogues that inhibit the HSV-1 enzyme have been characterized; however, these compounds have low affinity for the enzyme and are not clinically useful.

As shown in Table 1, dUTPases are also encoded by poxviruses, avian adenovirus as well as A-, B-, and D-type retroviruses. The occurrence of the enzyme among the DNA viruses is not surprising since these viruses often encode other enzymes involved in dNTP metabolism, such as thymidine kinase and ribonucleotide reductase. This complement of enzymes facilitates viral replication in non-proliferating host cells because cellular dNTP metabolism and DNA replication enzymes, including dUTPase, tend to be cell-cycle regulated (Kunz *et al.*, 1994; Strahler *et al.*, 1993). Thus, analogous to mutants of HSV-1, swine poxvirus and various lentiviruses (EIAV, FIV, CAEV, visna) defective in dUTPase replicate with near-normal kinetics in proliferating cells but display moderate to severely reduced replication in non-proliferating hosts (Jöns & Mattenleiter, 1996; Lichtenstein *et al.*, 1995; Threadgill *et al.*, 1993; Turelli *et al.*, 1996). Mutant lentiviruses defective in dUTPase exhibit 10- to 100-fold reduction in viral titres following infection of whole animals (Lichtenstein *et al.*, 1995). The reason for this effect is unknown but may also involve uracil glycosylase-mediated DNA damage since uracil has been found to be incorporated into the cDNAs of such mutants (Steagall *et al.*, 1995).

The presence of dUTPase genes among various retroviruses was an unexpected discovery. Unlike the much larger pox- and herpesviruses, retroviruses do not encode any other enzymes involved in dNTP metabolism and so it is unclear why these viruses encode dUTPases. Perhaps most remarkable is the fact that non-primate lentiviruses (EIAV, CAEV, FIV and visna virus) contain dUTPase genes whereas the primate lentiviruses such as HIV do not. A theory to account for the existence of dUTPase genes among retroviruses and the absence of this gene from HIV has recently been presented (McIntosh & Haynes, 1996). Essentially, it proposes that dUTP, naturally synthesized in the cytoplasm of mammalian cells, functions as a genotoxic metabolite or "cellular defence mechanism" directed against viral parasites. It also suggests that induction of elevated cytoplasmic dUTP levels may be lethal to HIV. Whether or not this theory is correct, it is evident that cellular dUTP levels represent a problem for the replication of certain viruses and more remains to be learned about this particular aspect of dNTP metabolism.

dUTPase INHIBITORS

Although few inhibitors of dUTPases are currently known, some have been characterized. In an early study, Beck *et al.* (1986) described 6 uracil analogues which, when added to crude cell extracts, were metabolized to compounds that inhibited the human dUTPase. Unfortunately, K_i determinations were not reported. The unmetabolized base analogues did not exhibit any inhibitory ac-

tivity against purified enzyme. These compounds, however, were found to enhance methotrexate-induced increases in cellular dUTP:dTTP ratios and cytotoxicity against PHA-stimulated lymphocytes.

5-Mercuri-dUTP (HgdUTP) has a weak inhibitory effect on dUTPases from various sources (Williams, 1986) with the HSV-1 and HSV-2 enzymes having the lowest K_i values (27 μM and 21 μM, respectively). The analogue has less affinity for the human enzyme (K_i = 53 μM) and is of no pharmacological use. The crystal structure of the human dUTPase reveals that the uracil moiety of the substrate hydrogen bonds to main-chain amides and carbonyl groups within a β-hairpin region of the polypeptide backbone (Mol *et al.*, 1996). The proximity of the C-5 proton of the pyrimidine ring to three conserved amino--acid residues within this pocket preclude binding of analogues with bulky substitutions at this position. This accounts for the low affinity for HgdUTP and also the finding that 5-mercurithioethylene glycol-dUTP is not a substrate for the human enzyme (Williams, 1986). The latter analogue weakly inhibits the HSV-1 and HSV-2 dUTPases (K_i = 526 μM and 483 μM respectively) which indicates that subtle differences exist between the uracil recognition properties of the human and herpesvirus dUTPases. The same is true for 5-mercaptoguanosine mercuri-dUTP which specifically inhibits the HSV-1 and HSV-2 enzymes (K_i = 223 μM and 188 μM; Williams *et al.*, 1993). Various mercaptan derivatives of 5-mercuri-deoxyuridine effectively block HSV-1 and HSV-2 replication *in vitro*, however, it is unknown whether this effect is owing to inhibition of the viral dUTPase or some other aspect of viral replication (Holliday & Williams, 1991).

