The pivotal role of CTP synthetase in the metabolism of (deoxy)nucleosides in neuroblastoma
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Chapter 7

Abstract

A non-radioactive procedure to measure the deoxycytidine kinase activity in crude cell free homogenates was developed. 2-Chloro-deoxyadenosine (CdA) was used as the substrate and was separated from its 5'-monophosphate by reversed-phase HPLC. A complete separation of CdA and its metabolites was achieved in 40 minutes. The minimum amount of CdA that could be detected was 1 pmol. The assay was linear with reaction times up to at least 3 hours. With respect to the protein concentration, the reaction was linear with protein concentrations up to 760 μg/ml in the assay. An amount of $8 \times 10^3$ cells was already sufficient to determine the specific dCK activity in SK-N-BE(2)c cells.

CdA was not only converted to its 5'-monophosphate but also to 2-Chloroadenine and, surprisingly, also to 2-Chlorodeoxyinosine, in MOLT-3 cells. Therefore, our results demonstrate that CdA is also a substrate for adenosine deaminase.

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), 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 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 dCK is regulated via feedback inhibition by dCTP, thus low intracellular 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 mRNA encoding dCK proved to 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 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'-monophosphate. 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 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 developed a dCK activity assay, using non-radio-labelled CdA as a 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-chloro-deoxyinosine.

2. Materials and methods

2.1 Chemicals

2-Chloro-2'-deoxyadenosine and ATP were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). [3H]2-Chloro-2'-deoxyadenosine (21.1 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA). Dulbecco's Modified Eagles Medium, Bovine Fetal Serum and Penicillin/Streptomycin/Fungizone-mix were obtained from BioWhittaker Europe. L-glutamine and gentamycin were obtained from Gibco BRL. Isoton II was obtained from Beckman Coulter. Triton X-100 and saponine were from BDH Laboratory Supplies. All other chemicals were of analytical grade.

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 I.U./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 % CO2. The cells were maintained in 75 cm2 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 \times 10^{-7} % (v/v) Triton X-100 and 2.7 \times 10^{-3} % (w/v) saponin. The nuclei were counted with a Coulter Counter Z 1000 with a 100 µM orifice (Coulter Electronics Ltd, Buckinghamshire, England). 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 Arne and colleagues, with a few modifications [7]. Cells were homogenised in a buffer consisting of 50 mM Tris-HCl pH 7.4,
200 mM NaCl, 10 mM DTT, and 2.5 mM phenylmethylsulphonyl fluoride, by sonication (4 x 10 sec 40 J/Ws) at a density of 10 x 10^9 cells/ml. After 15 minutes incubation on ice, the homogenates were centrifuged for 15 minutes at 10,000 x g at 4 °C. The supernatant was used for dCK activity assays.

The assay mix 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. To 25 μl of the assay mix, 25 μl of cell homogenate was added. The final concentrations were 0.5 mM CdA, 5 mM MgCl_2, 5mM ATP, 200 mM NaCl, 10 mM NaF, 6 mM DTT in 10 mM Tris-HCl pH 7.4. After 60 minutes incubation at 37 °C the reaction was terminated by placing the reaction tube on ice and adding 50 μl of ice-cold methanol. The samples were then stored at -20 °C until analysis. Reaction blanks were created by directly performing the methanol-precipitation after mixing the assay mix with the cell homogenates. Prior to analysis, the samples were centrifuged for 5 minutes at 10,000 x g at 4 °C and the supernatant was diluted 2-fold with 50 mM NH_4H_2PO_4. 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, as described previously [8].

2.4 HPLC analysis

CdA and its metabolites were separated by HPLC using a 250 x 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) and 50% (v/v) methanol in 50 mM NH_4H_2PO_4 (buffer B). The gradient used was: 10 minutes at 90% buffer A, in 10 minutes to 50% buffer A, hold for 10 minutes. Detection of CdA and CdAMP was performed at 265 nm and at 253 nm. CdA and CdAMP concentrations were calculated using pure CdA as a standard. Because of slight variation in the 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, respectively, 1.3 for CAde and 0.6 for Cdl.

For purpose of identification of metabolites, spectra were recorded on line using a Gynkotek UVD 340S photo diode array detector (Gynkotek, Germering, Germany) 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 x 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 minutes at 90% buffer A, in 10 minutes to 50% buffer A, hold for 10 minutes. Detection of CdA was performed at a wavelength of 265 nm. Pure[^3]H]CdA was obtained by collecting the fraction eluting at 27 minutes. The purified[^3]H]CdA was concentrated by evaporation of methanol.

2.6 Assay validation

The intra-assay variation of the procedure was assessed by determining the activity of dCK in ten replicate cell pellets of a culture of SK-N-BE(2)c cells, which was harvested 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 replicate cell pellets of SK-N-BE(2)c cells from the same culture. The reproducibility of the assay is expressed at the relative standard deviation.
2.7 Synthesis of 2-Chloro-deoxyinosine

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 and incubated for 16 hrs at 37 °C.

