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ORIGINAL ARTICLE

Decitabine triphosphate levels in peripheral blood mononuclear cells from patients receiving prolonged low-dose decitabine administration: a pilot study

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Abstract
Purpose Decitabine is a nucleoside analog used in the treatment for myelodysplastic syndrome. The compound requires intracellular conversion to its triphosphate to become active. Decitabine triphosphate has, however, never been quantified in peripheral blood mononuclear cells (PBMCs) from patients.
Method This article describes a method for the quantitative determination of decitabine triphosphate in PBMCs using liquid chromatography coupled to tandem mass spectrometry. The method was applied to ex vivo incubated whole blood samples and samples from three patients receiving prolonged low-dose decitabine treatment.
Results We successfully quantitated decitabine triphosphate in PBMCs. Considerable levels were detected in PBMCs from two patients that responded well to therapy, whereas only low levels were present in a non-responding patient. Moreover, the data show that, in contrast to plasma decitabine, intracellular decitabine triphosphate accumulates during a treatment cycle of nine infusions at a dose of 15 mg/m^2.
Conclusions The results suggest a relationship between decitabine triphosphate levels and response to therapy. Based on the observed accumulation of decitabine triphosphate during a treatment cycle, a less intensive dose scheme could be feasible.

Keywords Decitabine · Nucleotide analogs · Myelodysplastic syndromes · Resistance · LC–MS

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Introduction

Epigenetic therapy is a promising approach in cancer therapy because aberrant DNA methylation plays a role in many cancer types [12]. Decitabine (5-aza-2′-deoxycytidine, aza-dC; Fig. 1) is a classic epigenetic agent that is registered for the treatment for patients with myelodysplastic syndrome (MDS). Aza-dC is a deoxycytidine (Fig. 1) analog that has a plasma half-life of only 15–25 min due to deamination to 5-aza-2′-deoxyuridine (aza-dU) [27]. After uptake in cells by nucleoside transporters, conversely, aza-dC is converted to its mono-, and subsequently di- and triphosphate (aza-dCMP, aza-dCDP, and aza-dCTP, respectively). The metabolism of aza-dC is depicted in Fig. 2. The active metabolite aza-dCTP is incorporated into DNA leading to the inhibition of DNA methyltransferases, thereby causing global DNA hypomethylation. Moreover, specific tumor suppressor genes, such as *p15* can become hypomethylated, which is thought to result in their re-expression [6]. Studies on the relationship between *p15* and patient response are, however, conflicting [11, 13].

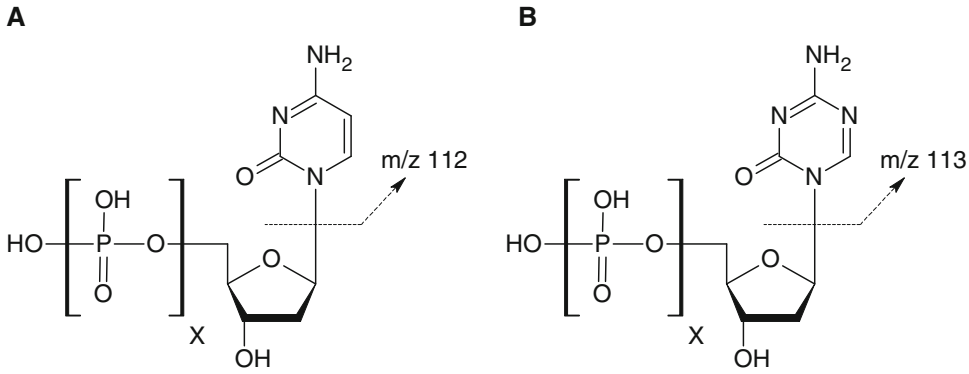

Fig. 1 Molecular structure of deoxycytidine (dC) (**a**) and decitabine (**b**) (X = 0) and their mono- (X = 1), di- (X = 2) and triphosphate (X = 3)

