Anodic Oxidation of Cladribine and In Situ Evaluation of DNA-Cladribine Interaction

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Abstract
Cladribine (CLD), 2-chlorodeoxyadenosine, was investigated at a glassy carbon electrode using cyclic, differential pulse and square wave voltammetry over a wide pH range. The oxidation of CLD is an irreversible, pH-dependent with the transfer of two electrons and two protons mechanism, leading to the formation of a hydroxylated species which undergoes reversible redox reactions with the transfer of one electron and one proton. The in situ evaluation of DNA-CLD interaction using a DNA-electrochemical biosensor was investigated. The CLD interaction causes dsDNA structural modifications in a time-dependent manner, but no DNA oxidative damage caused by CLD was observed.

Keywords: Cladribine, DNA, Oxidation, Oxidative damage
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1 Introduction
Nucleoside analogues and nucleobases are a pharmaceutically diverse family, which includes cytotoxic compounds, antiviral agents, and immunosuppressive molecules. The purine nucleoside analogs represent an important group of cytotoxic drugs active in the treatment of cancer, especially acute leukemia and autoimmune diseases [1].

Cladribine (CLD), 2-chlorodeoxyadenosine, Scheme 1, a catabolite of deoxyribonucleic acid (DNA) is a nucleoside analog used in anticancer therapy with high activity against hairy cell chronic lymphocytic leukemias [2] and auto-immune diseases such as multiple sclerosis [3] and systemic lupus erythematosus [4]. CLD requires intracellular metabolic activation but once present in the cell, CLD is phosphorylated and then incorporated into DNA leading to inhibition of nucleic acid synthesis, DNA strand breaks and finally apoptosis [5–7].

Besides the benefits obtained during treatment with CLD, the drug has potentially significant toxic side effects. Severe bone marrow suppression especially at high doses [8], teratogenicity in mice and rabbits and consequently the potential to cause fetal harm [9] as well as clastogenicity both in vitro and in vivo [10] have been described. Although no animal carcinogenicity studies have been conducted with CLD, its carcinogenic potential cannot be excluded.

Due to the importance of the drug in treatment of several diseases, methods are being developed for its rapid determination in body fluids. However, their number is limited and the detection of CLD has been mostly done with liquid chromatography with mass-spectrometry [11–14] and voltammetry [15]. Previous studies on the electrochemical reduction behavior of CLD have been undertaken using d.c. and a.c. polarography [16]. The oxidation process of CLD has also been mentioned in the study of different halogenated adenosine derivatives at graphite electrodes. Some redox mechanisms have been proposed [17] but they are only focused on understanding the metabolic processes.

As already mentioned, CLD is undergoing evaluation for its activity/toxicity. As a result of these evaluations there is an ongoing need for estimation of CLD pharmacokinetics so that issues such as drug-drug or drug-biomolecular complex interactions can be evaluated. Most of the studies concerning the metabolic process and the
The effect of CLD on biologically important molecules has been carried out with cell lines.

Although the DNA damage mechanism of CLD apparently involves a cascade of secondary biochemical reactions, there are several studies indicating that CLD is an agent that may lead to processes giving rise to or inducing disruption or breakages in the genetic material which in turn may result in chromosome aberrations. Accumulation of DNA damage increases carcinogenic or teratogenic risks and may result in a malignant transformation or cell death. Therefore, a direct interaction between dsDNA and CLD should be studied.

Electrochemical DNA biosensors can be used for the detection of DNA strand breaks and base damage; i.e., for detection of electroactive substances that interact with DNA or its bases. Such a kind of device has been applied to study the interaction of several substances with DNA in order to elucidate the mechanism by which DNA is damaged by hazardous compounds [18–21].

The electrochemical oxidation and the interaction with DNA of halogenated adenosine derivatives, such as fludarabine [22] and clofarabine [23], has been studied. The presence of different substituents in different positions in the adenine moiety may interfere in the redox mechanisms and interaction with DNA.

Therefore, a systematic investigation of CLD redox electrochemical behavior is essential in order to understand the DNA-CLB interaction mechanism.

In this study, electrochemical oxidation of CLD and the mechanism of interaction of CLD with dsDNA were prepared out at a glassy carbon electrode using cyclic (CV), square wave (SWV) and differential pulse (DPV) voltammetry.

