Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 805 (2004) 339–346
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Determination of the deoxycytidine kinase activity in cell homogenates with a non-radiochemical assay using reversed-phase high performance liquid chromatography Identification of a novel metabolite of 2-chlorodeoxyadenosine

Jörgen Bierau[a,1], René Leen[a], Albert H. van Gennip[a,1], Huib N. Caron[b], André B.P. van Kuilenburg[a,*]

[a] *Academic Medical Center, Department of Clinical Chemistry, University of Amsterdam, Emma Children's Hospital, P.O. Box 22700, 1100 DE Amsterdam, The Netherlands*
[b] *Academic Medical Center, Department of Paediatric Oncology and Haematology, Emma Children's Hospital, Amsterdam, The Netherlands*

Received 3 March 2003; received in revised form 2 March 2004; accepted 17 March 2004

Available online 24 April 2004

Abstract

A non-radioactive procedure to measure the deoxycytidine kinase (dCK) activity in crude cell free homogenates was developed. 2-Chlorodeoxyadenosine (CdA) was used as the substrate for dCK and was separated from its product 2-chlorodeoxyadenosine-5′-monophosphate (CdAMP) by reversed-phase HPLC. A complete separation of CdA and its metabolites was achieved in 30 min. The minimum amount of CdAMP that could be detected was 1 pmol. The assay was linear with reaction times up to at least 3 h. With respect to the protein concentration, the reaction was linear with protein concentrations up to 760 μg/ml in the assay. An amount of 8×10^3 cells was already sufficient to determine the specific dCK activity in SK-N-BE(2)c cells. CdA was not only converted to CdAMP but also to 2-chloroadenine and, surprisingly, also to 2-chlorodeoxyinosine, in MOLT-3 cells. The deamination of CdA was completely inhibited by deoxycoformycin, which clearly demonstrates that CdA is a substrate for adenosine deaminase.

Keywords: Deoxycytidine kinase; Enzymes; 2-Chlorodeoxyadenosine

1. Introduction

Deoxycytidine kinase (dCK) is a deoxynucleoside kinase with a broad substrate specificity. The natural substrates of dCK are dCyd, dAdo and dGuo [1]. However, dCK also phosphorylates therapeutically important deoxynucleoside analogues, such as 1-β-D-arabinofuranosyl cytosine (cytarabine, AraC), 2′,2′-difluorodeoxycytidine (gemcitabine) and 2-chlorodeoxyadenosine (cladribine, CdA). In fact, it is the rate-limiting enzyme in the activation of these cytotoxic nucleoside analogues [2]. Resistance of cancer cells to these cytotoxic deoxynucleoside analogues is often associated with a reduced dCK activity. Recently, it was demonstrated that resistance of acute myeloid leukaemia towards AraC is caused by alternative splicing of dCK-encoding mRNA, which leads to expression of inactive dCK proteins [3].

The regulation of the activity and expression of dCK is complex and depends on multiple factors. The activity of

Abbreviations: ADA, adenosine deaminase; APRT, adenine phosphoribosyl transferase; AraC , 1-β-D-arabinofuranosyl cytosine, cytarabine; CAde, 2-chloroadenine; CdA, 2-chloro-2′-deoxyadenosine, cladribine; CdAMP, cladribine-5′-monophosphate; CdADP, cladribine-5′-diphosphate; CdATP, cladribine-5′-triphosphate; CdI, 2-chloro-2′-deoxyinosine; Cl-AMP, 2-chloroadenosine-5′-monophosphate; Cl-ADP, 2-chloroadenosine-5′-diphosphate; Cl-ATP, 2-chloroadenosine-5′-triphosphate; dAdo, 2′-deoxyadenosine; dCyd, 2′-deoxycytidine; dGuo, 2′-deoxyguanosine; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; MTAP, methylthioadenosine phosphorylase; NMPK, nucleoside monophosphate kinase; NDPK, nucleoside diphosphate kinase; PNP, purine nucleoside phosphorylase; XDH, xanthine dehydrogenase

* Corresponding author. Present address: Academic Medical Center, Laboratory for Genetic Metabolic Diseases, F0-224, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. Tel.: +31-205665958; fax: +31-206962596.
E-mail address: a.b.vankuilenburg@amc.uva.nl (A.B.P.v. Kuilenburg).
[1] Present address: Department of Biochemical Genetics, Academic Hospital Maastricht, The Netherlands.

1570-0232/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2004.03.036

dCK is regulated via feedback inhibition by dCTP, thus low intra-cellular levels of dCTP increase the activity of dCK [4], and by post-translational modification. While the dCK activity may be cell-cycle dependent, the expression of dCK-encoding mRNA proved to be independent of the cell-cycle. Cell-cycle dependent regulation of dCK activity is a much-debated subject, but appears to depend on the cell line model studied [1]. Furthermore, inhibition of DNA synthesis and DNA damage cause the expression of dCK-encoding mRNA to increase and, consequently, the dCK activity [5,6].

