J. Serb. Chem. Soc. 70 (6) 823–831 (2005)
JSCS – 3318

UDC 615.33+547.963.32:616–006
Original scientific paper

AFM studies of DNA structures extracted from adriamycin treated and non-treated Ehrlich tumor cells

M. AVRAMOV IVIĆ[1*#], S. D. PETROVIĆ[2,3#], E. KALMÁN[6], T. MILOSAVLJEVIĆ[3], I. RELJIN[4], B. RELJIN[4], G. BOGDANOVIĆ[5], V. VIT. BALTIĆ[5] and Z. KERESZTES[6]

[1]ICTM, Institute of Electrochemistry, University of Belgrade, Njegoševa 12, Belgrade (e-mail: milka@elab.tmf.bg.ac.yu), [2]Faculty of Technology and Metallurgy, Department of Organic Chemistry, Karnegijeva 4, P. O. Box 494, Belgrade, [3]Hemofarm Group, Pharmaceutical-Chemical Industry, Vršac, [4]Faculty of Electrical Engineering, University of Belgrade, Bulevar Kralja Aleksandra 73, Belgrade, [5]Institute of Oncology Sremska Kamenica, 21204 Sremska Kamenica, Institutski put 4, Serbia and Montenegro and [6]Chemical Research Center, Department of Nanostructures and Surface Modification, Budapest 1025, Pusztaszeri ut. 59-67, Hungary

(Received 6 August, revised 12 October 2004)

Abstract: Atomic force microscopy (AFM), a unique tool to investigate drug treatment of cancer cells, was used to analyze the anti-neoplastic activity of adriamycin by comparing DNA structures of non-treated and adriamycin-treated Ehrlich tumor cells. The non-treated cells exhibited a highly branched intact chromatin structure, related to the intensive DNA replication in cancer cells. Images from adriamycin-treated tumor cells showed that the DNA chains were broken and the chromatin structure had been destroyed. Possible explanations for these effects of adriamycin are considered: breakage of hydrogen bonding, oxidation and intercalation effects, as well as the poisoning of topoisomerase enzyme. DNA fractal and multifractal analyses, performed in order to evaluate the degree of bond scission, showed that the treated DNA had become more fractal compared to non-treated DNA.

Keywords: adriamycin, Ehrlich tumor cells, atomic force microscopy, topoisomerase, fractal and multifractal analysis.

INTRODUCTION

Adriamycin (ADR), the first anthracycline drug, has been used in clinical practice since the 1960's and still remains the most widely used anticancer therapeutic with a wide spectrum of anti-neoplastic action.[1] However, its mode of action is not yet fully understood, although it is known that it intercalates strongly with the DNA double helix, mainly at CG-GC steps, the amino sugar being determinant for intercalation to occur.[2] In addition, ADR interferes with the DNA regulation machinery in several ways, thus promoting the

* Author for correspondence.
\# Serbian Chemical Society active member.

generation of reactive oxygen species, which ultimately cause oxidative damage to biomolecules.[3,4] In fact, a high level of 8-oxoguanine (a main biomarker of DNA oxidation) was detected in cancerous cells treated *in vitro* with adriamycin,[5] but it is not known whether or not ADR, after intercalation, directly oxidizes DNA *in vivo*.

In addition, ADR is referred to as a "topoisomerase II poison".[6–8] Eukaryotic type II topoisomerase is a multifunctional nuclear enzyme responsible for relieving DNA from the topological constraints by cleaving both DNA strands, which facilitates various cellular processes, such as transcription, replication, sister chromatid disjunction and chromosome segregation.[9] "Topoisomerase II poisons" significantly increase the levels of enzyme-mediated DNA cleavage, thus leading to the topoisomerase II inactivation.[6,10] It is believed that this is the most important mechanism of anthracyclines cytotoxicity.[1]

Different electrochemical DNA-biosensors for the detection of DNA–drug interactions and DNA oxidative damage have recently been investigated.[11–13] Additionally atomic force microscopy (AFM), a unique tool for the high-resolution characterization of biological objects, can be used to explain interactions between DNA molecules and different anti-neoplastic drugs.[14,15] AFM enables the recognition and identification of molecular interactions occurring during functional rearrangements *in situ*. The great advantage of AFM compared to other microscopic methods is that high vacuum is no longer required and samples can be analysed in their hydrated form, under liquid. An atomically flat mica surface serves well as a support for biomolecules in AFM investigations. However, the non-specific physical adsorption of biologically important macromolecules (proteins and nucleic acid) to this hydrophilic surface may interfere with the preservation of their native state.[14]

