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Phosphorylation of deoxycytidine kinase on Ser-74: Impact on kinetic properties and nucleoside analog activation in cancer cells

Rachid Amsailale [a], Eric Van Den Neste [a,b], Angélique Arts [a], Eliza Starczewska [a], Françoise Bontemps [a,1,*], Caroline Smal [a,1]

[a] Laboratory of Physiological Chemistry, de Duve Institute & Université catholique de Louvain, B-1200 Brussels, Belgium
[b] Department of Hematology, Cliniques universitaires Saint-Luc & Université catholique de Louvain, B-1200, Brussels, Belgium

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ABSTRACT

Deoxycytidine kinase (dCK) (EC 2.7.1.74) is a key enzyme in the activation of several therapeutic nucleoside analogs (NA). Its activity can be increased in vivo by Ser-74 phosphorylation, a property that could be used for enhancing NA activation and clinical efficacy. In line with this, studies with recombinant dCK showed that mimicking Ser-74 phosphorylation by a S74E mutation increases its activity toward pyrimidine analogs. However, purine analogs had not been investigated. Here, we show that the S74E mutation increased the k_{cat} for cladribine (CdA) by 8- or 3-fold, depending on whether the phosphoryl donor was ATP or UTP, for clofarabine (CAFdA) by about 2-fold with both ATP and UTP, and for fludarabine (F-Ara-A) by 2-fold, but only with UTP. However, the catalytic efficiencies (k_{cat}/K_m) were not, or slightly, increased. The S74E mutation also sensitized dCK to feed-back inhibition by dCTP, regardless of the phosphoryl donor. Importantly, we did not observe an increase of endogenous dCK activity toward purine analogs after in vivo-induced increase of Ser-74 phosphorylation. Accordingly, treatment of CLL cells with aphidicolin, which enhances dCK activity through Ser-74 phosphorylation, did not modify the conversion of CdA or F-Ara-A into their active triphosphate form. Nevertheless, the same treatment enhanced activation of gemcitabine (dFdC) into dFdCTP in CLL as well as in HCT-116 cells and produced synergistic cytotoxicity. We conclude that increasing phosphorylation of dCK on Ser-74 might constitute a valuable strategy to enhance the clinical efficacy of some NA, like dFdC, but not of CdA or F-Ara-A.

1. Introduction

Deoxycytidine kinase (dCK) (EC 2.7.1.74) is a cytosolic enzyme with broad substrate specificity, catalyzing the phosphorylation of deoxycytidine (dCyd), deoxyadenosine (dAdo) and deoxyguanosine (dGuo) with ATP or UTP as phosphoryl donor. This reaction constitutes the first and rate-limiting step of the deoxynucleoside salvage pathway, which provides cells with deoxynucleotides for DNA synthesis as an alternative to the de novo synthesis [1]. In addition, dCK phosphorylates a number of therapeutic nucleoside analogs including anticancer drugs such as fludarabine (F-Ara-A), cladribine (CdA), clofarabine (CAFdA), gemcitabine (dFdC), cytarabine (Ara-C), and antiviral compounds, such as zalcitabine (ddC) and lamivudine (3TC) [2,3]. Phosphorylation of these compounds by dCK is most often the rate-limiting step in the pathway that converts them to their active triphosphate form. Given its key role in the activation and therapeutic efficacy of nucleoside analogs [4], dCK has been the subject of many functional and structural investigations in the last years.

The finding that several genotoxic agents, including aphidicolin, etoposide (VP-16), nucleoside analogs, UV-C- and γ-radiation, were able to increase dCK activity without change in dCK protein level led to the hypothesis of a regulation of dCK through post-translational modification [5–10]. Previous work of our group revealed that dCK is a phosphoprotein containing at least four phosphorylation sites: Thr-3, Ser-11, Ser-15 and Ser-74, the latter being the major phosphorylated residue. Site-directed mutagenesis demonstrated that phosphorylation of the three first sites does not influence dCK activity, whereas phosphorylation of Ser-74 increases its activity [11,12]. Moreover, use of a specific anti-phospho-Ser-74 antibody showed that activation of dCK by genotoxic agents in leukemic cells is related to an increase of Ser-74 phosphorylation [11,13]. In all these experiments, dCK activity was measured using 10 μM dCyd as substrate, as usually performed [14]. Unexpectedly, further investigations showed

* Corresponding author. Tel.: +32 2 764 7539; fax: +32 2 764 7598.
E-mail address: francoise.bontemps@uclouvain.be (F. Bontemps).
[1] Shared senior co-authorship.

