The nucleotidohydrolases DCTPP1 and dUTPase are involved in the cellular response to decitabine

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ABSTRACT

Decitabine (5-aza-2´-deoxycytidine, aza-dCyd) is an anticancer drug used clinically for the treatment of myelodysplastic syndromes and acute myeloid leukemia that can act as a DNA-demethylating or genotoxic agent in a dose-dependent manner. On the other hand, DCTPP1 and dUTPase are two "house-cleaning" nucleotidohydrolases involved in the elimination of non-canonical nucleotides. Here we show that exposure of HeLa cells to decitabine up-regulates the expression of several pyrimidine metabolic enzymes including DCTPP1, dUTPase, dCMP deaminase and thymidylate synthase thus suggesting their contribution to the cellular response to this anticancer nucleoside. We present several lines of evidence supporting that, in addition to the formation of aza-dCTP, an alternative cytotoxic mechanism for decitabine may involve the formation of aza-dUMP, a potential thymidylate synthase inhibitor. Indeed, dUTPase or DCTPP1 down-regulation enhanced the cytotoxic effect of aza-dCyd producing an accumulation of nucleoside triphosphates containing uracil as well as uracil misincorporation and double-strand breaks in genomic DNA. Moreover, DCTPP1 hydrolyzes the triphosphate form of decitabine with similar kinetic efficiency than its natural substrate dCTP and prevents decitabine-induced global DNA demethylation. The data suggest that the nucleotidohydrolases DCTPP1 and dUTPase are factors involved in the mode of action of decitabine with potential value as enzymatic targets to improve decitabine-based chemotherapy.

SUMMARY STATEMENT

The nucleotidohydrolases DCTPP1 and dUTPase modulate the cytotoxic activity of the antitumoral agent decitabine. We propose an additional mode of action for decitabine involving the potential inhibition of thymidylate synthase and the genotoxic incorporation of uracil in DNA.

Short title: Role of dUTPase and DCTPP1 on decitabine action

Keywords: DCTPP1, XTP3-TPA, dUTPase, nucleotide pool, decitabine

Abbreviations: DCTPP1, dCTP pyrophosphatase 1; aza-dCyd, 5-aza-2′-deoxycytidine, DNMT, DNA methyltransferase; DCTD, dCMP deaminase; TMPK, thymidylate kinase; TS, thymidylate synthase; TK, thymidine kinase; CDA, cytidine deaminase; DCK, deoxycytidine kinase; NDPK, nucleoside diphosphate kinase; siRNA, small interfering RNA; BER, base excision repair; ara-Cyd, cytarabine; dCyd, 2′-deoxycytidine; dF-CTP, 2′-difluorocytidine-5′-triphosphate; ddCTP, 2′,3′-dideoxycytidine-5′-triphosphate.

INTRODUCTION

Nucleoside analogues are effective antimetabolites commonly used as anticancer agents. The cytidine analog decitabine (5-aza-2´-deoxycytidine, aza-dCyd) is a DNA demethylating agent and genotoxic drug used clinically for the treatment of myelodysplastic syndromes and acute myeloid leukemia [1, 2]. Aza-dCyd is metabolically activated *in vivo* through consecutive phosphorylations into aza-dCTP that is readily incorporated into DNA and extended by the DNA polymerase [3]. Once in DNA, aza-dCyd acts as a suicidal substrate by covalently trapping DNA methyltransferase (DNMT) molecules that attempt to initiate cytosine methylation. The resulting DNA-protein cross-links trigger the proteosomal degradation machinery and lead to the depletion of the DNA methylation activities of the cell. Consequently, the replacement of deoxycytidine by aza-dCyd results in hypomethylation at the promoter DNA regions and the reactivation of epigenetically repressed genes [4]. The transcriptional activation of tumor suppressor genes which are aberrantly silenced in cancer cells constitutes the basic principle of the epigenetic cancer therapy.

The extent to which the *in vivo* antitumor properties of decitabine and the clinical response to decitabine treatment depend on epigenetic activities remains unclear. It has not been possible to establish a definitive correlation between the inhibition of cell proliferation and clinical response with the reversal of methylation and gene re-expression [5]. In fact, it is generally agreed that decitabine has dual effects on neoplastic cells in a dose-dependent manner; at low doses, cells survive but reactivation of genes that control proliferation may lead to differentiation, cell cycle arrest and increased apoptosis [6]. At high doses, decitabine induces genome-wide DNA damage and cytotoxicity [7]. Most of the DNA damage arises from the DNA-DNMT crosslinks which can block DNA synthesis and induce directly or indirectly DNA double-strand

breaks, eventually leading to cell death [8]. Decitabine has also been reported to enhance mutagenesis, mainly point mutations and genome rearrangements most likely due to protein-DNA crosslinks [9, 10]. The biochemical pathway that initiates repair of DNA-DNMT adducts has not been yet described in detail, however, it may involve DNA double-strand break repair factors at its late stages. It is well established that decitabine treatment induces the activation of a specific DNA damage response that includes the phosphorylation of histone H2AX [7]. Additionally, the cytotoxic and mutagenic properties of decitabine may be partially derived from its intrinsic chemical instability once it is incorporated into DNA or by the accumulation of DNA repair intermediates [3]. Recently, it has been proposed that the base excision repair mechanism (BER) might be initiating the repair of decitabine-induced DNA base lesions although the precise nature of the damage or the DNA glycosylases involved remains unknown [11].

The all-α NTP pyrophosphatase DCTPP1 (dCTP pyrophosphatase 1) is a pyrophosphohydrolase that contributes to the homeostasis of the dNTP pool in human cells by controlling the levels of dCTP, one of its major substrates. DCTPP1 can also hydrolyze C5-modified dNTPs such as 5-halogenated, 5-methyl and 5-formyl deoxycytidines, and therefore may have an additional 'house-cleaning' function [12]. The enzyme deoxyuridine triphosphate nucleotidohydrolase (dUTPase) also plays two roles, providing dUMP for *de novo* biosynthesis of dTTP and sanitizing the deoxynucleotide pool by the specific removal of dUTP [13-15].

In this study, we have investigated the role of DCTPP1 and dUTPase in the metabolic response to decitabine. We found that DCTPP1 or dUTPase down-regulation increased aza-dCyd-induced toxicity in HeLa and MRC-5 cells at a wide range of doses. DCTPP1 can hydrolyze the activated form of decitabine, 5-aza-2´-deoxycytidine-5´-triphoshate

and might constitute a novel route in the detoxification of this DNA methylation inhibitor. On the other hand, both, dUTPase- and DCTPP1-deficient cells, exhibited an accumulation of dUTP in the nucleotide pool and uracil in their genomic DNA as a consequence of the treatment, suggesting an alternate mode of action for decitabine in which the metabolism of aza-dCyd may ultimately lead to the genotoxic accumulation of dUTP in the cellular pool.

MATERIALS AND METHODS

Nucleotides, nucleosides and antibodies.

Decitabine (aza-dCyd), cytarabine (ara-Cyd) and 2′-deoxycytidine (dCyd) were purchased from Sigma; 5-aza-2′-deoxycytidine-5′-triphosphate (aza-dCTP), ara-CTP, 2′-difluorocytidine-5′-triphosphate (dF-CTP) and 2′,3′-dideoxycytidine-5′-triphosphate (ddCTP) were obtained from Jena Bioscience. Anti-DCTPP1 [12] and anti-dUTPase (this work) are anti-rabbit polyclonal antibodies generated in our laboratory. dCMP deaminase (DCTD), thymidylate kinase (TMPK), thymidylate synthase (TS) and thymidine kinase (TK) detection was carried out with anti-rabbit polyclonal antibodies from Santa Cruz Biotechnologies Inc. Cytidine deaminase (CDA) and deoxycytidine kinase (DCK) were detected with anti-rabbit anti-CDA and anti-mouse anti-DCK antibodies respectively **(**Abcam). Anti-α-tubulin was purchased from Ab Frontier.