The aim of this work was to gain an understanding of the interactions between adriamycin and DNA leading to ADR antitumor activity in malignant cells *in vitro*. Atomic force microscopy was used to study this interaction in DNA samples extracted from ADR-treated and untreated Ehrlich ascites tumor cells. Additionally, the AFM images were analyzed using fractal (FG) and multifractal (MF) geometry in order to determine the degree of DNA discontinuity caused by the drug treatment.

EXPERIMENTAL

Chemicals

Adriablastina (ADR), commercially available powder for injection, was from Pharmacia & Upjohn S.p.A. (Milan, Italy). A working solution of ADR was prepared one hour before each series of experiments. The final concentration of ADR was 10^{-7} M. RPMI 1640 culture medium was purchased from GIBCO Brl (Life Technologies, Scotland), fetal calf serum (FCS) from NIVNS (Novi Sad, Serbia and Montenegro) and antibiotics from ICN Galenika (Belgrade, Serbia and Montenegro). All other chemicals used in the study were special grade commercial products or reagents of analytical grade.

Cell culture

Ehrlich ascites tumor cells were grown in the abdominal cavity of NMRI mice, age approximately five–seven weeks. Ten days after inoculation, the cells were isolated by needle aspiration, washed in saline, and the erythrocytes were removed with a lysing solution (Becton Dickinson).

Approximately $5 – 7 \times 10^6$ cells were cultured in 10 ml of RPMI 1640 supplemented with 10 % of fetal calf serum and antibiotics: 100 IU/ml of penicillin and 100 mg/ml of streptomycin. The cells were treated with ADR (10^{-7} M) while control samples were treated with the corresponding volume of complete medium. All samples were cultured for two hours at 37 ºC in a 100 % humidity atmosphere containing 5 % of CO_2. After treatment, the cells were pelleted (200 × g / 10 min), washed once in 10 ml of PBS (pH 7.2), resuspended in 1 ml of PBS, and counted in a hemocytometer. The cell density and percentage of viable cells were determined by the trypan blue dye exclusion test.[16]

Extraction and precipitation of DNA

DNA was extracted from the ADR-treated and untreated Ehrlich ascites tumor cells using mixtures of phenol, chloroform and isoamyl alcohol.[17] Briefly: at least 2×10^6 / ml of viable cells were washed in cold PBS by centrifugation (1000 × g for 10 min) and lysed with cold lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM sodium chloride, 10 mM EDTA) for three minutes on ice with gentle vortexing. Sodium dodecyl sulfate (SDS, 1 %) and proteinase K (200 μg/ml) were added and the mixture was incubated (1.45 h) at 50 ºC.

The fragmented DNA was extracted twice using phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v). The DNA was precipitated with ethanol and high salt. The DNA pellet was washed once in 70 % ethanol and stored at –20 ºC.

For the AFM analysis, the ethanol was poured off and the DNA was resuspended in Tris EDTA (TE) buffer (pH 8.0) by gentle mixing.

AFM measurements

The measurements were carried out using a Nanoscope IIIa controller with a Multimode AFM (Digital Instruments, Santa Barbara, CA). The DNA sample was dissolved in Tris buffer and placed in drops on a Mg^{2+} - treated mica surface. After half an hour of adsorption, the sample was rinsed with Tris buffer and dried. The investigation was carried out in the contact mode using a DI cantilever of 0.12 N/m spring constant.

RESULTS AND DISCUSSION

As previously reported, DNA – adriamycin interaction at charged interfaces represents the *in vivo* situation much better than a solution of these two compounds themselves. An electrochemical DNA-biosensor enabled the detection and a better understanding of DNA/molecule/ion interactions, *i.e.*, *in situ* adriamycin caused oxidative damage to DNA.[18] The DNA extracted from untreated Ehrlich tumor cells were adsorbed onto mica (according to the described experimental procedure) and visualized by AFM.

