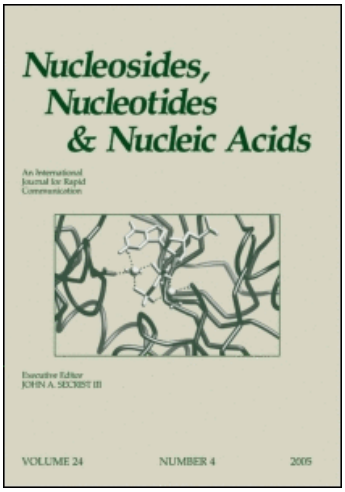

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F-ara-AMP IS A SUBSTRATE OF CYTOPLASMIC 5′-NUCLEOTIDASE II (cN-II): HPLC AND NMR STUDIES OF ENZYMATIC DEPHOSPHORYLATION

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□ *Intracellular accumulation of triphosphorylated derivatives is essential for the cytotoxic activity of nucleoside analogues. Different mechanisms opposing this accumulation have been described. We have investigated the dephosphorylation of monophosphorylated fludarabine (F-ara-AMP) by the purified cytoplasmic 5′-nucleotidase cN-II using HPLC and NMR. These studies clearly showed that cN-II was able to convert F-ara-AMP into its non phosphorylated form, F-ara-A, with a* K_m *in the millimolar range and* $V_{max} = 35$ *nmol/min/mg, with both methods. Cytoplasmic 5′-nucleotidase cN-II can degrade this clinically useful cytotoxic nucleoside analogue and its overexpression is thus likely to be involved in resistance to this compound.*

Keywords 5′-Nucleotidase; Fludarabine; cN-II; HPLC; NMR; Resistance

INTRODUCTION

Fludarabine (9-β-D-arabinosyl-2-fluoroadenine monophosphate; F-ara-AMP) is an active agent for the treatment of chronic lymphocytic leukemia (CLL). Although fludarabine monotherapy produces responses in 63–79%

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of untreated patients and 32–48% of pretreated patients, clinical resistance exists and limits the efficacy of this agent.[1] Different hypothesis are studied as the mechanism to fludarabine resistance as well as to other clinically used nucleoside analogues.[2]

Several studies have shown an implication of the cytoplasmic IMP-selective 5′-nucleotidase (cN-II) in the *in vitro* and *in vivo* resistance to nucleoside analogues.[3–6] A weak dephosphorylation of the monophosphorylated derivative of cladribine by purified cN-II was observed *in vitro*.[7] In addition, cN-II interacts with the antiviral nucleoside analogues acyclovir and carbovir.[8,9] However, whether cN-II dephosphorylates fludarabine inducing thereby drug resistance to this agent is not known. Given the widespread use of fludarabine in CLL and the preference of cN-II towards purine derivatives, we studied the possible enzymatic conversion of F-ara-AMP into F-ara-A by cN-II by using two non radioactive methods, one based on high performance liquid chromatography (HPLC) and one based on nuclear magnetic resonance (NMR). We show that cN-II is able to convert F-ara-AMP into its nonphosphorylated form 9-β-D-arabinofuranosyl-2-fluoro-Adenine (F-ara-A) and is thus likely to be involved in resistance to the cytotoxic effect of this drug.

MATERIALS AND METHODS

Reagents

F-ara-AMP (fludarabine, Fludara®) was from Schering Laboratories (Lysles-Lannoy, France). F-ara-A, NaCl, Tris, KCl, ethylene-bis (2-aminoethyl-ether)-N,N,N′,N′-tetraacetic acid (EGTA), phenylmethanesulfonyl fluoride (PMSF), $MgCl_2$, 2-mercaptoethanol, lysozyme, DTT, D_2O, and imidazole were purchased from Sigma Aldrich (Saint-Quentin, France) and isopropyl β-D-l-thiogalactopyranoside (IPTG) and LB Broth base from Invitrogen (Cergy Pontoise, France).

Purification of cN-II

The pET24b vector containing the C-terminal His-tagged and N-terminal deleted (30 amino acids) cN-II (cN-II-ΔN) was a generous gift from Dr. J. Spychala (Chapel Hill, North Carolina). Bacterial expression and protein purification were performed as previously described with only minor modifications.[10] The fraction containing cN-II-ΔN was analyzed for purity by silver staining of a polyacrylamide gel according to the manufacturer's instructions (Amersham, Orsay, France) and protein concentration was determined using the Bradford assay (Pierce, Montluçon, France).