More recently, Zalud *et al.* (1995) described the synthesis of a few dUTP analogues with low K_i values as measured against purified *E. coli* dUTPase. M-dUTP (K_i = 2.5 μM) is a substrate derivative in which the oxygen atom between the α and β phosphate groups is replaced by a methylene bridge resulting in a non-hydrolyzable substrate. BM-dUTP (K_i = 0.3 μM) is a derivative of M-dUTP in which a benzyl group is covalently attached to the γ-phosphate. A second derivative containing two benzyl groups was also synthesized (DBM-dUTP; K_i not reported). These latter two compounds were designed to reduce the charge on the analogue in an attempt to increase cell permeability. α-Thio-dUTP (ASD) was also found to be inhibitory but with complex kinetics that precluded a K_i determination. Each of these analogues exhibited cytostatic and/or cytotoxic effects comparable to, or greater than that of methotrexate when tested against human lymphoma, melanoma and glioma tumor cell lines. Of these analogues, BM-dUTP and ASD displayed the greatest degree of cytotoxicity in these preliminary studies. Notably, fast-growing normal fibroblasts were largely resistant to the effects of the analogues (with the exception of BM-dUTP). Thus, tumor cell lines in particular seem to be especially sensitive to these dUTPase inhibitors.

A very recent report has also described an additional inhibitory dUTP analogue (Persson *et al.*, 1996). dUPNPP is similar to the aforementioned compounds, however, in this case replacement of the α-β bridging oxygen atom by an imido group results in a non-hydrolyzable substrate. This analogue also acts as a competitive inhibitor of the *E. coli* dUTPase (K_i = 5 μM), however, its toxicity toward human tumor cells was not determined.

Apart from synthetic compounds, naturally occuring protein inhibitors of dUTPases have been characterized for *Drosophila* and the *Bacillus subtilis* phage PBS2 (Dudley *et al.*, 1992; Nation *et al.*, 1989; Price & Frato, 1975). In the case of *Drosophila*, it is believed that the inhibitor is developmentally regulated and its role is to promote uracil incorporation into the DNA of cells destined for programmed cell death during the histolyzation process associated with pupation (Dudley *et al.*, 1992; Nation *et al.*, 1989). The *B. subtilis* phages PBS1 and PBS2 normally contain uracil in their DNA in the place of thymine (Takahashi & Marmur, 1963). Thus, the phage encoded inhibitor of the cellular dUTPase facilitates this process. Although these inhibitors are irrelevant from the pharmacological standpoint, they further illustrate the importance of dUTPase and dUTP metabolism in DNA replication and cell death.

dUTPase AS A CHEMOTHERAPEUTIC TARGET

The availability of dUTPase clones from various cellular organisms and viruses can facilitate purification of these enzymes in sufficient quantity for crystallization and structural analysis. Indeed, as mentioned above, this has already been accomplished for the human and *E. coli* dUTPases and is ongoing for the enzyme from the human pathogenic fungus *C. albicans*. In addition, the HSV-1 dUTPase has been overexpressed and purified from *E. coli* (Björnberg *et al.*, 1993). The low degree of amino-acid sequence identity between various cellular and viral dUTPases suggests that significant structural differences may exist among these enzymes such that species- or viral-specific inhibitors can be designed through molecular modelling techniques (Shoichet *et al.*, 1993), or by design based on knowledge of the "foliage" surrounding the substrate binding site or the mechanism of catalysis. In this regard, structural information of the human and *E. coli* dUTPases should prove to be of considerable benefit.

Perhaps the most promising target for drug development among these enzymes is the human dUTPase. Inhibitors of this enzyme may be useful cancer chemotherapeutic agents either alone or in combination with thymidylate synthase or dihydrofolate reductase inhibitors. Of special significance are observations that the level of dUTPase varies considerably between individual cell types (Beck *et al.*, 1986; Strahler *et al.*, 1993). This is consistent with Northern analysis which shows a large degree of variation in the levels of the major dUTPase mRNAs between different human tissues (McIntosh *et al.*, 1992). Particularly noteworthy is the report by Strahler *et al.* (1993) that dUTPase levels are unusually low in breast, lung and colon cancers whereas neuroblastomas and hematopoietic malignancies have high levels of the enzyme. Thus, the former group of cancers, which are currently difficult to treat, may be exquisitely sensitive to cell killing by dUTPase inhibition.