Enzymatic assays and kinetic analysis

The pyrophosphohydrolase activity of DCTPP1 was determined using a continuous spectrophotometric assay previously described [12]. In a standard reaction (1 ml final volume), 10-250 μM of the nucleotide substrate was incubated in reaction buffer (20 mM $MgCl_2$, 100 mM KCl, 0.75 mg ml^{-1} BSA and 4 mM DTT) with concentrations ranging from 0.1 to 1 μM of DCTPP1. All reactions were carried out at 25 ºC. Kinetic parameters resulted from adjusting the data to the Hill equation:

$V_0 = (V_{max}[S]^{nH})/(K_m{}^{nH}+[S]^{nH})$. V_0, initial rate; V_{max}, maximum reaction rate; [S], substrate concentration; n_H, Hill coefficient; K_m, Michaelis-Menten constant.

Cell cultures and transfections

Two wild-type p53-expressing cell lines were used, the normal human fibroblast line MRC-5 (ATCC, CCL 171) and the tumor epithelial line HeLa (ATCC, CCL-2). Both cell lines were obtained from the American Type Culture Collection (ATCC) and authenticated by STR (Short Tandem repeat) DNA profiling. In this study, cells were not passaged for more than 6 months following purchase. MRC-5 was cultured in Eagle's Minimum Essential Medium (Gibco, Life Technologies) with 2 mM L-glutamine (GlutaMAX^{TM}, Gibco, Life technologies) and HeLa cell line was cultured in Dulbecco's Modified Eagle´s Medium (PAA, GE Healthcare) supplemented with 1X MEM Non-essential Amino Acids Solution (Gibco, Life technologies) and 2 mM L-glutamine. Media were supplemented with 10% fetal bovine serum (FBS), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Gibco, Life technologies). Cell lines were cultured in a humidified atmosphere of 5 % CO_2 at 37 ºC.

DCTPP1 and *dUTPase* silencing were carried out with small interfering RNA (siRNA) oligonucleotide pool (ON-TARGETplus smart pool, Dharmacon). The DCTPP1-specific siRNAs have been previously validated [12]. For *dUTPase* silencing, the following mRNA sequences were targeted: 5´-GCUCAUUUGCGAACGGAUU-3´, 5´-UGUAGGAGCUGGUGUCAUA-3´, 5´-UAGAGGAAAUGUUGGUGUU-3´ and 5´-UGCCUAUGAUUACACAAUA-3. The negative control consisted of four non-targeting siRNAs oligonucleotides (ON-TARGETplus non-targeting pool, Dharmacon). Transient transfections were carried out according to the manufacturer's instructions.

Proliferation and clonogenic assays.

10^3 cells were exposed to increasing doses of decitabine, deoxycytidine or cytarabine at 37 ºC for 24h (HeLa) or 72 h (MRC-5). Incubation times with the nucleoside analogs were based on differences in cell proliferation and sensitivity to decitabine. The medium

was exchanged and the drug replenished every 24 h. Viable cell number was determined by Resazurin (Sigma) reduction. For the clonogenic assay, cells were seeded in a 6-well plate at a density of 300 cells per well and treated with decitabine. After completion of the treatments, fresh growth medium was added to the wells and cells were left to proliferate for 7 (Hela) or 12 (MRC-5) additional days. Colonies were stained with a solution of 0.5% crystal violet in methanol.

Quantification of DNA methylation by liquid chromatography-tandem mass spectrometry

DNA was isolated using the Tissue & Cell GenomicPrep MiniSpin Kit (GE Healthcare), quantified and subjected to hydrolysis with formic acid as previously described [12]. UPLC/MS/MS analysis was carried out using a Waters XEVO TQ-S spectrometer at the Center of Scientific Instrumentation (University of Granada, Spain).

Intracellular dNTP pool size determination

dNTP levels were measured using a DNA polymerase assay with minor modifications [16-18]. 10^6 cells were extracted with 1 ml of 1:1 (v/v) methanol/water at -20 ºC and the suspension mixed vigorously by vortexing. Samples were then subjected to two freeze-thaw cycles (10 min each at dry ice/ethanol and ice), before centrifugation at 16,000 *g*, 20 min and 4 ºC. The supernatants were collected, dried under vacuum and dissolved in 40 µL of dUTPase buffer (34 mM Tris–HCl, pH 7.8, 5 mM $MgCl_2$) or dUTPase buffer plus 30 ng of human dUTPase [19] for 20 min at 37 ◦C. To stop the reaction, samples were precipitated with methanol, centrifuged for 20 min at 16,000 *g*, dried and used for the quantification of the dNTP pool size as described [12].

Quantitative determination of uracil in DNA

Uracil incorporation into DNA was measured using a qPCR-based assay following the protocol described by Horváth and Vértessy (2010) with minor variations [20, 21]. Genomic DNA was extracted using MasterPure DNA Purification Kit (Epicentre), digested with SacI-HF (NE Biolabs) and 3-5 kb fragments containing the target template (*GAPDH* gene), isolated and purified from 1% agarose gel. Two-fold dilution series of the DNA samples were amplified with PfuTurbo Hotstart (Agilent Technologies) or Taq polymerase (Bioline) and primers: 5'- CTC CTGCCCTTTGAGTTTGATG-3' and 5'- CAGCAGAGAAGCAGACAGTTATG-3'. qPCR was performed with CFX96 Real Time System C1000 Thermal Cycler (BioRad). Cq values obtained were used to determine uracil content as described [20]. All values are referred to non-treated control siRNA-transfected cells.

Immunofluorescence analysis of γ-H2AX foci

Cells were grown on sterile glass coverslips, fixed with 4% paraformaldehyde for 15 min at RT, and permeabilized in PBS containing 0.1% Triton X-100 for 10 min at RT. Cells were then incubated with 1:250 diluted monoclonal anti-γH2AX antibody (clone JBW301, Millipore) for 30 min at 37 ºC and detected with secondary Alexa 488–conjugated goat anti-mouse IgG (Invitrogen, Life Technologies). Coverslips were dehydrated in methanol and mounted in Vectashield with DAPI (Vector Laboratories, Inc.). Digital images were captured using a LEICA TCS SP5 confocal microscopy system and analyzed with FIJI software.

Statistics

Results are expressed as mean ± standard deviation of at least three independent

replicates. One or two-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test (referred to control or non-treated cells) was used to analyze the data. Normality and homogeneity of variance assumptions were checked for each analysis. When the variances were different, Welch test followed by Games-Howell post-test were used. A Chi-square test was performed for γH2AX-positive cells analysis. Fisher test was applied when the assumptions did not fulfill. Differences were considered significant when *P*-value was less than 0.05.

RESULTS

DCTPP1 and dUTPase expression is up-regulated in response to decitabine

We have previously shown that media supplementation with an excess of deoxycytidine induces an increase in the expression of DCTPP1 in HeLa cells [12]. Hence, we expected that exposure to decitabine might induce a similar modulation of enzyme levels in response to the accumulation of aza-dCTP. As shown in Figure 1, the amount of DCTPP1 protein increased up to three-fold at 24 h after addition to the medium of 10 μM aza-dCyd. At the same time, CDA, DCTD, TS and dUTPase expression was also up-regulated, suggesting the generation of metabolic intermediates different from those leading to the formation of aza-dCTP.

Depletion of DCTPP1 or dUTPase sensitizes cells to aza-dCyd.