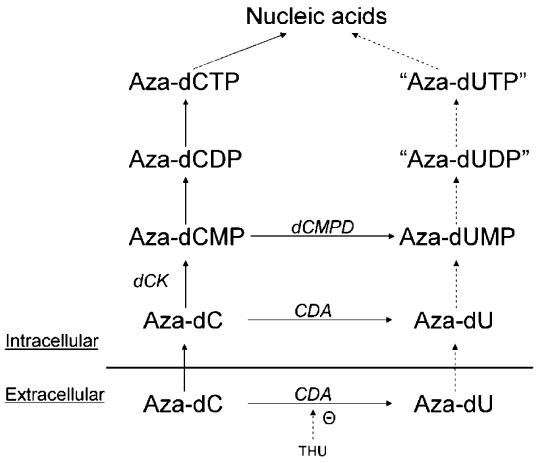

Fig. 2 Schematic representation of the metabolism of aza-dC. Hypothesized metabolites are indicated by quotation marks. *dCK* deoxycytidine kinase; *CDA* cytidine deaminase, *dCMPD* deoxycytidine monophosphate deaminase; *THU* tetrahydrouridine

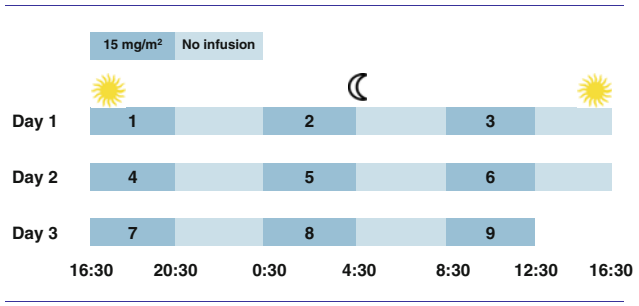

Fig. 3 Schematic representation of the applied decitabine dosing scheme

Much effort has been spent on finding the optimal aza-dC dose and dosing schedule. Based on classical maximum tolerated dose determinations, the drug was first tested at high (1,500–2,500 mg/m^2 per cycle) doses. Lower doses (100–150 mg/m^2 per cycle), however, were later found to be effective as well, but without causing the myelosuppression observed at high doses [14, 36]. The cytotoxic effect observed at higher doses reduces the hypomethylating effect, resulting in *U*-shaped dose-methylated DNA curves [40, 41]. Thus, hypomethylation is the main effect at low doses, whereas high doses are cytotoxic [21]. This dual mechanism of action and the fact that the agent is S-phase-specific make that the dose schedule is of extreme importance.

For that reason, various dosing schedules have been tested, ranging from long low-dose continuous [52] or intermittent [13] infusions to short, high-dose infusions [42]. Promising results have been obtained with low-dose infusions at a high dose intensity (total dose per week). Several of these dose schemes have been tested in relatively large clinical trials [13, 19, 20, 43], leading to the registered dosing scheme of 15 mg/m^2 administered as a 3-h intravenous infusion repeated every 8 h during 3 days for MDS (Fig. 3).

Several pharmacokinetic and pharmacodynamic parameters have been determined to assess the effect of the different dosing schemes. Plasma aza-dC pharmacokinetic data are available [1, 26, 29, 33, 42, 46], but only one study involved the approved dose [3]. Global and specific gene methylation have been used as pharmacodynamic end points in many studies. Both approaches have, however, shown mixed correlations with clinical outcome [11, 37, 53].

Despite these efforts, the optimal aza-dC dosing schedule remains a subject of debate [23, 31]. Furthermore, some patients are, or become, resistant to aza-dC treatment [40, 43].

For other nucleoside analogs, measurements of the active triphosphate have been used to determine optimal dosing schemes [10, 51]. Additionally, it has been suggested that aza-dC resistance is related to the lack of intracellular aza-dCTP formation [40]. Therefore, the determination of intracellular aza-dCTP is crucial to design an optimal dosing scheme as well as to detect and to understand the source of resistance to therapy.

Quantitative determination of aza-dC and its metabolites is, however, analytically challenging because of their instability and low concentrations [25, 26, 38, 40]. As a result, data on the intracellular concentrations of aza-dCTP are extremely sparse [9]. In addition, these measurements were

taken in vitro and in animals receiving high doses, whereas patients currently receive low doses of the drug.

Recently, the formation of deaminated nucleotides has been shown in patients who were treated with the deoxycytidine analogs cytarabine and gemcitabine (2′-2′-difluorodeoxycytidine, dFdC) [8, 48, 50]. These metabolites can be incorporated into nucleic acids and are cytotoxic, but their exact role in deoxycytidine treatment is unclear [49]. Cytarabine and gemcitabine use the same metabolic pathways as aza-dC (Fig. 2). Aza-dCMP is, for example, a substrate for deoxycytidine monophosphate deaminase (dCMPD) [34]. Indeed, early work with radioactive aza-dC showed in vivo and in vitro formation of the deaminated aza-dC monophosphate (aza-dUMP) and possibly of higher phosphates [5]. We hypothesized that deaminated nucleotides might also be formed during aza-dC therapy (Fig. 2).