The results presented lead to the proposal of a mechanism through which CLD causes direct in vivo damage to DNA.

2 Experimental

2.1 Materials and Reagents

Cladribine (CLD), double stranded DNA (dsDNA), polyadenylic acid (poly[A]) and polyguanylic acid (poly[G]) were obtained from Sigma-Aldrich and used without further purification. A stock solution of 1.3 mM CLD was prepared in deionized water and stored at 5°C.

Solutions of different concentrations of CLD were prepared by dilution of the appropriate quantity in supporting electrolyte. Stock solutions of 151μM dsDNA, 477μM poly[G], and 587μM poly[A] were prepared in deionized water and diluted to the desired concentrations in pH 4.5 0.1 M.

All supporting electrolyte solutions (Table 1) were prepared using analytical grade reagents and purified water from a Millipore Milli-Q system (conductivity < 0.1 μS cm⁻¹).

<table>
<thead>
<tr>
<th>pH</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>HCl + KCl</td>
</tr>
<tr>
<td>3.4</td>
<td>HAcO + NaAcO</td>
</tr>
<tr>
<td>4.3</td>
<td>HAcO + NaAcO</td>
</tr>
<tr>
<td>4.5</td>
<td>HAcO + NaAcO</td>
</tr>
<tr>
<td>5.4</td>
<td>HAcO + NaAcO</td>
</tr>
<tr>
<td>6.1</td>
<td>NaH₂PO₄ + Na₂HPO₄</td>
</tr>
<tr>
<td>7.0</td>
<td>NaH₂PO₄ + Na₂HPO₄</td>
</tr>
<tr>
<td>8.0</td>
<td>NaH₂PO₄ + Na₂HPO₄</td>
</tr>
<tr>
<td>9.2</td>
<td>NH₃ + NH₄Cl</td>
</tr>
<tr>
<td>10.5</td>
<td>NH₃ + NH₄Cl</td>
</tr>
<tr>
<td>12.0</td>
<td>NaOH + KCl</td>
</tr>
</tbody>
</table>

Table 1. Supporting electrolyte buffer solutions.

The pH measurements were carried out using a Crison micropH 2001 pH-meter with an Ingold combined glass electrode. All experiments were done at room temperature (25±1°C) and microvolumes were measured using EP-10 and EP-100 Plus Motorized Microliter Pipettes (Rainin Instrument Co. Inc., Woburn, USA).

2.2 Voltammetric Parameters and Electrochemical Cells

Voltammetric experiments were carried out using a μAutolab III running with GPES 4.9 software, Eco-Chemie, Utrecht, The Netherlands. Measurements were carried out using a three-electrode system in a 0.5 mL one-compartment electrochemical cell (Cypress System Inc., USA). A glassy carbon electrode (GCE, d = 1.5 mm) was the working electrode, a Pt wire the counter electrode and an Ag/AgCl (3 M KCl) reference electrode.

The experimental conditions for differential pulse (DP) voltammetry were: pulse amplitude 50 mV, pulse width 70 ms and scan rate 5 mVs⁻¹. For square wave (SW) voltammetry a frequency of 50 Hz and a potential increment of 2 mV, corresponding to an effective scan rate of 100 mVs⁻¹.

The GCE was polished using diamond spray (particle size 3 μm) (Kemet, UK) before each electrochemical experiment. After polishing, it was rinsed thoroughly with Milli-Q water. Following this mechanical treatment, the GCE was placed in buffer supporting electrolyte and voltammograms were recorded until a steady state baseline voltammogram was obtained. This procedure ensured very reproducible experimental results.

2.3 Acquisition and Presentation of Voltammetric Data

All the voltammograms presented were background-subtracted and baseline-corrected using the moving average application with a step window of 5 mV included in GPES version 4.9 software. This mathematical treatment improves the visualization and identification of peaks over the baseline without introducing any artifact, although the peak intensity is, in some cases, reduced (<10%) relative to that of the untreated curve. Nevertheless, this mathematical treatment of the original voltammograms was used in the presentation of all experimental voltammograms for a better and clearer identification...
tion of the peaks. The values for peak current presented in all plots were determined from the original untreated voltammograms after subtraction of the baseline.