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that mimicking phosphorylation of Ser-74 by a Ser-74-Glu mutation increases dCK activity for dCyd and dCyd analogs, but decreases it for dAdo and dGuo, suggesting that Ser-74 phosphorylation could positively influence dCK activity toward pyrimidine, but not purine nucleosides [15]. Nevertheless, the influence of Ser-74 phosphorylation on dCK activity toward purine analogs, which are widely used in the treatment of hematological malignancies and particularly chronic lymphocytic leukemia (CLL) [16], remained to be analyzed. Also, implication of Ser-74 phosphorylation in the activation of nucleoside analogs, either purine or pyrimidine, inside cancer cells had not been investigated yet.

In the present study, we further analyzed the effects of mimicking Ser-74 phosphorylation on the kinetic properties of recombinant dCK, particularly toward purine analogs. We also investigated the effect of an increase of the phosphorylation of endogenous dCK on Ser-74 on its activity toward various substrates and on the intracellular conversion of nucleoside analogs into their active triphosphate form. The results show that increasing dCK activity via Ser-74 phosphorylation can enhance the intracellular activation of some nucleoside analogs, like gemcitabine, but not of CdA or F-Ara-A.

2. Materials and methods

2.1. Materials

Ficoll-Paque Plus (density: 1.077) was from GE Healthcare (Buckinghamshire, UK). Dulbelcco's Modified Eagle Medium (DMEM), ultraglutamine, FCS and penicillin–streptomycin were purchased from Lonza (Basel, Switzerland). RPMI-1640 was from Gibco/Invitrogen (Carlsbad, CA, USA). [5-^{3}H]-dCyd (30 Ci/mmol), [5-^{3}H]-dFdC (21.9 Ci/mmol), [2,8-^{3}H]-dAdo (10.4 Ci/mmol), [8-^{3}H]-CdA (7 Ci/mmol), [8-^{3}H]-CAFdA (2.4 μCi/mmol), [8-^{3}H]-F-Ara-A (4.4 Ci/mmol) and [5-^{3}H]-Ara-C (20.6 Ci/mmol) were from Moravek Biochemicals (Brea, CA, USA). dCyd, dAdo, F-Ara-A, Ara-C, etoposide (VP-16), genistein (GNT), aphidicolin (APC) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St Louis, MO, USA). CdA was synthesized and supplied by Prof. J. Marchand (Laboratory of Organic Chemistry, Université catholique de Louvain, Louvain-la Neuve). dFdC from Eli Lilly (Indianapolis, USA) was kindly provided by V. Gregoire (Centre for Molecular Imaging and Experimental Radiotherapy, Université catholique de Louvain, Brussels). Ac-DEVD-AMC (Ac-Asp-Glu-Val-Asp-AMC) and AMC (7-amino-4-methyl coumarin) were purchased from Alexis Biochemicals (San Diego, CA, USA). Other chemicals, materials and reagents were from Sigma–Aldrich (St Louis, MO, USA), Merck Biosciences (Gibbstown, NJ, USA) or Bio-Rad (Hercules, CA, USA) Laboratories.