The observation that cell exposure to decitabine translates into the overexpression of two house-cleaning nucleotidohydrolases with capacity to hydrolyze non-canonical nucleotides, led us evaluate their potential protective role against the cytotoxic action of aza-dCyd once activated and incorporated into the nucleotide pool. HeLa and MRC-5 cells were transfected with a siRNA pool against *DCTPP1* or *dUTPase* and then treated with increasing concentrations of the nucleoside analog. In all the experiments performed, a non-targeting siRNA pool was used as negative control. SiRNA-mediated depletion of the proteins was checked by Western blot (Figure 2A). Strong down-regulation of DCTPP1 or dUTPase increased the toxic effect of aza-dCyd in HeLa or in the non-tumoral cell line MRC-5 (Figure 2B). In contrast, cells transfected with the non-targeting siRNA pool did not exhibit a decrease in viability even in the presence of 100 μM aza-dCyd. The protective effect was specific for decitabine since the absence of

DCTPP1 or dUTPase did not have a significant impact on cell proliferation after exposure to the canonical nucleoside deoxycytidine or to the cytidine analog cytarabine (Figure 2B).

DCTPP1 or dUTPase-deficient cells incubated with decitabine also displayed a reduced colony forming capacity compared to control cells (Figure 2C). To investigate a potential resistance response to decitabine mediated by DCTPP1 and dUTPase, we tested whether the overexpression of these proteins might confer additional protection to aza-dCyd by comparing clonogenic survival of HeLa cells both parental and overproducing DCTPP1 or dUTPase after exposure to a range of decitabine doses (Figure 3). At 1 μM of aza-dCyd, the proportion of surviving cells expressing dUTPase was significantly enhanced compared to the number of viable colonies obtained with cells transfected with the empty vector. The overexpression of DCTPP1 had no impact on cell survival at any of the concentrations tested, suggesting that endogenous levels of DCTPP1 but not of dUTPase are sufficient to deal with potentially cytotoxic metabolites generated from aza-dCyd.

DCTPP1 modulates DNA demethylation induced by decitabine.

Since DCTPP1 catalyzes the hydrolysis of dCTP to dCMP and pyrophosphate, we investigated its potential role in detoxification of aza-dCTP (activated form of decitabine). *In vitro*, DCTPP1 is active on aza-dCTP (K_m = 54.04 ± 0.34 μM and k_{cat} = 7.54 ± 0.03 s^{-1}) (Figure 4A), exhibiting kinetic parameters very similar to those obtained for dCTP (K_m = 47.63 ± 2.66 μM and k_{cat} = 5.69 ± 0.18 s^{-1}) [12]. The sigmoidal character of the aza-dCTP saturation curve and the double reciprocal plot showing non-linear kinetics indicate that substrate binding is occurring with positive cooperativity (n_H = 2.29 ± 0.03) (Figure 4A). No activity was detected with other nucleotide analogs

tested such as ara-CTP (cytarabine triphosphate), dF-CTP (gemcitabine triphosphate) or ddCTP (zalcitabine triphosphate).

The ability of DCTPP1 to act upon aza-dCTP suggests that under decitabine exposure, this enzyme may be preventing the accumulation of azacytosine in DNA and consequently the level of DNA methylation. To address this question, we determined changes in global methylation by measuring the ratio of 5-methylcytosine to cytosine by LC-MS/MS (Figure 4B). Under our experimental conditions, we observed a moderate decrease in global genome demethylation in control cells only after exposure to the highest dose of decitabine for 48 hours (P = 0.026). Depletion of DCTPP1 further increased the DNA demethylating activity of decitabine, which in the absence of the nucleotidohydrolase, caused significant reductions in the level of methylcytosine at 10 and 100 μM concentrations. These data support a potential role for DCTPP1 in preventing the incorporation of azacytosine in cell treatments with decitabine.

Decitabine treatment alters the nucleotide pool composition

It has been suggested that deaminated derivatives of aza-dCyd could interfere with the pyrimidine biosynthesis pathway by inhibiting TS [22]. To investigate whether exposure to decitabine promotes the expansion of the dUTP pool in a similar way to other TS inhibitors such as 5-fluorouracil [23], we measured perturbations in the nucleotide pool upon treatment with aza-dCyd and the role of the nucleotidohydrolases DCTPP1 and dUTPase in these potential perturbations (Figure 5).

The DNA polymerase-based assay used to quantify dNTPs does not allow discrimination between dCTP and aza-dCTP nor dUTP and aza-dUTP so we will refer to them as (aza-)dCTP or (aza-)dUTP. In control cells, only the treatment with the highest dose of decitabine induced an important increase in the concentration of

intracellular (aza-)dCTP (from 3.9 to 4.1 and 8.2 pmol/10^6 cells at 10 μM or 100 μM respectively) probably due to the conversion of aza-dCyd to aza-dCTP and the saturation of catabolic activities. On the other hand, the depletion of DCTPP1 promoted a substantial accumulation of aza-(dCTP) by treatment with decitabine (from 5.6 to 13.8 and 17.3 pmol/10^6 cells) suggesting that DCTPP1 is likely the main catabolic activity of the activated form of decitabine. Unexpectedly, in the absence of decitabine, DCTPP1-silenced cells also exhibited a significant pool of dUTP (0.9 pmol/10^6 cells) which cannot be detected in control cells. This pool increased by two-fold after exposure to 100 μM of decitabine (1.9 pmol/10^6 cells). We hypothesize that dUTP might be generated as a result of the allosteric activation of dCMP deaminase by the abnormally high levels of dCTP that accumulate in these cells [12] rendering an excess of dUMP that is subsequently converted to dUTP.

In the case of cells subjected to siRNA-mediated depletion of dUTPase and exposed to aza-dCyd, important alterations in the nucleotide pool were observed. These alterations consisted in a strong increase in the intracellular amount of pyrimidine nucleotides: (aza-)dCTP (from 4.5 to 7.3 and 9.6 pmol/10^6 cells), dTTP (from 8.6 to 12.1 and 11.5 pmol/10^6 cells) and (aza-)dUTP (from 0.4 to 1.6 and 2.2 pmol/10^6 cells). While it is true that the expansion of the dCTP pool induced by decitabine can be mostly attributed to the formation of aza-dCTP, the increase of dTTP pools might be the consequence of an up-regulation of dNTP synthesis in response to DNA damage as reported for other genotoxic agents [24]. Indeed, in DNA-damage-stressed cells, the synthesis of dTTP needed for recovery from DNA damage has been reported to be essentially mediated by TK [24]. In spite of such expanded dTTP pool, decitabine exposure caused a two-fold increase in the dUTP/[dUTP+dTTP] ratio that elevates substantially the risk of uracil misincorporation into DNA.

Assessment of uracil and DNA damage induced by aza-dCyd in DCTPP1- and dUTPase-deficient backgrounds

An expected consequence of the accumulation of dUTP in the nucleotide pool as a result of the treatment with decitabine would be the misincorporation of uracil into DNA, especially in the absence of dUTPase. To monitor changes in the uracil genome content in the genome, we have used a real-time PCR assay which utilizes the B-type DNA polymerase of Pyrococcus furiosus (Pfu) and the Taq DNA polymerase for amplifications [20]. The Pfu/Taq PCR method is based on the fact that the DNA polymerase Pfu is strongly inhibited by uracil-containing DNA, whereas Taq DNA polymerase can replicate through the deaminated base. The differences in product formation by the two enzymes provides a mean to quantify the amount of uracil in the DNA sample [25]. Genomic DNA was obtained from HeLa cells transfected with control, *DCTPP1* or *dUTPase* siRNAs and treated with 0, 1, 10 and 100 μM of aza-dCyd for 24 or 48 hours. Treatment of control cells with aza-dCyd for 24 hours produced a significant increase in the DNA uracil content which was further enhanced after exposure to the nucleoside analog for 48 hours (P<0.00001) (Figure 6A). We next analyzed the presence of uracil in *DCTPP1*- or *dUTPase*-silenced cells. Consistently with the existence of an endogenous dUTP pool, DCTPP1-deficient cells contain higher constitutive levels of uracil in their genomic DNA and accumulate higher amounts of uracil after exposure to aza-dCyd than control cells.

