Anal Bioanal Chem (2013) 405:3783–3790
DOI 10.1007/s00216-012-6546-x

ORIGINAL PAPER

In situ electrochemical evaluation of anticancer drug temozolomide and its metabolites–DNA interaction

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Received: 7 August 2012 / Revised: 30 October 2012 / Accepted: 2 November 2012 / Published online: 13 November 2012

Abstract Temozolomide (TMZ) is an antineoplastic alkylating agent with activity against serious and aggressive types of brain tumours. It has been postulated that TMZ exerts its antitumor activity via its spontaneous degradation at physiological pH. The in vitro evaluation of the interaction of TMZ and its final metabolites, 5-aminoimidazole-4-carboxamide (AIC) and methyldiazonium ion, with double-stranded DNA (dsDNA) was studied using differential pulse voltammetry at a glassy carbon electrode. The DNA damage was electrochemically detected following the changes in the oxidation peaks of guanosine and adenosine residues. The results obtained revealed the decrease of the dsDNA oxidation peaks with incubation time, showing that TMZ and AIC/methyldiazonium ion interact with dsDNA causing its condensation. Furthermore, the experiments of the in situ TMZ and AIC/methyldiazonium ion–dsDNA interaction using the multilayer dsDNA-electrochemical biosensor confirmed the condensation of dsDNA caused by these species and showed evidence for a specific interaction between the guanosine residues and TMZ metabolites, since free guanine oxidation peak was detected. The oxidative damage caused to DNA bases by TMZ metabolites was also detected electrochemically by monitoring the appearance of the 8-oxoguanine/2,8-dyhydroxyadenine oxidation peaks. Non-denaturing agarose gel electrophoresis of AIC/methyldiazonium ion–dsDNA samples confirmed the occurrence of dsDNA condensation and oxidative damage observed in the electrochemical results. The importance of the dsDNA-electrochemical biosensor in the in situ evaluation of TMZ–dsDNA interactions is clearly demonstrated.

Keywords Temozolomide · Spontaneous chemical degradation · Oxidation · Multilayer dsDNA-electrochemical biosensor · DNA oxidative damage

Published in the topical collection in *Bioelectroanalysis* with guest editors Nicolas Plumeré, Magdalena Gebala, and Wolfgang Schuhmann.

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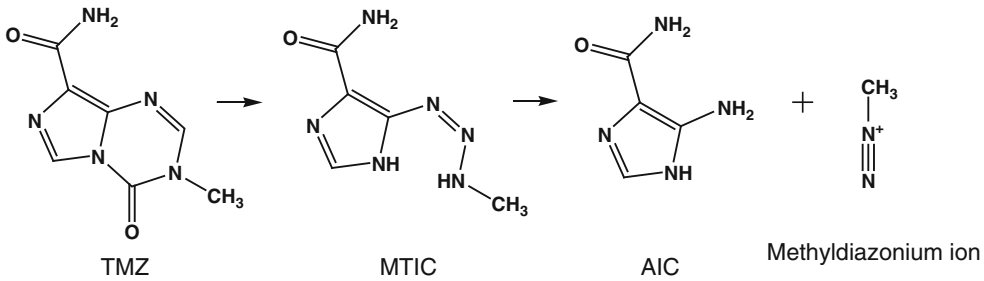
Introduction

Temozolomide (TMZ) belongs to the group of triazene compounds of clinical interest, they are alkylating agents whose active moiety is represented by the triazenyl group, i.e. three adjacent nitrogen atoms responsible for the chemical, physical, antitumour and mutagenic properties of the molecule [1]. Alkylating drugs are the oldest class of anticancer drugs which are still commonly in use, and they remain important in the treatment of several types of cancers [2].

TMZ (8-carbamoyl-3-methylimidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3*H*)-one), Scheme 1, is an oral methylating agent of the imidotetrazine class [3]. This compound readily crosses the blood–brain barrier and it is one of the most commonly compounds used in chemotherapy for the treatment of malignant primary brain tumours (e.g. glioblastoma) [4, 5]. TMZ also has significant activity against recurrent glioma [6] and metastatic melanomas [1].

The alkylating agents are well known for their reactivity and instability [7], and TMZ is an anticancer prodrug that undergoes spontaneous chemical degradation at physiologic pH to form the highly unstable 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC), Scheme 1. MTIC is further hydrolyzed to 5-aminoimidazole-4-carboxamide (AIC), which is known to be an intermediate in purine and nucleic acid biosynthesis, and to methyldiazonium ion which is the active alkylating specie [4]. This highly reactive cation reacts with DNA purinic bases forming methyl adducts, especially at



Scheme 1 Hydrolysis cascade at physiological pH of TMZ to MTIC and their metabolites, AIC and methyldiazonium ion

N7-guanine, N3-adenine and O6-guanine. It is noticeable that 70 % of alkylation involves N7 position of guanine. However, N3-methyladenine (9 %) and N7-methylguanine are much less toxic lesions than O6-methyguanine (5 %), which is mainly responsible for the cytotoxic and genotoxic effects of TMZ and hence is one of the major determinants on the drug clinical response [1, 8–11]. Methylation of O6-guanine causes base pair mismatch. Unsuccessful cycles of mismatch repair eventually lead to breaks in the daughter strand of DNA and the cell undergoes apoptosis [4]. Nevertheless, the precise mechanism of cytotoxicity of TMZ is still unclear, and so far there is no previous report in the literature on oxidative damage to DNA caused by TMZ.