Because of the low intracellular levels and many interfering endogenous nucleotides, aza-dC nucleotides have only been analyzed in a radioactive form [9]. High-performance liquid chromatography coupled with tandem mass spectrometry (HPLC–MS/MS) currently allows the sensitive and specific detection of nucleotide analogs [17]. The primary aim of this pilot study was to develop a sensitive method for the quantitative determination of non-radioactive aza-dC nucleotides, making studies in patients feasible.

In this article, we present a novel sensitive method for the quantification of aza-dC nucleotides in peripheral blood mononuclear cells (PBMCs) using HPLC–MS/MS. We employed this method to determine intracellular aza-dCTP concentrations in PBMCs from patients with MDS for the first time. Furthermore, we investigated the presence of deaminated aza-dC nucleotides in plasma and PBMCs.

Materials and methods

Patients and dosing

Three patients diagnosed with MDS received 15 mg/m^2 aza-dC without prior treatment, administered as a 4-h intravenous infusion repeated every 8 h during 3 days (Fig. 3) as previously described [43]. These treatment cycles were repeated every 6 weeks. A single sample was collected from patient 1 and 2 after the last (9th) infusion of the first cycle. Additional samples were collected only from patient 2 and 3 after infusion 3, 6, and 9 of cycle 5. All patients gave informed consent prior to collection of the samples.

Patient sample collection

Blood samples (15–20 ml) were collected within 30 min after the end of the infusion, except the last sample from patient 3, which was collected 25 min before the end of the infusion. All samples were immediately placed on ice. A 10 mg/ml tetrahydrouridine solution was added (final concentration of 100 µg/ml) to the samples collected after the first cycle to prevent plasma ex vivo deamination of aza-dC [3, 38].

The samples were centrifuged for 5 min at 1,500*g* (4°C) to separate the plasma from the buffy coat containing the PBMCs. The plasma of the samples collected after the first cycle was stored at −70°C until analysis. The buffy coat was diluted in cold phosphate-buffered saline (PBS) and layered over ficoll paque density gradient. After centrifugation for 20 min at 550*g* (4°C), the PBMCs were collected and washed with cold PBS. The number of PBMCs isolated was determined, and the cells were finally suspended in PBS. Cold methanol was added (2.33 times the volume of the suspension) to lyse the cells and to extract the nucleotides. Finally, the samples were vortexed for 30 s and stored at −70°C until analysis. Blank PBMCs were isolated from donor buffy coat as previously described [47], and lysed with methanol as described above.

Ex vivo incubations

Blood was collected from two healthy volunteers. Aliquots of approximately 8 ml were incubated at 1, 10, or 100 µM aza-dC for 1 h (batch 1) or at 1 and 100 µM for 5 h (batch 2) at 37°C. The samples were then transferred to cell preparation tubes (BD Vacutainer CPT; BD, Franklin Lakes, NJ, USA) and centrifuged for 20 min at 1,500*g*. The plasma and PBMCs were collected and processed further as described under "Patient sample collection."

Aza-dC deamination

A solution containing 1 mg/ml aza-dC (Sigma, St. Louis, MO, USA) in 10 mM tris(hydroxymethyl)aminomethane (TRIS)-HCl buffer pH 7.5 was treated with 20 µl (134 µg/ml) cytidine deaminase (Eli lilly and company, Indianapolis, IN, USA). The mixture was incubated at 37°C, and 10 µl samples were taken at time zero and after up to 1 h. These samples were diluted with 90 µl methanol, centrifuged, and directly analyzed with the qualitative method described below.

Synthesis of aza-dC nucleotides

A mixture containing aza-dCMP, aza-dCDP, and aza-dCTP was synthesized from aza-dC. About 25 µmol aza-dC was dissolved in 2.5 ml trimethylphosphate by brief gentle heating. Proton sponge (1,8-bis(dimethylamino)naphthalene) (50 µmol) was added, and the mixture was cooled on ice. A large excess (15 equivalents) of phosphorous oxychloride was then added dropwise over 6 min, after which the

mixture was stirred at room temperature for 2.5 h. After adding an excess (20 equivalents) of cold tributylammonium phosphate and tributylamine (250 μM in dimethylformamide) to the cooled reaction mixture, it was poured into 15 ml cold 1 M triethylammonium bicarbonate. The solution was lyophilized, redissolved in water, and stored at −70°C.