Similarly to DCTPP1, the lack of dUTPase provoked an increase in the basal levels of uracil in DNA, supporting the notion that dUTP is being constantly generated by the normal metabolism of the cell and removed by the dUTPase from the nucleotide pool. Upon decitabine treatment, *dUTPase*-silenced cells were highly susceptible to

accumulate uracil suggesting a major role for this enzyme in counteracting the formation of aza-dUTP.

Former studies have reported that cell treatment with aza-dCyd leads to the formation of DNA double-strand breaks in a direct or indirect manner and also promotes the accumulation of γH2AX foci (H2AX phosphoserine-139), a hallmark of the cellular response to this type of DNA damage [7]. We proceeded to monitor γH2AX foci by immunofluorescence microscopy in DCTPP1 and dUTPase-depleted HeLa cells after incubation with decitabine (Figure 6B). Control cells exposed to 10 μM of aza-dCyd only exhibited 7% of the population with γH2AX foci, and this percentage increased up to 10% and 12% in the absence of DCTPP1 and dUTPase respectively. At 100 μM aza-dCyd, the number of γH2AX positive cells raised from 21% in control cells to 33% and 38% in DCTPP1- and dUTPase-depleted cells respectively, observation that supports the notion that at least some of the DNA breaks in these genetic backgrounds can arise as a consequence of the incorporation of uracil or aza-uracil during replication.

DISCUSSION

Catabolic activities may influence metabolism and thereby, the pharmacological efficacy of anticancer nucleoside analogs. Mammalian cells can uptake and incorporate decitabine into the nucleotide pool and be potentially channeled to different biochemical pathways (Figure 7). Decitabine can be deaminated by CDA to aza-deoxyuridine, which is poorly phosphorylated by thymidine kinase [26] or alternatively, aza-dCyd can be converted into its active form via a phosphorylation pathway that includes DCK, UMP/CMP kinase and nucleoside diphosphate kinase (NDPK). The ability of DCTPP1 to hydrolyze the activated form of decitabine and the hypersensitivity of DCTPP1-deficient cells exposed to this nucleoside analog suggest that this enzyme may be involved in the catabolism of aza-dCTP. Indeed, our data showing an increase in cytosine demethylation in *DCTPP1*-silenced cells support the notion that this nucleotidohydrolase is interfering with the primary mode of action of decitabine and prevents the incorporation of aza-dCTP into DNA. Since the action of DCTPP1 entails the reversion of the phosphorylation steps involved in aza-dCyd activation, it is plausible that the relative activity ratio of the kinases UMP/CMP kinase and NDPK with regard to DCTPP1 may have predictive clinical value in treatments with this nucleoside analog.

Importantly, a number of evidences presented in this work suggest that aza-dCMP can also be deaminated by dCMP deaminase to produce aza-dUMP. Early reports have demonstrated the formation of aza-dUMP *in vivo* after exposure to [^{3}H]aza-dCyd [27]. The reported increase in dCMP deaminase expression would certainly contribute to the generation of aza-dUMP. Moreover, the presence of elevated levels of aza-dCTP resulting from decitabine metabolic activation would further favor deamination due to the allosteric activation of dCMP deaminase [28]. In addition to the potential formation

of aza-dUTP, it is likely that aza-dUMP itself acts as a competitive TS inhibitor. In agreement with this notion, a recent screening of TS inhibitors using a cellular thermal shift assay led to a product of decitabine metabolism which was later identified as 5-aza-2'-deoxyuridine 5'-monophosphate [29]. On the other hand, the up-regulation of TS is a well-known mechanism of resistance to short-term exposure to TS inhibitors [30]. It has been proposed that TS controls its own translation by binding its own mRNA while the interaction with the inhibitor disrupts this autoregulatory loop, leading to the translational derepression and increased TS protein levels [31, 32].

Numerous studies have established that the DNA damage associated to misincorporation of uracil in DNA is a significant mechanism of cytotoxicity induced by TS-inhibiting chemotherapeutic agents [23]. In agreement with an alternate genotoxic mechanism for decitabine based on the formation of aza-dUMP, we have detected a significant amount of intracellular (aza-)dUTP after exposure to aza-dCyd, which correlated with an increased level of genomic uracil, especially in the absence of dUTPase or DCTPP1. While the dUTPase activity preserves genomic integrity by removing (aza-)dUTP from the nucleotide pool, the absence of DCTPP1 might be promoting decitabine cytotoxicity by two different mechanisms: first, it allows the accummulation of aza-dCTP in the cellular pool and second, the excess of (aza-)dCTP stimulates the synthesis of toxic aza-dUMP derivatives through the allosteric activation of dCMP deaminase (Figure 7).

We postulate that decitabine enhances uracil misincorporation into DNA and activates base excision repair mechanisms. Indeed, a recent study has demonstrated that XRCC1 mutant cells, defective in BER, are hypersensitive to aza-dCyd and this phenotype is associated with an accumulation of abasic sites and DNA strand breaks in genomic DNA [11]. It is therefore possible that some of these breaks are an indication of the

formation of DNA repair intermediates induced by the presence of uracil and aza-uracil. In summary, the data from this work suggest a novel mode of action for decitabine in which aza-dCyd may ultimately lead to the toxic accumulation of (aza-)dUTP in the nucleotide pool and its incorporation into the genome. Further research will allow us to establish whether inhibition of these nucleotidohydrolases may constitute a novel approach to improve the efficacy of antitumor therapy with decitabine and circumvent potential drug resistance phenotypes. Indeed, novel molecules have been recently identified with capacity to specifically inhibit the activity of dUTPase and DCTPP1 that enhance the cytotoxic effect of pyrimidine anticancer agents [33, 34] thus highlighting the relevance of these nucleotidohydrolases in the mode of action of pyrimidine derivatives currently used in antitumour therapy.

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FIGURE LEGENDS

Figure 1. Modulation of enzyme expression by aza-dCyd. HeLa cells were exposed to 10 μM aza-dCyd and enzyme levels in 10 μg of protein cell extract were determined by Western blot. DCTPP1, dCTP pyrophosphatase 1; CDA, cytidine deaminase; DCK, deoxycytidine kinase; DCTD, dCMP deaminase; dUTPase, deoxyuridine triphosphate nucleotidohydrolase; TS, thymidylate synthase; TK, thymidine kinase; TMPK, thymidylate kinase. Expression at 24 hours was quantified with ImageQuant software (GE Healthcare) from a representative gel before representation as a diagram bar.

Figure 2. Down-regulation of DCTPP1 and dUTPase sensitizes cells to decitabine. A, Western blot analysis of HeLa and MRC-5 cells transfected with *DCTPP1*-specific siRNAs, *dUTPase*-specific siRNAs or a non-targeting (Control) siRNA. Cell extracts were prepared at the fourth day post-transfection. Protein loading was normalized with anti-α-tubulin. B, HeLa (upper row) and MRC-5 cells (lower row) were exposed to decitabine (aza-dCyd), 2′-deoxycytidine (dCyd), and cytarabine (ara-Cyd). Percentage of viability was calculated relative to the number of viable cells in the absence of exogenous nucleosides. All values are mean ± SD (N=3). Data concerning the sensitivity of *DCTPP1* siRNA-transfected cells to dCyd have been published elsewhere [12] and are included for comparison purposes. C, Clonogenic survival of HeLa and MRC-5 cells transfected with Control, *DCTPP1*- or *dUTPase*-specific siRNAs and treated with increasing doses of decitabine. All values are mean ± SD (N=4).