2.2. Cell culture

Peripheral blood from CLL patients was obtained after written informed consent in accordance with the hospital ethics committee. All patients had an established diagnosis of CLL, were free of any anticancer treatment for at least 3 months, and had lymphocytes $>30 \times 10^9$/L. Mononuclear cells (≥95% CLL cells) were isolated by Ficoll-Paque sedimentation, washed in PBS, suspended in RPMI-1640 supplemented with 10% heat-inactivated FCS and 1% penicillin–streptomycin, and cultured at a concentration of 10×10^6 cells/ml. EHEB cells, a B-cell line established from CLL lymphocyte [17], purchased from DSMZ-German Collection of Microorganisms and Cell Culture (Braunschweig, Germany), were cultured in RPMI-1640, supplemented with 10% heat-inactivated FCS at 37 °C in an atmosphere of 5% CO_2 in air. Before experiments, cells were counted and suspended at a concentration of 0.5×10^6 cells/ml. Hydrophobic activators of dCK (aphidicolin, etoposide and genistein) were dissolved in DMSO and equal amounts of DMSO were added in control cells. In each independent experiment, control condition was performed in duplicate.

HCT-116 and HT-29 colon cancer cell lines, kindly provided by G. Bommer (de Duve Institute, Brussels) were maintained in DMEM supplemented with 10% heat-inactivated FCS, 1% penicillin–streptomycin, and 2 mM ultraglutamine in an atmosphere of 5% CO_2 in air.

2.3. Kinetic studies of recombinant dCK

Human wild-type (WT) dCK or the mutant S74E were expressed in *Escherichia coli* and purified as previously described [18]. The purified proteins were stored at −80 °C in the presence of 20% (w/v) glycerol. dCK activities were determined using a radiochemical method [14]. All measurements were done at 37 °C in a buffer containing 50 mM Tris–HCl, pH 7.6, 2 mM dithiothreitol, 100 mM KCl, 5 mM $MgCl_2$, 2 mM ATP or UTP and labeled nucleosides, at concentrations that varied according to the nucleoside. The enzyme concentration varied between 5 and 20 nM. The K_m and k_{cat} values were calculated using non-linear regression with the GraphPad Prism 5.0 software. To investigate inhibition by dCTP, we measured dCK activity at variable concentrations of labeled dCyd or CdA in the presence of different fixed dCTP concentrations (ranging from 2 to 200 μM). K_i values were calculated with the GraphPad Prism 5.0 software from secondary plots of inhibitor concentration versus intercept or slope of the double reciprocal plots using linear least-squares regression analysis to determine the best-fit line describing the data points.

2.4. dCK activity in cell lysates

Activity of dCK in cell lysates was measured as reported previously [10], using 20–80 μg of cell extract protein depending on the cell line, with 10 μM [5-^{3}H]-dCyd (∼900 cpm/pmol), 50 μM [5-^{3}H]-dFdC (∼300 cpm/pmol), 50 μM [2,8-^{3}H]-2′-dAdo (∼200 cpm/pmol), 50 μM [8-^{3}H]-CdA (∼200 cpm/pmol) or 50 μM [8-^{3}H]-F-Ara-A (∼200 cpm/pmol) as substrates. When dAdo was used, 10 μM erythro-9-(2-hydroxy-3-nonyl)adenine was included in the assay to prevent its deamination by adenosine deaminase. In some experiments, dCyd and CdA were used at several concentrations as indicated. The protein content of samples was determined by the method of Bradford by using bovine serum albumin as standard [19].

2.5. Analysis of deoxycytidine and nucleoside analog metabolism in intact cells

To investigate the influence of the phosphorylation state of Ser-74 on the metabolism of nucleoside (analogs) in CLL cells, samples containing 30×10^6 cells were preincubated without or with 3 μM aphidicolin for 30 min and incubated for additional 2 h with labeled nucleosides (∼2 μCi/ml) at the indicated concentrations. For analysis of dFdC activation in HCT-116 cells, cells were plated at a concentration of 0.5×10^6 cells/well in 6-well plates and incubated without or with 3 μM aphidicolin for 24 h. Incubation medium was then replaced by fresh medium to remove aphidicolin and cells were incubated for additional 24 h in the presence of [5-^{3}H]-dFdC at the indicated concentrations. Preparation of perchloric cell extracts and separation of labeled nucleotides by HPLC were performed as described previously [20]. The amounts of nucleotides synthesized were calculated from the specific radioactivity of the precursor. To analyze the incorporation of labeled nucleosides into nucleic acids, radioactivity was measured in the