TMZ is rapidly and completely absorbed from the gastrointestinal tract after oral administration and the time necessary to reach peak plasma concentration (t_{max}) is 1 h. It is rapidly eliminated with a half-life ($t_{1/2}$=ln 2/k, where k is the elimination rate constant) of 1.7–1.9 h and exhibits linear kinetics over the therapeutic dosing range [12, 13]. In the in vitro serum studies, the TMZ half-life was found to be only 15 min [14] and 33 min (and 28 min in water at pH 7.9) [15]. The MTIC half-life has been determined 1.9 h in human plasma in vivo [16, 17], 25 min in human plasma in vitro [14, 16], and 13 min in water pH 7.9 [15]. AIC is stable in human plasma [16] and in water at pH 7.9 [15], at room temperature.

Relatively few methods have been described for the analysis of TMZ–DNA interaction, such as pulsed field gel electrophoresis [18], positron emission tomography (PET) [10] and immunoassays [8, 9, 11, 19, 20]. Therefore, an electrochemical study of TMZ direct interaction with DNA has not been investigated.

Electrochemical techniques, such as pulse techniques, are suitable for studies of biological systems, since they are fast, low cost and have high sensitivity. One advantage of the use of pulse techniques is that they lead to a great improvement in the signal-to-noise ratio compared to steady-state techniques and in many cases greater selectivity [21]. The DNA electrochemical biosensor, using differential pulse voltammetry, has been successfully utilized to investigate the interaction of DNA with anticancer drugs [22, 23], cyanotoxins [24], heavy metals [25, 26], and hydroxyl free radicals [27], and comparing with other methods shows great sensitivity towards detecting small perturbations of the double-helical structure and DNA oxidative damage [22].

A dsDNA-electrochemical biosensor consists of an electrochemical transducer with dsDNA immobilized on the surface [22]. Among the electrochemical transducers, carbon electrodes demonstrate several unique properties. The extensive potential window in the positive direction allows sensitive electrochemical detection of dsDNA conformational changes and oxidative damage caused to DNA by monitoring the appearance of the oxidation of the DNA components such as, nucleotides, nucleosides, purine and pyrimidine bases and the oxidation products of guanine, 8-oxoguanine (8-oxoGua), and of adenine, 2,8-dyhydroxyadenine (2,8-oxoAde), that are biomarkers of DNA base oxidative damage [22].

Different immobilization procedures by adsorption, electrostatic adsorption or evaporation, of a monolayer or multilayer DNA films have been used. The multilayer dsDNA-electrochemical biosensor consists of an electrode with dsDNA deposited on the transducer surface by successive monolayer coverage. Mac Mode AFM images have shown that the multilayer immobilization method leads to total coverage of the carbon electrode surface, thus the adsorption of undesired substances on the transducer surface does not occur, and the biosensor response is due only to the interaction of the substance with dsDNA [22].

Since it is believed that TMZ exerts its antitumor activity via its spontaneous and rapid degradation at physiological pH, a systematic study has been undertaken to investigate the interaction of the TMZ and its final metabolites, AIC and methyldiazonium ion, with dsDNA in aqueous solution by differential pulse voltammetry, using two approaches: (a) incubated samples of dsDNA with TMZ or AIC/methyldiazonium ion, and (b) the multilayer dsDNA-electrochemical biosensor. Gel-electrophoresis also was used to investigate the interaction of TMZ metabolites with dsDNA.

Experimental

Materials, reagents and solutions

The sodium salt of highly polymerized calf thymus dsDNA (length 10,000–15,000 bp) and TMZ (≥98 %) were

purchased from Sigma-Aldrich and used without further purification.

Stock solutions of 600 $\mu g mL^{-1}$ dsDNA and 1 mM TMZ were prepared in deionised water and kept at 4 °C until further utilization. A stock solution of 1 mM AIC/methyldiazonium ion was obtained from degradation of 1 mM TMZ solution in 0.1 M phosphate buffer pH 7.0, Scheme 1, during 7 days and stored at room temperature. The supporting electrolyte used in all experiments was 0.1 M phosphate buffer pH 7.0.