Figure 3. Effect of DCTPP1 and dUTPase overexpression on the survival against decitabine. *Left panel*, Western blot showing the levels of dUTPase and DCTPP1 proteins in HeLa cells transfected with pcDNA3-DCTPP1 (DCTPP1 OE), pcDNA3-

dUTPase (DUT OE) or the empty pcDNA3 vector (Control). *Right panel*, Clonogenic survival of HeLa cells expressing normal or increased levels of DCTPP1 or dUTPase and treated with increasing doses of decitabine. All values are mean ± SD (N=3).

Figure 4. DCTPP1 hydrolyzes aza-dCTP and prevents DNA demethylation in human cells. A, *Left panel*, The enzymatic assay was performed by incubating DCTPP1 (0.5 μM) with increasing concentrations of aza-dCTP (20-250 μM) under optimized reaction conditions as previously described [12]. Kinetic parameters and the Hill's coefficient (n_H) resulted from adjusting the data to the Hill´s equation. All values are mean ± SD (N=3). *Right panel*, Double reciprocal plot showing non-linear kinetics for aza-dCTP. B, Global DNA methylation in HeLa cells exposed to increasing concentrations of aza-dCyd for 24 or 48 h. All values are mean ± SD (N=3).

Figure 5. Effect of decitabine on the intracellular dNTP pool. Nucleotide concentration was measured in HeLa cells transfected with non-targeting, *DCTPP1* or *dUTPase*-specific siRNA pools. Treatments were carried out with 0-10-100 μM of decitabine for 24 h. All values are mean ± SD (N=4).

Figure 6. Decitabine induces uracil accumulation and DNA strand breaks specially in the absence of DCTPP1 or dUTPase. A, Uracil content in genomic DNA from HeLa cells transfected with Control, *DCTPP1* or *dUTPase* siRNAs and treated with increasing concentrations of aza-dCyd for 24 or 48 h. Values resulting from genomic DNA isolated from non-treated Control siRNA-transfected cells were used as reference. All values are mean ± SD (N=3). *P*-values underlined with dotted line are referred to treated Control siRNA cells. B, *Left panel,* Representative microscopy images showing

γH2AX staining in non-treated or aza-dCyd-treated HeLa cells. All chosen fields contain a similar number of cells as determined by DAPI staining (not shown). *Right panel,* Percentage plots of γH2AX-positive cells in Control, *DCTPP1*, and *dUTPase* siRNA cells treated with aza-dCyd. γH2AX-positive cells (> 5 foci) were scored from a total of two hundred cells per cell line and dose.

Figure 7. Role of DCTPP1 and dUTPase in the response to decitabine. Aza-dCyd is converted into its active form via a phosphorylation pathway initiated by deoxycytidine kinase (DCK). DCTPP1 can hydrolyze aza-dCTP and could prevent its incorporation into DNA and the formation of DNMT-DNA cross-links. Alternatively, aza-dCyd can be deactivated by cytidine deaminase (CDA) to aza-deoxyuridine (aza-dUrd) which is poorly phosphorylated by thymidine kinase (TK). The aza-dCMP intermediate can also be deaminated by dCMP deaminase (DCTD) to produce aza-dUMP, which might potentially inhibit thymidylate synthase (TS). The accumulation of genotoxic (aza-)dUTP would promote uracil misincorporation and DNA damage.

Figure 1

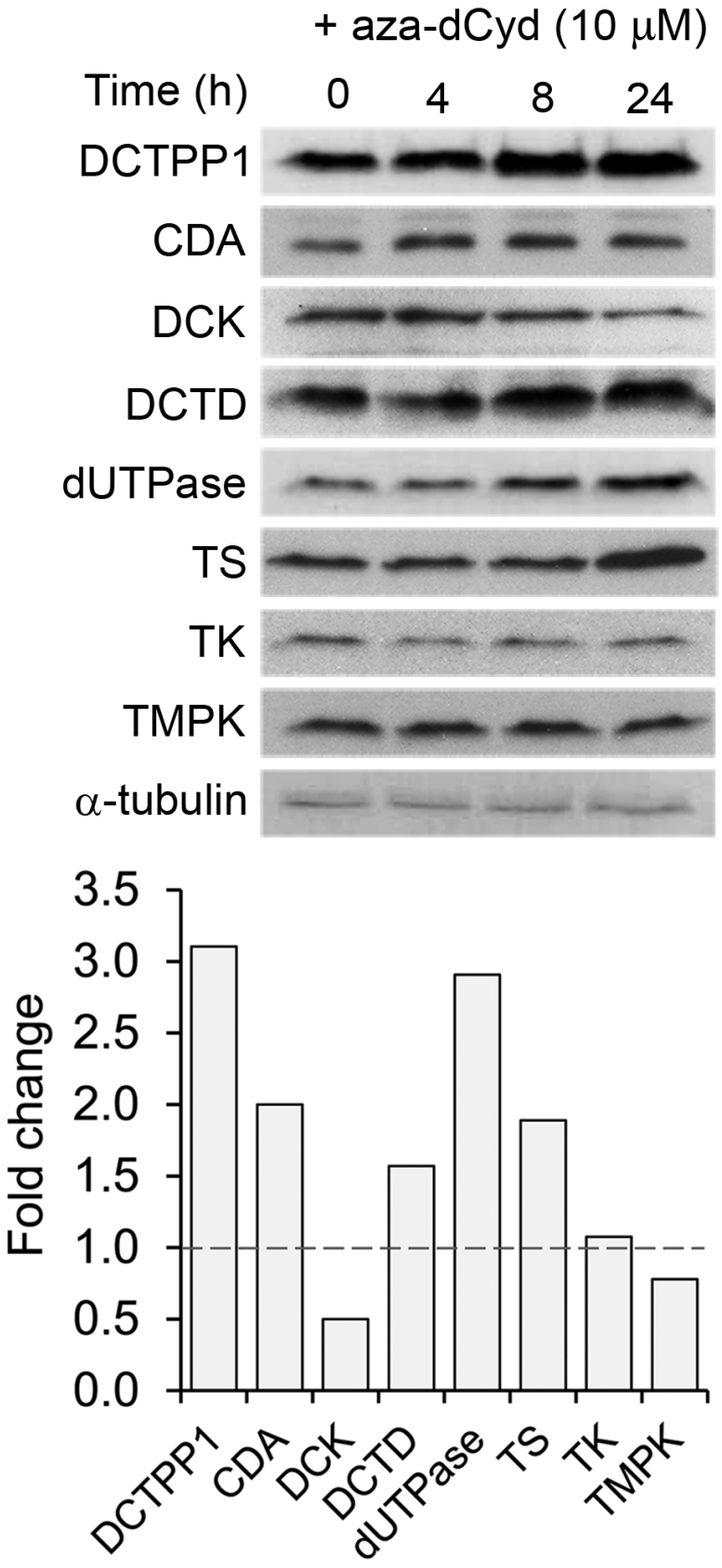

+ aza-dCyd (10 μM)
Time (h)
0
4
8
24
DCTPP1
CDA
DCK
DCTD
dUTPase
TS
TK
TMPK
α-tubulin
Fold change
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
DCTPP1
CDA
DCK
DCTD
dUTPase
TS
TK
TMPK