It is evident that a reliable method to measure the dCK activity in cell homogenates is a prerequisite when studying the salvage metabolism of (cytotoxic) deoxynucleoside analogues. To date, all procedures to measure dCK activity are based on the method described by Ives and Durham [4], and rely on thin-layer chromatography or weak ion-exchange paper chromatography to separate the radioactive substrate (CdA or dCyd) from the corresponding nucleoside-5′-monophospate. These procedures, using radio-labelled substrates, proved to be extremely laborious and time consuming. Another major disadvantage of these traditional analytical methods is that the formation of other metabolites, which might hamper accurate measurement of dCK activity, may not be detected.

CdA, is an analogue of deoxyadenosine and is phosphorylated to cladribine 5′-monophosphate (CdAMP) with high specificity by dCK [7]. CdA is thought to be resistant to degradation by adenosine deaminase and is highly toxic to proliferating and non-proliferating lymphocytes. CdA is successfully used in the treatment of several haematological malignancies, such as hairy cell leukaemia and chronic lymphatic leukaemia.

In this paper, we present a dCK activity assay, using non-radio-labelled CdA as substrate combined with reversed-phase HPLC for analysis. By using HPLC, we observed that CdA-5′-monophosphate is not the only metabolite formed during the dCK assay. We describe the identification of the metabolites formed in the reaction-mixture of the dCK assay, including the novel metabolite of CdA: 2-chlorodeoxyinosine (CdI).

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade. 2-Chloro-2′-deoxyadenosine, inosine, ATP, bicinchoninic acid, adenosine deaminase, phenylmethylsulphonylfluoride and bovine serum albumin were obtained from Sigma–Aldrich Chemie (Zwijndrecht, The Netherlands). Deoxycoformycin was a generous gift from Prof. Dr. J. Balzarini, Rega Institute for Medical Research, Catholic University of Leuven, Belgium. $NH_4H_2PO_4$, Tris, NaCl, NaF, NaOH, $MgCl_2$, perchloric acid, and methanol were purchased from Merck (Darmstadt, Germany). Dithiothreitol was purchased from Boehringer (Mannheim, Germany). [^{3}H]2-chloro-2′-deoxyadenosine (21.1 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA, USA). Dulbecco's Modified Eagles Medium, bovine fetal serum and penicillin/streptomycin/fungizone mix were obtained from BioWhittaker Europe (Verviers, Belgium). L-Glutamine and gentamycin were obtained from Gibco BRL (Paisley, Scotland). Isoton II was obtained from Beckman Coulter (Krefeld, Germany). Triton X-100 and saponine were from BDH Laboratory Supplies (Poole, UK).

2.2. Cell culture

The SK-N-BE(2)c neuroblastoma cell line, the MOLT-3 and HL-60 leukaemia cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) The cells were routinely cultured in Dulbecco's Modified Eagles Medium (DMEM, BioWhittaker Europe, Verviers, Belgium), supplemented with 2 mM L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, 0.2 mg/ml gentamycin, 0.25 μg/ml fungizone and 10% (v/v) bovine fetal serum at 37 °C in humidified (90%) air with 5% CO_2. The cells were maintained in 75 cm^2 loosely capped culture flasks (Co-star Corp, Cambridge, MA, USA) and maintained in logarithmic growth phase. Cell cultures were consistently free of mycoplasma (tested with Mycoplasma PCR–ELISA, Boehringer Mannheim).

SK-N-BE(2)c cells were harvested by trypsinisation and cell numbers were determined after solubilizing the cells in isoton II containing 2.7×10^{-7}% (v/v) Triton X-100 and 2.7×10^{-3}% (w/v) saponin. The nuclei were counted with a Coulter Counter Z 1000 with a 100 μM orifice (Coulter Electronics Ltd, Buckinghamshire, UK). Molt-3 and HL-60 cells were harvested by centrifugation and washed once with phosphate-buffered saline and counted. Cell pellets were snap-frozen in liquid nitrogen and stored at −80 °C until use.

2.3. Standard dCK-assay procedure

The dCK assay conditions were essentially as described by Arnér and colleagues, with a few alterations [7]. Cell pellets were resuspended to a density of 10×10^6 cells/ml in lysis buffer containing 50 mM Tris–HCl pH 7.4, 200 mM NaCl, 10 mM DTT, and 2.5 mM phenylmethylsulphonylfluoride. Cells were sonicated three times at 40 W (Vibra-cell Sonificator, output control 20%) for 10 s with intervals of 30 s. During this procedure, the samples were constantly kept in ice-water. After sonification, the samples were incubated for 15 min on ice, and were then centrifuged for 15 min at 10,000 × g at 4 °C. The supernatant was used for dCK activity assays, while the pellet was discarded.

