Electrochemical behavior of Methotrexate upon binding to the DNA of different cell lines

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Abstract: Methotrexate (MTX) is a widely used anticancer agent whose DNA binding properties are well known. Despite its consolidated usage in the therapeutics of cancer, the physicochemical features of MTX binding to healthy and neoplastic DNA are still not fully understood. Therefore, this work showcases the electrochemical study of MTX binding to distinct DNA sequences through voltametric approaches.

1. Introduction

Methotrexate (MTX) is an anticancer agent whose main therapeutic property relies on the impairment of cellular metabolism[1]. This compound is considered an antifolate derivative due to its structural similarity to folic acid, what hinders folate-dependent enzymatic activity[2]. Notwithstanding, literature reports that MTX might also interact with nucleic acids, what nonetheless further supports its antineoplastic appeal[3].

Considering the distinction between the constitution and physicochemical behavior of healthy and neoplastic DNA sequences, it can be inferred that anticancer agents might couple differently according to the nature of the DNA segment[4]; what is nonetheless supported by other authors[4–6]. In this regard, a comparative study between the binding of MTX to distinct cell lines would likely provide remarkable information to shed light on the pharmacodynamics of anticancer agents whose DNA is the main biological target.

Regarding the DNA-binding properties of anticancer agents, redox mechanisms may play a distinct role on their pharmacology[7–9]. Owing to the occurrence of electron transfer in covalent-based DNA-binding, as well as the impairment of charge transfer upon intermolecular interactions; the detection of changes in faradaic currents may provide information regarding the physicochemistry of the anchoring of the ligand to the DNA[7]. In this sense, electrochemical methods such as voltammetry may be useful to evaluate the dynamics of charge transfer in reaction media, and by consequence, provide information regarding the binding of anticancer agents to DNA[10–12].

Therefore, in view of the importance of differentiating the binding of anticancer agents to the DNA from distinct cell lineages, this study showcases the investigation of the electrochemical behavior of anticancer drug MTX upon binding to the DNA of melanoma BRAFV600E (SK-Mel-28) and wild type (WM852) cell lines, as well as a commercial double-stranded calf thymus DNA (ds-CT).

2. Materials and Methods

2.1. Reagents and Solutions

MTX (Sigma) was diluted in purified water (conductivity ≤ 0.1 µS.cm⁻¹) obtained from Milli-Q purification system Millipore S/A (Molsheim, France) in order to render a stock solution of 0.01 mol L⁻¹. Potassium chloride, disodium hydrogen phosphate, potassium hydrogen phosphate, and sodium chloride were used to prepare 0.1 mol L⁻¹ phosphate buffered saline (PBS) solution, pH 7.0. Furthermore, 1.5 g of ds-CT was diluted in 2 mL PBS, pH 7.0, in order to render a concentrated mixture for the studies.

2.2. DNA extraction

The following cell lines were used in this study: melanoma BRAFV600E (SK-Mel-28) and wild type (WM852). The cells were incubated in culture plates with penicillin, streptomycin, and 10% fetal bovine serum. Incubator temperature was maintained at 37°C under 5% CO_2 pressure until cell monolayer was formed. Thereafter, DNA was extracted according to standard protocols[13,14].

2.3. Electrochemical assays

Voltametric experiments were carried out in a potentiostat/galvanostat Autolab III® integrated to the GPES 4.9® software, Eco-Chemie, Utrecht, Netherlands. The measurements were performed in a 1.0 mL one-compartment/three-electrode system electrochemical cell consisting of glassy carbon electrode (GCE) - 0.785 mm² area, a Pt wire and Ag/AgCl/KClsat electrode (Lab solutions, São Paulo, Brazil), representing the working electrode, the counter electrode and the reference electrode, respectively.

Experimental conditions for Cyclic Voltammetry (CV) were: scan rate (υ) of either: 12.5; 25; 50; 100; 250 or 500 mV s⁻¹, and scan range of 0.5 to 1.2 V. All voltametric assays were performed in 0.1 mol L⁻¹ PBS solution, pH 7.0.

