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Efficacy of DNA hypomethylating capacities of 5-aza-2′-deoxycytidine and its alpha anomer

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Abstract

In this paper, we have compared hypomethylating ability of classical beta-D-anomer of 5-aza-2′-deoxycytidine (5-aza-CdRf) and its alpha anomer in cell cultures. Alpha anomers of nucleosides generally exhibit low biological activity compared to their beta counterparts. It is reported that alpha anomer of 5-aza-CdRf efficiently hypomethylated genomic DNA in human T-lymphoblastoid CCRF-CEM cells. Satellite 2 and 18S rDNA were hypomethylated by alpha anomer at concentrations comparable to the beta form. However, the toxicity of the alpha anomer was 4-fold less than that of beta form. Contrast to CCRF-CEM the A549 lung carcinoma cells, possessing negligible level of methylation at repetitive loci, were highly resistant to 5-aza-CdRf treatment suggesting that global genomic methylation might be needed to mediate cytotoxic effect of the drug. Possible mechanisms of inhibition of DNA methylation by alpha anomer are discussed. In conclusion, alpha anomer of 5-aza-CdRf displaying lower host cytotoxicity than the classical beta form may be of potential use in epigenetic therapy.

Keywords: DNA methylation; Hypomethylation drug; 5-aza-2′-Deoxycytidine; Anomers; Cancer

1. Introduction

Cytosine methylation is so far most common modification of DNA in higher eukaryots. Its role in organism is rather pleiotropic as it has been shown that methylation can control gene expression, recombination, transposon activity and genome integrity (for recent review see, e.g. [1]). At the cellular level developmental processes are tightly linked with methylation changes at specific genomic regions [2].

It has become apparent over the past several years that DNA methylation might be a target of drug design [3,4]. For example, aberrant hypermethylation of promoter regions has been implicated in the transcriptional inactivation of the tumour suppressor genes (review [5]) and transgenes [6]. The deoxycytidine analog 5-aza-2′-deoxycytidine (5-aza-CdRf), clinically known as decitabine is a potent chemotherapeutic agent effective against various types of leukaemias [7–11] and a myelodysplastic syndrome [12]. Despite numerous clinical and experimental studies the anticancer effect of the drug remains elusive. The drug has been used as a popular DNA hypomethylation agent in many systems including animals [7,8,13,14] and plants [15]. In the cell 5-aza-CdRf is readily phosphorylated by cellular kinases. The three-step intracellular phosphorylation results in 5-aza-2′-deoxycytidine 5′-triphosphate, which is the substrate for DNA polymerases and then replaces 2′-deoxycytidine 5′-monophosphate in DNA [13]. It is believed that incorporation of 5-aza-CdRf into DNA during a replication cycle is essential for hypomethylation effect of the drug [16]. DNA methyltransferases would become covalently bound to the 5-azacytosine moiety and irreversibly inactivated [12,17–19]. Alternatively, recent observations suggest that the drug can induce selective enzyme degradation by formation of non-DNA complexes in absence of DNA replication [20]. In this connection, it should be mentioned that 5-aza-CdRf as well as its alpha anomer can also function as formylating agents by covalent addition of hydroxyl, thiol or amino groups of regulatory proteins to the reactive double bond of the 5-azacytosine ring followed by a prototropic rearrangement and hydrolytic cleavage of the intermediate to

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form the respective formyl-protein and carbamoylguanidine [21]. Formylation of catalytic groups of enzymes would lead to their irreversible inactivation.

Alpha anomers of nucleosides generally do not interfere with cellular metabolism and possess lower biological activity than beta anomeres. Nevertheless rare instances of biological effects of alpha nucleosides were reported [22]. We have previously shown that alpha anomer of 5-aza-CdRf was effective in preventing growth of cells *in vitro* and displayed an antileukaemic effect in mouse albeit at higher concentrations than the beta anomer [23]. Importantly, the *in vivo* toxicity of the alpha anomer was at least 100-fold less than its beta counterpart. In this paper we have addressed the question of hypomethylating properties of alpha and beta anomers of 5-aza-CdRf. The methylation of two genetic loci was studied in two cell lines using CG-methylation-sensitive restriction enzymes and Southern blot hybridisation.

2. Materials and methods

2.1. Chemicals

Alpha and beta anomers of 5-aza-CdRf were synthesized as described by Piskala and Sorm [24]. The formulas are given in Fig. 1. The purity of both anomers was established by 500 MHz ^{1}H NMR spectra and HPLC analysis using a commercially packed octadecylsilane column (Spherisorb ODS, 5 μm), which was eluted at a flow rate of 1 ml min^{-1} with 10^{-2} M phosphate buffer (pH 7.0). The column eluate was monitored at 244 nm.