The chromatin structure of untreated cells is shown in Fig. 1, which indicates a helix form of "superstructure" above the basic DNA double helix.[19] The basic DNA fibers (3–4 nm) are packed with proteins into bigger fibers, *i.e.*, a chromatin "superstructure" 30–40 nm wide. These numerical values correspond to the microscopically observed parameters.

A larger scale AFM image is presented in Fig. 2 where proteins bound at the junctions of the fibers can be identified.

An AFM image of DNA extracted from *in vitro* ADR-treated Ehrlich ascites tumor cells is presented in Fig. 3. It can be clearly seen that the original structure has been destroyed under adriamycin activity. The interaction of DNA with adriamycin resulted in broken chains of double helices, which is probably the main anti-neoplastic effect of this drug.

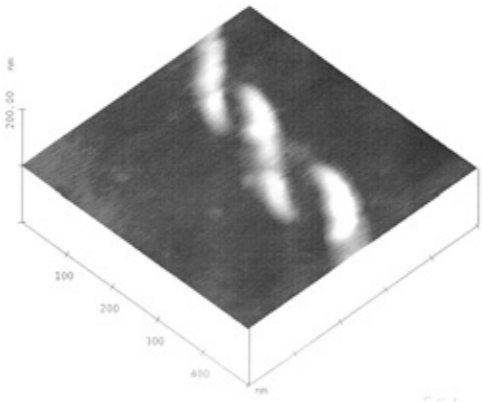

Fig. 1. AFM image of chromatin structure isolated from untreated Ehrlich ascites tumor cells. The measurement was taken in the contact imaging mode under ambient conditions.

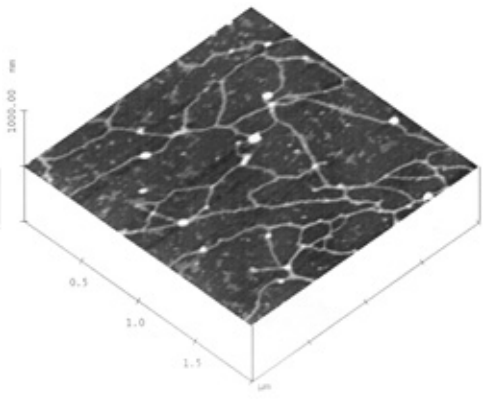

Fig. 2. AFM image of DNA isolated from untreated Ehrlich ascites tumor cells. The measurement was taken in the contact imaging mode under ambient conditions.

There are few possible explanations for the observed phenomenon of the degradation of native DNA.

The degradation can be caused by the destruction of hydrogen bonds as they are essential components of the structure and function of biological molecules. These bonds are very rigid and not easily broken and yet strong hydrogen bonds are rare in biological structures. The strongest hydrogen bonds are so called salt bridges, NH···O=C in proteins and P–OH···O=P bonds in nucleic acids.[20] Molecules of water or any potent organic substance, such as the anthracycline antibiotic, adriamycin, can interrupt hydrogen bonds and thus destroy them. Subsequently, this can lead to the induction of breaks in DNA single and double strands and considerable death of tumor cells.[18]

A second possibility is the structural modification of DNA strands caused by intercalation of adriamycin molecules. This mode of ADR action is not yet fully understood, but

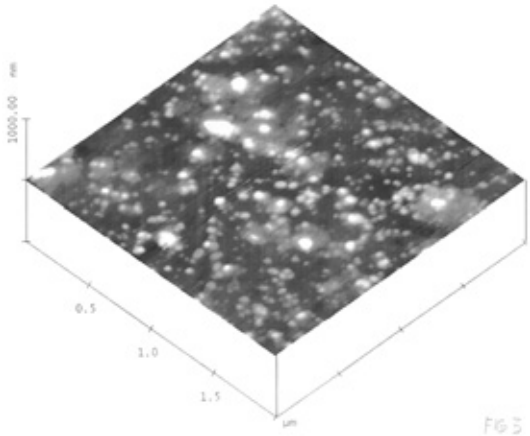

Fig. 3. AFM image of DNA isolated from Ehrlich ascites tumor cells treated with adriamycin. The measurement was taken in the contact imaging mode under ambient conditions.