Sample Preparation for HPLC Assays

For HPLC studies, purified enzyme (75 ng/μL) was incubated for 8 h at 37°C in a final volume of 15 μL containing 10 mM $MgCl_2$, 500 mM NaCl, 50 mM imidazole pH 6.5, and 0.2–3.5 mM F-ara-AMP. The pH of this solution was 6.7 at 25°C.

Sample Preparation for NMR Assays

For the kinetic studies, F-ara-AMP was introduced in NMR tubes at a final concentration of 0.5 to 7.0 mM in solutions containing purified enzyme (500 ng/μL), 10 mM $MgCl_2$, 500 mM NaCl, 130 mM imidazole, 7.7 mM Tris, 1 mM DTT, and 5% D_2O. The pH of the solution was 8.3 at 25°C (without correction for D_2O). Prior to F-ara-AMP addition, each sample was incubated at 37°C ± 0.1°C for 10–15 min. NMR acquisitions were started immediately after addition of F-ara-AMP.

HPLC Experiments

Identification of F-ara-A and F-ara-AMP and quantification of F-ara-A were performed using a validated HPLC method with diode array UV detection (Thermo electron chromatogram system). The column used was a C18 Symmetry Shield column (5 μm particle size, 250 × 4.6 mm, Waters). The mobile phase was constituted of acetonitrile and a potassium dihydrogenophosphate (50 mM) buffer with octane sulfonic acid (250 mM) adjusted to pH 7.0 (5:95, v/v). The separation of compounds was achieved at 30°C with an isocratic solvent delivery system at a flow rate of 1.0 mL/min. Detection was performed at 272 nm. Chromatographic peak purity and identification of compounds were verified using the spectral analysis function of Chromquest software (Thermo electron).

NMR Experiments

NMR spectra were acquired on a Bruker *Avance DRX500* spectrometer, equipped with a 5 mm $^{1}H/^{13}C/^{15}N$ triple resonance probe head with z-field gradients. F-ara-AMP ^{1}H assignments were verified from 1D-proton, homonuclear multiple bond coherence (HMBC) and phase-sensitive double quantum filtered correlated spectroscopy (DQF-COSY) experiments performed at 5°C on a 7.0 mM F-ara-AMP solution. The ^{1}H chemical shifts were calibrated using the water signal as a reference (4.96 ppm at 5°C). 1D-proton experiments were acquired over a sweep width of 6,000 Hz into 6k complex datapoints. The acquisition time was 1 s and the relaxation delay was 2.6 s. For water suppression a W5 scheme was used with WATERGATE pulse-train delays of 400 μs.[11] The free induction decays were weighted by an exponential function (line broadening of 3 Hz) prior

to Fourier transformation. Gradient-selected HMBC[12] were acquired with sweep-widths of 16,000 × 4,500 Hz and 64 × 1024 complex points, using an 80 ms delay for the evolution of long-range ^{13}C-^{1}H couplings. Phase-sensitive DQF-COSY spectra[13,14] were acquired with a water-suppression sequence using excitation sculpting.[15] For this purpose, a 2,900 μs, 180° rectangular-shaped pulse was employed. The spectra were acquired over a 4,500 Hz sweep-width in both dimensions, using 128 × 1,024 complex points. Typical 2D-spectra were recorded with a 0.23 s acquisition time and a 2 s relaxation delay. For kinetic studies, 128 scan 1D-proton experiments were acquired as described above at 37.0°C. The ^{1}H chemical shifts were recalibrated using the water signal as a reference (4.66 ppm at 37°C). The data were processed and analysed using XWINNMR (Bruker Analyti GmgH).

Calculation of Reaction Rates and Kinetics Parameters from HPLC Data

The concentration of F-ara-A formed was calculated using the height of the corresponding peak for each initial F-ara-AMP concentration. The velocity of F-ara-A production was calculated from F-ara-A concentrations after 8-h incubations (F-ara-A production was linear over this timespan). Results are given as a mean value of triplicate experiments. K_m and V_{max} values were derived from Lineweaver-Burk plots obtained with Microsoft® Excel.