The reaction solution consisted of 1 mM CdA, 10 mM ATP, 10 mM $MgCl_2$, 200 mM NaCl, 20 mM NaF, 2 mM DTT

in 10 mM Tris–HCl pH 7.4. In a typical assay, to 25 μl of the reaction solution, 25 μl of cell homogenate was added. The final concentrations were 0.5 mM CdA, 5 mM $MgCl_2$, 5 mM ATP, 200 mM NaCl, 10 mM NaF, 6 mM DTT in 10 mM Tris–HCl pH 7.4. The reaction was started by putting the test tube containing the reaction solution and the cell lysate in a waterbath at 37 °C. After an appropriate incubation time (usually 60 min) at 37 °C, the reaction was terminated by placing the reaction tube on ice and adding 50 μl of ice-cold methanol, causing precipitation of the protein. After 10 min of incubation on ice, the samples were either directly prepared for HPLC analysis or stored at −20 °C until analysis. Reaction blanks were created by performing the methanol-precipitation immediately after mixing the cell extract with the reaction solution. Prior to HPLC analysis, the samples were centrifuged for 5 min at 10,000 × *g* at 4 °C and diluted two-fold with a 50 mM $NH_4H_2PO_4$ (pH not adjusted) solution.

The protein pellet obtained after centrifugation of the methanol precipitated reaction mixture was dissolved in 50 μl 0.2 M NaOH and the protein content was determined using a bicinchoninic acid solution containing 0.1% (w/v) $CuSO_4$, using bovine serum albumin as the standard, as described previously [8].

The specific dCK activity is expressed as the amount of CdAMP formed from CdA per amount of protein per hour and is calculated by dividing the amount of picomoles CdAMP formed in the assay mixture during the incubation by the amount of protein (μg) in the assay mixture (pmol CdAMP/(μg protein h)).

2.4. HPLC analysis

CdA and its metabolites were analysed by HPLC at ambient temperature using a 250 mm × 4.6 mm Supelcosil LC-18-S column at a flow rate of 1 ml/min, using a gradient of 50 mM $NH_4H_2PO_4$ (buffer A, pH unadjusted) and 50% (v/v) methanol in 50 mM $NH_4H_2PO_4$ (buffer B). The gradient used was: 10 min at 90% buffer A, in 10 min to 50% buffer A, hold for 10 min. Detection of CdA and CdAMP was performed at 265 and 253 nm. CdA and CdAMP concentrations were calculated using pure CdA as a standard. Because of slight variation in elution times, the ratio A_{265}/A_{253} was determined for the compounds of interest in order to confirm their identity. The A_{265}/A_{253} ratios were 1.4 for CdA and CdAMP, 1.3 for 2-chloroadenine (CAde) and 0.6 for CdI.

The HPLC system used consisted of a Perkin-Elmer Series 410 LC pump (Perkin-Elmer, Norwalk, CT, USA) including a Gilson 401C dilutor and a Gilson 231 XL autosampler equipped with a Gilson 832 temperature regulator (Gilson Medical Electronics, Villiers Le Bel, France). The detector was a Gynkotek UVD 340S photodiode array detector (Gynkotek GmbH, Munich, Germany) with Gynkosoft chromatography software version 5.30. For purpose of identification of metabolites, spectra were recorded using the photodiode array detector and, where possible, compared to the spectra of pure standards.

2.5. Purification of radio-labelled cladribine

[^{3}H]CdA was purified by HPLC using a 250 mm × 4.6 mm Supelcosil LC-18-S column at a flow rate of 1 ml/min. Purification was performed using a gradient of H_2O (buffer A) and 50% methanol (buffer B). The gradient used was: 10 min at 90% buffer A, in 10 min to 50% buffer A, hold for 10 min. Detection of CdA was performed at a wavelength of 265 nm. Pure [^{3}H]CdA was obtained by collecting the fraction eluting at 27 min. The purified [^{3}H]CdA was concentrated by evaporation of methanol.

2.6. Radiochemical assay procedure

In order to be able to detect all metabolites formed from CdA, the dCK assay procedure was also performed with purified radio-labelled CdA, under the same reaction conditions as the non-radiochemical assay. Radioactivity was detected online with a Radiomatic 525TR Flow Scintillation Analyzer with a 500 μl TR-LCS cell (Packard Instruments, Meriden, CT, USA) using Ultima Flow AP (Packard, Dowers Grove, IL, USA) at an effluent to scintillation fluid ration of 1:1.

2.7. Assay validation

The intra-assay variation of the procedure was assessed by determining the activity of dCK in ten replicate cell pellets of a large culture of SK-N-BE(2)c cells, which was harvested and divided over multiple cell pellets. The inter-assay (between-day) variation of the procedure was determined by measuring the activity of dCK on 8 different days in these cell pellets of SK-N-BE(2)c cells from the same culture. The reproducibility of the assay is expressed at the coefficient of variation.

2.8. Synthesis of 2-chlorodeoxyinosine

CdI was synthesised both chemically and by enzymatic deamination of CdA.

