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Interaction of antimutagenic 1,4-dihydropyridine AV-153-Na with DNA and DNA-damaging molecules and its impact on DNA repair activity

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Abstract
1,4-dihydropyridines (1,4-DHP) possess important biochemical and pharmacological properties, including antioxidant and antimutagenic activities. Interaction of some 1,4-DHP with DNA was recently reported. AV-153-Na, an antimutagenic and DNA-repair-enhancing compound appeared to be able to interact with DNA by intercalation.

The aim of the current study was to characterize DNA’s capacity for the binding of AV-153-Na, and using different approaches, to test intracellular distribution of the compound, to test the ability of the compound to scavenge peroxynitrite and hydroxyl radical and to assess the ability of the compound to modify the activity of DNA repair enzymes.

The DNA binding activity of AV-153-Na was determined by means of fluorescence assay. Titration of the AV-153-Na solutions with DNA gradually increased fluorescence of the solution, indicating direct interactions of the molecule with DNA. AV-153-Na quenched the fluorescence of ethidium bromide and DNA complex, which points to intercalation binding mode. Binding via intercalation was confirmed by means of cyclic voltammetry and circular dichroism spectroscopy. The compound could interact with the four DNA bases in vitro, manifesting a higher affinity to guanine. Some ability to scavenge hydroxyl radical by AV-153-Na was detected by the EPR method. AV-153-Na turned out to be incapable of reacting chemically with peroxynitrite. However, AV-153-Na effectively decreased DNA damage produced by peroxynitrite in cultured HeLa cells. The effects of AV-153-Na on the activity of DNA repair enzymes were tested using Glyco-SPOT and ExSy-SPOT assays. The Glyco-SPOT test essentially revealed an inhibition by AV-153-Na of the enzymes involved thymine glycol repair. Results with ExSy-SPOT chip indicate that AV-153-Na significantly stimulates excision/synthesis repair of 8-oxoguanine (8-oxoG), abasic sites (AP sites) and alkylated bases. Laser confocal scanning fluorescence microscopy demonstrated that within the cells AV-153-Na was found mostly in the cytoplasm; however, a stain in nucleolus was also detected. Binding to cytoplasmic structures might occur due to high affinity of the compound to protein, revealed by fluorescence spectroscopy titration and circular dichroism. Activation of DNA repair enzymes after binding to DNA appears to be the basis for the antimutagenic effects of AV-153-Na.

Key words: 1,4-dihydropyridines, DNA repair, DNA binding, AV-153-Na
1. Introduction

Synthetic derivatives of 1,4-dihydropyridine (1,4-DHPs) possess important biochemical and pharmacological properties. They show modulating activity on cardiovascular and neuronal processes as well as anticancer, genoprotective and radioprotective effects. In the present investigation we have focused our attention on a representative of the 1,4-DHP derivatives, which is considered to be “unusual”. These compounds are water-soluble molecules without the activity of blockers of calcium channels or with a very weak blocking activity. 1,4-DHPs of this group manifest different biological activities, including genome-protecting effects; for example, glutapyrone is an antineoplastic and anticlastogenic agent, antimutagen and enhancer of DNA repair (Goncharova et al. 2001; Kuzhir et al. 1999; Vartanian et al. 2004). Our interests were focused on the compound AV-153-Na, possessing antimutagenic activity and being an enhancer of DNA repair (Ryabokon et al. 2009a; Ryabokon et al. 2008; Ryabokon et al. 2005; Ryabokon et al. 2009b). Recently we have revealed the DNA binding capacity of this compound (Buraka et al. 2014). The aim of the current study was to reproduce data on DNA binding using different approaches to test the DNA-protective capability of the compound in formerly unstudied systems, to test the ability of the compound to scavenge peroxynitrite and hydroxyl radical, and to assess the ability of the compound to modify the activity of DNA repair enzymes. To achieve these goals, the study was designed as follows. The first work package was aimed at the verification of the interaction of AV-153-Na with DNA and the evaluation of possible mechanisms of interaction, comprising spectrofluorometric study of interactions of the compound with DNA; confirmation of capacity of the AV-153-Na to bind DNA by cyclic voltammetry, and evaluation of possible mechanism of interaction; further study of the DNA and the compound interaction mode with DNA by circular dichroism spectroscopy; evaluation of the possibility of AV-153-Na to interact with DNA bases and an attempt to visualize AV-153-Na in the cells. The second work package was aimed at evaluation of possible direct interaction of the compound with DNA-damaging agents, hydroxyl radical and peroxynitrite in vitro, to reveal the role of direct chemical interactions in antimutagenic activity of AV-153-Na. Finally, possible impact of the AV-153-Na on the dynamics of DNA breakage in living cells, and activity of DNA repair enzymes was studied by means of single cell electrophoresis and functional repair assays (Glyco-SPOT and ExSy-SPOT assays).
2 Materials and Methods