The experiments consisted of two approaches, namely: *i.* investigate the electrochemical behavior of MTX without DNA, and *ii.* Evaluate the electroanalytic signal of MTX upon the addition of DNA in the test solution. Henceforth 100 µL of MTX solution was added in the electrochemical cell containing 850 mL of PBS buffer (i); followed by the addition of 5 µL of either 7.8 ng mL⁻¹(WM852); or 5.6 ng mL⁻¹(SK-Mel-28); or 0.75 g mL⁻¹ (ds-CT) DNA solution (ii).

Thereafter, the mixture of MTX and DNA from each lineage was submitted to several CV under crescent υ, in order to obtain the plots of the faradaic currents versus the square root of the voltametric scan rate, as stated by Randles-Sevcik equation (1). This mathematical treatment was used given that it allows us to draw comparisons regarding diffusional aspects of the electrochemical reaction[15].

$$\frac{I_{pa}}{v^{1/2}} = 2.69 \ 10^5 \ A \ n^{3/2} \ D^{1/2} \ c$$

Wherein: I_{pa} is the anodic peak current, A is electrode area in cm², n is the number of transferred electrons, D is the diffusion coefficient, c is the concentration of MTX in mol L⁻¹, and v is scan rate in V s⁻¹.

All experiments were performed in triplicates, and a strenuous cleaning of working electrode surface was conducted prior each assay. Furthermore, all voltametric data was analyzed and treated with Origin 8® software.