Calculation of Reaction Rates and Kinetic Parameters from NMR Data

Initial rates of F-ara-A production were derived from the increase rates of a characteristic peak integral corresponding to the H8 proton in F-ara-A. The increase of this peak was followed by 8-min steps over the first 80 min of the reaction, and a linear equation was fitted to the data (Microsoft® Excel). Integral values for the H8 peaks were correlated to concentration values, using a reference sample of 2.50 mM F-ara-AMP under the same conditions as for the other studies. Background F-ara-A production was assessed from a control experiment using a 3.5 mM F-ara-AMP sample, incubated at 37.0°C during 80 min without cN-II-ΔN.

Kinetic parameters were derived from the initial rates of F-ara-A production, at different concentrations of substrate, using the Lineweaver-Burk representation. All experiments were duplicated. Standard errors were estimated from experimental conditions.

RESULTS

Purification of cN-II-ΔN

We obtained approximately 5 mg of purified cN-II-ΔN from 1 l of bacteria culture using the purification method described in the Methods section. Silver staining of SDS-PAGE showed that the protein fraction obtained was of high purity (data not shown). The identity of the protein was verified using a monoclonal antibody against cN-II (generated in our laboratory, data not shown). The purified protein was stable for over 3 months when stored at 4°C.

HPLC Study of F-ara-AMP Dephosphosphorylation by cN-II-ΔN

As shown in Figure 1, the HPLC method described above was useful to study the dephosphorylation of F-ara-AMP into the non-phosphorylated derivative F-ara-A in presence of purified cN-II-ΔN. Under these conditions, retention times were 5.6 and 14.9 min for F-ara-AMP and F-ara-A, respectively. No significant degradation of F-ara-AMP into F-ara-A was observed in the absence of cN-II-ΔN.

Based on a kinetic study, further experiments were performed using 8-h incubations of different concentrations of F-ara-AMP and purified cN-II-ΔN. The Lineweaver-Burk plot obtained with the HPLC experiments showed a K_m of 4 mM and a V_{max} of 35 nmol/min/mg for F-ara-AMP dephosphorylation by cN-II-ΔN (Figure 2).

NMR Study of F-ara-AMP Dephosphosphorylation by cN-II-ΔN

F-ara-AMP proton chemical shifts were assigned from the experiments mentioned in the Methods section. At 5°C, the ^{1}H resonance assignment is the following: H1′, 6.24 ppm (*d*); H2′, 4.55 ppm (*t*); H3′, 4.42 ppm (*t*); H4′, 4.10 ppm (*m*); H5′ and H5″, 4.10 and 4.03 ppm (*m*); H8, 8.49 ppm (*s*).

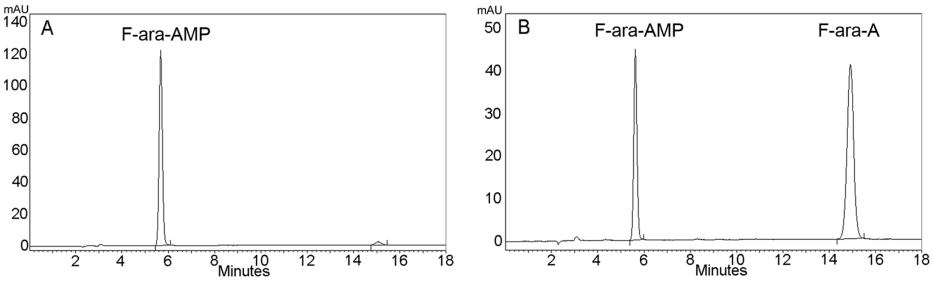

FIGURE 1 HPLC analysis of dephosphorylation of F-ara-AMP to F-ara-A. F-ara-AMP (3.5 mM) was incubated without (A) or with (B) cN-II-ΔN (75 ng/μL) at 37°C for 8 h.