The mixture was analyzed using the method for the qualitative analysis coupled to an LTQ mass spectrometer (MS) and photodiode array detector (both from Thermo Fisher Scientific Inc., Waltham, MA, USA). The synthesis products were identified by their m/z values (m/z 229, 309, 389, and 469 for aza-dC, aza-dCMP, aza-dCDP, and aza-dCTP, respectively) and UV-absorption spectra (absorption maximum at 245 nm). Because nucleosides and nucleotides have an identical molar absorption, the products could be quantitated using UV-detection (245 nm), with an external aza-dC reference standard.

Sample preparation

Cell lysate and plasma samples were thawed in ice water. To 90 μl of the samples, 5 μl water (unknown samples) or 5 μl diluted synthesized aza-dC nucleotide mixture (calibration standards) was added. Calibration standards were spiked with concentrations ranging from 1.20 to 120, 0.263–26.3, and 0.310–31.0 nM for aza-dCMP, aza-dCDP, and aza-dCTP, respectively.

Stable isotope (^{13}C,$^{15}N_2$)-labeled dFdC (*dFdC), its deaminated metabolite 2′-2′-difluorodeoxyuridine (dFdU), and their nucleotides (5 μl) were added as internal standards. Finally, the sample was centrifuged for 5 min at 23,100*g* (4°C) and transferred to an autosampler vial.

Qualitative analysis of aza-dC, aza-dCMP, aza-dCDP, and aza-dCTP and their deaminated metabolites

We recently developed a HPLC–MS/MS method to separate dFdC, dFdU, and their mono-, di-, and triphosphates compatible with MS detection [16]. With minor adaptations, this method was used to qualitatively screen for aza-dC, aza-dU, and their mono-, di-, and triphosphates in plasma and PBMCs. In brief, 25 μl processed sample was injected onto a porous graphitic carbon column (Hypercarb, 100 × 2.1 mm ID, 5 μm particles; Thermo Fisher Scientific Inc.). Separation was achieved using a gradient of 1 mM ammonium acetate in acetonitrile/water (15:85, v/v) pH 5 and 25 mM ammonium bicarbonate in acetonitrile/water (15:85, v/v), at a flow of 0.25 ml/min, and detection was performed employing an API4000 triple quadrupole MS (Applied Biosystems, Foster city, CA, USA) using the transitions and polarities presented in Table 1.

Table 1 Mass transitions monitored with the API4000 triple quadrupole mass spectrometer for the qualitative analysis of aza-dC and it metabolites

Compound	Parent ion (m/z)	Product ion (m/z)	Polarity
Aza-dC	229	113	+
Aza-dU	228	112	–
Aza-dCMP	309	113	+
Aza-dUMP	310	114	+
Aza-dCDP	389	113	+
Aza-dUDP	390	114	+
Aza-dCTP	469	113	+
Aza-dUTP	470	114	+

Table 2 Mass transitions monitored with the API4000 triple quadrupole mass spectrometer for the quantitative analysis of the aza-dC nucleotides

Compound name	Type	Parent ion (m/z)	Production (m/z)
dCMP	Interference	308	112
Aza-dCMP	Analyte	309	113
*dFdCMP	Internal standard	347	249
dCDP	Interference	388	112
Aza-dCDP	Analyte	389	113
*dFdCDP	Internal standard	427	329
dCTP	Interference	468	112
Aza-dCTP	Analyte	469	113
*dFdCTP	Internal standard	507	329

The asterisk indicates that it is stable isotope-labeled dFdC

Quantitative analysis of aza-dCMP, aza-dCDP, and aza-dCTP

The quantitative analysis of aza-dCMP, -DP, and -TP in PBMC cell lysates was adapted from a method previously described for cladribine [15] and gemcitabine [47] nucleotides. A volume of 25 μl processed cell lysate was injected onto a weak anion exchange column (Biobasic AX column, 50 × 2.1 mm, 5 μm particles; Thermo Fisher Scientific Inc,). The analytes were separated using a fast gradient of 10 mM ammonium acetate in acetonitrile/water (30:70, v/v) pH 6.0 and 1 mM ammonium acetate in acetonitrile/water (30:70, v/v) pH 10.5, at a flow of 0.25 ml/min. Detection was performed with an API4000 triple quadrupole mass spectrometer operated in the positive ionization mode with the mass transitions presented in Table 2. To assess the reproducibility of the method, standards ranging from 1.55 to 31.1 nM aza-dCTP were each injected 6 times in a single analytical batch.

