

Modes of action of the DNA methyltransferase inhibitors azacytidine and decitabine

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The cytosine analogues 5-azacytosine (azacytidine) and 2'-deoxy-5-azacytidine (decitabine) are the currently most advanced drugs for epigenetic cancer therapies. These compounds function as DNA methyltransferase inhibitors and have shown substantial potency in reactivating epigenetically silenced tumor suppressor genes *in vitro*. However, it has been difficult to define the mode of action of these drugs in patients and it appears that clinical responses are influenced both by epigenetic alterations and by apoptosis induction. To maximize the clinical efficacy of azacytidine and decitabine it will be important to understand the molecular changes induced by these drugs. In this review, we examine the pharmacological properties of azanucleosides and their interactions with various cellular pathways. Because azacytidine and decitabine are prodrugs, an understanding of the cellular mechanisms mediating transmembrane transport and metabolic activation will be critically important for optimizing patient responses. We also discuss the mechanism of DNA methyltransferase inhibition and emphasize the need for the identification of predictive biomarkers for the further advancement of epigenetic therapies.

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Key words: azacytidine; decitabine; DNA methyltransferase; DNA methylation; cancer

More than 40 years ago, the azanucleosides 5-azacytidine (azacytidine) and 2'-deoxy-5-azacytidine (decitabine) were developed as classical cytostatic agents.¹ Several years later, it was shown that these compounds inhibit DNA methylation in human cell lines, which provided a mechanistic explanation for their differentiation-modulating activity.² In addition, this observation also initiated the development of azanucleosides as epigenetic drugs. After substantial refinements in their clinical dosing schedules, both azacytidine and decitabine have now shown significant clinical benefits in the treatment of myelodysplastic syndrome (MDS), a preleukemic bone marrow disorder.^{3,4} As a consequence, these drugs have now received FDA approval for the treatment of MDS. There are substantial ongoing efforts to identify and develop novel DNA methyltransferase inhibitors.^{5,6} However, the currently available compounds appear to have weaker gene reactivation potencies than azanucleosides^{7,8} and none of the candidate drugs has reached an advanced clinical testing stage for epigenetic indications yet. This has established azacytidine and decitabine as archetypal drugs for epigenetic cancer therapies.⁹

Despite the renewed interest in azacytidine and decitabine, surprisingly little is known about the molecular mode of action of these drugs. It is clear that azanucleosides have cytotoxic effects and that they can cause DNA demethylation, but the relationships between these characteristics and their respective significance for clinical responses has not been established yet. A comprehensive understanding of drug characteristics will be critically important for defining their modes of action and to further advance their clinical development.

Demethylation therapies: Proof of mechanism and proof of concept

Azanucleosides are established molecular tools for the induction of DNA demethylation in cellular model systems. However, it is also known that high doses of these drugs can induce pronounced toxicities in patients. When dosing schedules were adapted to optimize epigenetic effects it became increasingly

important to provide proof of mechanism data, *i.e.*, to demonstrate DNA demethylation in patients. Several studies have now shown that decitabine can induce significant demethylation in the approved indication^{10,11} and additional results suggest that azacytidine might have comparable epigenetic effects in patients.^{12,13}

The rationale behind demethylation therapies is the ability of DNA methyltransferase inhibitors to revert hypermethylation-induced gene silencing.¹⁴ Hypermethylation-induced gene silencing of tumor suppressor and other cancer-related genes plays a fundamental role in human tumorigenesis.¹⁵ The reversion of these epigenetic mutations can therefore restore proliferation control and apoptosis sensitivity. The identification of such events in patients undergoing demethylation therapy has been notably difficult. Most studies in this context have focused on the p15 tumor suppressor gene, which can be hypermethylated in MDS and AML patients and can be demethylated and reactivated in patients undergoing decitabine therapy.¹⁶ Similar observations were also made in other clinical studies with azacytidine, but a close connection between demethylation and reactivation of p15 and clinical responses could not be confirmed.^{12,13} The identification of hypermethylated genes that become demethylated and reactivated by drug treatment and the establishment of statistically robust associations between epigenetic reactivation events and patient responses will be an important area for future research.