Enzymatic preparation: Enzymatic preparation of 2-chloroinosine from 2-chloroadenosine was previously described by Antonino and Wu [9]. To obtain CdI, an 8 mM CdA solution was mixed with an equal volume of a 144 U/ml solution of adenosine deaminase (ADA) and incubated for 16 h at 37 °C. *Chemical synthesis*: CdI was synthesised analogously to the synthesis of 2-chloroinosine as described by Suzuki et al. [10]. Briefly, 1 mM dGuo was incubated with 100 mM $NaNO_2$ and 2 M NaCl in 3 M sodium acetate at pH 3.2 at 37 °C for 2 h. The chemical preparation of CdI yielded 5.9% CdI, as determined by HPLC analysis of the reaction mixture. The spectra of enzymatically

and chemically prepared CdI were recorded an used for identification of metabolites formed in the dCK assay.

2.9. Preparation of 2-chloroadenine and 2-chlorohypoxanthine

CAde and 2-chlorohypoxanthine were prepared by incubating CdA and CdI, respectively, in 0.1 M $HClO_4$ for 2 h at 37 °C, followed by neutralisation with a 5 M K_2CO_3 solution.

3. Results

3.1. Synthesis of CdA metabolites

The enzymatic deamination of CdA resulted in 100% degradadation of CdA and yielded 95% CdI and 5% 2-chlorohypoxanthine. The UV-Vis spectrum of the enzymatically prepared CdI corresponded with the spectra of chemically synthesised CdI and the enzymatically synthesised 2-chloroinosine published by Suzuki and colleagues and Antonino and Wu, respectively [9,10]. No spontaneous deamination of CdA was observed under the conditions described in the materials and methods section. The deamination of CdA by purified ADA was thus confirmed. The UV-Vis spectrum of CdI showed an absorption maximum at 253 nm, at which wavelength CdI was detected in further experiments. When the enzymatically prepared CdI was incubated with 100 mM $HClO_4$ for two hours at 37 °C, CdI was hydrolysed to 2-chlorohypoxanthine with 100% efficiency. Analogous to the UV-Vis spectrum of CdI, the UV-Vis spectrum of 2-chlorohypoxanthine also showed an absorption maximum at a wavelength of 253 nm. The acid hydrolysis of CdA caused 100% breakdown of CdA and yielded >95% CAde. The UV-Vis spectrum of CAde showed an absorption maximum at a wavelength of 265 nm. The chemical structures and UV-Vis absorption spectra of CdA and its metabolites are shown in Fig. 1.

3.2. HPLC analysis

In order to determine the amount of CdAMP formed in the reaction mixture during the incubation time, we separated CdA and CdAMP by reversed-phase HPLC. Using the gradient described, we achieved a complete baseline separation in 30 min (Fig. 2B). The optimal wavelength for the detection of CdA and CdAMP proved to be 265 nm, as determined by recording the UV-Vis spectra of these compounds. The minimum amount of CdA that could be detected was approximately 1 pmol. The CdAMP peak was identified by comparison of absorption spectra and retention time. In addition, competition experiments in which the reaction was performed in the presence of 5 mM dCyd, caused a near complete inhibition of the phosphorylation of CdA to CdAMP.

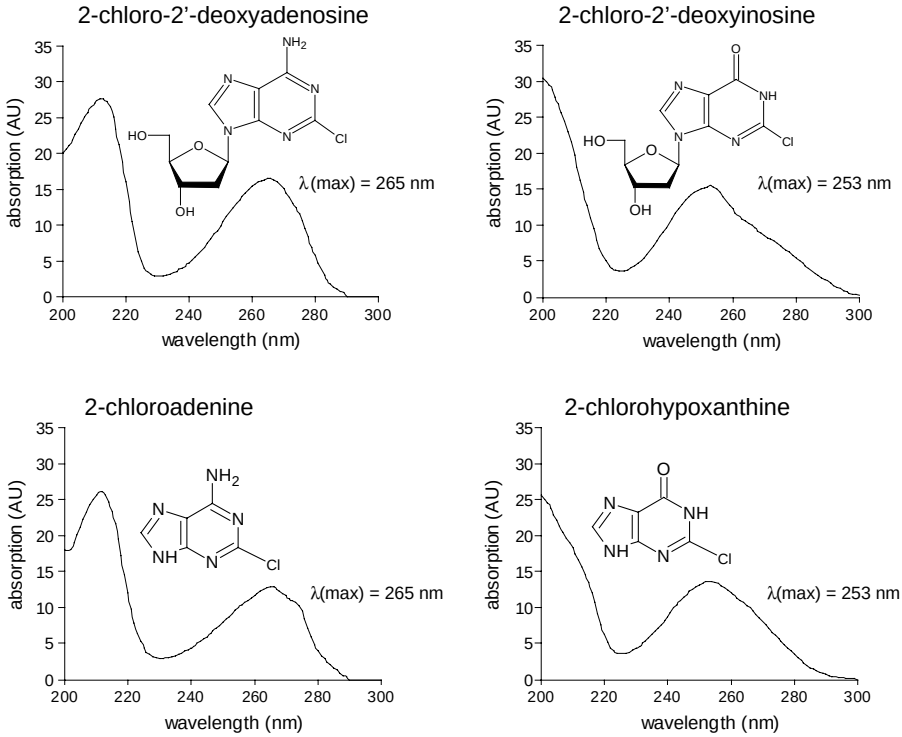

Fig. 1. UV-Vis absorption spectra and structural formulas of 2-chloro-2′-deoxyadeosine (cladribine), 2-chloroadenine, 2-chloro-2′-deoxyinosine and 2-chlorohypoxanthine (AU: arbitrary units).