2.2. Cell cultivation and inhibitors treatments

Cell growth experiments were performed on CCRF-CEM T lymphoblastoid cells (human acute lymphoblastic leukaemia, ATCC:CCL 119) and human small lung carcinoma cells A549 (ATCC:CCL 185). The CCRF-CEM line has been repeatedly used for biochemical and pharmacological characterization of anticancer and virostatic drugs [25,26]; the A549 line has been recently used in epigenetic studies of DNA [27] and histone methylation [28]. The CCRF-CEM T cells were cultivated in RPMI 1640 medium supplemented with 10% calf fetal serum. Cells were counted in Celltac MEK 5208 (NIHON KOHDEN) haematological analyzer on days indicated. The A549 cells were plated at a density of 2×10^5 ml^{-1} in D-MEM (PAN Germany) supplemented with 10% fetal calf serum. Number of adherent A549 cells was estimated from total protein content. The methylation inhibitors were used at concentrations of 0.5–30 μM; Trichostatin A (TSA) at 150 nM. Cells were harvested after the 72 h (methylation inhibitors) or 48 h (TSA) of treatment.

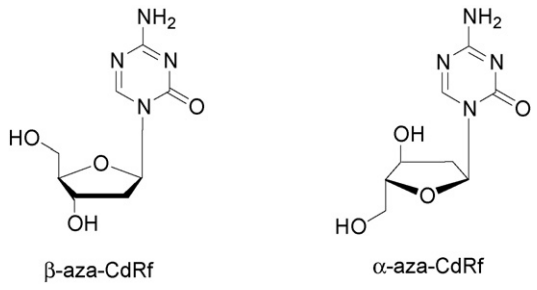

Fig. 1. Structure of 5-aza-2′-deoxycytidines.

2.3. Southern blot hybridisation

Total genomic DNA was isolated from approximately 5×10^7 cells using a standard cetyltrimethylammonium bromide (CTAB) method [29]. After the lysis the DNA was extracted by phenol–chloroform–isoamylalcohol (25:24:1, v/v) and ethanol precipitated. Purified genomic DNAs (1–3 μg) were digested with an excess of the enzymes (5 U μg^{-1} DNA) and subjected to electrophoresis on a 1.0% agarose gel. After electrophoresis, the gels were alkali-blotted onto Hybond-XL membranes (GE Healthcare, Little Chalfont, UK). Southern hybridisation was carried out in 0.25 M Na-phosphate buffer, pH 7.0, supplemented with 7% sodium dodecyl sulfate (SDS) at 65 °C for 16 h followed by washing with 2× SSC (1× SSC = 150 mM NaCl, 15 mM Na_3-citrate, pH 7.0), 0.1% SDS (twice 5 min), 0.2× SSC, and 0.1% SDS (twice 15 min). The hybridisation bands were visualized with a PhosphorImager STORM and the data were processed by ImageQuant software (GE Healthcare, Little Chalfont, UK).

2.4. Western blotting

Cells were washed with PBS and lysed in sodium dodecyl sulfate (SDS) lysis buffer (50 mM Tris–HCl, pH 7.5; 1% SDS; 10% glycerol) and subjected to SDS-PAGE. After being electrotransferred onto a polyvinylidene difluoride membrane (Immobilon-P, Sigma, Czech Republic), proteins were immunodetected using appropriate primary and secondary antibodies, and visualized by ECL+Plus reagent (GE Healthcare) according to the manufacturer's instructions. To monitor apoptosis goat polyclonal antibody against the carboxy terminus of mouse lamin B (sc-6217, Santa Cruz Biotechnology, CA) which cross-reacts with the human homolog, was used at a 1:500 dilution. Western blots were performed according Pachernik et al. [30] and loading was monitored by staining of total proteins in lanes.

2.5. DNA probes

[18S] rDNA probe was generated by PCR amplification using the following primers designed according to the sequence in database (M10098): 18S_for: 5′-CTGGATTTGCTGGTGATGAT-3′; 18S_rev: 5′-TGGCCTCAGTTCCGAAAACC-3′.

Human satellite 2 probe was generated by amplification of genomic DNA using primers derived from conserved region of the repeat (X06199): SAT2_for: 5′-TTCGAGTCCATTCGATGAT-3′; SAT2_rev: 5′-ATGGAATCAACATCAAATGG-3′. The PCR products were gel purified, sequenced and used as probes.