Figure 2

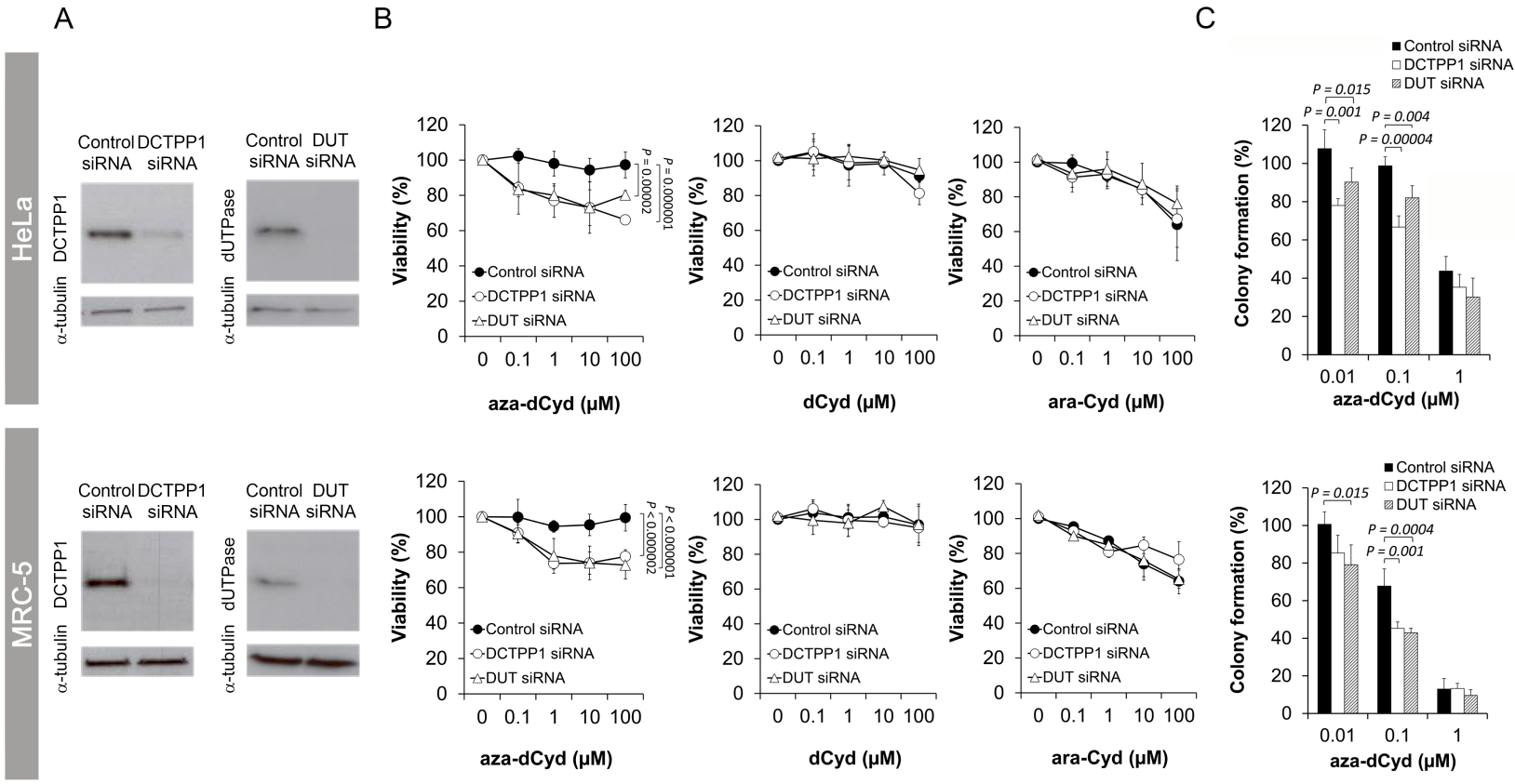
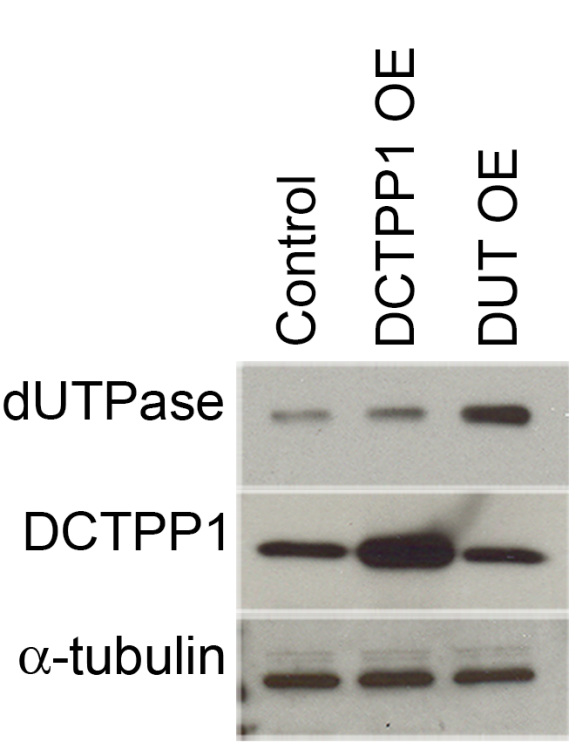

A
B
C
HeLa
MRC-5
Control siRNA
DCTPP1 siRNA
DUT siRNA
α-tubulin
DCTPP1
dUTPase
Viability (%)
aza-dCyd (μM)
dCyd (μM)
ara-Cyd (μM)
Colony formation (%)
P = 0.015
P = 0.001
P = 0.004
P = 0.00004
P = 0.0004

Figure 3


Control
DCTPP1 OE
DUT OE
dUTPase
DCTPP1
α-tubulin

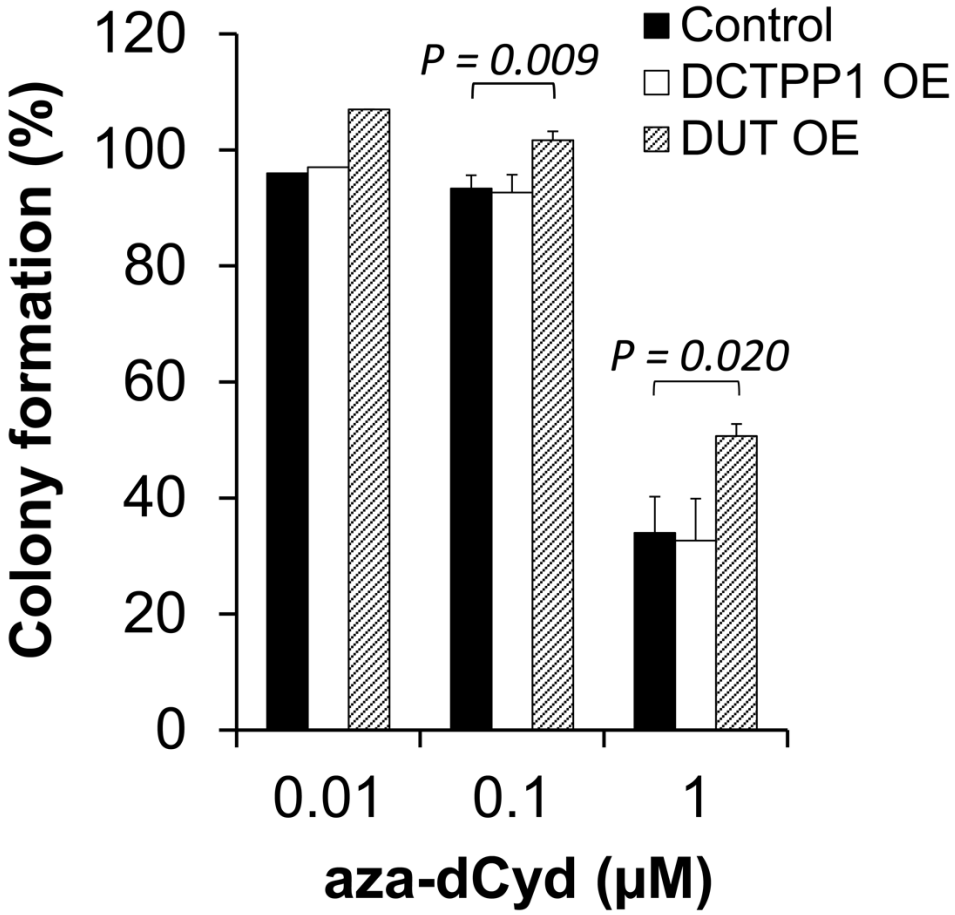

Control
DCTPP1 OE
DUT OE
P = 0.009
P = 0.020
Colony formation (%)
120
100
80
60
40
20
0
0.01
0.1
1
aza-dCyd (μM)