2.4 Incubation Procedure and DNA-Biosensor Preparation

For the incubation procedure, CLD-dsDNA, CLD-poly[G] and CLD-poly[A] solutions were prepared by incubation of 100 μg mL⁻¹ dsDNA or poly[G] or poly[A] in pH 4.5 0.1 M acetate buffer with 2 μM CLD solution, during different periods of time at room temperature. Control solutions of dsDNA, poly[G] and poly[A] in pH 4.5 0.1 M acetate buffer were also prepared and stored during the same periods of time in similar conditions as incubated solutions.

The thin layer dsDNA-modified electrode was prepared with three drops of 5 μL each containing 50 μg mL⁻¹ dsDNA on the electrode surface. After placing each drop, the dsDNA-electrochemical biosensor was allowed to dry with a constant flux of N₂. The dsDNA biosensor was incubated in 250 μM CLD solution during different times. Then, the electrode was rinsed with deionized water to remove unbound molecules and transferred to supporting electrolyte and voltammograms were recorded. After each measurement the dsDNA film was removed from the electrode surface. Poly[G] and poly[A] biosensors were also prepared same way (25 μg mL⁻¹, each). Systematic studies to elucidate the mechanisms of interactions of CLD with dsDNA, poly[A] and poly[G] were carried out on a glassy carbon electrode using DP voltammetry.

3 Results and Discussion

3.1 Redox Behavior

3.1.1 Cyclic Voltammetry

Cyclic voltammetry (CV) was performed in aqueous buffered solutions containing 60 μM CLD in the pH range from 2.0 to 12.0 at a scan rate v = 100 mV s⁻¹, and only one anodic peak was observed. Scanning in the negative direction, no reduction peak was observed, indicating that the adsorption of CLD and/or its oxidation product on the GCE surface.

For electrolytes with pH < 3.0 and pH > 10.0 no oxidation peak for CLD occurred. For 3.0 < pH < 10.0, the CVs always showed one irreversible peak. The peak potential was shifted to less positive values with pH and the peak current decreased with increasing number of scans due to the adsorption of CLD and/or its oxidation product on the GCE surface.

CV in pH 7.0 0.1 M phosphate buffer showed the anodic peak 1a, at Epa = +1.30 V, Figure 1, and peak 1a current in the first scan increased with increasing scan rate, following a linear dependence with square root of scan rate, in agreement with a diffusion-controlled process [24]. The effect of scan rate was evaluated and the CVs were recorded at scan rates between 5 and 500 mV s⁻¹ in 60 μM CLD.

Fig. 1. CV in 100 μM CLD, N₂ saturated, in pH 7.0 0.1 M phosphate buffer, (→) first and (—–) second scans, v = 100 mV s⁻¹.

Successive CVs were recorded and between measurements, the electrode surface was always polished in order to ensure a clean surface to avoid possible problems due to adsorption. The results obtained always showed peak 1a, on the first scan and, on subsequent scans corresponding to the oxidation of product formed on peak 1a, a pair of reversible peaks 2a–2c.

3.1.2 Differential Pulse Voltammetry

The electrochemical oxidation of CLD was studied using DP voltammetry in aqueous buffer supporting electrolytes for 2.0 < pH < 9.2 with 0.1 M ionic strength, in 100 μM CLD solutions, Figure 2A.

CLD occurs in neutral form in the whole pH range studied since the pK value is 1.3 [25]. For strong acid electrolytes, CLD is very unstable and in pH 2.0 0.1 M hydrochloric solution CLD undergoes hydrolysis leading to the formation of 2-chloroadenine [26]. However, no CLD oxidation peak has been observed in these conditions.

For pH > 3.0 was observed the occurrence of peak 1a, and the peak potential was shifted to less positive values with increasing pH. The relationship was linear following the equation Epa(V) = 1.540−0.059 pH, Figure 2B. The slope of the line, −0.59 mV per pH unit, shows that the mechanism of oxidation involves the same number of electrons and protons. The number of electrons transferred, n, was determined by the peak width at half height, W1/2 ≈ 61 mV, close to the theoretical value corresponding to an electrochemical reaction involving the transfer of two electrons and two protons.