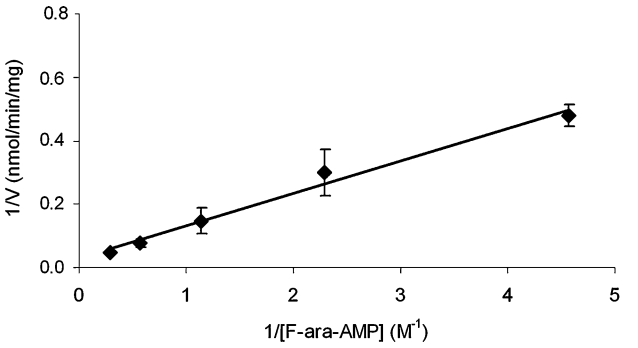

FIGURE 2 Lineweaver-Burk plot for the conversion of F-ara-AMP to F-ara-A by cN-II-ΔN with different initial concentrations of F-ara-AMP using HPLC detection. The figure shows the velocity of the conversion of F-ara-AMP into F-ara-A by cN-II-ΔN using 75 ng/μL cN-II-ΔN at 37°C for 8 h. The figure shows the mean of 3 separate experiments with standard errors. Determination coefficient = 0.99.

For the kinetic study, ^{31}P NMR experiments could have been used to overcome problems arising from the buffer conditions (water and imidazole). However, ^{31}P NMR is inherently less sensitive than ^{1}H-NMR and quantitative analysis by ^{31}P NMR was not as reliable as by ^{1}H-NMR, in the concentration and time conditions of our experiments. Therefore, ^{1}H-NMR was used to follow the reaction with maximum sensitivity. The H8 NMR signals in F-ara-AMP and F-ara-A appeared as sensitive probes to follow the kinetic reaction. The H8 peaks are well resolved and give sharp singlets in the ^{1}H-NMR spectra (Figure 3). Moreover, the H8 chemical shifts difference between the substrate and the product is −0.18 ppm, a value large enough to allow good signal separation and detection. It was then necessary to work at pH 8.3 because

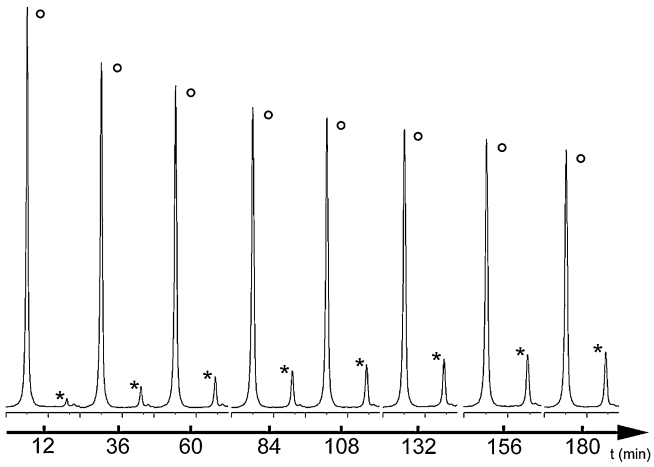

FIGURE 3 Evolution of F-ara-AMP (o) and F-ara-A (*) H8 NMR peaks over time. In this example, the initial F-ara-AMP concentration was 3.8 mM.

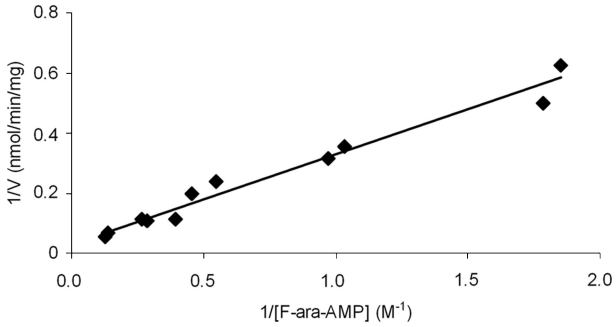

FIGURE 4 Lineweaver-Burk plot for the conversion of F-ara-AMP to F-ara-A by cN-II-ΔN with different initial concentrations of F-ara-AMP using NMR detection. The figure shows the velocity of the conversion of F-ara-AMP into F-ara-A by cN-II-ΔN using 500 ng/μL cN-II-ΔN at 37°C. The figure simultaneously shows two separate experiments. Determination coefficient = 0.97.