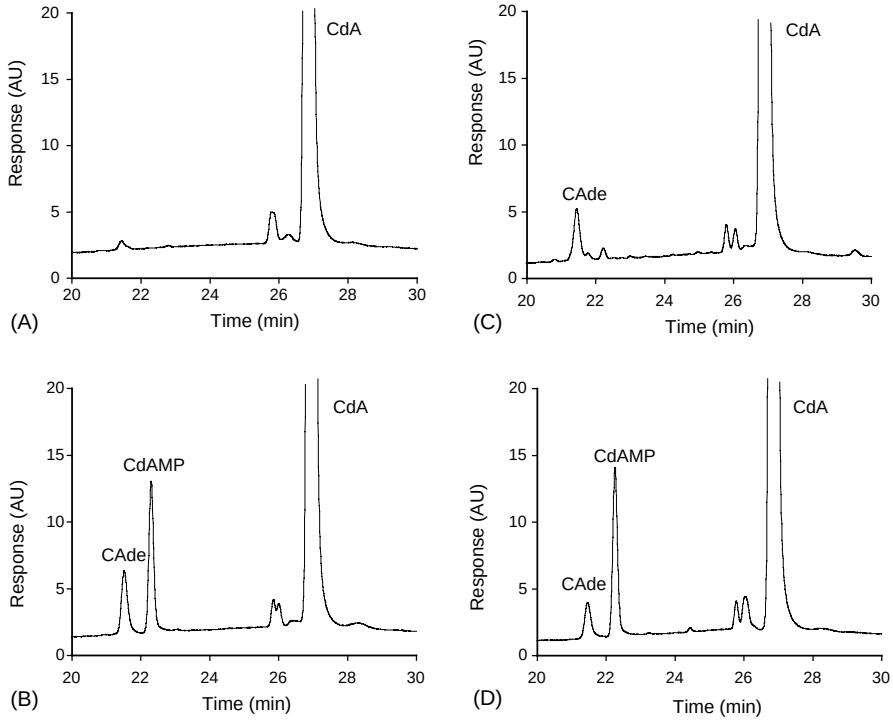

Fig. 2. HPLC traces of the dCK assay performed in a lysate of SK-N-BE(2)c cells under various conditions. (Panel A) Reaction blank. (Panel B) Standard reaction conditions. (Panel C) Reaction in presence of 5 mM dCyd. (Panel D) Reaction in presence of 10 mM inosine (AU: arbitrary units).

The intra-assay C.V. and the inter-assay C.V. for the complete assay, HPLC detection and protein determination, were 4.3% ($n = 10$) and 10.9% ($n = 8$), respectively.

3.3. Formation of 2-chloroadenine

During the optimisation of the assay procedure described in this paper, we noticed that when the assay was performed using SK-N-BE(2)c neuroblastoma cells, another peak eluted closely to CdAMP with a similar UV-Vis spectrum. When the reaction mixture was spiked with CAde, the added CAde co-eluted with the additional product peak, confirming the presence of CAde in the reaction mixture. The ratio of CAde to CdAMP formed was about 0.6 under standard assay conditions. In order to characterise the formation of CAde during the assay procedure, the assay was repeated under various conditions. The omission of NaF from the reaction buffer caused a slight increase in the amount of CAde formed, while the addition of excess dCyd (5 mM) inhibited the phosphorylation of CdA to CdAMP by more than 90%, but not the formation of CAde (Fig. 2C). The addition of inosine, the preferred susbtrate of purine nucleoside phosphorylase, inhibited the formation of CAde by 30–60% (Fig. 2D). With the exception of the addition of dCyd, the formation of CdAMP was unaltered by the presence or absence of both NaF and inosine. By performing the standard assay with radio-labelled CdA as the substrate, the sum of the radioactivity from the CdAMP, CAde and CdA peaks accounted for all the radioactivity added to the assay mixture. This indicated that CdAMP and CAde were the only metabolites formed under the assay conditions in extracts of SK-N-BE(2)c cells.