Figure 4

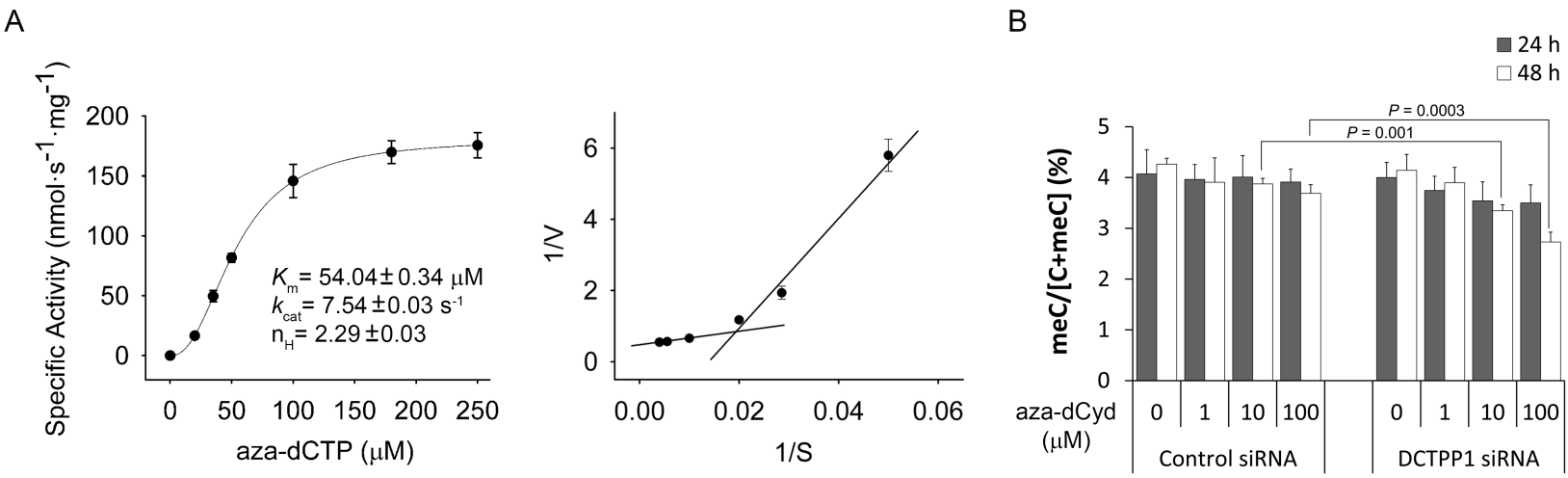

A
Specific Activity (nmol·s-1·mg-1)
200
150
100
50
0
0 50 100 150 200 250
aza-dCTP (μM)
Km = 54.04± 0.34 μM
kcat = 7.54 ±0.03 s-1
nH = 2.29 ±0.03
1/V
6
4
2
0
0.00 0.02 0.04 0.06
1/S
B
24 h
48 h
P = 0.0003
P = 0.001
meC/[C+meC] (%)
5
4
3
2
1
0
aza-dCyd (μM)
0 1 10 100 0 1 10 100
Control siRNA
DCTPP1 siRNA

Figure 5

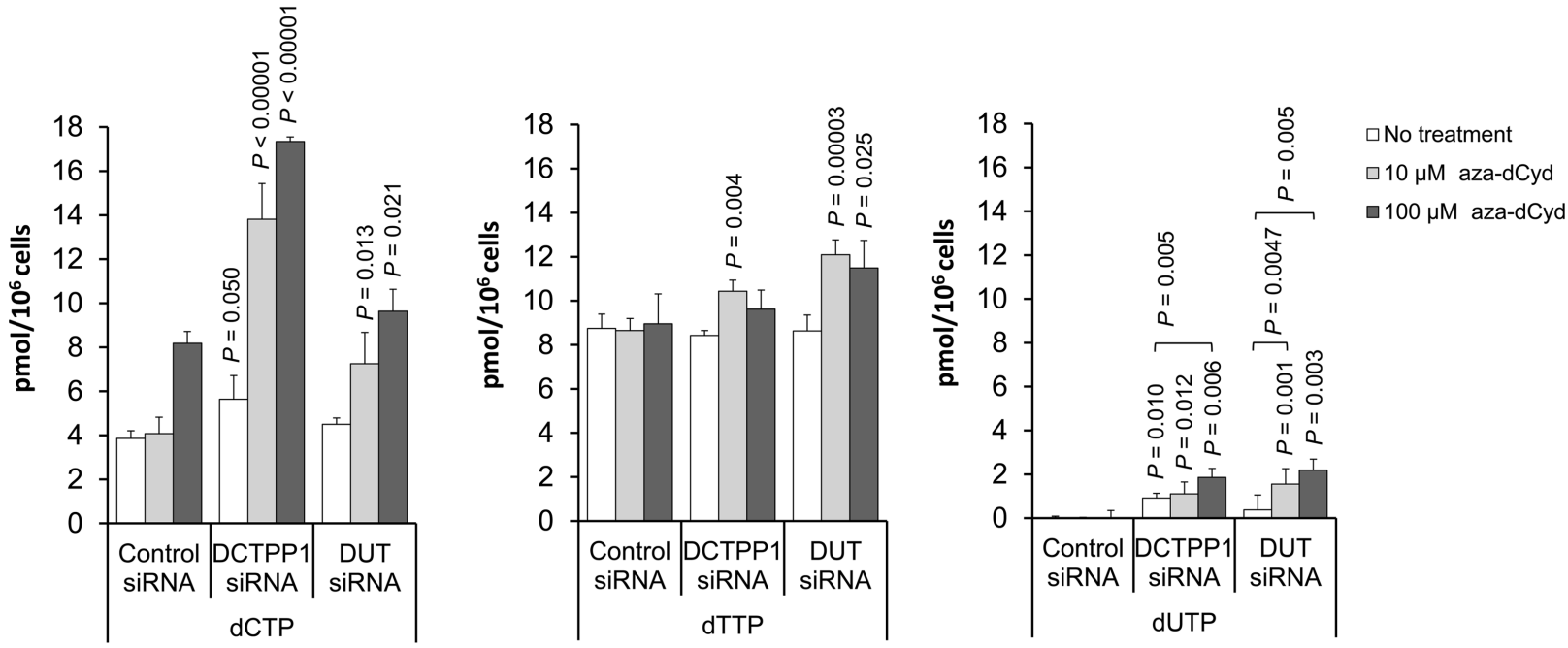

pmol/10^6 cells
18
16
14
12
10
8
6
4
2
0
P = 0.050
P < 0.00001
P < 0.00001
P = 0.013
P = 0.021
Control siRNA
DCTPP1 siRNA
DUT siRNA
dCTP
P = 0.004
P = 0.00003
P = 0.025
dTTP
P = 0.010
P = 0.012
P = 0.006
P = 0.005
P = 0.001
P = 0.003
P = 0.0047
P = 0.005
dUTP
No treatment
10 μM aza-dCyd
100 μM aza-dCyd