at this pH, the H8 peaks were not hidden by imidazole resonances as is the case at pH 6.5, owing to a strong pH-dependent shift. Under these conditions, during the reaction course in the presence of 0.5 μg/μL cN-II-ΔN and at the time points and substrate concentrations studied, the H8 F-ara-A peak increased linearly and the H8 F-ara-AMP peak decreased concomitantly (Figure 3). An 80-min control experiment using a 3.5 mM F-ara-AMP sample without cN-II-ΔN evidenced neither F-ara-AMP degradation nor F-ara-A production. Following the F-ara-A-H8 peak intensity increase by 8-min steps over the first 80 min of the reaction, kinetic parameters were derived as described in the Methods section. A K_m of 10 ± 4 mM was obtained for the system cN-II-ΔN/F-ara-AMP (Figure 4). The V_{max} of the F-ara-AMP to F-ara-A conversion was 34 ± 9 nmol/min/mg cN-II-ΔN.

DISCUSSION

Fludarabine is a widely used nucleoside analogue in the treatment of hematological malignancies such as refractory chronic lymphocytic leukemia. Because of low solubility, this compound is administered as a monophosphorylated derivative that is dephosphorylated by the ectoplasmic 5′-nucleotidase eN (CD73) to form F-ara-A. This latter compound enters the cell by the mean of nucleoside transporters, and is phosphorylated intracellularly by deoxycytidine kinase (dCK) and other kinases to form the active triphosphorylated form of the drug, F-ara-ATP. Conversely, the first phosphorylation step is likely to be reversed by the activity of cytoplasmic 5′-nucleotidases such as cN-II. Because of the preference of cN-II for purine analogues, we studied the dephosphorylation of F-ara-AMP by this enzyme.

Using two different methods we show that the cytoplasmic 5′-nucleotidase cN-II is able to catalyze the conversion of F-ara-AMP to F-ara-A. The V_{max} values derived from HPLC and NMR methods were statistically identical and

the K_m were reasonably similar. The small differences observed in K_m may be attributed to the methods used or to the differences in buffer conditions.

The enzymatic characteristics found in this study (K_m = 4–10 mM and V_{max} = 34–35 nmol/min/mg) should be compared to those obtained during similar studies with related compounds. Inhibition studies of purified cN-II from rat liver with 5′-deoxy-5′-isobuthylthio derivatives of adenosine and inosine, showed K_i in the same order of magnitude as the K_m of F-ara-AMP found in our study.[16] Skladanowski et al. determined kinetic parameters for different nucleotides using purified cN-II from rabbit heart.[17] Their results (AMP: K_m = 18.4 mM, V_{max} = 22.6 μmol/min/mg protein, IMP: K_m = 2.8 mM, V_{max} = 50.2 μmol/min/mg protein, GMP: K_m = 7.2 mM, V_{max} = 82.5 μmol/min/mg protein, XMP: K_m = 5.3 mM, V_{max} = 37.1 μmol/min/mg protein) suggest K_m constants in the same millimolar range as for F-ara-AMP with our protein preparation but the maximum velocity in our case is 1,000-fold slower than what was observed with rabbit heart cN-II. Similarly, Spychala et al. found a V_{max} of 21.2 μmol/min/mg for the dephosphorylation of IMP, the preferred substrate of cN-II, using the same protein as in this study.[10] The dephosphorylation of F-ara-AMP described here is about 1,000-fold slower than that observed with IMP. Nevertheless, cN-II in our protein preparation is able to dephosphorylate F-ara-AMP. Whether the dephosphorylation of F-ara-AMP by cN-II would still occur in a cellular environment remains uncertain. If such an enzymatic reaction was possible, cN-II could be involved in the resistance to fludarabine. The NMR method we used is well suited for the study of interactions between our purified protein and substrates or molecules developed to be enzyme inhibitors. Further studies will show whether such molecules have inhibitory effects on the dephosphorylating reaction of F-ara-AMP by cN-II.

In conclusion, we show for the first time that the cytoplasmic 5′-nucleotidase cN-II is able to dephosphorylate a clinically cytotoxic nucleoside analogue. It is therefore possible that this enzyme is involved in clinical resistance to fludarabine in patients with chronic lymphocytic leukemia.

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