Results

Qualitative analysis of aza-dC, aza-dCMP, aza-dCDP, and aza-dCTP and their deaminated metabolites

A qualitative method was developed to search for the presence of aza-dU and its mono-, di-, and triphosphate. Deoxycytidines (dFdC and aza-dC and their nucleotides) cause a signal in the mass transition of their uridine variants (dFdU, aza-dU, and their nucleotides) [16]. For correct detection of the uridine variants, these analytes must, therefore, be separated chromatographically. Using the described HPLC system, dFdC, dFdU, and their nucleotides were all separated. The retention times of aza-dC and its nucleotides were very similar to those of dFdC and its nucleotides. Therefore, we assumed that aza-dU, aza-dUMP, aza-dUDP, and aza-dUTP were also separated from their aza-dC counterparts. The mass transitions of aza-dU and its nucleotides were based on those of dFdU and its nucleotides. To confirm that, aza-dU was detectable with the described system we deaminated aza-dC with CDA and analyzed the product. Deaminated aza-dC products were indeed detected and separated from aza-dC, using the selected conditions.

Ex vivo incubations

We performed ex vivo incubations with aza-dC under different conditions (individuals, concentration, and incubation time) and analyzed these samples with the qualitative method. In plasma and cell lysate, we detected aza-dC only in the samples incubated with 10 and 100 μM aza-dC. We could, however, detect aza-dC nucleotides in all cell lysates. Like aza-dC, deaminated aza-dC was only detected in the samples incubated with high aza-dC concentrations. Aza-dU nucleotides were, on the other hand, not detected in plasma or cell lysate (Fig. 4).

Patient samples

We sought to detect deaminated aza-dC metabolites in the samples collected after the 9th infusion of the first treatment cycle. Aza-dU and its nucleotides could not be detected in these plasma or cell lysate samples. In contrast to the ex vivo experiments, aza-dC was not detected in these plasma and cell lysate samples, whereas its nucleotides were observed.

Quantitative analysis of aza-dCMP, aza-dCDP, and aza-dCTP

Because aza-dU and its nucleotides were not detected in any of the patient samples, we could restrict the quantitative analysis to aza-dCMP, aza-dCDP, and aza-dCTP, with the focus being on the active metabolite aza-dCTP. Therefore, we analyzed cell lysates with a less specific, but more sensitive HPLC method. Figure 5 shows a chromatogram from a patient sample. Using this method, we noticed a peak in the aza-dCTP mass transition of the blanks. This interference was caused by deoxycytidine triphosphate (dCTP), the natural variant of aza-dCTP. Although the masses of dCTP (467 Da) and aza-dCTP (468 Da) are slightly different, dCTP molecules containing a naturally occurring ^{13}C- or ^{15}N-isotope have the same parent and fragment mass as aza-dCTP (Fig. 1). Similarly, interferences caused by deoxycytidine have been observed in the HPLC–MS/MS analysis of aza-dC by others [33]. Since the number of isotopes in dCTP, and thus its relative interference, are constant, the interference can be determined if the dCTP signal is known. We, therefore, additionally monitored the dCTP mass transition in each sample. Theoretically, 5.5% of the dCTP molecules will give a signal in the aza-dCTP mass transition. This percentage is in close agreement with the 6% we determined by spiking blank cell lysate with different dCTP concentrations. With this percentage, we calculated the interference caused by dCTP for each patient sample and found that 15.4% of the aza-dCTP signal could be attributed to dCTP in the sample containing the lowest aza-dCTP concentration. All other calculated interferences were lower than 7.20%. The coefficients of variance obtained by repeated injections of standards were between 4 and 14% showing that the method is reproducible.

Ex vivo incubations

Aza-dCTP was the main metabolite detected in all samples analyzed. Aza-dCMP and aza-dCDP accounted for 21.4–23.4% and 3.91–42.3% of the total aza-dC nucleotide concentration, respectively. The aza-dCTP concentrations determined in the ex vivo incubated samples are presented in Fig. 6a. The intracellular aza-dCTP levels increased with higher extracellular aza-dC concentrations. This increase was, however, not proportional. Most likely, cellular aza-dC uptake or phosphorylation was saturated at higher concentrations, as also observed with other nucleoside analogs [10]. Finally, the data of the ex vivo incubations showed a considerable inter-individual variability. This variability was even more pronounced between the patients, as discussed below.