Chemical stability of azacytidine and decitabine

Azanucleoside drugs are widely considered to be unstable, and have therefore been handled with considerable care, both in the laboratory and in the clinic. In alkaline solutions azanucleosides undergo a rapid and reversible opening of the 5-azacytosine ring, followed by irreversible decomposition.^{17,18} In acidic solutions the glycosidic bond of azanucleosides is cleaved, which also interferes with a potential oral administration of these drugs.¹⁹

To determine the half-life times of azacytidine and decitabine in neutral aqueous solutions, we used a capillary electrophoresis-based analytical assay. Both azacytidine and decitabine, respectively, were dissolved in neutral buffer, together with a chemically stable internal standard (adenine). Solutions were stored at 4, 20 and 37°C, respectively, and samples were taken at various time points. The results revealed that both drugs were stable at 4°C with half-life times of 21 days (azacytidine) and 7 days (decitabine; Fig. 1a). At 20°C, compound degradation became more rapid and half-life times were calculated to be 37 hr for azacytidine and 96 hr for decitabine (Fig. 1a). At 37°C the half-life times were 7 hr for azacytidine and 21 hr for decitabine (Fig. 1a). A very similar half-life time (20 hr) for decitabine solutions stored at 37°C was also found in an independent, recent study.²¹ Our results

Grant sponsors: German National Genome Research Network (NGFN-2); Helmholtz Association of National Research Center (Ideenwettbewerb Gesundheit).

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Received 21 December 2007; Accepted after revision 14 March 2008

DOI 10.1002/ijc.23607

Published online 18 April 2008 in Wiley InterScience (www.interscience.wiley.com).

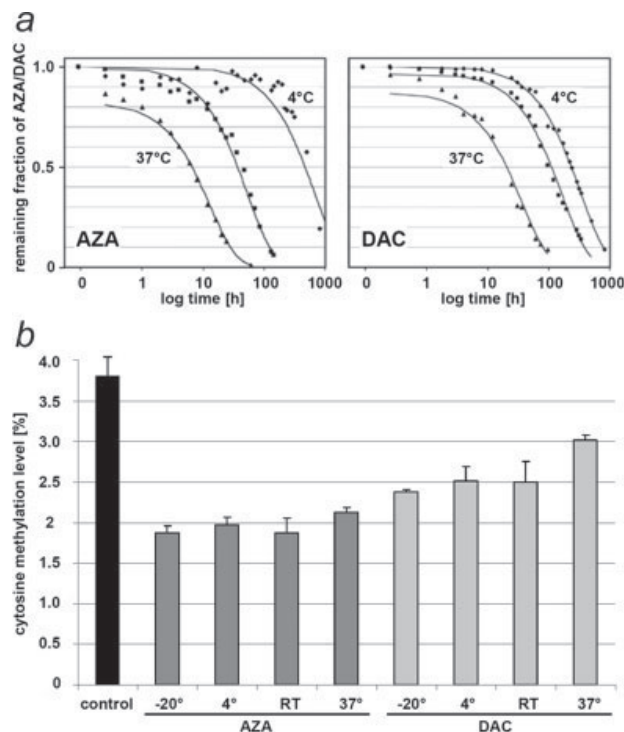


FIGURE 1 – Chemical stability of neutral azacytidine and decitabine solutions. (a) Temperature-dependent decomposition of azacytidine (AZA) and decitabine (DAC). Compounds were dissolved in neutral 0.9% NaCl solutions, stored at 4, 20 and 37°C, respectively, and snap-frozen in liquid nitrogen at the time points indicated. Samples were then diluted to 0.45 mg/mL and mixed with adenine as an internal standard (400 μ M final concentration). Analyses were performed on a Beckman Coulter capillary electrophoresis system (MDQ Molecular Characterization System) with UV detection at 254 nm. Separation occurred in an untreated fused-silica column of 60 cm (effective length 50 cm) in a 10 mM phosphate buffer system, pH 7.0, with 150 mM SDS. Analyses were performed at 25 kV and a capillary temperature of 25°C. (b) Pharmacological potency of stored azacytidine and decitabine solutions in inhibiting DNA methylation. Genomic cytosine methylation levels were analyzed by capillary electrophoresis.²⁰ Drug solutions were dissolved in neutral aqueous buffer and stored under the conditions indicated for 24 hr. HCT116 cells were treated with 2.0 μ M azacytidine (AZA) or 0.5 μ M decitabine (DAC). A significant reduction in pharmacological potency could only be observed after storage of decitabine at 37°C.