All solutions were diluted to the desired concentration by mixing buffer supporting electrolyte, and prepared using analytical grade reagents and purified water from a Millipore Milli-Q system (resistivity 18.2 MΩ cm, conductivity ≤ 0.1 $\mu S\ cm^{-1}$). All experiments were performed at room temperature (25±1 °C).

The concentration of nucleic acids in aqueous solution was determined by measuring their absorbance at 260 nm and considering the relation between optical units and micrograms given by the Sigma-Aldrich certificate of analysis. The absorbance of the nucleic acid solutions were measured using a spectrophotometer SPECORD S100, running with Aspect Plus Version 1.5 (Analytik Jena GmbH, Jena, Germany). The purity level of calf thymus dsDNA samples was checked spectrophotometrically by determining their UV 260/280 nm absorbance ratio and was always higher than 1.8.

Microvolumes were measured using P20, P200 and P1000 Microliter Pippettes (Gilson S.A., Villiers-le-Bel, France). The pH measurements were carried out using a Crison micropH 2001 pH-meter (Barcelona, Spain) with an Ingold combined glass electrode.

Voltammetric parameters and electrochemical cells

Voltammetric experiments were carried out using an IVIUM CompactStat potentiostat in combination with IviumSoft program version 1.84 (Ivium Technologies, Eindhoven, The Netherlands). The experimental conditions for differential pulse (DP) voltammetry were: pulse amplitude 50 mV, pulse width 80 ms and scan rate 5 $mV s^{-1}$. Measurements were carried out using a glassy carbon electrode (GCE, d= 1.5 mm), with a Pt wire counter electrode, and an Ag/AgCl (3 M KCl) electrode as reference, in a one-compartment electrochemical cell of 2 mL capacity, all from Cypress System, Inc., USA.

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

Acquisition and presentation of voltammetric data

All voltammograms presented were baseline-corrected using the moving average application included in Ivium version 1.84 software. This mathematical treatment improves the visualization and identification of peaks over the baseline without introducing any artefact, 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 of the peaks.

Electrochemical experimental procedures

The electrochemical evaluation of the dsDNA interaction with TMZ and AIC/methyldiazonium ion was undertaken using the two experimental procedures:

Procedure 1—incubated samples

TMZ–dsDNA and AIC/methyldiazonium ion–dsDNA samples were prepared by incubation of 250 μM TMZ or 100 μM AIC/methyldiazonium ion with 250 $\mu g mL^{-1}$ dsDNA in 0.1 M phosphate buffer pH 7.0 during different time periods: 0, 1, 2, and 4 h (TMZ–dsDNA) and 0, 24, and 48 h (AIC/methyldiazonium ion–dsDNA). Control samples of dsDNA in 0.1 M phosphate buffer pH 7.0 were also prepared and stored during the same periods of time in similar conditions as TMZ–dsDNA and AIC/methyldiazonium ion–dsDNA incubated samples. DP voltammograms were recorded with a clean GCE in these samples after each incubation time period.

Procedure 2—multilayer dsDNA-electrochemical biosensor

The multilayer dsDNA-electrochemical biosensors were prepared by covering successively the GCE surface with three drops each of 5 μL from a 250 $\mu g mL^{-1}$ dsDNA solution diluted in 0.1 M phosphate buffer pH 7.0. After placing each drop on the electrode surface the biosensor was dried under a constant flux of N_2.

The multilayer dsDNA-electrochemical biosensors prepared were immersed and allowed to incubate in solutions of 250 μM TMZ or 100 μM AIC/methyldiazonium ion in 0.1 M phosphate buffer pH 7.0, during different time periods: 10 min, 4 h, 24 h and 72 h. Afterwards, the biosensors were removed from the solution, washed with deionised water in order to remove the unbounded TMZ, AIC or methyldiazonium ion, and placed in the electrochemical cell containing only the supporting electrolyte 0.1 M phosphate buffer pH 7.0, where the transduction was performed by DP voltammetry.

DNA gel electrophoresis

Nondenaturing agarose (1 %, ultrapure DNA grade from Sigma) gel was prepared in TAE buffer (10 mM Tris base, 4.4 mM acetic acid and 0.5 mM EDTA, pH 8.0). Forty microliters dsDNA control solution and AIC/methyldiazonium ion-dsDNA samples aliquots (with 0.25 % bromophenol blue in water) were loaded into wells, and electrophoresis was carried out in TAE buffer for 1 h at ~100 V. After 0.5 % ethidium bromide (EtBr) stained DNA was visualized and photographed under UV (312 nm) transillumination to visualize DNA mobility.

Results and discussion

Electrochemical behaviour of dsDNA, TMZ and AIC

The anodic behaviours of dsDNA, a multilayer dsDNA-electrochemical biosensor, TMZ and AIC, using DP voltammetry were investigated in 0.1 M phosphate buffer pH 7.0 (Fig. 1a and b) as necessary controls to enable clear identification of the oxidation peaks occurring after the interaction of TMZ–dsDNA and AIC/methyldiazonium ion–dsDNA.

