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An electrochemical study of interaction of an anticancer alkaloid ellipticine with DNA

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ABSTRACT *Ellipticine (5,11-demethyl-6H-pyridol(4,3-b)carbazole) and its derivates belong to prototype compounds acting predominantly by DNA-damaging mechanisms and serving as cytotoxic agents. The aim of this work was to study the intercalation of the ellipticine into DNA. For this purpose we utilized adsorptive transfer stripping technique (AdTS) coupled with square wave voltammetry (SWV). Primarily, we investigated the influence of ellipticine on genomic DNA electrochemical signals. Decrease in redox signal of cytosine/adenine (at -1.4 V) was observed as a result of ellipticine intercalation into DNA. The decrease was proportional to increasing concentration of ellipticine. In additional experiments we investigated the changes in the reduction signal of cytosine/adenine (CA) in a genomic dsDNA, dsDNA treated with ellipticine in vitro and dsDNA isolated from UKF-NB-3 neuroblastoma cells treated with 1 μM ellipticine. In the mentioned cases, the changes were determined.*

INTRODUCTION

Ellipticine (5,11-demethyl-6H-pyridol(4,3-b)carbazole) and its derivates belong to prototype compounds acting predominantly by DNA-damaging mechanisms (1). This alkaloid found in *Apocyanaceae* plants (i.e. *Ochrosia borbonica, Excavatia coccinea*), is one of the simplest naturally occurring alkaloids, having a planar structure (8). It was first isolated in 1959 from the leaves of the evergreen tree *Ochrosia elliptica*, which grows wild in Oceania (2). Numerous studies have reported the involvement of p53 tumour suppressor protein in ellipticine cytotoxic effects (3-7). Ellipticine has been found to restore the transcription function of mutant p53. This property may contribute to the selectivity of ellipticine-derived compounds against tumour cell lines expressing mutant p53 (6). Ellipticine might also function as a modulator of p53 nuclear localization; in HCT116 colon cancer cells ellipticine increased the nuclear localization of endogenous p53 with a resultant increase in the transactivation of the p21 promoter (7). Recently, we have found that ellipticine also activates the p53 pathway in glioblastoma cells; its impact on these cancer cells depends on the p53 status. In a U87MG glioblastoma cell line expressing p53wt, ellipticine provoked an early G0/G1 cell cycle arrest, whereas in a U373 cell line expressing p53mt it caused arrest in S and G2/M phases (4).

However, the precise molecular mechanism responsible for these effects has not yet been explained. Chemotherapy-induced cell cycle arrest was shown to result from DNA damage caused by a variety of chemotherapeutics. In the case of ellipticine, it was suggested that the prevalent DNA-mediated mechanisms of their anti-tumour, mutagenic and cytotoxic activities are (i) intercalation into DNA, (ii) inhibition of DNA topoisomerase II activity and formation of covalent DNA adducts mediated by cytochromes P450 (CYPs) and peroxidases (8-20). Deoxyguanosine was identified as the target base to which reactive species of ellipticine metabolites generated by CYPs and peroxidases are bound (16). In our previously published paper (21) we utilized adsorptive transfer stripping technique (AdTS) coupled with square wave voltammetry (SWV) to study interaction of doxorubicin, an anti-tumour drug, with dsDNA. Based on these results, we employ AdTS SWV to investigate interactions between ellipticine and DNA in this study.

MATERIAL AND METHODS

Chemicals and material

Ellipticine, parafilm and all chemicals of ACS purity used were purchased from Sigma-Aldrich (Sigma Aldrich corp., USA). Lyophilised polymerized DNA isolated from chicken erythrocytes (MW = 400 000 g/mol) was from Reanal (Hungary). Final concentration DNA was determined spectrophotometrically at 260 nm, (Spectronic Unicam, type Helios Beta, England).