Figure 6

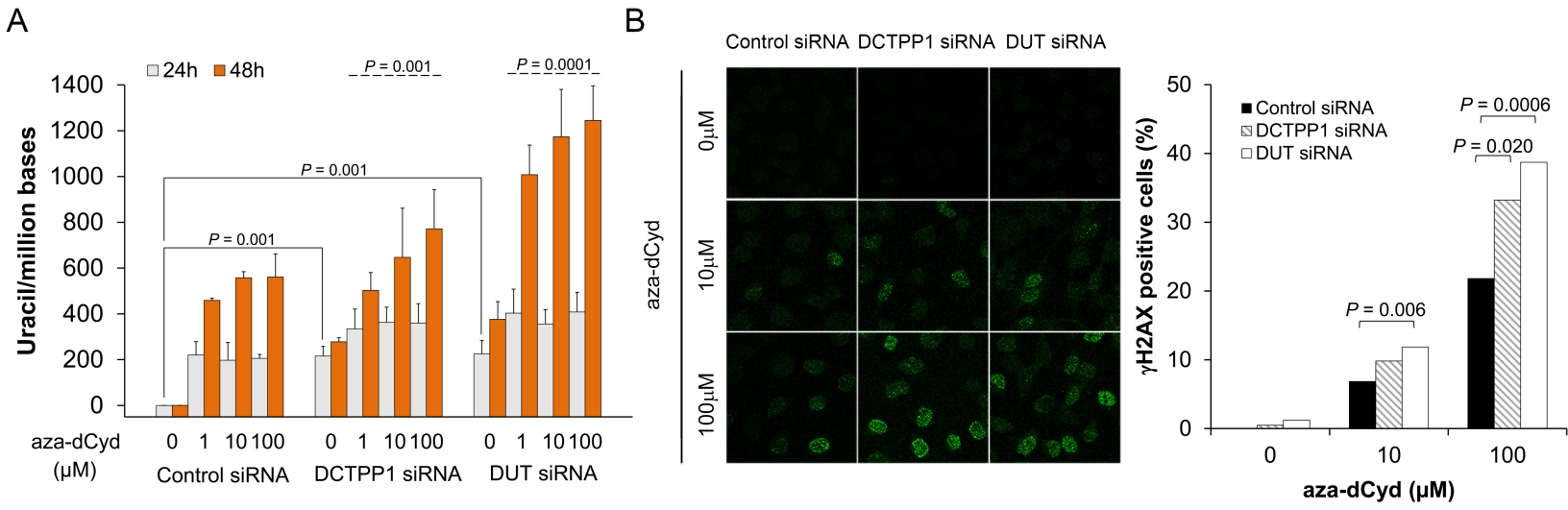
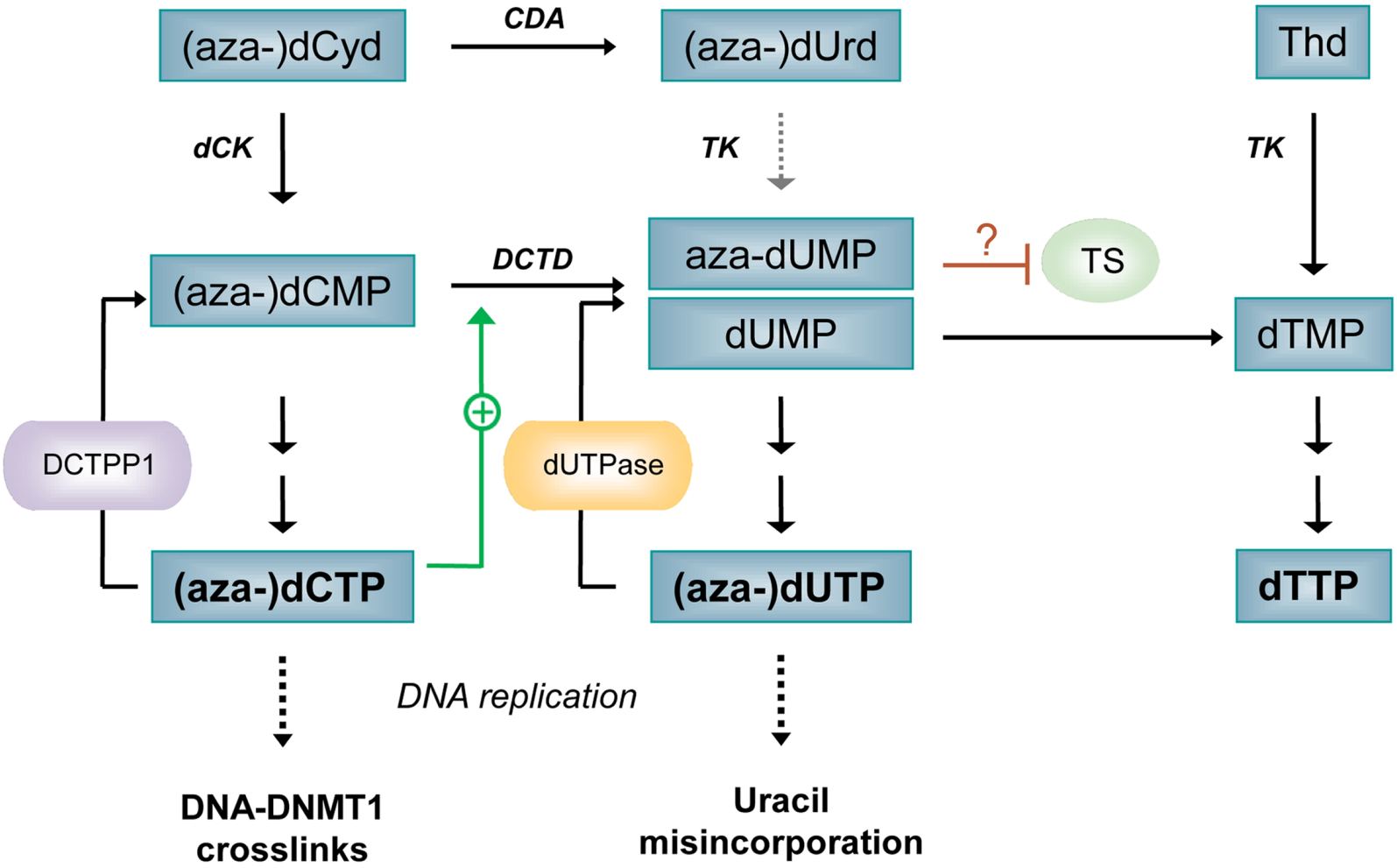

A
24h
48h
P = 0.001
P = 0.0001
P = 0.001
P = 0.001
Uracil/million bases
1400
1200
1000
800
600
400
200
0
aza-dCyd (μM)
0 1 10 100 0 1 10 100 0 1 10 100
Control siRNA
DCTPP1 siRNA
DUT siRNA
B
Control siRNA DCTPP1 siRNA DUT siRNA
aza-dCyd
0μM
10μM
100μM
γH2AX positive cells (%)
50
40
30
20
10
0
Control siRNA
DCTPP1 siRNA
DUT siRNA
P = 0.0006
P = 0.020
P = 0.006
0
10
100
aza-dCyd (μM)

Figure 7


(aza-)dCyd
CDA
(aza-)dUrd
Thd
dCK
TK
TK
(aza-)dCMP
DCTD
aza-dUMP
?
TS
dUMP
dTMP
DCTPP1
dUTPase
(aza-)dCTP
(aza-)dUTP
dTTP
DNA replication
DNA-DNMT1 crosslinks
Uracil misincorporation