indicate a considerable chemical stability of azanucleosides at temperatures that are relevant for their general handling and use.

To confirm the stability of azanucleoside solutions we tested their ability to inhibit DNA methylation in the human HCT116 colon carcinoma cell line. Drug samples were stored for 24 hr at -20, 4, 20 or 37°C and then added to the tissue culture medium. Cells were harvested after 72 hr and their global DNA methylation level was analyzed by capillary electrophoresis. This revealed that azanucleoside solutions stored at room temperature were still effective in inhibiting genomic cytosine methylation (Fig. 1b) and provided an important confirmation for the relative stability of these drugs.

Drug pharmacokinetics

In vivo, several additional factors limit the stability and bioavailability of azanucleosides. Refinements in dosing and administration have continuously improved the clinical performance of

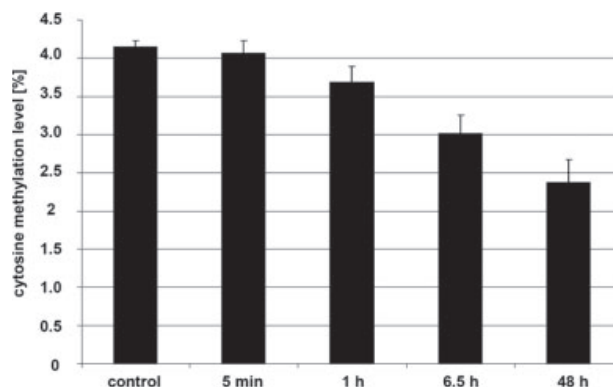


FIGURE 2 – Azacytidine-induced DNA demethylation requires extended drug exposure. Global methylation analysis was performed by capillary electrophoresis,²⁰ after treatment of HCT116 cells with 2 μ M azacytidine. Cells were incubated in drug-containing medium for the time indicated. The medium was then exchanged for drug-free medium and cells were grown for a total of 48 hr.

these drugs. For example, it has been shown that subcutaneous administration of azacytidine results in an $\sim$ 2-fold higher beta (substance elimination) half-life time compared to intravenous administration.²²

Both azacytidine and decitabine show wide distribution in body fluids of rabbits and dogs, with a comparably low alpha (substance distribution) half-life time of about 5 min.²³ Nevertheless, both compounds are rapidly cleared from systemic circulation. Systemic clearance exceeds the glomerular filtration rate and total renal blood flow, which suggests an important role of nonrenal elimination. In this context it has been proposed that deamination by cytidine deaminase in the human liver and spleen appears to be an important pathway.²⁴ A detailed quantification of azacytidine plasma levels in 3 patients treated s.c. with 25 mg/m² showed a maximal plasma concentration of 374 ng/mL (1.5 μ M), which occurred in the first hour after administration and the observed beta half-life time in plasma was 1.8 hr.²⁵ When patients were treated with the established standard treatment schedule for MDS patients (75 mg/m²), plasma levels of 5-azacytidine in the range of 3–11 μ M could be achieved.²² Similar analyses with samples from AML patients that had been treated intravenously with 15 mg/m² decitabine revealed a maximum concentration of 103 ng/mL (0.5 μ M) and a beta half-life time of less than 1.5 hr.²⁶