it may be through intercalation in the DNA double helix and interaction with CG-GC base pairs.[2] As was recently shown,[21] the oxidation and reduction of adriamycin molecules intercalated in double helix DNA is potential-dependent, causing contact between guanine and adenine bases of DNA. It is assumed that the adriamycin amino sugar moiety plays a very important role in this intercalation. The existence of irregular cyclic- or stellar-shaped DNA, or random coil conformations in a treated sample (Fig. 2) indicate that native DNA is degraded probably to double stranded fragments by both double strand scissions at one locus and single strand breaks. This means that the result of this interaction is a specific association of the two broken chains of the double helix. The possibility that adriamycin intercalated to double helix DNA reacts specifically with the guanine moiety is known and may lead to base pairing mismatch.

As a third possibility, it is believed that anthracycline drugs stabilize a covalent topoisomerase IIα – DNA intermediate. This effect is achieved by forming a ternary enzyme–poison–DNA complex in which both DNA strands are broken and covalently linked to enzyme subunits.[22] This intermediate disables repair of broken strands and the double helix breaks remain intact, as can be seen in Fig. 3. Topoisomerase II activity increases significantly during periods of rapid cell proliferation,[23] which is the situation shown in Fig. 2 (intensive DNA replication in Ehrlich ascites tumor cells). When treated with adriamycin, native DNA is significantly degraded and the breakage points probably correspond to activity of topoisomerase IIα.

The AFM images of untreated and ADR-treated DNA, shown in Figs. 2 and 3, were also analyzed by the fractal geometry and multifractal spectrum approaches.[24–28] It is important to point out that fractal analysis in biology and medicine has already been successfully used by several authors.[29–32] Fractal and multifractal analyses were applied to a part of the AFM image shown in Fig. 4. The two-dimensional grey-level signals were converted to the corresponding one-dimensional (*i.e.*, scanned) signals and fractal and multifractal analyses applied. The fractal nature of the signal was checked by the value of the Hurst index, *H*. It is known that processes having a value of the Hurst index between 0.5 and 1.0 exhibit self-similarity (repeated structure in all scales), *i.e*, these processes have fractal behaviour. The Hurst index[24,26] was determined by using the periodogram method. The calculated values were H_{cntrl}=0.696 and H_{treat}=0.699 for CONTROL (non-treated) and TREATED sample, respectively, indicating the fractal nature of both samples. Although the fractal analysis enabled a number of non-regular phenomena to be described, this method suffers from at least one drawback: it assumes self-similarity but uses binary "yes-no" logic. For many phenomena, it is more appropriate to use a *scale of measures* between limiting (max and min) values. In this case fractal analysis can be extended to multifractal (MF) approach.[24] The MF method assumes two quantities are derived: the values of so-called *Hölder exponents*, α, describing the *local regularity* of signal points, and the value of the *distribution* of *these coefficients* – usually known as the *spectrum* of α, or the *multifractal* (MF) *spectrum, f*(α), in short. The MF spectrum gives the *global description* of the signal (or, more generally, of the phenomenon under investigation). The so-called coarse Hölder exponent at point (x) is given by