Patient samples

In patient samples, aza-dCTP was the main metabolite detected, with aza-dCMP and aza-dCDP accounting for 2.08–28.8% and 2.33–21.2% of the total aza-dC nucleotide concentration, respectively.

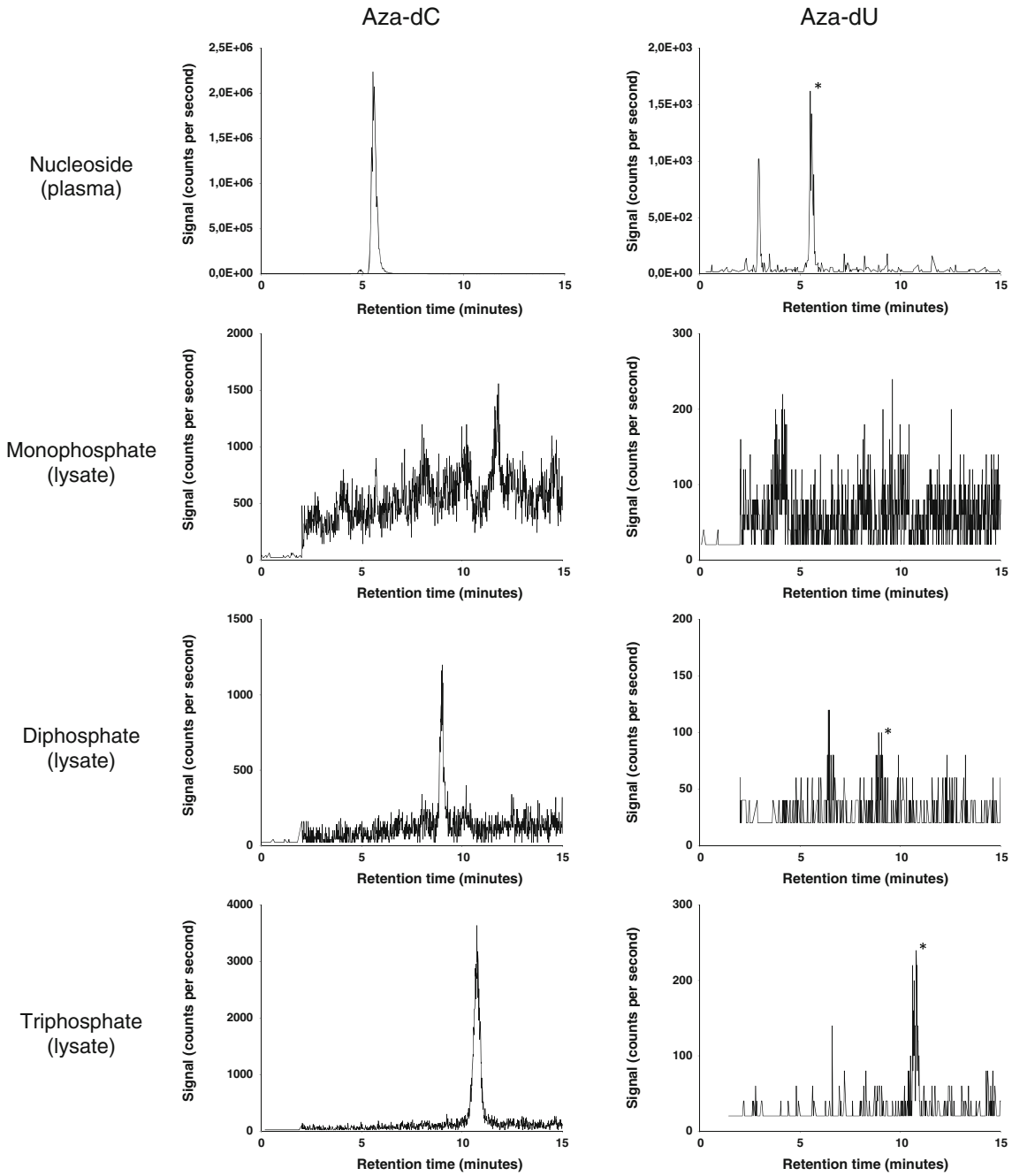

Fig. 4 Chromatogram of aza-dC, aza-dU, and their nucleotides in plasma and cell lysate of ex vivo incubated whole blood (100 μM aza-dC). Peaks in the aza-dU mass transitions caused by isotopic interference from their aza-dC counterparts are marked with an *asterisk*