The oxidation in pH 7.0 0.1 M phosphate buffer solution of 250 $\mu g mL^{-1}$ dsDNA and of multilayer dsDNA biosensor control showed two small oxidation peaks (Fig. 1a) corresponding to the oxidation of desoxyguanosine (dGuo), at E_{pa}=+0.90 V [22, 28], and desoxyadenosine (dAdo), at E_{pa}=+1.17 V [22, 29], oxidised nucleosides in the dsDNA polynucleotide chain. The DP voltammogram of dsDNA shows small oxidation peaks due to the difficulty of the electron transfer from the inside of the double-stranded rigid form of DNA to the electrode surface [22]. However, the current of the dsDNA oxidation peaks obtained in the DP voltammogram using multilayer dsDNA biosensor are several-fold higher when compared with dsDNA in solution, since the dsDNA multilayer molecules are pre-concentrated on the electrode surface (Fig. 1a).

DP voltammograms of 250 μM TMZ in 0.1 M phosphate buffer pH 7.0 shown only one oxidation peak at E_{pa}=+0.42 V (Fig. 1b) associated with the oxidation of the tetrazinone ring [30]. While, DP voltammograms of 250 μM AIC oxidation showed two large oxidation peaks, peak 1_a, at E_{pa}=+0.41 V, and peak 2_a, at E_{pa}=+0.79 V (Fig. 1b) [31].

Voltammetric evaluation of TMZ or AIC/methyldiazonium ion–dsDNA interaction

In order to investigate the action of TMZ and its metabolites, AIC and methyldiazonium ion, to induce conformational changes, hydrogen bonding cleavage and/or oxidative damage to dsDNA the study by DP voltammetry of the interaction between dsDNA and TMZ or AIC/methyldiazonium ion was performed.

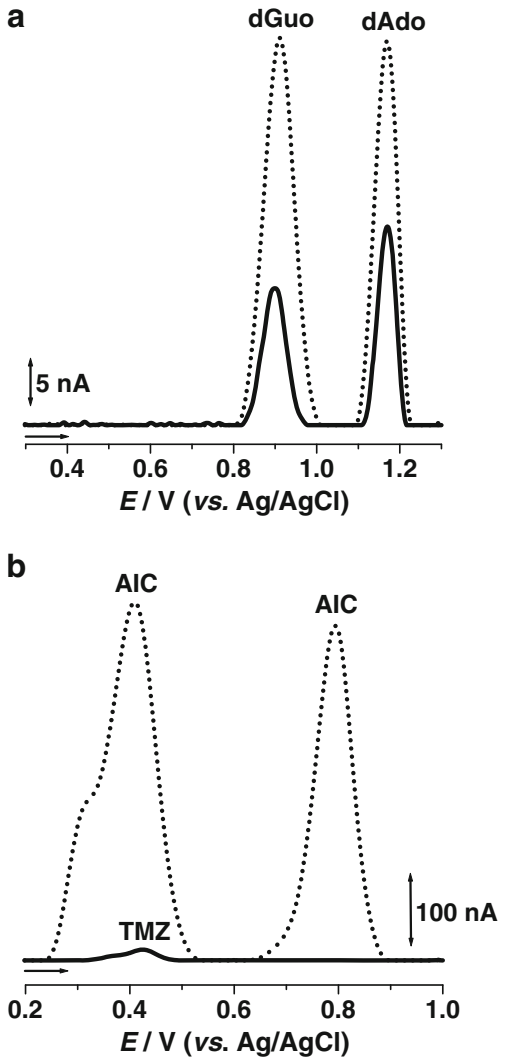


Fig. 1 DP voltammograms baseline-corrected in 0.1 M phosphate buffer pH 7.0: **a** (*solid line*) 250 μg mL^{-1} dsDNA solution and (*dotted line*) a multilayer dsDNA biosensor; **b** (*solid line*) 250 μM TMZ solution freshly prepared and (*dotted line*) after 7 days incubation

The effect of TMZ–dsDNA and AIC/methyldiazonium ion–dsDNA interaction was followed comparing the changes of the DNA oxidation peaks, dGuo and dAdo, in the absence/presence of these species, and monitoring the appearance of the oxidation products of guanine (8-oxoGua) or/and adenine (2,8-oxoAde), which both peaks occur at E_{pa}=+0.35 V in 0.1 M phosphate buffer pH 7.4, and their occurrence is an indication of DNA oxidative base damage [22, 26, 27].

Evidence for a specific interaction between dGuo and dAdo residues also is verified electrochemically by the appearance of free guanine (Gua), at E_{pa}=+0.68 V, and adenine (Ade), at E_{pa}=+0.98 V, in 0.1 M phosphate buffer pH 7.4 [22]. The oxidation peaks of guanine and adenine

correspond to the oxidation of the C1′ carbon of deoxyribose that causes the liberation of the bases [26, 27].