Stationary electrochemical measurements

Electrochemical measurements were performed with AUTOLAB PGS30 Analyzer (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland) and 797

VA computrace (Metrohm, Switzerland) using a standard cell with three electrodes. A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was employed as the working electrode. An Ag/AgCl/3M KCl electrode served as the reference electrode. Glassy carbon electrode was used as the auxiliary electrode. For smoothing and baseline correction the software GPES 4.9 supplied by EcoChemie was employed. Square wave voltammetric (SWV) measurements were carried out in the presence of acetate buffer pH 5.0. SWV parameters: potential step 5 mV, frequency 260 Hz on HMDE) and (50 Hz on carbon electrode) (21). The analysed samples were deoxygenated prior to measurements by purging with argon (99.999 percent), saturated with water for 120 s. All experiments were carried out at room temperature.

Neuroblastoma cell line of malignant tumour

The UKF-NB-3 neuroblastoma cell line, established from bone marrow metastases of high risk neuroblastoma, were a gift of J. Cinatl (J. W. Goethe University, Frankfurt, Germany). The doxorubicin resistant cell subline designated UKF-NB-3 (DOXO) was established by incubation of parental cells with increasing concentrations of respective drug by the procedure as described (22-24). Cells were cultivated in DMEM cultivation medium supplemented with 5 percent foetal serum and subcultivated in four-day intervals. Own cultivation was proceeding in controlled atmosphere incubator at 37°C and 5 percent CO_2. Neuroblastoma cell lines were seeded 24 hr prior to treatment at a density of 1 × 10^5 cells/ml in two 75 cm^2 culture flasks in a total volume of 20 ml of Iscove's Modified Dulbecco's Media. Ellipticine was dissolved in 20 ml of DMSO, the final concentration was 0, 1 or 10 mM. After 48 h the cells were harvested after trypsinizing by centrifugation at 2000 × g for 3 min and two washing steps with 5 ml of PBS yielded a cell pellet, which was stored at -20 °C until DNA isolation.

DNA isolation and ^{32}P-postlabeling of DNA adducts

DNA isolation was done using Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to manufacturer's instructions. The cells were harvested and transferred to a 1.5 ml microcentrifuge tube. For adherent cells, the cells were trypsinized before harvesting. The cells was centrifuged at 16,000 × g for 10 seconds. The obtained supernatant was removed and the cell pellet plus 10.5 µl of residual liquid was leaving behind. Consequently 200 µl PBS to wash the cells were added and the mixture was centrifuged at 16,000 × g for 10 seconds. Then, PBS was removed. Cells were subsequently vigorously vortexed to resuspend. Nuclei Lysis Solution (600 µl) were added and pipetted to lyse the cells until no visible cell clumps remained. RNase Solution (3 µl) were added to the nuclear lysate and the sample was mixed by inverting the tube. Mixture was further incubated for 930 seconds at 37°C; sample cooling to room temperature for 5 minutes before proceeding followed. To the room temperature sample, 200 µl of Protein Precipitation Solution were added and vortexed vigorously at high speed for 20 seconds. In the next step the sample was chilled on ice for 5 minutes and centrifuged for 4 minutes at 16,000 × g. Supernatant containing DNA was carefully removed and transferred into a clean 1.5 ml microcentrifuge tube containing 600 µl of room tempered isopropanol. Solution was gently mixed by inversion until the white thread-like strands of DNA form a visible mass. Centrifugation for 1 minute at 16,000 × g at room temperature followed. The obtained supernatant was carefully decanted. Room tempered 70 percent ethanol (600 µl) were added and the tube was gently inverted several times to wash the DNA. Centrifugation for 1 minute at 16,000 × g at room temperature was carried out. Subsequently ethanol was carefully aspirated using either a drawn Pasteur pipette or a sequencing pipette tip. Tube was inverted on clean absorbent paper and the pellet was air-dried for 15 minutes. DNA Rehydration Solution (100 µl) were added and DNA was rehydrated by incubating at 65°C for 1 hour. Solution was periodically mixed by gently tapping the tube. ^{32}P-postlabeling analyses were performed using nuclease P1 enrichment as described previously (8, 10, 25).