Of the samples collected after the first cycle, a very low aza-dCTP concentration (6.62 fmol/10^6 PBMCs) was detected in the sample from patient 1, whereas an aza-dCTP concentration of 308 fmol/10^6 PBMCs was detected in patient 2 (Fig. 6b). Interestingly, this striking difference in active metabolite concentration seemed associated with the clinical response later observed. Patient 1 did not show any objective response to the therapy, whereas patient 2 responded well and no longer required blood transfusions. Patient 3 already showed a good response to therapy when samples were collected, which was concurrent with the high intracellular aza-dCTP levels detected for that patient. A possible mechanism of resistance is an increase in intracellular dCTP, the natural competitor of aza-dCTP [30]. The dCTP levels monitored in patient 1 were, however, not increased compared to those in other samples (mean dCTP peak area/10^6 cells of 1,130, 1,010, and 5,210 for patient 1, 2, and 3, respectively).

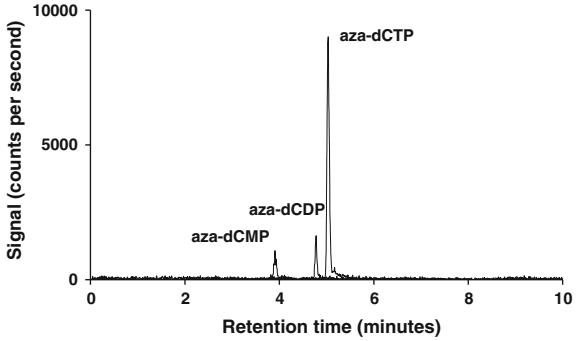

Fig. 5 Chromatogram of a PBMC sample collected from patient 2 after the last infusion of the 5th treatment cycle

To assess aza-dCTP accumulation, we collected additional samples from patient 2 and 3 at the end of infusion 3, 6, and 9 (Fig. 3). These data are presented in Fig. 6b. The aza-dCTP levels of patient 2 and 3 were not significantly different ($P = 0.49$; two-sided paired sample t test).

Discussion

The lack of detectable aza-dC in patient plasma is most likely caused by a combination of the low dose administered and rapid metabolism [3]. The absence of aza-dU and its nucleotides can be explained by a lack of formation or their instability. We and others have shown that aza-dC is a substrate for CDA [4, 7, 22, 24] and that aza-dCMP is a substrate for dCMPD [35]. A lack of formation, therefore, seems unlikely. More likely, aza-dU and its nucleotides rapidly degrade to other metabolites, such as those proposed by Patel et al. [38].

Resistance to nucleoside analogs can be caused by several mechanisms [18]. Aza-dC resistance has been associated with low dCK and nucleoside transporter activity, and high activity of CDA [7, 28, 32, 39, 40, 45]. Conversely, increased sensitivity was observed in dCK overexpressing cells [2] and when the drug was administrated in combination with a CDA inhibitor [24]. These mechanisms of resistance result in less intracellular aza-dCTP formation (Fig. 2) as observed in patient 1. A third mechanism of resistance is an increase in intracellular dCTP, the natural competitor of aza-dCTP [30]. The dCTP levels monitored in patient 1 were, however, not increased compared to those in other samples. Thus, increased dCTP levels were not the source of resistance in this patient. The low aza-dCTP level in patient 1 is striking and might be the reason why this patient was resistant to therapy. Since we did not determine mRNA transcript or protein levels of CDA, nucleoside transporters or dCK the source, or combination of sources [39, 40], of the low aza-dCTP level remains unknown.

Currently, resistance to therapy can only be determined based on clinical response after several 6-week cycles. If a lack of aza-dCTP formation is indeed the main source of resistance, determination of aza-dCTP levels in PBMCs earlier during therapy might offer a marked improvement in the assessment of response to therapy.