3.4. Formation of CdI

Analogous to our findings in cell extracts of SK-N-BE(2)c cells, we also found that in extracts of MOLT-3 cells CdAMP was not the only metabolite formed from CdA. In this cell line, however, the additional metabolite was not CAde. The unidentified metabolite eluted closely to CdAMP, but had a slightly longer retention time (Fig. 3A). The UV-Vis spectrum stongly resembled that of inosine. Spiking the reaction mixture with enzymatically prepared CdI confirmed the presence of CdI in the reaction mixture. The ratio of CdI to CdAMP formed was about 0.3 under standard assay conditions. The formation of CdI was studied under a variety of conditions. The deamination of CdA to CdI was not influenced by the presence or absence of NaF, nor by excess dCyd. However, when the assay was repeated in the presence of deoxycoformycin (0.8 mM) the deamination of CdA was inhibited by 100% (Fig. 3B). The formation of CdI was inhibited by 15% in the presence of 10 mM inosine. The deamination of CdA by purified, commercially available, ADA (14 U/ml) was inhibited by 100% by deoxycoformycin (0.8 mM) and by 20% by inosine (10 mM), respectively.

By performing the standard assay with radio-labelled CdA as the substrate, the sum of the radioactivy from the CdAMP, CdI and CdA peaks accounted for all the radioactivity added

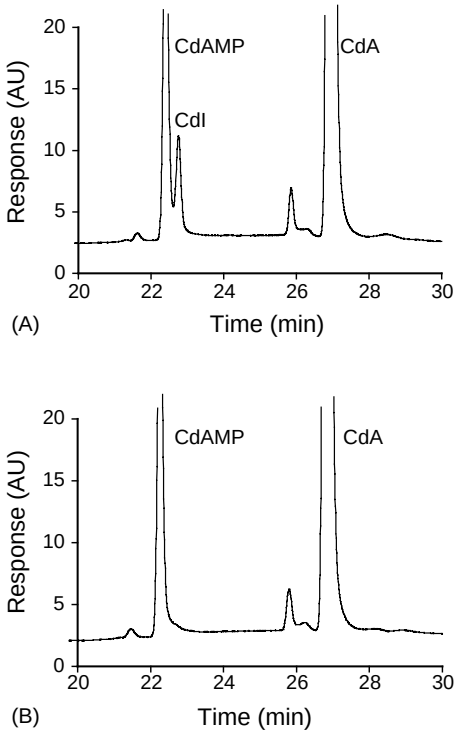

Fig. 3. HPLC traces of the dCK assay performed in a lysate of MOLT-3 cells. (Panel A) Standard reaction conditions. (Panel B) Reaction in presence of 0.8 mM deoxycoformycin (AU: arbitrary units).

to the assay mixture. This indicated that CdAMP and CdI were the only metabolites formed under the assay conditions in extracts of MOLT-3 cells.

3.5. Reaction conditions

Fig. 4 shows that the amount of CdAMP produced by dCK from SK-N-BE(2)c cells increased linearly with amount of protein added to the reaction mixture in the range of 0.6–18 μg, which corresponded with a concentration in the assay mixture of 12–380 μg/ml. The highest amount of protein in Fig. 4 corresponds with 0.25×10^6 SK-N-BE(2)c cells added to the reaction mixture. The reaction was linear up to approximately 15×10^6 SK-N-BE(2)c cells/ml, which corresponded with 0.75×10^6 SK-N-BE(2)c cells in the assay mixture. At higher cell densities, a deviation from linearity was observed with respect to the amount of CdAMP formed and the amount of protein added (data not shown). Approximately 8×10^3 cells proved to be sufficient to measure the dCK activity in SK-N-BE(2)c neuroblastoma cells. With respect to the time-dependence of the dCK assay, the formation of CdAMP from CdA by dCK was linear with reaction times up to at least 180 min (Fig. 5).

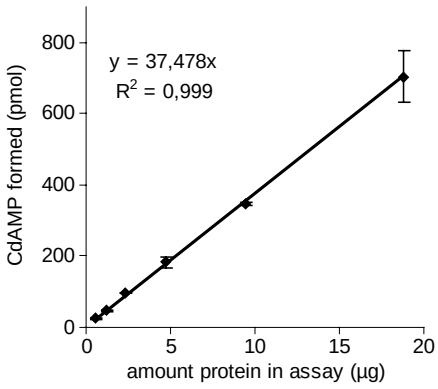

Fig. 4. Protein dependence of the dCK reaction. The figure shows the amount of CdAMP produced by dCK from SK-N-BE(2)c cells. The reactions were allowed to proceed for 1 h at 37 °C. Each data point represents the mean of three experiments ± S.D.

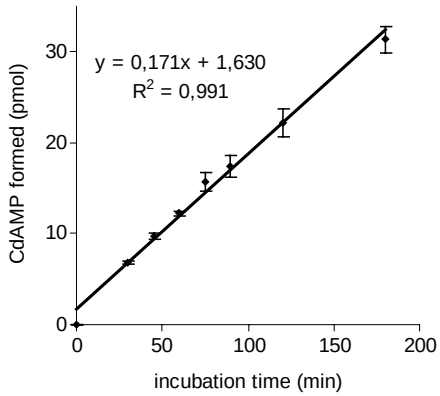

Fig. 5. Time dependence of the dCK reaction. The figure shows the amount of CdAMP formed by dCK from SK-N-BE(2)c cells. The dCK activity was measured at a protein concentration of 0.39 mg/ml (19.7 μg of protein added to the reaction mixture). Each data point represents the mean of three experiments ± S.D.