RESULTS AND DISCUSSION

Adsorptive transfer technique as a tool to study intercalation of ellipticine into DNA

Convenient electroanalytical tool for DNA studies is the modification of working electrode both liquid and/or solid. The electrode can be modified with the molecule of interest. An easy and elegant way how to modify a working electrode represents technique called adsorptive transfer stripping discovered at the end of eighties of the last century (26, 27). Figure 1A shows the scheme of the technique. To modify HMDE with DNA organized layer (maximum electrochemical response of nucleic acids) the electrode surface is immersed in solution with DNA. Subsequently, the modified electrode is transferred to a drop with an intercalating molecule. There is interaction between DNA and

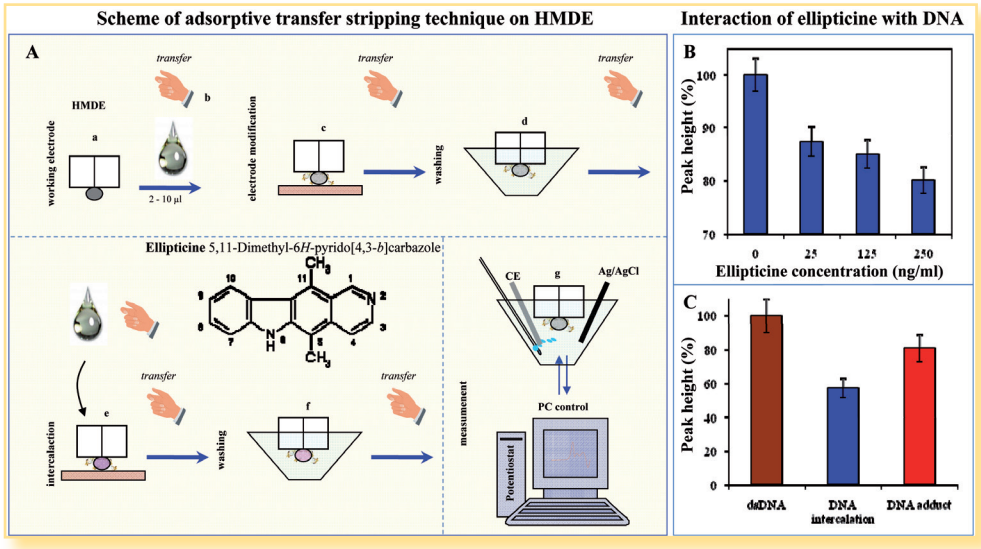

Figure 1. (A) Scheme of adsorptive transfer stripping technique used for studying ellipticine-dsDNA interactions; (a) renewing of the hanging mercury drop electrode (HMDE) surface; (b) pipetting of a drop (5 µl) of dsDNA onto cooled parafilm (30); (c) adsorbing of dsDNA in a drop solution onto the HMDE surface at open circuit (240 s); (d) washing electrode in water of ACS purity (Sigma); (e) interaction of ellipticine in a drop solution (5 µl) with the dsDNA modified HMDE surface at open circuit (300 s); (f) washing electrode in water of ACS purity (Sigma); (g) square wave voltammetric measurement. (B) Dependence of cytosine/adenine signal height on concentration of ellipticine intercalating to dsDNA. Intercalation experiment was performed in triplicates (n = 5). (C) SWV signal of genomic dsDNA (standard, no ellipticine), dsDNA (DNA intercalation, intercalated ellipticine) treated with ellipticine in vitro (5 µl, 2.5 µg/ml, 300 s) and dsDNA isolated from UKF-NB-3 neuroblastoma cells treated with ellipticine (DNA adduct, covalently bound ellipticine).