TMZ–dsDNA or AIC/methyldiazonium ion–dsDNA incubated samples

The interaction between 250 μM TMZ and 250 $\mu g mL^{-1}$ dsDNA, in incubated samples, in 0.1 M phosphate pH 7.0 buffer, following *Procedure 1* (Fig. 2a) was carried out.

DP voltammogram obtained immediately after the addition of TMZ to the dsDNA solution showed a decrease of the dGuo and dAdo oxidation peaks, when compared with the results from the control dsDNA sample (Fig. 2a). Moreover, the absence of the TMZ oxidation peak confirmed that the decrease of the dsDNA oxidation peaks is indeed due to the dsDNA condensation, and is not caused by blocking of the GCE by TMZ oxidation products. Also, since no peaks corresponding to 8-oxoGua/2,8-oxoAde and free Gua/Ade were observed, it was concluded that, for these experimental conditions used, there was no oxidative damage.

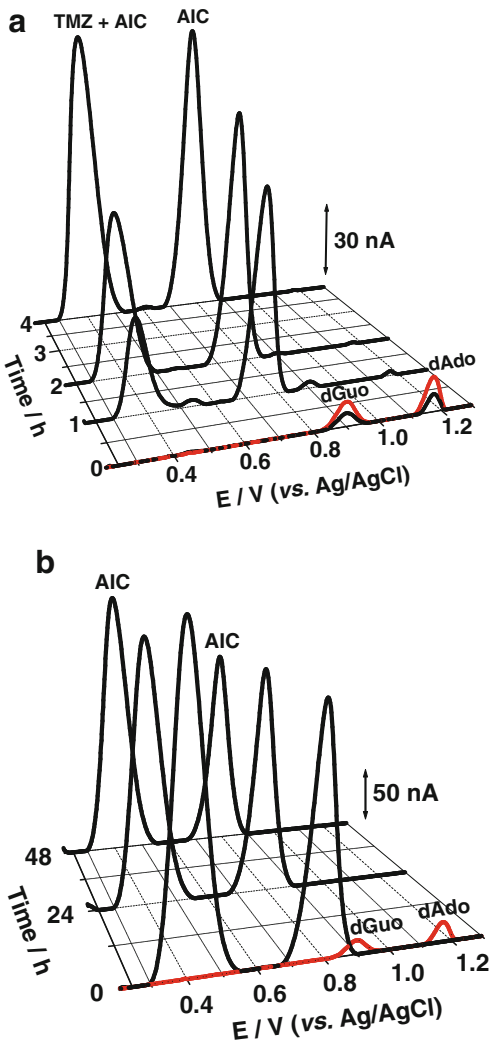


Fig. 2 DP voltammograms baseline-corrected in 0.1 M phosphate buffer pH 7.0: 250 $\mu g mL^{-1}$ dsDNA (*red line*) control and (*black lines*) incubated with: **a** 250 μM TMZ during 0, 1, 2 and 4 h and **b** 100 μM AIC/methyldiazonium ion during 0, 24 and 48 h

TMZ–dsDNA incubated samples were investigated after 1, 2, and 4 h. The DP voltammograms obtained showed a significant decrease of the dsDNA oxidation peaks with time and its complete disappearance after 4 h incubation (Fig. 2a). On the other hand, two new oxidation peaks appeared corresponding to the oxidation of TMZ/AIC degradation, at E_{pa}=+0.38 V, and AIC, at E_{pa}=+0.77 V (Fig. 2a) which increased with increasing incubation time. However, no peaks corresponding to 8-oxoGua/2,8-oxoAde were observed in the DP voltammogram, due to the high oxidation current of TMZ/AIC and since the oxidation of TMZ, AIC, and DNA biomarkers 8-oxoGua/2,8-oxoAde, occur at exactly the same potential.

When the incubated TMZ–dsDNA samples were investigated after 24 and 48 h incubation the DP voltammograms showed the complete disappearance of the dsDNA oxidation peaks and that the peak currents of TMZ/AIC and AIC increased due to still occurring TMZ degradation.

The above experiments were repeated using AIC/methyldiazonium ion–dsDNA samples for different incubation times in order to investigate if theses TMZ metabolites induced conformational changes, hydrogen bonding cleavage and/or oxidative damage to DNA.