Aza-dC plasma concentrations reached in patients during a similar dosing scheme are about 0.25 μM [3]. Based on the ex vivo experiments performed at 1, 10, and 100 μM, relatively low aza-dCTP levels were therefore expected in the patient samples. The aza-dCTP concentration in the sample from patient 2 was, however, remarkably high compared to those found in the ex vivo experiments (Fig. 6). Since the sample was taken at the end of the 9th infusion, this indicated that aza-dCTP accumulated during the subsequent infusions of a treatment cycle. To assess the accumulation, we collected additional samples. The data presented in Fig. 6b show that aza-dCTP indeed accumulated in PBMCs during a treatment cycle. The accumulation rate indicates a half-life that exceeds the 4-h dosing interval. The last sample from patient 3, which contains a relatively low aza-dCTP concentration, was collected

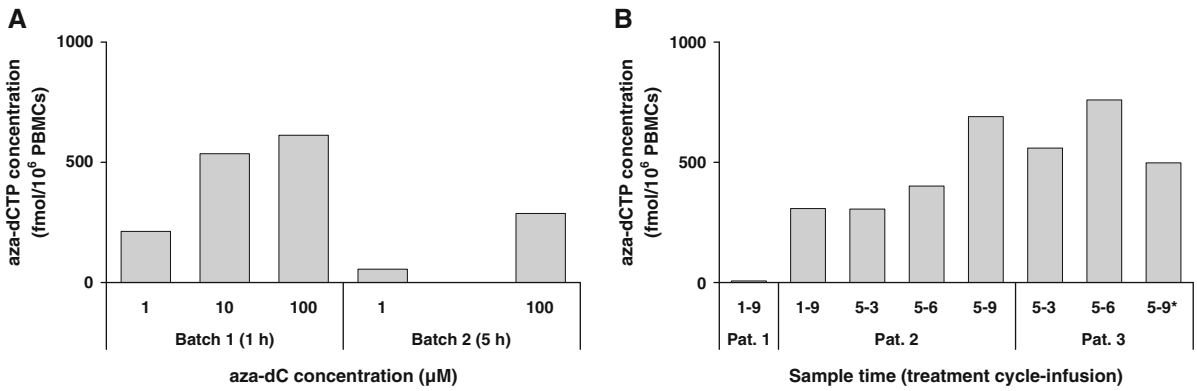

Fig. 6 Intracellular aza-dCTP levels found in PBMCs after ex vivo incubations of whole blood obtained from healthy volunteers with aza-dC (**a**) and in three patients receiving prolonged low-dose aza-dC treatment (**b**). *Asterisk* sample was collected 25 min before the end of the infusion

25 min before the end of the infusion. Assuming a long half-life, it is, however, very unlikely that the aberrant sampling time was the cause of the low aza-dCTP levels detected. Pharmacokinetic studies performed in larger groups of patients should confirm whether this unexpected result can be considered an outlier, or whether there is a pharmacokinetic explanation.

Cashen et al. [3] found identical plasma aza-dC curves on day 1, 2, and 3 of a treatment cycle, showing that aza-dC does not accumulate in plasma of patients on a similar dose scheme. Our results show that, in contrast to aza-dC, the active metabolite aza-dCTP does accumulate during a treatment cycle.

Optimal dosing scheme

Knowledge of the intracellular pharmacology of aza-dC is critical in designing the optimal dosing scheme. The optimal dosing scheme should be aimed at reaching effective intracellular aza-dCTP levels for a period sufficiently long for all cells to pass the S-phase, in which aza-dCTP is incorporated into DNA. The concentration at which optimal hypomethylation occurs is, however, unknown and requires further investigation. The accumulation of aza-dCTP during therapy suggests that the current dosing scheme might not be optimal. More constant intracellular aza-dCTP levels could be reached by the administration of a high loading dose followed by low maintenance doses, administered at intervals based on the aza-dCTP half-life. Theoretically, a loading dose should not result in extra toxicity, but caution should be taken when testing the approach. Using such a scheme, a sufficiently long half-life of aza-dCTP would allow once-daily, or even less frequent dosing, making the current patient hospitalization unnecessary. In line with these findings, it was recently shown that once-daily decitabine administration during 5 days in an outpatient setting resulted in an efficacy and safety, which were comparable to the currently recommended inpatient regimen [44].

Conclusion

We successfully quantified aza-dC nucleotides in PBMCs of two patients receiving prolonged low-dose aza-dC. The absence of detectable aza-dCTP levels in a non-responding patient and the presence of significant aza-dCTP levels in 2 responding patient might suggest that a lack of intracellular aza-dCTP formation is a source of resistance to therapy. This, however, needs conformation in a larger patient cohort. Aza-dCTP accumulated during the 9-infusion cycle suggesting that a less intensive dosing scheme is feasible. Finally, deaminated aza-dC metabolites have not been detected in patient samples, which is probably due to their instability. Future research should corroborate our findings and determines the relationship between intracellular aza-dCTP levels, hypomethylation, and clinical response.

Conflict of interest None.

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