the intercalating molecule under open circuit. Interaction is terminated by washing the electrode. The washed electrode is transferred into a supporting electrolyte, in which own electrochemical measurement takes place.
Genomic dsDNA (5 µl, 10 µg/ml) was adsorbed onto surface of HMDE for 240 s. After washing, the electrode was immersed to a drop (5 µl) with ellipticine for 300 s. Then, the electrode was washed and transferred to pure supporting electrolyte. Similar to doxorubicin decrease in redox signal of cytosine/adenine (at -1.4 V) was observed as a result of ellipticine intercalation into DNA (Figure 1B). The decrease was proportional to increasing concentration of ellipticine. The resulting changes show fast process of intercalation and high affinity of ellipticine to dsDNA.

Adsorptive transfer technique as a tool for study of DNA interaction with ellipticine in vitro and DNA covalently modified by ellipticine in neuroblastoma cells in culture

In additional experiments we investigated the changes in the reduction signal of cytosine/adenine (CA) in a genomic dsDNA (see sample assigned as standard in Figure 1C), dsDNA treated with ellipticine *in vitro* (see sample assigned as DNA intercalation in Figure 1C) and dsDNA isolated from UKF-NB-3 neuroblastoma cells treated with 1 µM ellipticine (see sample assigned as DNA adduct (covalently bound ellipticine) in Figure 1C). The changes were determined by using AdTS SWV. The experiments were carried out according to the following protocol. Genomic dsDNA (5 µl, 10 µg/ml) was adsorbed onto surface of HMDE for 240 s. The modified electrode was then immersed into pure supporting electrolyte and measured. Brown column shows the height of the resulted signal of control dsDNA (n = 10, Figure 1C). We also determined control DNA isolated from untreated cells. The differences between standard DNA and DNA isolated from untreated cells was negligible. Then, the surface of HMDE was renewed and immersed into the same concentration of genomic dsDNA for the same time. After washing, the electrode was immersed to a drop with ellipticine (5 µl, 2.5 µg/ml) for 300 s. Then, the electrode was washed and transferred to pure supporting electrolyte. The resulted signal of dsDNA containing intercalated ellipticine is shown in Figure 1C as blue column (n = 10). We also tried to answer the question whether it would be possible to observe changes in CA signal in DNA isolated from UKF-NB-3 neuroblastoma cells treated with ellipticine. Neuroblastoma cells that have been exposed to ellipticine were homogenized according to standard protocol and dsDNA isolated as shown in Material and Methods. Using the nuclease P1 version of ^{32}P-postlabeling assay (8, 10, 25), ellipticine-derived covalent adducts were detected in the DNA sample of these cells and their levels were evaluated to be 5.9 adducts per 10^7 normal nucleotides. This neuroblastoma dsDNA was adsorbed onto surface of HMDE and, after washing step, immersed into pure supporting electrolyte and measured. CA signal and signal of ellipticine, which clearly showed the presence of ellipticine in dsDNA molecule, were detected. dsDNA isolated from the ellipticine-treated cells gave the signal shown in Figure 1C as red column. It is obvious that the height is lowered for more than 20 percent compared to dsDNA standard signal. Even though this change is lower than that found in the DNA sample, in which elipticine was intercalated into DNA *in vitro* (blue column in Figure 1C), indicating that modification of DNA might be different than the pure intercalation process, this finding is the confirmation of ellipticine binding into nuclear dsDNA in neuroblastoma cells. Therefore, electrochemical detection can be considered as a tool for rapid monitoring of ellipticine adducts in DNA. Nevertheless, studies examining differences in electrochemical signals of DNA with intercalated ellipticine and DNA covalently modified by this antitumor agent in details await further investigation.

CONCLUSION

Electrochemical detection represents easy-to-use and inexpensive tool for a study of dsDNA interaction with prototype of DNA-damaging drugs (28, 29). Here, we show the possibility of square wave voltammetry not only to sensitively detect modification of dsDNA by ellipticine (DNA-ellipticine adducts and ellipticine intercalation complex with DNA).

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