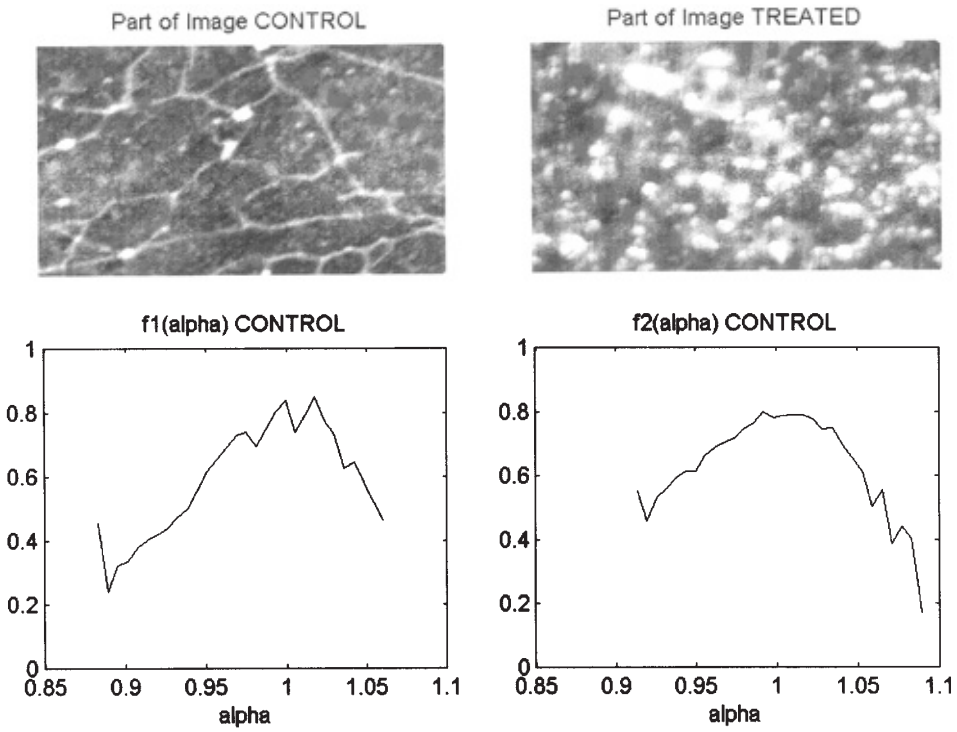

Fig. 4. Part of DNA images (extracted from Figs. 2 and 3), respectively denoted as CONTROL (not treated) and TREATED, and their multifractal spectra.

$\alpha_{(x)}(i) = [\ln c_{(x)}(i)]/[\ln(i)]$,where $c_{(x)}(i)$ is the signal *measure* (or, the *capacity*)[25] describing some signal feature (for instance, the grey level of the image) acting on a measure domain, and i is the *dimension* of the surrounding across the observed point (the measure domain). From this, the pointwise digital Hölder α exponent is determined as: $\alpha_{(x)} = \lim_{i\to 0} \alpha_{(x)}(i) = \lim_{i\to 0} [\ln c_{(x)}(i)] / [\ln(i)]$. Once the coefficients α have been found, the distribution of these quantities, usually known to as the multifractal spectrum, $f(\alpha)$, may be derived, describing the global behaviour of the signal. Probably a simpler way of deriving an MF spectrum is that based on the *Hausdorff measure* (or, Hausdorff dimension). This procedure first assumes that the whole range of α ($\alpha_{\min}$ to $\alpha_{\max}$) be divided into N subranges. Subsequently, the Hausdorff dimension is calculated for every subrange, particularly. Usually, a uniform subdivision is assumed $\varepsilon = (\alpha_{\max} - \alpha_{\min})/N$. If α_k denotes the k-th subrange, then its scope is $[\alpha_k - \varepsilon\ \alpha_k + \varepsilon]$.

The so-called *box-counting* algorithm is the most often used method for the estimation of the Hausdorff dimension.[26] The α matrix is covered by a regular grid of boxes. After covering with boxes, the number of non-empty boxes N_i (α_k) is counted. A box is non-empty if at least one value from a given subrange α_k is found within it, which means if $\alpha_k - \varepsilon < \alpha(x) < \alpha_k + \varepsilon$ holds for at least one point (x) within the box. Boxes of different sizes are recursively taken, and from a bilogarithmic diagram $\ln i \leftrightarrow \ln$

$N_i(\alpha_k)$, the Hausdorff dimension α_k of a subrange is calculated by linear fitting: $f(\alpha_k) = \lim_{i\to 0} [\ln N_i(\alpha_k)/\ln(1/i)]$. A simplified version of this method, known as the histogram method[24,26] has been developed as a computer program HISTMF.[28] This program was used for determining the MF spectra of the AFM images, shown in the second row of Fig. 4.

From the MF spectra, Fig. 4, it is evident that the DNA after treatment exhibits more discontinuities since the spectrum becomes more right-sided, *i.e.*, the main part of the MF spectrum is to the right of its maximum.[27] The obtained results clearly demonstrate that fractal and multifractal analyses can be successfully applied for the evaluation of drug efficiency in cancer research.