2.1 Chemicals. AV-153-Na and AV-154-Na were synthesized in the Laboratory of Membrane Active Compounds at the Latvian Institute of Organic Synthesis. Structures of the AV-153 salts are given in Figure 1 inserts and structures of AV-154-Na in Figure 8; the synthesis of the compounds was performed essentially as described (Dubur, Uldrikis, 1969). Tris base, sucrose, ethidium bromide, acridine orange, Triton-X-100, Hind III/λ DNA digest, human serum albumin (HSA), ethidium bromide (EtBr), calf thymus DNA (ct-DNA) Na₂EDTA, LiCl, NaCl, CaCl₂ and other inorganic salts were purchased from Sigma-Aldrich (Taufkirchen, Germany). 2-mercaptoethanol was obtained from Ferak Berlin (Germany), sodium dodecyl sulphate was supplied by Acros Organics (Pittsburg, USA), isoamylic alcohol was obtained from Stanlab (Lublin, Poland), and 6×Orange loading solution, RNase A and Proteinase K were purchased from Thermo Fisher Scientific (Pittsburg, USA). Peroxynitrite was synthesized as described by Robinson and Beckman (2005).

2.2 Cell culture. HeLa cells (Biomedical Research and Study Centre, Riga, Latvia) were grown in DMEM + GlutamaxTM – I, F-12 Nut-Mix (1x) (Sigma-Aldrich, Taufkirchen, Germany) + 10% fetal bovine serum (Sigma-Aldrich, USA), at 37°C in a humidified atmosphere containing 5% CO₂.

2.3 Fluorescence spectroscopic measurements. Spectrofluorimetric analyses were performed on a Fluoromax-3 (Horiba JOBIN YVON, China). Fluorescence spectra of a 25 µM solution of the 1,4-DHP in 5 mM Tris-HCl; 50 mM NaCl at pH 7.4 or other buffer were recorded over a range of 365-600 nm at an excitation wavelength of 350 nm. An aliquot containing 12.5 µM DNA was sequentially added at each step until saturation. Scatchard binding constants were calculated using modified Scatchard method (Strothkamp & Stothkamp 1994). Fluorescence spectroscopic experiments on the interaction of 1,4-DHP with the DNA-EtBr complex were carried out at room temperature in 5 mM Tris HCl; 50 mM NaCl at pH 7.4 or other buffer using a 1 cm cuvette (2ml). The complex calf thymus DNA (74.8 µM) and ethidium bromide (1.26 µM) was titrated with 8 µl aliquots of the 2.5 mM solution of the compound. After each titration, the solution was mixed thoroughly and allowed to equilibrate for 5 min prior to fluorescence measurement. Fluorescence intensity of the DNA-EtBr complex was recorded at 600 nm using an indirect excitation wavelength of EtBr at 260 nm (Geall & Blagbrough 2000). Quenching constants were calculated using linear Stern–Volmer equation as described (Geethanjali et al. 2015).

2.4 UV/VIS spectroscopic measurements. These were applied for the study of the compound interaction with bases (Sadeghi et al. 2016). UV-VIS spectra were recorded with a Perkin Elmer Lambda 25 UV/VIS spectrophotometer in the absence of bases and in the presence of increasing
amounts of bases in 50 mM NaCl and 5 mM Tris HCl at pH 7.4. A 25 µM solution of the tested compound was diluted out of a 1mM stock solution in the buffer in a quartz cell (2 ml). A reference cell was filled with 1 ml of the buffer. The mixture was mixed thoroughly and titrated by base solutions, 10 µM each time to both sample and reference cells. Binding constants were calculated as described (Buraka et al. 2014).