Chemical synthesis: CdI was synthesised analogous to the synthesis of 2-chloroinosine as described by Suzuki et al. [10]. Briefly, dGuo (1 mM) was incubated with 100 mM NaNO₂ and 2 M NaCl in 3M sodium acetate at pH 3.2 at 37 °C for 2 hr. The chemical preparation of CdI yielded 5.9% CdI (peak purity).

The spectra of enzymatically or chemically prepared CdI were recorded and used for identification of metabolites formed in the dCK assay.

2.8 Preparation of 2-Chloroadenine and 2-Chlorohypoxanthine

CAde and 2-Chlorohypoxanthine were prepared by incubating CdA and CdI, respectively, in 0.1 M HClO₄ for 2 hrs at 37 °C, followed by neutralisation with K₂CO₃.

![Figure 1: HPLC elution profiles and UV-VIS absorption spectra of CdA (panel A), CdI (panel B) and 2-Cl-Hypoxanthine (panel C). The chromatograms were at 265 nm. The optimum absorption wavelength was 265 nm for CdA and 253 nm for CdI and 2-Cl-Hypoxanthine.](image-url)
3. Results

3.1 Synthesis of CdA metabolites

The enzymatic deamination of CdA resulted in 100% degradation of CdA and yielded 95% CdI and 5% 2-Chloro-hypoxanthine (figure 1). The UV-VIS spectrum of enzymatically prepared CdI corresponded with that of chemically prepared CdI, thus confirming the deamination of CdA by ADA. The maximum absorption wavelength of CdI was 253 nm (figure 1), at which wavelength CdI was detected in further experiments. When the enzymatically prepared reaction mixture was treated with 0.1 M HClO₄ for two hours, CdI was hydrolysed to 2-Chloro-hypoxanthine with 100% efficiency (figure 1).

Acid hydrolysis of CdA resulted in 100% breakdown of CdA and yielded > 95% CAde. The preparation of CAde by acid hydrolysis of CdA is depicted in figure 2.

![Figure 2: CAde was prepared from CdA by acid hydrolysis (panel A) and the UV-VIS absorption spectrum was recorded (panel B) and the absorption maximum was determined to be 265 nm.](image)

3.2 HPLC analysis and identification of metabolites

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 system described in the materials and methods section, we achieved complete baseline separation (figure 3). The optimal detection wavelength of CdA and CdAMP proved to be 265 nm, as determined by recording the spectra of these compounds under the conditions described. The minimum amount of CdA that could be detected was 1 pmol. The identity of the CdAMP peak was confirmed by comparison of absorption spectra and the retention time. Figure 3 shows that CdAMP is formed during the dCK assay, as it was not detected in
reaction blanks. When the reaction was performed in the presence of 5 mM dCyd, the phosphorylation of CdA by dCK was nearly completely inhibited.

**Figure 3:** Elution profile of a standard dCK assay performed with SK-N-BE(2)c cells. Panel A shows the elution profile of a reaction blank. The elution profile of the reaction mixture is shown in panel B.

Besides CdAMP, another metabolite, which eluted before CdAMP, was formed when the dCK assay was performed in SK-N-BE(2)c cells. The formation of this metabolite proved to be inhibited by the presence of 10 mM NaF. Therefore, the assay was repeated without the presence of NaF, in order to facilitate the identification of this unknown metabolite. Based on the retention time, the match of the UV-VIS absorption spectrum with chemically prepared CAde and spiking the reaction mixture with chemically prepared CAde, it was demonstrated that CAde was formed from CdA during the assay procedure. In order to determine whether CAde was formed from CdA or CdAMP, the assay was repeated in the presence of excess dCyd (5 mM) which completely inhibited the phosphorylation of CdA. Figure 4 shows that CAde was also formed when the assay was performed in the presence of 5 mM dCyd, indicating that CdA is directly metabolised to CAde.
When the assay was performed with MOLT-3 cells, a metabolite was observed that eluted after CdAMP (figure 5). The UV-VIS absorption spectrum of this spectrum resembled that of inosine, and had an absorption maximum at 253 nm. Therefore, we prepared CdI from CdA both enzymatically and chemically, with the purpose to record the UV-VIS absorption spectrum of CdI. The UV-VIS spectrum of the unknown metabolite formed in lysates of MOLT-3 cells corresponded with that of chemically and enzymatically prepared CdI. Subsequently a sample was spiked with enzymatically prepared CdI, which confirmed that CdA was deaminated to CdI in lysates of MOLT-3 cells. When the assay was repeated in the presence of 5 mM dCyd, the phosphorylation of CdA was >95% inhibited. However, the deamination of CdA to CdI was unaffected, which indicated that CdI is directly formed from CdA.

Repeating the dCK-assay with radioactive CdA as a substrate confirmed that CdAMP, CAde and CdI were the only metabolites formed and thus the sum of the substrate and metabolites accounted for all radioactivity added to the assay mixture.