Successive DP voltammograms in the same solution without cleaning the GCE surface, showed a new small peak 2, corresponding to the oxidation of the CLD oxidation product, Figure 3.
The variation of peak 2a potential with the pH was also investigated. In each supporting electrolyte, two consecutive voltammograms were recorded and the peak 2a potential was plotted vs. pH. Increasing the pH, peak 2a potential decreased linearly and the slope of the line, \( \frac{dE}{dpH} = 59 \text{ mV per pH unit} \), shows that the mechanism of this oxidation process, peak 2a, in aqueous media also involves the same number of electrons and protons. Considering that \( E_{1/2} = 90 \text{ mV} \) for peak 2a, it was concluded that the oxidation reaction occurs with the transfer of one electron and one proton.

3.1.3 Square Wave Voltammetry

Square wave (SW) voltammetry experiments were carried out over the same pH range. SW voltammetry permits to see during only one scan if the electron transfer reaction is reversible or not. Since the current is sampled in both positive and negative-going pulses, peaks corresponding to the oxidation and reduction of the electroactive species at the electrode surface can be obtained in the same experiment.

The occurrence of anodic peak 1a was observed in all buffer supporting electrolytes. The irreversibility of peak 1a was confirmed by plotting the forward and backward components of the total current obtained in 500 \( \mu \text{M} \) CLD in pH 7.0 0.1 M phosphate buffer, Figure 4A.

The anodic peak 2a of CLD oxidation product always occurred on subsequent scans but over a smaller pH interval. The reversibility of peak 2a is confirmed by plotting the forward and backward components of the total current voltammograms obtained in the same solution without cleaning the GCE surface, Figure 4B. In this case, the oxidation and reduction components of peak 2a are equal. SW voltammetry showed a higher sensitivity than DPV since much faster scan rates can be used.

3.1.4 Oxidation Mechanism

The oxidation of CLD is an irreversible process, the electroactive centre is the adenine moiety, involving the transfer of two electrons and two protons to produce a radical cation, which reacts with water and yields a final hydroxylated product, Scheme 2. Similar results have been obtained with fludarabine [22] and clofarabine [23].

The hydroxylated product also undergoes oxidation and the process is reversible, peak 2a, involving the transfer of one electron and one proton.

3.2 DNA-CLD Interaction

3.2.1 Analysis of Incubated Solutions

Initial studies concerning the interaction between CLD and dsDNA were carried out incubating 2 \( \mu \text{M} \) CLD with 100 \( \mu \text{g mL}^{-1} \) dsDNA in pH 4.5 0.1 M acetate buffer. DP voltammograms were recorded after different incubation times. The GCE surface was cleaned between each mea-
surement to avoid the blocking of the GCE surface by adsorption of the compound and dsDNA.

Control solutions of 100 μg mL⁻¹ dsDNA and 2 μM CLD were also prepared in buffer and analyzed after the same periods as the CLD-dsDNA incubated solutions. A DP voltammogram obtained in dsDNA showed two anodic peaks due to the oxidation of desoxyguanosine, dGuo, at $E_{pa} = +1.03$ V, and desoxyadenosine, dAdo, at $E_{pa} = +1.28$ V, Figure 5. For 2 μM CLD, without dsDNA only one oxidation peak appears at $E_{pa} = +1.34$ V.

The DP voltammogram obtained immediately after the addition of CLD to the solution of dsDNA showed the two peaks due to dsDNA bases and the CLD oxidation peak. Increasing the incubation time, the dGuo and dAdo peaks, corresponding to DNA oxidation, showed a large, time-dependent decrease, Figure 5.

This experiment shows that structural modifications occur in dsDNA upon interaction with CLD. The CLD oxidation peak also decreased in height owing to fewer free CLD molecules due to the CLD-DNA interaction. The decrease of DNA oxidation peaks with increasing incubation time is explained considering the formation of a condensed more rigid DNA structure, with a greater difficulty for the transition of electrons from the purine residues, than from the more flexible structure, where the dGuo and dAdo can reach the surface, leading to higher peak currents. However, during the experiments, CLD in the bulk solution also diffused and adsorbed non-specifically on the electrode’s uncovered regions leading to the two different contributions to the electrochemical signal, one from the simple adsorbed CLD and the other due to the CLD that interacted with dsDNA.
3.2.2 In Situ Study of CLD-DNA Interaction

During the evaluation of dsDNA-CLD interaction in incubated solutions, the formation of a thin and incomplete network film of co-adsorbed dsDNA-CLD, free dsDNA and free CLD molecules on the electrode surface occurs [20].