2.5 Circular dichroism spectroscopy. CD spectra were recorded on a Chirascan CS/3D spectrometer (Applied Photophysics, Surrey, UK); DNA and compound binding measurements were done in 10 mM HEPES buffer, pH 7.4 in a quartz cell of 10 mm path-length at room temperature. CD spectra of DNA were recorded in a range of 200-300nm, spectra of compound in a range of 300-420 nm and spectra of human serum albumin in a range of 200-260 nm. The parameters for all spectra were as follows: scan rate - 200 nm min⁻¹, averaging time - 0.125 s, bandwidth - 1 nm; one recorded spectrum is the average of four scans. Titration in the DNA region was carried out by adding progressively increasing amounts of AV-153-Na (10 µM at each step) to 50 µM DNA solution. Titration in the induced CD region of the compound was performed by adding DNA (62.5 µM at each step) to 500 µM AV-153-Na solution. CD spectra of HSA in the absence or in the presence of AV-153-Na salts were recorded in PBS buffer, pH 7.4. A 300 nM HSA solution was titrated with AV-153-Na (1 µM at each step).

2.6. Cyclic voltammetry. Voltammetric experiments were performed using an EcoChemie Autolab PGSTAT 302T potentiostat/galvanostat (Utrecht, The Netherlands) with the electrochemical software package Nova 2.0. A three-electrode system was used: a 2 mm-sized Pt disk working electrode, an Ag/AgCl reference electrode (3 M KCl) and a Pt wire counter electrode. Electrodes were purchased from Metrohm Co (Herisau, Switzerland). AV-153-Na solution was added to 0.1 M Tris-HCl (pH = 7.4) solution up to a final concentration 5 mM, and voltammograms were recorded. After that 10 µM of DNA was added to solution and measurements were repeated. The step was repeated at least twice. A scan rate of 100 mV/s was used throughout the experiments. All electrodes were washed with double distilled water prior to each measurement. Oxygen-free nitrogen was bubbled through the solution for 5 min before each experiment. All experiments were carried out at 25°C.

The binding constant was determined according to the following equation:

\[
\log (1/DNA) = \log (K) + \log (I_{\text{free}}/I_{\text{free}} - I_{\text{bond}}),
\]

where K – the apparent binding constant; I_{\text{free}} – the peak current of free compound; and I_{\text{bond}} – the peak current of compound in the presence of DNA (Feng et al. 1997).

The number of the binding sites was determined according to the equation:

\[
I - I_{\text{DNA}}/I_{\text{DNA}} = K [DNA]/2s
\]

where I – the peak potential of compound in the absence of DNA; A, I_{\text{DNA}} – the peak potential of...
compound in the presence of DNA; A, K – the binding constant of compound-DNA complex; [DNA] – concentration of DNA, mol/L; s – number of binding sites (Aslanoglu 2006; Carter et al. 1989).

The number of electrons (n) was calculated using equation:

\[ \text{Ep} - \frac{\text{Ep}}{2} = 47.7 \text{ mV/} \alpha n \]

Where \( \text{Ep} \) – peak potential of compound; mV; \( \frac{\text{Ep}}{2} \) – half wave potential of compound; mV, \( \alpha \) – the assuming value = 0.539; n – number of electrons (Wang et al. 2011).

2.7 **Fenton reaction – ESR measurements.** For the Fenton reaction (\( \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{•OH} + \text{OH}^- \)), 80 µl of reaction mixture containing 250 µM ferrous sulphate, 250 µM H₂O₂, 80 mM spin trap 5,5-dimethylpyrroline-N-oxide (DMPO), and 1 mM of 1,4 DHP was transferred to a micro pipettes tube for measurement of the electron spin resonance (ESR) spectra of DMPO-OH radicals. ESR spectra of the spin trap and radical complex were recorded at room temperature using an EMX-plus EPR spectrometer (Bruker, Germany). The EPR instrumental settings for field scan were as follows: field sweep – 100G; microwave frequency – 9.84 GHz; microwave power – 15.9 mW; modulation amplitude – 1 G; conversion time – 163 ms; time constant – 327 ms; sweep time – 83 s; receiver gain – 1·10⁴; resolution – 512 points for 1 scan.