The peak plasma concentrations observed in patients are comparable to the concentrations that are being used to achieve DNA demethylation *in vitro*.⁸ However, substance elimination half-life times in patients are substantially shorter than the drug incubation times used for *in vitro* experiments (usually 48 or 72 hr). We therefore analyzed the possibility that short incubation times in drug-containing medium would be sufficient for the induction of azacytidine-mediated DNA demethylation. To this end, HCT116 cells were incubated with 2 μ M azacytidine. After various time points ranging from 5 min to 48 hr, cells were washed and incubated in drug-free medium for the remaining time until the end of the experiment was reached (after 48 hr). Analysis of global cytosine methylation levels in genomic DNA from these cells failed to indicate any demethylation after 5 min of drug incubation, but showed progressive demethylation after 1, 6.5 and 48 hr (Fig. 2). The maximum demethylation was observed after 48 hr, which confirmed that prolonged drug exposures cause more pronounced demethylation responses.²⁷

Because of the association between length of drug exposure and DNA demethylation, various clinical studies have tried to maximize demethylation responses in patients by continuous infusion of azanucleosides over several days. Analysis of DNA

methylation levels showed that continuous administration of decitabine caused pronounced demethylation, but the study design did not permit the analysis of correlations between demethylation and clinical responses.²⁸ More comprehensive studies may become feasible through the development of oral decitabine.²⁹ Alternatively, more prolonged drug exposure might also be achieved by chemical modifications that improve plasma stability of azanucleosides. One example for this approach is the development of the decitabine-containing dinucleotide S110. This compound showed comparable growth inhibiting and demethylating effects in tumor cell lines and improved resistance to enzymatic deamination.²¹

A potential safety concern for continuous DNA demethylation is the increased induction of illegitimate transcription events. Mouse models with strongly and permanently reduced DNA methylation levels have shown genetic amplification and insertional activation of oncogenic loci.^{30,31} These events are probably linked to the epigenetic reactivation of mobile DNA elements and need to be monitored and minimized during demethylation therapy. In this context, it is interesting to notice that clinical decitabine administration schedules with relatively high dose intensity have shown better response rates than schedules that continuously maintain low plasma drug levels.³² Thus, downstream effects of demethylation might also be important for clinical drug activity and optimized clinical schedules will probably cause balanced DNA demethylation and apoptosis induction.

Cellular uptake of azanucleosides

Conceivably, the effectiveness of azanucleoside therapy is also influenced by the relative transport capacities of the target tissue. Of particular interest are the membrane transporters that mediate cellular drug uptake. Early studies in a leukemic mouse model showed a connection between azacytidine resistance and impaired uptake of uridine and cytidine, which supported an important role of cellular transport mechanisms in mediating drug effects.³³ However, the role of transport processes in the treatment with azanucleosides has not been characterized on the molecular level yet.

In human cells, four different classes of proteins mediate the transport of nucleosides across membranes (Fig. 3)³⁴: (i) equilibrative uniporters (SLC29A family), (ii) substrate exchange transporters (SLC22 and SLC15 family), (iii) concentrative transporters (SLC28 family), and (iv) ATP-dependent exporters (ABC family). The transporter family members responsible for equilibrative uniporter (ENTs/SLC29A) or the concentrative uptake (CNTs/SLC28A) of nucleosides have been directly linked to the uptake of chemotherapeutic nucleoside analogues in the treatment of leukemias.^{35–38}

Experimental data from *in vitro* treated patient cells showed a significant correlation between the expression levels of nucleoside transporters and the sensitivity to nucleoside chemotherapeutics, like gemcitabine,^{36,39,40} fludarabine³⁷ or cytarabine,³⁵ which might indicate a role of these proteins in mediating azanucleoside uptake. A statistically significant correlation between the expression level of the equilibrative transporter ENT-1 and the sensitivity of *ex vivo* cultivated mononuclear cells from 50 AML patients could also be demonstrated for decitabine.³⁵ Similarly, an array-based study of transport-associated genes in 60 human cancer cell lines also identified a positive correlation between ENT-1 expression and azacytidine chemosensitivity.⁴¹ A role of ENT-1 in azacytidine uptake was also suggested by the observation that azacytidine-induced cytotoxicity could be reduced by nitrobenzylmercaptapurine ribonucleoside, a specific inhibitor of ENT-1.⁴¹ Nevertheless, functional data demonstrating a role of specific nucleoside transporters in mediating the cellular uptake of azanucleosides is still lacking. This is an important area for future research, because nucleoside transporters expression patterns could potentially be used as predictive biomarkers for therapy responses with nucleoside therapeutics.⁴²