3. Results and discussion Results and Discussion

3.1. Redox behavior of MTX

In order to primarily investigate the redox behavior of MTX, CV was assayed on a bare MTX solution in PBS buffer, pH 7.0. Results are showcased in figure 1.
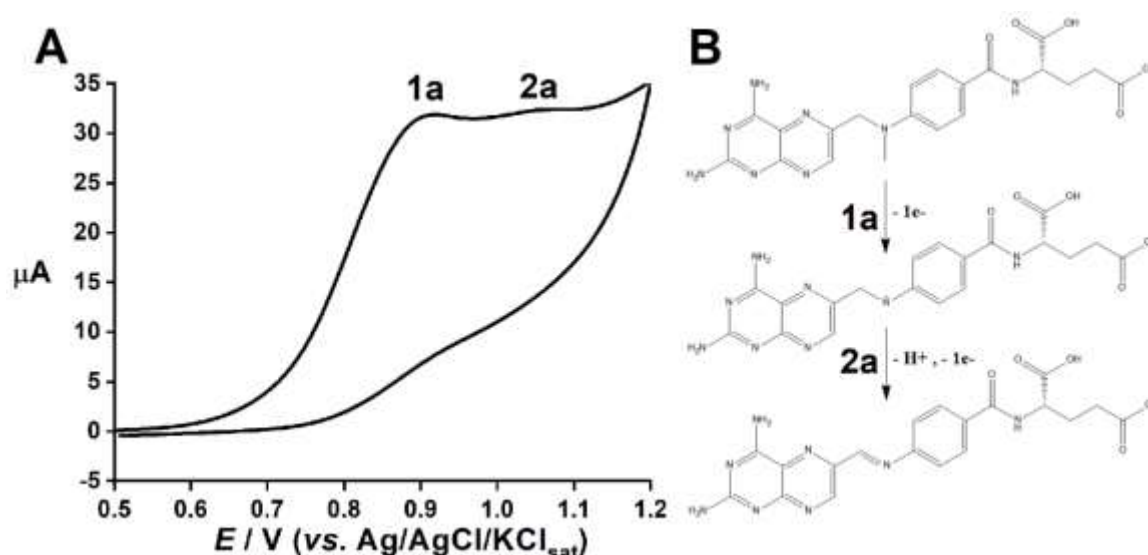
Figure 1. Cyclic voltammogram of MTX showcasing two anodic processes (1a and 2a); B. Proposed electrooxidation reaction for MTX. All voltametric assays were performed in 0.1 mol L^{-1} PBS solution, pH 7.0.
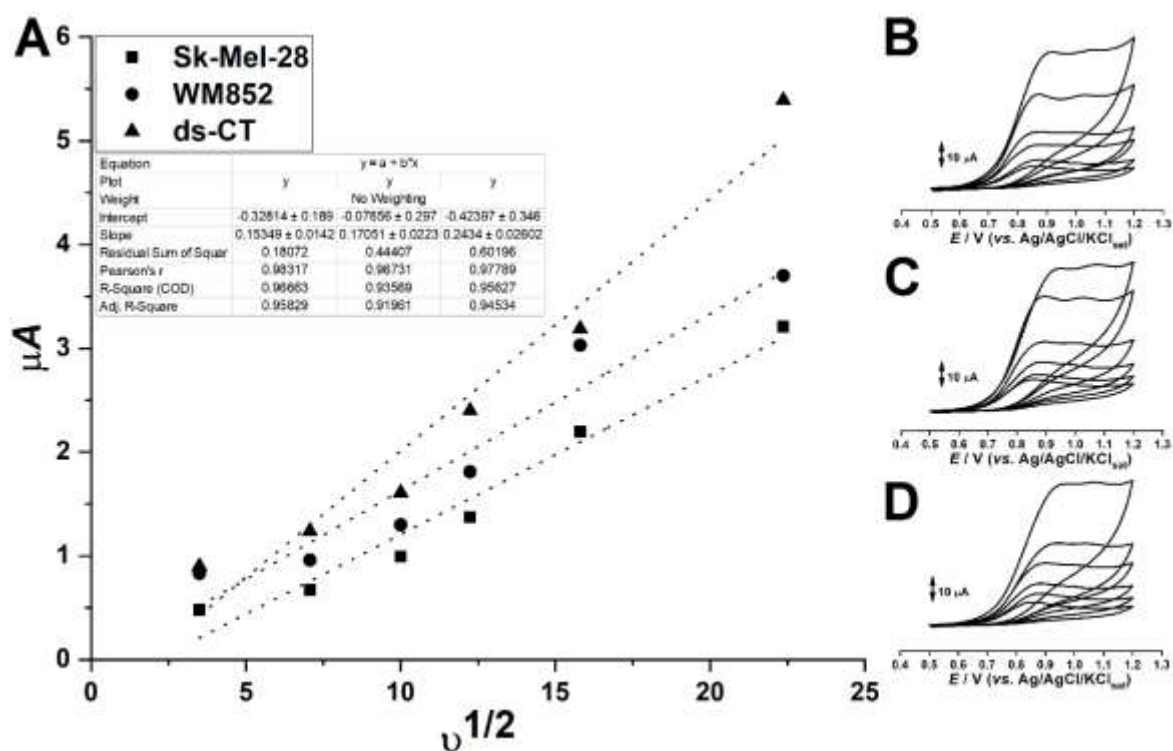
Results showcased that MTX exhibits two anodic signals at $E_{p1a} \approx 0.9$ V and $E_{p2a} \approx 1.05$ V (Figure 1A), which correlates to the oxidation of electroactive moieties in MTX molecule (Figure 1B).

The findings showcased in Figure 1 are in consonance to literature, wherein is reported that MTX undergo irreversible electrooxidation at $E_{p1a} \approx 0.9$ V and $E_{p2a} \approx 1.05$ V[16–18]. The reaction was deemed irreversible hence the absence of visible cathodic peaks in the voltammogram[19–21]. Considering that the peaks showcased broadening, it can be suggested that MTX oxidation products might undergo adsorption on the working electrode surface, what was in fact observed during experiments, leading us to adopt a strenuous electrode surface renewal protocol. Furthermore, this behavior was also reported by other authors[16,22].