The interaction between 100 μM AIC/methyldiazonium ion and 250 $\mu g mL^{-1}$ dsDNA was investigated immediately after the addition of AIC/methyldiazonium ion to the dsDNA solution. The DP voltammogram, when compared with the results obtained from the control dsDNA (Fig. 2b) showed the total disappearance of the dGuo and dAdo oxidation peaks and the appearance, as expected, of the AIC oxidation peaks at E_{pa}=+0.41 V and E_{pa}=+0.79 V. After 24 and 48 h incubation no dsDNA oxidation peaks were observed and the peak currents of the AIC remained constant (Fig. 2b). The disappearance of the dsDNA oxidation peaks demonstrated that during the incubation time a strong condensation of the double helix occurred. As the DNA bases are hindered inside the compact structure and less exposed to the electrode surface, their oxidation is more difficult, which can explain the decrease of the oxidation peak currents of dGuo and dAdo. However, in the DP voltammograms, no peak related to the presence of oxidative damage biomarkers, 8-oxoGua or 2,8-oxoAde, since the oxidation of AIC occurs at exactly the same potential, was observed.

The DP voltammograms showed that TMZ–dsDNA and AIC/methyldiazonium ion interaction occurred. However, the strong adsorption of AIC and its oxidation product blocking the GCE surface are responsible for the total disappearance of the dsDNA oxidation peaks.

In situ sensing with a multilayer dsDNA-electrochemical biosensor

The multilayer dsDNA-electrochemical biosensor was used to evaluate the interaction between TMZ–dsDNA and AIC/methyldiazonium ion-dsDNA. The multilayer dsDNA biosensor completely covers the electrode surface [22], avoiding non-specific adsorption and binding of TMZ or AIC molecules to the electrode surface.

The effect of TMZ and its metabolites was followed by DP voltammetry, comparing the dsDNA-electrochemical biosensor control with the changes observed with a dsDNA-electrochemical biosensor after the interaction. For each experiment a new multilayer dsDNA-electrochemical biosensor was prepared and incubated during different time periods with 250 μM TMZ or 100 μM AIC/methyldiazonium ion solutions in 0.1 M phosphate buffer pH 7.0, following *Procedure 2*, subsequently washed with deionised water in order to remove the unbound TMZ or AIC/methyldiazonium ion and transferred to 0.1 M phosphate buffer pH 7.0 where the DP voltammograms for each multilayer dsDNA-electrochemical biosensor were obtained.

Newly prepared multilayer dsDNA-electrochemical biosensor were incubated in a 250 μM TMZ solution during 10 min and 4 h, and the DP voltammograms obtained compared with the results from the control dsDNA biosensor, showed a decrease of the oxidation currents of dGuo and dAdo with incubation time (Fig. 3a).

Multilayer dsDNA-electrochemical biosensors in 250 μM TMZ solutions were investigated for long incubation times, 24 and 72 h, and both DP voltammograms showed four oxidation peaks (Fig. 3a).

The current decrease of the oxidation peaks of dGuo and dAdo is observed, due to the TMZ–dsDNA and AIC/methyldiazonium ion–dsDNA interaction, since that the degradation of TMZ to its AIC/methyldiazonium ion metabolites occurred for long incubation periods. The oxidation peak at E_{pa}=+0.35 V corresponds to a superposition of peaks, since both the oxidation of TMZ/AIC and 8-oxoGua/2,8-oxoAde occur at exactly the same potential.

The peaks of 8-oxoGua/2,8-oxoAde are due to dsDNA oxidative damage and TMZ/AIC peaks to their preconcentrated on the multilayer dsDNA-electrochemical biosensor. The peak at E_{pa}=+0.60 V corresponds to the oxidation of free Gua released from the dsDNA.

The results clearly showed that TMZ and/or AIC/methyldiazonium ion interact with dsDNA leading to condensation in the double-helical structure. Also, a specific interaction between the dGuo residues and TMZ and/or AIC/methyldiazonium ion is evidenced based on the occurrence of the free Gua oxidation peak, at E_{pa}=+0.60 V, due the oxidation of the C1′ carbon of deoxyribose [26, 27] by TMZ or