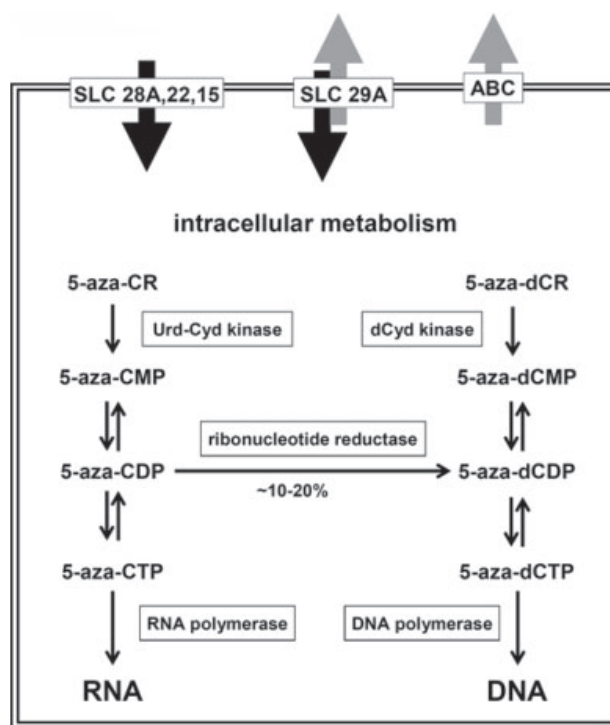


FIGURE 3 – Membrane transport and intracellular metabolism of azanucleosides. Four candidate transporter protein families (black and gray arrows) are believed to mediate the transport of nucleosides and nucleoside metabolites across the cell membrane (double line). After cellular uptake, azacytidine (5-aza-CR) and decitabine (5-aza-dCR) are modified by different metabolic pathways. It is assumed that 80–90% of azacytidine is incorporated into RNA, because ribonucleotide reductase limits the conversion of 5-aza-ribonucleotides to 5-aza-deoxyribonucleotides.

Intracellular metabolism of azanucleosides

After their cellular uptake, azanucleosides need to be activated and metabolically converted into the active nucleotide for DNA methylation inhibition, 5-aza-2'-deoxycytidine-5'-triphosphate (Fig. 3). A first limiting step in this cascade is the ATP-dependent phosphorylation of the nucleoside to the monophosphorylated nucleotide. It is generally assumed that this reaction is catalyzed by different enzymes for azacytidine (uridine-cytidine kinase) and decitabine (deoxycytidine kinase), and phosphorylation of decitabine by deoxycytidine kinase has been confirmed experimentally.⁴³ However, recombinant human uridine cytidine kinase 1 and 2 enzymes failed to phosphorylate azacytidine, while showing detectable activity for cytidine, uridine and some derivatives.⁴⁴ These results suggested that azacytidine phosphorylation is mediated by different enzymes. Selection for decitabine resistance in rat leukemic cell lines has been shown to be associated with the occurrence of mutations in the deoxycytidine kinase gene.⁴⁵ This suggested that deoxycytidine kinase plays an essential role in the metabolic activation of decitabine. Similarly, enzymes that negatively regulate the conversion of azanucleosides to azanucleotides could also play an important role in modulating drug responses. It has been shown that cytidine deaminase can deaminate azacytidine and decitabine to inactive aza-uradine nucleosides,²⁴ and retroviral overexpression of human cytidine deaminase in murine cells caused a significant drug resistance against decitabine.⁴⁶ It should be noted, however, that there is presently no published data supporting a role of human cytidine deaminase or deoxycytidine kinase in modifying decitabine responses in patients.