Table 1
Specific dCK activities in SK-N-B(2)c, HL-60 and MOLT-3 cells

Cell line	Specific dCK activity (pmol/μg protein/h)
SK-N-BE(2)c	32.0 ± 1.1
HL-60	73.3 ± 1.1
MOLT-3	158.6 ± 6.0

The data shown represent the mean of three experiments ± S.D.

Using the assay we have developed, we have measured the specific activity of dCK in SK-N-BE(2)c neuroblastoma cells, HL-60 acute promyolocytic leukaemia and MOLT-3 acute lymphoblastic leukaemia cells (Table 1). The specific activity in extracts of MOLT-3 cells proved to be two and five times higher than in extracts of HL-60 and SK-N-BE(2)c cells, respectively.

4. Discussion

In this paper we describe a fast and sensitive assay to measure the activity of dCK in crude cell homogenates, based on a simple, non-radioactive, reversed-phase HPLC-method using UV detection. The reproducibility of the assay procedure was acceptable. The major advantages of the assay

procedure presented in this paper are that it is considerably less expensive and less elaborate than the standard procedures, which require the use of radio-labelled CdA or dCyd as substrates and analysis by thin layer chromatography or weak ion-exchange paper chromatography.

The phosphorylation reaction in our standard assay procedure was performed at a concentration of 200 mM NaCl. Usova and Eriksson previously demonstrated that the activity of purified recombinant dCK increased when the assay mixture contained a high concentration of NaCl, the optimum concentration being 400 mM NaCl [11]. However, the addition of NaCl in concentrations greater than 200 mM NaCl to the cell lysates caused an unacceptably high standard deviation in our assay (data not shown). This may be caused by denaturation and precipitation of protein due to the high ion-strength of the buffer.

Because of the fact that we analysed our samples by HPLC, we observed that, under the assay conditions described, CdA was not only metabolised to CdAMP, but also to CAde and CdI. The fact that in the presence of excess dCyd, for which dCK has a much higher affinity, the phosphorylation of CdA is more than 90% inhibited while the formation of CAde and CdI is unaffected, strongly indicates that CdA is directly metabolised to CAde and CdI. To date, these metabolites of CdA have not been observed by using thin layer- or weak ion-exchange paper chromatography. Allthough the formation of CdA and CdI did not affect the phosphorylation of CdA by dCK, the metabolites need to be properly separated in order to accurately measure the activity of dCK.

One might argue that CdA may not be the appropriate substrate to measure dCK activity, as it is also metabolised to CAde and CdI. However, the concentration of CdA under our assay conditions is sufficiently high to maintain a saturating concentration of CdA. Moreover, the advantage of using CdA instead of dCyd as the substrate for dCK is that CdA has a much higher specificity for dCK than dCyd [7]. Not only is dCyd a substrate for dCK, it is also a substrate for mitochondrial thymidine kinase, TK-2 [1]. Furthermore, when dCyd is used as the substrate, the addition of an inhibitor of (d)Cyd deaminase is required. Tetrahydrouridine is a potent inhibitor of (d)Cyd deaminase and is often added to the dCK reaction mixture. If CdA is used as the susbtrate, the addition of tetrahydrouridine is not necessary.

With respect to the formation of CAde from CdA, our results are in accordance with the *in vitro* observations made by Bontemps and colleagues, who have previously shown that CAde was the major metabolite of CdA in a leukaemic cell line [12]. It has been suggested that the formation of CAde is catalysed by methylthioadenosine phosphorylase (MTAP). Our experiments show that the formation of CAde is inhibited by inosine, the natural substrate of purine nucleoside phosphorylase. We suggest that purine nucleoside phosphorylase (PNP) may also catalyse the formation of CAde from CdA.