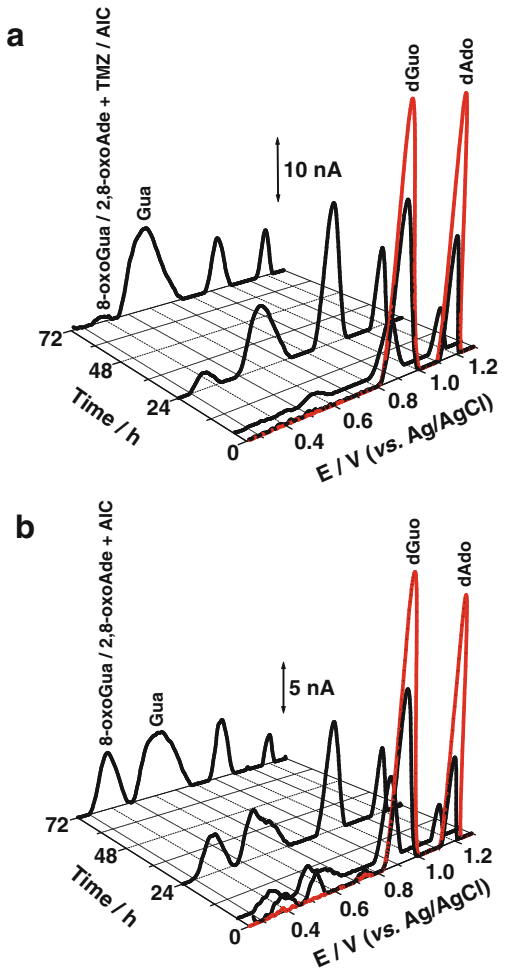


Fig. 3 DP voltammograms baseline corrected in 0.1 M phosphate buffer pH 7.0 using a multilayer dsDNA-electrochemical biosensor (*red line*) control and after (*black lines*) 10 min, 4, 24 and 72 h incubation in: **a** 250 μM TMZ solution and **b** 100 μM AIC/methyldiazonium ion solution

AIC/methyldiazonium ion that caused the liberation of guanine.

The oxidative damage caused to DNA by TMZ and its metabolites was also detected electrochemically by monitoring the appearance of the 8-oxoGua/2,8-oxoAde oxidation peak, at E_{pa}=+0.35 V.

The above experiments were repeated using multilayer dsDNA-electrochemical biosensors incubated for different time periods in 100 μM AIC/methyldiazonium ion solutions in pH 7.0 in order to investigate if AIC/methyldiazonium ion induced modifications in the double-helical structure, hydrogen bonding cleavage and oxidative damage to DNA.

The DP voltammograms obtained of the multilayer dsDNA-electrochemical biosensors after 10 min and 4 h of incubation in the AIC/methyldiazonium ion solution showed a progressive decrease of dGuo and dAdo oxidation peaks, when compared with the results obtained with the control multilayer dsDNA-electrochemical biosensor (Fig. 3b). Furthermore, two small oxidation peaks, at

E_{pa}=+0.38 V and E_{pa}~+0.60 V, were also detected when compared with the results with the control multilayer dsDNA-electrochemical biosensor and when compared with the results obtained for the TMZ–dsDNA interaction in the same conditions. The first oxidation peak is attributed to the oxidation of 8-oxoGua/2,8-oxoAde and AIC bound to DNA. The second oxidation peak is due to the oxidation of free Gua molecules released from dsDNA after 10 min and 4 h incubation.

It is concluded that the AIC/methyldiazonium ion, under the experimental conditions studied, caused DNA condensation and oxidative damage to DNA after 10 min interaction. When AIC/methyldiazonium ion–dsDNA solutions were investigated after 24 and 72 h of incubation (Fig. 3b), the DP voltammograms showed a progressive decrease of dsDNA oxidation peaks. On the other hand, the peaks attributed to 8-oxoGua/2,8-oxoAde + AIC and free Gua oxidation increased with increasing incubation time. These results indicate that the DNA condensation and oxidative damage to DNA caused by AIC/methyldiazonium ion are proportional to the incubation time.

The Gua oxidation peak is due to the oxidation of the C1′ carbon of deoxyribose [26, 27] by AIC/methyldiazonium ion, that causes the liberation of guanine.

The oxidative base damage caused to DNA by AIC/methyldiazonium ion species was also detected electrochemically by monitoring the appearance of the 8-oxoGua/2,8-oxoAde oxidation peak, at E_{pa}=+0.35 V.

The TMZ–dsDNA interaction was not due to direct TMZ interaction with dsDNA but to the direct interaction of DNA with its metabolites, AIC and methyldiazonium ions, formed by TMZ chemical degradation at physiological pH.

The multilayer dsDNA-electrochemical biosensors clearly showed that for TMZ–dsDNA and AIC/methyldiazonium ion–dsDNA interaction (Fig. 3a and b), the DNA oxidative damage caused by TMZ was only observed after a long incubation time, when the AIC and highly reactive methyldiazonium ion were present.

Besides, the results showed that the very strong condensation and oxidative damage caused to dsDNA are due to the methyldiazonium ion–dsDNA interaction, considering the low oxidation potentials of AIC (Fig. 1b) that shows its lower electron-donor character when compared with DNA (Fig. 1a) the high electron acceptor character of the reactive methyldiazonium ion, and the high affinity between the methyldiazonium cation at physiological pH with the DNA backbone negatively charged phosphate groups.

Electrophoresis evaluation of AIC/methyldiazonium ion–dsDNA interaction

Gel-electrophoresis was performed as described in the "Experimental" section to detect conformation changes in dsDNA during incubation with AIC/methyldiazonium ion solution. The main objective of the electrophoresis experiments was to compare the relative migration profile of the dsDNA after interaction with AIC/methyldiazonium ion solution with that of the control native dsDNA. Consequently, the use of markers for molecular mass determination was not considered necessary. To visualize the DNA mobility and possible damage, the EtBr binding assay for DNA damage was used [32].