2.8 **The single cell electrophoresis (comet assay).** Cells in the exponential phase of growth were washed with Dulbecco's phosphate buffer (PBS) without glucose, MgCl₂, CaCl₂. The flasks were filled with phosphate buffer (50 mM Na₂HPO₄, 90 mM NaCl, 5 mM KCl, 0.1 mM CaCl₂, 8 mM MgCl₂, 5 mM glucose, pH 7.4) and DHPs were added to the buffer (0–100 nM). Incubations lasted for 45 min at 37°C (3 h in some experiments) in a humidified atmosphere containing 5% CO₂. Cells were washed with PBS and the bolus of peroxynitrite (6 µl) was added at a final concentration of 200 µM. During the peroxynitrite treatment, the cell plate was gently swirled for 30 s. The action was repeated 2 times (total duration of exposure to peroxynitrite was 1 minute). To assess the cell protection against peroxynitrite in the presence of the studied DHPs, the compounds were added before the peroxynitrite treatment or simultaneously with it. After the peroxynitrite treatment, cells were washed in ice-cold PBS 2 times, trypsinized and processed for comet assay. To assess the impact of medium on DNA breaks, a group of vehicle control was introduced (bolus of 10 mM NaOH, 6 µl, final concentration 60 µM). The comet assay was performed as described (Ryabokon et al. 2005; Tice et al. 2000) with minor modifications (Olive & Banath 2006). HeLa cells treated or not treated with peroxynitrite in the absence or presence of DHP were detached by trypsinization, washed, resuspended in ice-cold PBS and held on ice. Fifty microliters of cell suspension containing 10 000 cells were mixed with 100 µl of 1% low melting-point agarose (Sigma-Aldrich, USA) and placed on a microscope slide that had been
pre-coated with 0.5% normal melting-point agarose. The cell membranes were lysed by keeping the slides in cold lysing solution (pH 10.0) that contained 2.5 M NaCl, 10 mM Na₂EDTA, 10 mM Tris (AppliChem, Darmstadt, Germany), 1% Triton-X 100 (Sigma-Aldrich, Taufkirchen, Germany), for at least 1 h. Subsequently, the slides were placed in a horizontal tank filled with fresh electrophoresis buffer (1 mM Na₂EDTA, 300 mM NaOH, pH 13.2) for 40 min to allow the DNA to unwind. Then, horizontal electrophoresis was carried out for 30 min at 400 mA, 16 V/cm and 4°C. After electrophoresis, the slides were washed three times for 5 min with 0.4 M Tris buffer (pH 7.5) for neutralization and then fixed in ice-cold 96% ethanol for 10 min. Slides were dried and stained with ethidium bromide and analyzed with a fluorescence microscope equipped with 515–560 nm excitation filter and 590 nm barrier filter. Cells were visually graded into 5 classes (A₀ – A₄) (Ryabokon et al. 2005) from class 0 (undamaged, no discernible tail) to class 4 (almost all DNA in the tail, insignificant head). The mean value of DNA damage (D) in arbitrary units was calculated as $D = A_1 + 2 \times A_2 + 3 \times A_3 + 4 \times A_4$, giving D values from 0 to 400 for 100 cells.

2.9 UV/VIS spectroscopic measurement of peroxynitrite decomposition. The rate of peroxynitrite (0.38 mM) decomposition in the presence or in the absence of the 1,4-DHP (0.16 mM) was followed at 302 nm (absorbance peak for the peroxynitrite anionic form) in 10 mM Tris pH 10 buffer on Perkin Elmer Lambda 25 UV/VIS spectrophotometer (Carballal, Bartesaghi, and Radi 2014). The average rate of reactions were calculated according to the formula $V = \pm \left( \frac{(C_2 - C_1)}{(t_2 - t_1)} \right) = \pm \left( \frac{\Delta C}{\Delta t} \right)$, where $C_1$ was the concentration of peroxynitrite in the beginning of reaction, and $C_2$ the concentration of peroxynitrite at the end of the reaction; $\Delta t$: 20 min.