If the apparent variation in enzyme level between different tumor types is true of dUTPase *but not other chemotherapeutically targetted replication enzymes* (e.g. thymidylate synthase, DHFR, topoisomerase, DNA polymerases) then cancers with low levels of dUTPase may be best treated with dUTPase inhibitors rather than agents which target these other enzymes. For example, it is possible that the levels of thymidylate synthase, DHFR or other replication enzymes in tumors cells are the same as those in normal, non-transformed proliferating cells. Thus, 5-FU or methotrexate may be equally lethal to normal and tumor cells. However, if a reduced dUTPase level is an intrinsic property of particular tumors, then normal, non-transformed cell lines may be relatively resistant to dUTPase inhibitors — a feature that should confer selective killing of tumor cells at appropriate doses. A quantitative assessment of the levels of enzymes that are currently targetted by chemotherapeutic agents in various cancers would seem to be a useful endeavour in designing a therapeutic regime.

It is of interest to consider why dUTPase levels might be reduced in certain cancers. One explanation is that since dUTPase deficiency promotes genetic instability (i.e. enhanced recombination, mutation rates) in *E. coli* (Hochhauser & Weiss, 1978), viruses (Lerner *et al.*, 1995) and likely other systems as well, it is possible that mutations reducing, but not completely eliminating dUTPase, may act as tumor suppressors in humans. A low level of the enzyme, sufficient for cell viability, but insufficient to maintain a normal dUTP:dTTP ratio, might promote mitotic recombination events leading to loss of heterozygosity among tumor suppressor gene alleles, or gene rearrangements activating protooncogenes. Such a situation would be analogous to defects in mismatch repair genes such as occurs in hereditary non-polyposis colon cancers (Nicholaides, 1994). Notably, there are two isoforms of the human dUTPase, one mitochondrial and the other nuclear (Ladner *et al.*, 1996). Both isoforms are encoded by a single gene residing on chromosome 15 (Cohen *et al.*, 1997). Thus, two promoters plus differential transcript processing give rise to these isoforms. Mutations resulting in reduced expression of the

much more abundant nuclear isoform could account for a low intracellular level of the enzyme without resulting in lethality — assuming that the mitochondrial enzyme is just sufficient to maintain cell viability. This might explain the unusually low levels of dUTPase in breast, lung and colon cancers observed by Strahler *et al.* (1993).

Although methotrexate is a widely used cancer chemotherapeutic drug, resistance owing to DHFR gene amplification represents a significant problem. dUTPase inhibitors might be useful alternatives or adjuvants to methotrexate therapy to circumvent this problem, particularly in cells that are normally responsive to antifolate therapy. On the other hand, dUTPase over-expression could conceivably play a role in methotrexate or 5-FU resistance, as suggested by the results of Canman *et al.* (1993, 1994). Thus, dUTPase inhibitors might restore drug sensitivity to such resistant tumors.

Collectively, the results described above point to dUTPase as being a potentially useful target for drug discovery. The enzymes from various systems are readily available in large quantity and purified form. The characterization of appropriate substrate analogues or other inhibitory compounds is clearly needed, however, to fully address this issue.

SUMMARY

dUTPase has been largely overlooked as a potential target for drug development. This is likely owing to the proven success of using thymidylate synthase and DHFR as therapeutic targets, and may have been compounded by a lack of understanding of the importance of dUTP metabolism in DNA replication and thymineless death. Many studies have now correlated anti-thymidylate and antifolate agents with the induction of grossly elevated intracellular dUTP levels, uracil incorporation into DNA, and DNA damage associated with cell killing in mammalian cells. Genetic analyses have established that dUTPase — the primary modulator of cellular dUTP levels — is critical for the viability of bacteria and yeast. dUTPases are also encoded by certain viruses including the medically important herpesviruses, and the enzyme is important for optimal virus replication in whole animals. This indicates that the herpesvirus dUTPases may be useful targets for antiviral drug development and preliminary studies with various substrate analogues suggest that the binding determinants in the active sites of the human and herpesvirus enzymes may be quite different. The crystal structures of the human and *E. coli* dUTPases have recently been determined and some inhibitory substrate analogues have been developed. Initial results indicate that these analogues are toxic for human tumor cell lines. This finding, combined with indications that dUTPase levels are characteristically low in particular forms of cancer, suggest that these or other inhibitors of the enzyme may be useful cancer chemotherapeutic agents. Finally, the existence of naturally occuring inhibitors of cellular dUTPase, such as those found in *Drosophila* and phage PBS2-infected *B. subtilis*, combined with the relatively recent discovery that a variety of retroviruses encode dUTPases, supports the argument that cellular dUTP metabolism is of greater biological importance than previously realized and more remains to be learned about this particular aspect of DNA replication.

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