Because of its deoxyribonucleoside structure, decitabine is generally believed to be a more potent DNA methylation inhibitor

than the ribonucleoside analogue azacytidine. Early incorporation studies in L1210 leukemic cells have shown that 80–90% of azacytidine are incorporated directly into RNA.⁴⁷ A rate-limiting step for the conversion of ribonucleotides to deoxyribonucleotides is the activity of the ribonucleotide reductase enzyme. Surprisingly, treatment of cancer cell lines with hydroxyurea, a ribonucleotide reductase inhibitor, blocked the ability of both azacytidine and decitabine to induce DNA demethylation.⁴⁸ This block was linked to the overall depletion of the nucleotide pool and a concomitant cell cycle arrest. It will be important to use more specific inhibitors for similar experiments and to investigate the role of the nucleotide metabolism as a potential response modifier in demethylation therapies.

Incorporation of azanucleosides into nucleic acids and DNA demethylation

After azanucleosides have been metabolized to 5-aza-2'-deoxycytidine-triphosphate, they can become substrates for the DNA replication machinery and will be incorporated into DNA, where azacytosine can substitute for cytosine. Azacytosine-guanine dinucleotides are recognized by the DNA methyltransferases as natural substrate and the enzymes will initiate the methylation reaction by a nucleophilic attack. This results in the establishment of a covalent bond between the carbon-6 atom of the cytosine ring and the enzyme.^{49,50} The bond is normally resolved by beta-elimination through the carbon-5 atom, but the reaction is blocked with azacytosine, where carbon-5 is substituted by nitrogen (Fig. 4a). Thus, the enzyme remains covalently bound to DNA and its DNA methyltransferase function is blocked. In addition, the covalent protein adduction also compromises the functionality of DNA and triggers DNA damage signaling, resulting in the degradation of trapped DNA methyltransferases (Fig. 4b). As a consequence, methylation marks become lost during DNA replication.

Covalent trapping of mouse Dnmt1 has been confirmed experimentally by photobleaching approaches and has been shown to be dependent on the presence of the catalytic cysteine residue that is required for covalent complex formation.⁵¹ A different study indicated that the azacytidine-dependent degradation of DNA methyltransferases was not affected by mutations in the catalytic cysteine residue of human DNMT1 and that the drug induced specific proteasomal targeting of the enzyme.⁵² Whereas the latter results are difficult to reconcile with the covalent trapping paradigm, they raised the possibility that additional pathways could also contribute to azanucleoside-induced DNA demethylation. In this respect it is interesting to notice that a recent study has provided detailed insight into the DNA damage response induced by decitabine.⁵³ It was shown that decitabine caused the formation of double strand breaks in human cancer cell lines and that DNMT1 might play a role in mediating the cellular damage response to the drug. The induction of DNA damage by decitabine (and, presumably, also by azacytidine), combined with a role of DNMT1 in DNA repair⁵⁴ indicates that drug-induced demethylation patterns might be influenced by DNA repair mechanisms.

To characterize the mechanisms of drug-induced demethylation in greater detail, it will be critically important to quantitatively determine azanucleoside incorporation rates into genomic DNA. An early study with 10T1/2 mouse cells and radioactively labeled decitabine suggested that the substitution rate of 5-azacytosine for cytosine in genomic DNA could be as high as 10%.⁵⁵ However, the corresponding assays cannot be easily adapted to the requirements of clinical sample analysis and most laboratories use the drug-dependent depletion of DNMT1 protein as a non-quantitative substitute assay for confirming azanucleoside incorporation.^{56,57} It should be feasible to establish analytical methods for the quantitative determination of azacytosine levels in genomic DNA and it will be interesting to evaluate this parameter as a potential biomarker for response prediction.