CONCLUSIONS

Adriamycin is one of the most important anti-neoplastic agents used widely in clinical practice, especially in the treatment of human neoplasms. A number of important biochemical effects have been described but the exact mode of adriamycin antineoplastic action is not yet fully understood. Atomic force microscopy serves as a unique tool in the investigation of drug treatment of cancer cells. Hence, this method was used to analyze the anti-neoplastic activity of adriamycin by comparing DNA structures extracted from ADR-treated and non-treated Ehrlich tumor cells. Non-treated DNA exhibited an intact chromatin structure, *i.e.*, the chromatin had a highly branched appearance, related to the intensive DNA replication in cancer cells.

Images of DNA extracted from Ehrlich ascites tumor cells treated with adriamycin *in vitro* showed that the chromatin is destroyed under the effect of adriamycin and the chains of the double helix are broken. Possible explanations for this morphological effect of adriamycin activity are considered, such as the scission of hydrogen bonds, oxidation and intercalation effects, as well as the poisoning of topoisomerase enzyme by adriamycin.

Fractal and multifractal analyses of the AFM images of the DNA samples were performed in order to evaluate the degree of bond scission. Fractal analysis showed that treated DNA becomes more fractal than non-treated DNA. Moreover, the multifractal spectrum of an ADR-treated DNA sample, obtained by the histogram method, indicates the existence of more discontinuities of the signal and of its derivative in the image in comparison with that of the non-treated DNA. This type of analysis gives the possibility of numerically evaluating drug efficiency, which will be one of the guidelines in our further research activities.

As can be seen, both analyses showed a significant difference in the morphology of the chromatin of non-treated and adriamycin-treated cells. This combination of AFM and fractal analysis enabled adriamycin anticancer activity to be better understood and numerically evaluated and, thus, can be of great importance for the future development of anticancer therapeutics.

ИЗВОД

AFM МИКРОСКОПИЈА DNA ИЗОЛОВАНЕ ИЗ ЕРЛИХОВИХ ТУМОРСКИХ ЋЕЛИЈА ТРЕТИРАНИХ И НЕТРЕТИРАНИХ АДРИАМИЦИНОМ

М. АВРАМОВ ИВИЋ[1], С. Д. ПЕТРОВИЋ[2,3], E. KALMÁN[6], Т. МИЛОСАВЉЕВИЋ[3], И. РЕЉИН[4], Б. РЕЉИН[4], Г. БОГДАНОВИЋ[5], В. ВИТ. БАЛТИЋ[5] и Z. KERESZTES[6]

[1]ИХТМ, Институт за електрохемију, Универзитет у Београду, Његошева 12, Београд, [2]Технолошко-металуршки факултет, Катедра за органску хемију, Карнегијева 4, Универзитет у Београду, Београд, [3]Хемофарм Концерн, Фармацеутско-хемијска индустрија, Вршац, [4]Електротехнички факултет Универзитета у Београду, Булевар Краља Александра 73, Београд, [5]Институт за онкологију Сремска Каменица, Институтски пут 4, 21204 Сремска Каменица, Србија и Црна Гора и [6]Chemical Research Center, Department of Nanostructures and Surface Modification, Budapest 1025, Pusztaszeri ut. 59-67, Hungary

DNA изолована из Ерлихових туморских ћелија је AFM микроскопијом посматрана претходно третирана адриамицином и у нетретираном стању, ради поређења. AFM микроскопија пружа могућност добијања директне слике изгледа DNA структуре малигне ћелије која није третирана леком и исте структуре после деловања адријамицина, што даје нове податке о деловању цитостатика на малигну ћелију. Нетретиране ћелије (виђене AFM микроскопијом) су испољиле нетакнуту структуру хроматина која одговара интензивној DNA репликацији у малигној ћелији. Малигне ћелије третиране адриамицином су показале да су DNA ланци покидани, тј. даља репликација је спречена, што је један од основа активности цитостатика. Мултифрактална анализа AFM микроскопских слика третиране и нетретиране DNA је квантитативно потврдила закључке добијене микроскопијом јер су графици AFM слика узорака третираних адриамицином показали фракталнију структуру од AFM слика нетретираних малигних ћелија код којих график указује на потпуни губитак фракталности, што је знак присутног патолошког процеса у организму.

(Примљено 6. августа, ревидирано 12. октобра 2004)

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