2.10 Cell treatment and nuclear extract preparation for repair reactions. HeLa cells were incubated with 50 nM of AV-153-Na for 3, 12 or 24 hours, washed with PBS, trypsinized, suspended in PBS and pelleted by low-speed centrifugation. Cell pellets were incubated in ice for 20 min in 1.25 mL of ice-cold buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.01% Triton X-100, 0.5 mM DTT, 0.5 mM PMSF) and vortexed for 30 sec. After centrifugation for 5 min at 5000 rpm at 4°C, the nuclei were suspended in 31.25 µL of ice-cold buffer B (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 400 mM KCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT, antiproteases [Complete-mini, Roche, France] and 0.5 mM PMSF). The nuclear membrane lysis was completed by incubation for 20 min on ice, followed by two cycles of freezing-thawing at -80°C and 4°C respectively. Debris was eliminated by centrifugation for 10 min at 13000 rpm at 4°C. The supernatant was stored frozen in 10 µl aliquots at -80°C. Protein content was determined using the BCA kit (Interchim, Montluçon, France). Typical protein content was 0.8 mg/mL.
2.11 Assays of the activity of DNA repair enzymes. The impact of the tested compounds on activity of glycosylases/AP endonucleases belonging to Base Excision Repair and on Excision/Synthesis Repair activities was performed using Glyco-SPOT (Pons et al. 2010) and ExSy-SPOT assays (Forestier et al. 2012), respectively (LXRepair, Grenoble, France). These assays allowed quantifying different DNA repair activities from extracts prepared from treated and non-treated cells.

The former chip, which is a multiplex, on-support, oligonucleotide (ODN) cleavage assay, reveals excision activities against 8-oxoguanine paired with C (8-oxoG-C), A paired with 8oxoguanine (A-8oxoG), ethenoadenine (EthA-T), thymine glycol (Tg-A), uracil (paired either with G or A (U-G and U-A, respectively)), hypoxanthine (Hx-T), and abasic sites (THF-A). Cleavage of the lesions by the enzymes contained in the extracts released the fluorescence attached to the lesion containing ODNs.

Repair reactions were conducted for 1h at 37°C with 15 µg/mL of protein in 80 µL of excision buffer (10 mM Hepes/KOH pH 7.8, 80 mM KCl, 1 mM EGTA, 0.1 mM ZnCl₂, 1 mM DTT, 0.5 mg/mL BSA). After 3 washes, 5 min at room temperature in PBS containing 0.2 M NaCl and 0.1% Tween 20, the spots fluorescence was quantified using the Innoscan scanner from Innopsys (Toulouse, France). Each extract was run in duplicate. The results between the replicates (4 spot fluorescence) were normalized using the NormalizeIt software as described by Millau et al. (2008).

Wells incubated with the excision buffer served as reference (100% fluorescence) to calculate the lesions percentage of cleavage in the wells incubated with the extracts. Non-specific cleavage of the control ODN (Lesion_Free ODN) was also taken into account to calculate the percentage of excision of each lesion using the following formula: (100 x (1-percentage of fluorescence of Lesion_ODN/percentage of fluorescence of Lesion_Free ODN)).

The ExSy-SPOT assay quantified Excision/Synthesis Repair of 8-oxoguanine (8oxoG), alkylated bases (AlkB) and abasic sites (AbaS), incorporated into different supercoiled plasmid DNA. The principle of the methods is described by Millau et al. (2008). Extracts (0.1 mg/mL) incubated on the biochip where the different plasmid preparations were immobilized at specific sites for 3h at 30°C in reaction buffer (40 mM Hepes KOH pH 7.8, 7 mM MgCl₂, 0.5 mM DTT, 0.25 µM dATP, 0.25 µM dTTP, 0.25 µM dGTP, 3.4 % glycerol, 12.5 mM phosphocreatine [Sigma, Taufkirche, Germany], 2 mM EDTA, 50 µg/mL creatine phosphokinase, 0.1 mg/mL BSA) containing 1 mM ATP [Amersham, England] and 1.25 µM dCTP-Cy3. After washing for 3x5 min in H₂O (MilliQ), the total fluorescence intensity of each spot was quantified using the Innoscan scanner from Innopsys (Toulouse, France). Each extract was run in duplicate and data were normalized using the NormalizeIt software as described by Millau et al. (2008). Results
were expressed as Fluorescence Intensity (FI).