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

Figure 4: Elution profile of the dCK assay performed without NaF (panel A). Homogenised SK-N-BE(2)c cells were added to the reaction mixture to a density of 20 × 10⁶ cells/ml. The phosphorylation of CdA was inhibited > 90% by the addition of 5 mM dCyd, while CdA was still metabolised to CAde (panel B).
Determination of dCK activity

3.3 Reaction conditions

Figure 6 shows that the amount of CdAMP produced by dCK from SK-N-BE(2)c cells increased linearly with the amount of protein added to the reaction mixture in the range of 0.6 μg to 18 μg, which corresponded to a concentration in the assay mixture of 12 μg/ml to 380 μg/ml. The highest amount of protein in figure 2 corresponds with $0.25 \times 10^6$ SK-N-BE(2)c cells added to the reaction mixture. The reaction was linear up to approximately $0.75 \times 10^6$ cells in the assay, which corresponded with a cell density of $15 \times 10^6$ cells/ml. 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).

With respect to the time dependence of the dCK reaction, the formation of CdAMP from CdA by dCK from SK-N-BE(2)c cells was linear with reaction times up to at least 180 min (figure 7).

Approximately $8 \times 10^3$ cells proved to be sufficient to measure the specific dCK activity in SK-N-BE(2)c neuroblastoma cells. By 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 promylocytic leukaemia cells and MOLT-3 acute lymphoblastic leukaemia cells (table 1).
The specific activity of MOLT-3 cells proved to be 2 and 5 times higher than that of HL-60 and SK-N-BE(2)c cells, respectively.

**Figure 6:** 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 ± SD.

**Figure 7:** 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 ± SD.

### 4. Discussion

In this paper we developed a fast and sensitive assay of dCK activity in crude cell homogenates, based on a simple, non-radioactive, reversed phase HPLC-method using UV detection. The assay procedure was very reproducible, with an inter assay C.V of 10.9 %. The major advantages of the assay procedure presented in this paper are that it is much cheaper 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 paper chromatography. The advantage of using CdA as a substrate for dCK, in stead of dCyd, is that it has a higher specificity for dCK than dCyd [7]. Not only is dCyd a substrate for dCK, but it is also a substrate for thymidine kinase 2 [1]. Furthermore, in case that dCyd is used as the substrate for the dCK reaction, 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 when dCyd is used as the substrate. When CdA is used as the substrate, the addition of tetrahydrouridine in unnecessary.
CdA is also a substrate for dGK [11]. However, the dGK activity in our preparations proved to be negligible as the phosphorylation of CdA by dCK was completely inhibited by addition of excess dCyd.

<table>
<thead>
<tr>
<th>cell line</th>
<th>Specific dCK activity (pmol/µg protein/h)</th>
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</thead>
<tbody>
<tr>
<td>SK-N-BE(2)c</td>
<td>32.0 ± 1.1</td>
</tr>
<tr>
<td>HL-60</td>
<td>73.3 ± 1.1</td>
</tr>
<tr>
<td>MOLT-3</td>
<td>158.6 ± 6.0</td>
</tr>
</tbody>
</table>

Our standardised assay procedure, was performed at a concentration of 200 mM NaCl. Usova and Eriksson previously demonstrated that addition of high concentrations of NaCL increased the activity of purified recombinant dCK, the optimum concentration being 400 mM NaCl [12]. However, addition of NaCl in concentrations greater than 200 mM NaCl, caused an unacceptably high standard deviation in our assay (data not shown). This may be caused by denaturation and precipitation of protein due to high ion-strength in the buffer.

By using HPLC analysis, we observed that next to CdAMP, also CAde and CdI are formed under the assay conditions. CAde and CdI were metabolised directly from CdA, thus before its phosphorylation to CdAMP. This was confirmed by the fact that in the presence of excess dCyd, a better substrate for dCK than CdA, CAde and CdI were still formed from CdA. To date, these metabolites of CdA have not been observed by using thin-layer chromatography or weak ion-exchange paper chromatography. Although the formation of CAde and CdI apparently did not affect the dCK reaction, the metabolites of CdA need to be properly separated in order to make an accurate assessment of dCK activity.

With respect to CdA being metabolised to CAde, our results are in accordance with the in vitro observation made by Bontemps and colleagues, who have previously shown that CAde was the major metabolite of CdA in a leukaemic cell line [13]. The conversion of CdA to CAde is most likely catalysed by purine nucleoside phosphorylase.

In the present report, we demonstrate that CdA is deaminated to CdI in cell lysates prepared from MOLT-3 cells. Purified, commercially available, ADA also proved to efficiently deaminate CdA. Previously, Antonino and Wu have enzymatically prepared 2-Cl-Inosine from 2-Cl-adenosine and demonstrated this way that a chlorine atom at the 2-position of the adenosine-ring does not confer resistance to ADA [9]. This and our results are in contrast with the general consensus that CdA is resistant to ADA.

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 sensitivity of these types of cancer 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. The first and rate-limiting step in the anabolism of cytarabine is its phosphorylation to cytarabine monophosphate, catalysed by dCK. High activity of dCK in leukemic cells correlates with their sensitivity toward cytarabine.
Chapter 7

Acknowledgements

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