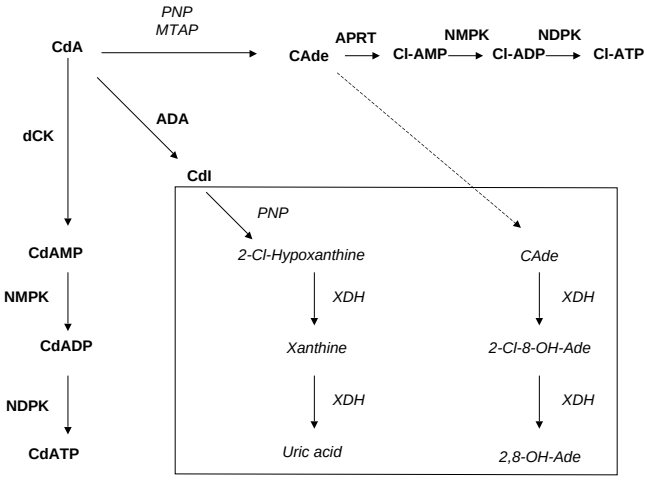

Fig. 6. Proposed metabolism of cladribine. The pathways depicted with bold text are established metabolic routes for cladribine, the boxed pathways and the enzymes depicted in italics are speculative. Abbreviations: CdA: 2-chlorodeoxyadenosine; CdAMP: CdA 5′-monophosphate; CdADP: CdA 5′-diphosphate; CdATP: CdA 5′-triphosphate; CAde: 2-chloroadenine; Cl-AMP: 2-chloroadenosine-5′-monophosphate; Cl-ADP: 2-chloroadenosine-5′-diphosphate; Cl-ATP: 2-chloroadenosine-5′-triphosphate; CdI: 2-chlorodeoxyinosine; dCK: deoxycytidine kinase; NMPK: nucleoside monophosphate kinase; NDPK: nucleoside diphosphate kinase; PNP: purine nucleoside phosphorylase; MTAP: methylthioadenosine phosphorylase; APRT: adenine phosphoribosyl transferase; ADA: adenosine deaminase, XDH: xanthine dehydrogenase.

In the present report, we demonstrate that CdA is deaminated to CdI in cell lysates of MOLT-3 cells. This deamination proved to be catalysed by ADA, as the addition of the ADA inhibitor deoxycoformycin completely inhibited the deamination of CdA. Inosine also inhibited the deamination of CdA via feedback inhibition of ADA. Purified, commercially available, ADA also proved to efficiently deaminate CdA. The deamination of CdA by purified ADA was also completely inhibited by deoxycoformycin and partially inhibited by inosine. Previously, Antonino and Wu have enzymatically prepared 2-chloroinosine from 2-chloroadenosine and thus demonstrated that the chlorine atom at the 2-position of the adenine-ring does not confer resistance to ADA [9]. This finding, and our novel finding that CdA is deaminated to CdI demonstrate that, in contrast with the general consensus, CdA is an alternative substrate for ADA.

Based on our present findings and the references cited, we postulate that CdA can be metabolised as depicted in Fig. 6. Whether or not CdI, 2-Chloro-inosine and CAde are substrates for xanthine dehydrogenase (XDH) and if these compounds have physiological significance remain subjects for future research.

The fact that the specific dCK activity in the leukaemic cell lines was 2-5 times higher than in SK-N-BE(2)c cells correlates well with the sensitivity of these types of cancer cells towards cytarabine. Cytarabine is an analogue of dCyd and is the drug of choice for the treatment of patients suffering from acute leukaemia as it is highly toxic to leukaemic cells.

Acknowledgements

This study was supported by the "Stichting Kindergeneeskundig Kankeronderzoek". The authors thank Prof. Dr. Jan Balzarini, Rega Institute for Medical Research, Catholic University of Leuven, Belgium for generously providing deoxycoformycin. The authors also thank Ms. Lida Zoetekouw and Mr. Henk van Lenthe (Department of Clinical Chemistry and Emma Kinder Ziekenhuis, Academic Medical Center, University of Amsterdam) for the useful discussions and their expert advice.

References

[1] E.S. Arner, S. Eriksson, Pharmacol. Ther. 67 (1995) 155.
[2] J.O. Liliemark, W. Plunkett, Cancer Res. 46 (1986) 1079.
[3] M.J. Veuger, M.W. Honders, J.E. Landegent, R. Willemze, R.M. Barge, Blood 96 (2000) 1517.
[4] D.H. Ives, J.P. Durham, J. Biol. Chem. 245 (1970) 2285.
[5] M. Sasvari-Szekely, T. Spasokoukotskaja, M. Szoke, Z. Csapo, A. Turi, I. Szanto, S. Eriksson, M. Staub, Biochem. Pharmacol. 56 (1998) 1175.
[6] Z. Csapo, M. Sasvari-Szekely, T. Spasokoukotskaja, I. Talianidis, S. Eriksson, M. Staub, Biochem. Pharmacol. 61 (2001) 191.
[7] E.S. Arner, T. Spasokoukotskaja, S. Eriksson, Biochem. Biophys. Res. Commun. 188 (1992) 712.
[8] P.K. Smith, R.I. Krohn, G.T. Hermanson, A.K. Mallia, F.H. Gartner, M.D. Provenzano, E.K. Fujimoto, N.M. Goeke, B.J. Olson, D.C. Klenk, Anal. Biochem. 150 (1985) 76.
[9] L.C. Antonino, J.C. Wu, Biochemistry 33 (1994) 1753.
[10] T. Suzuki, H. Ide, M. Yamada, T. Morii, K. Makino, Bioorg. Med. Chem. 9 (2001) 2937.
[11] E.V. Usova, S. Eriksson, Eur. J. Biochem. 248 (1997) 762.
[12] F. Bontemps, A. Delacauw, S. Cardoen, E. van den Neste, G. van den Berghe, Biochem. Pharmacol. 59 (2000) 1237.