Complete coverage of the electrode surface is necessary [20] to investigate the DNA-CLD interaction and can be obtained using the dsDNA-electrochemical biosensor, prepared as described in Section 2.4, which avoids the undesired CLD nonspecific binding to the electrode surface. A dsDNA-electrochemical biosensor was used showing in situ, and in real time, the changes occurring to the dsDNA immobilized on the electrode surface during interaction with CLD.

The dsDNA-electrochemical biosensor prepared was incubated in 250 μM CLD solution for different periods of time and afterwards transferred to acetate buffer where DP voltammograms were recorded, Figure 6. Before transferring into supporting electrolyte, the biosensor was washed carefully with deionized water to remove unbound CLD. In this way, the observed peaks in DP voltammograms are due to the interaction between CLD and dsDNA, Figure 6. The DP voltammogram showed the peaks due to the oxidation of dsDNA bases, dGuo and dAdo, and CLD oxidation peak 1a.

The experiment was repeated, always with a new biosensor, for incubation times of 5, 10 and 15 minutes. The DP voltammogram showed that increasing the incubation time in the CLD solution, the dsDNA oxidation peaks decreased in size. This is attributed to the aggregation of DNA immobilized on the electrode surface.

In order to obtain more information about the interaction between CLD and dsDNA, experiments using GCE modified with polynucleotides of known sequences were performed. The GCE surface was modified as described in Section 2.4 with polyhomonucleotides that contained only residues of guanine, poly[G] or only residues of adenine, poly[A].

![Fig. 6. DP voltammograms in pH 4.5 0.1 M acetate buffer: (—) before and after (-----) 5, (-----) 10 and (—) 15 min incubation in a solution of 250 μM CLD, using a dsDNA-electrochemical biosensor.](image)

![Fig. 7. DP voltammograms in pH 4.5 0.1 M acetate buffer: (—) before and after (-----) 5, (-----) 10 and (—) 15 min incubation in a solution of 250 μM CLD, using (A) poly[G]-electrochemical and (B) poly[A] -electrochemical biosensors.](image)
When the poly[G]-electrochemical biosensor was used the DP voltammograms obtained in pH = 4.5 0.1 M acetate buffer showed only one peak at $E_{pa} = +1.05 \text{V}$, corresponding to dGuo oxidation, Figure 7A. The poly[G]-electrochemical biosensor was incubated for different times in 250 μM CLD and two peaks, corresponding to poly[G] and CLD, appeared. The DP voltammogram showed the decreasing of the poly[G] and CLD oxidation peaks with increasing time.

Experiments were carried with the poly[A]-electrochemical biosensor and the DP voltammogram showed a peak at $E_{pa} = +1.25 \text{V}$, corresponding to dAdo oxidation, Figure 7B. After different incubation times in 250 μM CLD, the DP voltammogram showed the decrease of poly[A] and CLD oxidation peaks with increasing time.

These experiments using polyhomopurinenucleotide single stranded sequences, of poly[G] and poly[A]-electrochemical biosensors, showed that the interaction between CLD and DNA is not selective and can occur at any purine base. No additional peaks were observed showing that there was no oxidative damage to DNA bases.

4 Conclusions

CLD, a drug used to treat a number of lymphocytic malignancies, is irreversibly oxidized at a glassy carbon electrode. The oxidation of CLD is pH-dependent, occurs in one step with the transfer of two electrons and two protons, and leads to the formation of a hydroxylated product that adsorbs on the electrode surface. The CLD oxidation product undergoes reversible redox reactions. A redox mechanism was proposed.

The dsDNA-CLD interaction was investigated using dsDNA, poly[A] and poly[G]-electrochemical biosensors and incubation solutions. The decrease the dGuo and dAdo oxidation peaks with increasing incubation time in the CLD solution was observed, showing that CLD interacts with the dsDNA causing its condensation but no evidence of CLD oxidative damage to dsDNA was found.

The presence of different halogen substituents in the nucleoside analogues structure do not interfere with their redox behavior or DNA interaction mechanisms.

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