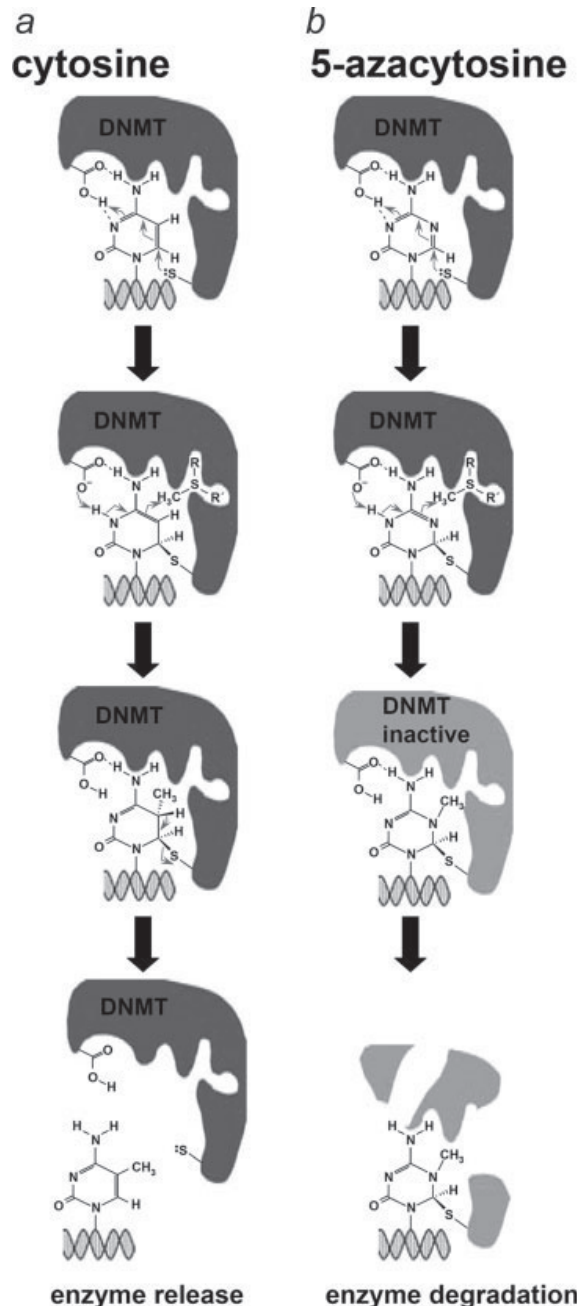


FIGURE 4 – Trapping mechanism of azacytosine. (a) A nucleophilic attack of the protein-thiol group (from a catalytic cysteine residue of the DNA methyltransferase enzyme, DNMT) at the C6 position of cytosine drives the subsequent transfer of the methyl group from the methyl donor S-adenosyl-L-methionine. The transfer proceeds through a covalent complex at position C6 between the DNA and the DNMT protein. The complex is resolved through a β -elimination reaction resulting in the release of the active DNA methyltransferase enzyme. (b) Mechanism-based inhibition of DNMTs by azacytosine-containing DNA. The covalent complex at C6 cannot be resolved through β -elimination, because of the presence of a nitrogen atom at position 5. Covalently trapped DNMTs are degraded, resulting in the depletion of cellular DNMTs.

Outlook

Understanding the mode of action of azanucleosides will require continued translational approaches on the molecular and the clinical level. It will be important to identify and validate biomarkers that predict the response of patients. Depending on their association with epigenetic regulation (*i.e.*, demethylation of specific markers) or apoptosis induction (*i.e.*, activation of damage signaling), these biomarkers might provide detailed insight into the cellular pathways that are influenced by azanucleosides. Similarly, determining the mechanisms of azanucleoside resistance and sensitivity will ultimately allow a better understanding of the drugs' mode(s) of action and facilitate the development of molecular markers for response prediction. For example, it will be inter-

esting to analyze potential associations between clinical responses and polymorphisms in the genes encoding the transporters for azanucleoside uptake. Lastly, it will be important to obtain a better understanding of the drug-induced epigenetic changes in patients. Genome-wide methylation profiling technologies should be used in order to maximize epigenetic reprogramming events at silenced tumor suppressor genes and to minimize epigenetic side effects, like the activation of silenced retroelements.

Acknowledgement

The author thank Mr. Dirk Stach for his support in determining the chemical stability of azacytidine and decitabine.

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