Figure 4 shows the electrophoretic migration profile of incubated samples of 100 μgmL^{-1} dsDNA with 100 μM AIC/methyldiazonium ion solution during 10 min (lane 2), 4 h (lane 3) and 72 h (lane 4). The control sample of 100 μgmL^{-1} dsDNA was incubated in the same experimental conditions than AIC/methyldiazonium ion–dsDNA samples and analyzed after 72 h (lane 1). The band observed for dsDNA in the absence of AIC/methyldiazonium ion corresponds to different-sized length fragments present in the loaded samples [27, 33].

In all experiments, it was observed that the intensity of the bands diminished with increasing incubation time of AIC/methyldiazonium ion (Fig. 4) when compared with results obtained from the control dsDNA sample (lane 1). This decrease in total fluorescence observed between the bands in lanes 2, 3 and 4 indicates that EtBr was excluded from binding sites due to conformational changes caused by

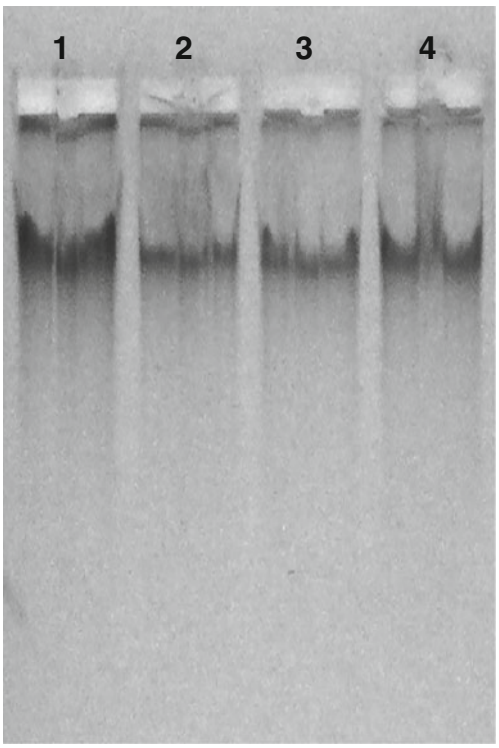


Fig. 4 Nondenaturing agarose (1 %) gel electrophoresis of 100 μg mL^{-1} dsDNA control (*lane 1*) and incubated with 100 μM AIC/methyldiazonium ion solution during 10 min (*lane 2*), 4 h (*lane 3*) and 72 h (*lane 4*)

AIC/methyldiazonium ion bound to the DNA double helix and that occurred accumulation of damage in the DNA exposed to AIC/methyldiazonium ion with incubation time.

These electrophoretic experiments confirmed the voltammetric results obtained for the AIC/methyldiazonium ion–dsDNA interaction, and were of crucial importance to confirm, interpret and explain the occurrence of oxidative damage to dsDNA.

Conclusions

TMZ is an anticancer prodrug that exerts its antitumor activity via its spontaneous degradation at physiological pH. The interaction of dsDNA with TMZ and its final metabolites, AIC and methyldiazonium ion, in neutral pH by DP voltammetry, in incubated samples, using multilayer dsDNA-electrochemical biosensors and gel electrophoresis, was investigated.

The voltammetric results obtained in the TMZ–dsDNA and AIC/methyldiazonium ion incubated samples showed the decrease of the dsDNA oxidation peaks with incubation time, corresponding to TMZ and AIC/methyldiazonium ion interaction with dsDNA causing the condensation of DNA strands. Furthermore, using the multilayer dsDNA-electrochemical biosensor the TMZ–dsDNA or AIC/methyldiazonium ion–dsDNA interaction showed oxidative damage caused to DNA by TMZ metabolites, since 8-oxoGua/2,8-oxoAde and free Gua were detected.

The electrophoresis results confirmed that a dsDNA conformational transition occurred during incubation with AIC/methyldiazonium ion solution, associated to the condensation and oxidative damage of DNA caused by AIC/methyldiazonium ion metabolites.

The sensitivity of the multilayer dsDNA-electrochemical biosensor offered the possibility to follow the interaction of TMZ and its metabolites with DNA under different conditions and the results enabled a better understanding of the molecular interaction mechanism between dsDNA–TMZ.

Acknowledgements Financial support from Fundação para a Ciência e Tecnologia (FCT), Post-Doctoral Grant SFRH/BPD/71965/2010 (S.C.B. Oliveira), project PTDC/QUI-QUI/098562/2008, POFC-QREN, POPH (co-financed by the European Community Funds FSE e FEDER/COMPETE), CEMUC-R (Research Unit 285), CNPq - Brazil, Post-Doctoral Grant/201487/2011-0 (I.C. Lopes) is gratefully acknowledged.

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