The proposed electrooxidation mechanism of MTX contemplates an irreversible demethylation step at $E_{p1a} \approx 0.9$ V. This oxidation process leads to the collection of a single electron from MTX by the working electrode surface[23,24]. Moreover, the second proposed electrooxidation at $E_{p2a} \approx 1.05$ V leads to the formation of an imine derivative, and follows 1:1 stoichiometry regarding MTX and the released electrons; as well as an equivalence of electrons and protons (*i.e.* Nernstian process) [23]. Owing to the fact that these electrons raise the total charge which is detected by the electric current detector coupled to the working electrode circuit, each oxidation is followed by raises in the electric current, what is nonetheless in consonance to literature. Furthermore, other authors proposed similar oxidation mechanisms to anticancer drugs bearing structural similarity to MTX[25,26].

3.1. Plots of the faradaic currents versus the square root of the voltametric scan rate, as stated by Randles-Sevcik equation

In order to draw information regarding the diffusional process of MTX-DNA adducts, plots of the faradaic currents *versus* the square root of the voltametric scan rate were performed according to Randles-Sevcik equation. Results are showcased in Figure 2.

Equation	y = a + b*x		
Plot	y	y	y
Weight		No Weighting	
Intercept	-0.32814 ± 0.189	-0.07856 ± 0.297	-0.42387 ± 0.346
Slope	0.15348 ± 0.0142	0.17051 ± 0.0223	0.2434 ± 0.02802
Residual Sum of Squar	0.18072	0.44407	0.60196
Pearson's r	0.98317	0.98731	0.97789
R-Square (COD)	0.96663	0.93569	0.95627
Adj. R-Square	0.95829	0.91961	0.94534

Figure 2. Handles-Sevcik plots of MTX signal in presence of different DNAs. Insert. Analytical parameters of each fitted curve. B. Plot of MTX-WM852 at successive υ; C. Plot of MTX-SK-Mel-28 at successive υ. D. Plot of MTX-ds-CT at successive υ. All voltametric assays were performed in 0.1 mol L^{-1} PBS solution, pH 7.0, at υ of: 12.5; 25; 50; 100; 250 and 500 mV s^{-1}.

Results showcased that the response of MTX signal upon the addition of each DNA was distinct under Randles-Sevcik treatment (Figure 2A). It could be noticed that all plots showcased linear profile, being response of MTX in the presence of SK-Mel-28 the lowest, while in the presence of ds-CT the highest (Figure 2A to D).

Literature states that MTX electrooxidation is adsorption controlled[25], though the addition of DNA shifts the rate-defining step of the electrooxidation of binding agents to the diffusion of adducts through the bulk solution[7]. In this sense, the Handles-Sevcik treatment can be applied even for molecules whose oxidation products promote extensive electrode fouling. Nonetheless, the presence of DNA in the solution leads to the formation of MTX-DNA adducts, which diffuse slower through the bulk solution and take longer to reach the working electrode surface[24]. Moreover, the DNA-binding leads to lesser amounts of faster-diffusing free MTX, thereby leading to smaller faradaic signals, as observed elsewhere to other DNA-binding agents[7].

Considering that the response of MTX upon the addition of SK-Mel-28 was the smallest of all assayed DNAs, and that this DNA was nonetheless the most diluted among them all, it can be suggested that SK-Mel-28 DNA leads to the smallest amount of free MTX in the reaction media, hence the higher affinity of this neoplastic DNA to the anticancer drug[27]. In this sense, our results are corroborated by literature, which describe the DNA-binding properties of MTX[26,27]. Moreover, it was noteworthy that highly concentrated ds-CT DNA did not promote major changes in the response when compared to WM852 DNA. However, owing to the small differences in the signals (*i.e.* in the order of μA), more investigations are needed in order to fully explore the applicability of electrochemical methods in the study of the DNA-binding properties of MTX.

3. Conclusion

This work showcased the electrochemical study of MTX binding to distinct DNA sequences through voltametric approaches. Results showcased that MTX exhibits two irreversible anodic peaks

at $E_{p1a} \approx 0.9$ V and $E_{p2a} \approx 1.05$ V, which correlate to the oxidation of electroactive moieties in its structure. Moreover, the investigation of MTX binding to DNAs through Handles-Sevcik equation evidenced that MTX response in presence of all DNAs led to a linear profile, being the response of MTX in the presence of SK-Mel-28 the lowest, while in the presence of ds-CT the highest.

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