2.12 Laser confocal scanning microscopy. For imaging, the HeLa cells were seeded in 4 well Nunc Lab-Tek Permanox Chamber slide (Thermo Scientific Nunc, Pittsburg, USA) and cultivated for 24 h as described above. Subsequently, the cells were washed twice with PBS for 5 min and then incubated with 1 mM AV-153-Na in PBS for 16 h in a CO₂ incubator at 37°C and 5% CO₂. After incubation, the cells were washed with PBS for 5 min and fixed in 70% ethanol for 0.5 h at room temperature. Slides were rinsed with PBS for 5 min and counterstain chromatin was dyed with 15 µM propidium iodide (PI) in PBS for 0.5 h, then washed twice with PBS for 5 min. Slides were analyzed using a Leica DM RA-2 microscope equipment with a TCS-SL confocal scanning head (Leica Microsystems, Bannockburn, USA). Images were collected with a Leica 40 X HCX PL Fluator objective (NA = 0.75) and 100x HCX PIAP0 oil immersion objective (NA = 1.40). AV-153-Na and propidium iodide were excited with a 488 nm band from a four-line argon ion laser. AV-153-Na fluorescence was detected between 510 and 560 nm, propidium iodide fluorescence was detected between 600 and 650 nm. Cell shapes were controlled with reflected light 475-505 nm. Cells were scanned along the Z-axis with a step size – 0.5 µm.

2.13 Statistical analysis. The values of DNA damage assayed by single gel electrophoresis are represented as the mean ± standard error of the mean (SEM). The data were subjected to the one-way analysis of variance (ANOVA), followed by the Tukey multiple comparisons test and the data were considered as significant at p < 0.05.
3. **Results**

3.1 *Fluorescence titration.* Fluorescence spectra of AV-153-Na with excitation at 350 nm and emission at 480 nm are given in Figure 1. Titration of the solutions with DNA increased the intensity of the fluorescence, and the Scatchard binding constant was equal to $7.4 \times 10^4$.

3.2 *Cyclic voltammetry.* Cyclic voltammograms of 5 mM AV-153-Na in the absence and presence of various concentrations of DNA in 0.1 M Tris-HCl buffer, pH = 7.4, are shown in Figure 2. The peak current increases upon the addition of increasing concentrations of DNA, due to the binding of the 1,4-DHP. The compound exhibited a single well-defined anodic peak, which corresponds to the oxidation of dihydropyridine ring (Augustyniak et al. 2010). In reverse scan, no peak was observed, indicating that oxidation of the compound is an irreversible process. The peak potential ($I_p$) of the oxidation wave AV-153-Na was proportional to the square root of the scan ($v^{1/2}$). The binding constant calculated on the basis of voltammetric measurements was equal to $5.01 \times 10^4$, number of electrons $-0.97$, and number of binding sites $-4.5$.

3.3 *Fluorescent intercalator displacement assay.* To assess the mode of DNA interactions with 1,4-DHP manifesting the highest affinity to DNA in previous series of measurements, we performed a fluorescent intercalator displacement assay. In this assay, the enhanced fluorescence of the DNA-EtBr complex is quenched by the addition of a second ligand, which is either an intercalator or a groove binder (Ghosh et al. 2010). As presented in Figure 3, AV-153-Na quenched the EtBr fluorescence up to 77%, evidently compounds competed with EtBr for intercalation sites in DNA. Stern-Volmer quenching constant was equal to $1.3 \times 10^5$. (Figure 3)

3.4. **Circular dichroism.** Circular dichroism spectra of ct-DNA in the presence of increasing concentrations of AV-153-Na are shown in Figure 4. DNA manifests a negative band at 245 nm due to helicity and a positive band at 270 nm because of base stacking, which is characteristic of the B form of DNA. Adding AV-153-Na to DNA increases the negative band intensity and decreases the positive band intensity. A 2 nm red shift of crossover point is also observed. These data clearly indicate interactions of the compound with DNA although changes in spectra are not typical for any binding mode. In an induced circular dichroism experiment, when measurements were done in the 1,4-DHP absorbance area, with fixed concentrations of the drug and increasing concentrations of DNA, a negative band with maximum at 340 nm was observed. Its intensity increased with each portion of added DNA, and a red shift of maximum was also observed. (Figure 4B). The increase of negative ICD signal in the region of compound absorbance spectra after DNA addition usually points to an intercalative-binding mode. (Garbett et al. 2007).

3.5 *UV/VIS spectroscopy, titration with bases.* The above results indicate that the binding between AV-153-Na and DNA occurs via intercalation. The influences of DNA bases, C, G, A and T on the UV/VIS absorption spectra of AV-153 were used to evaluate possible base-
specificity of binding. Data are presented in Figure 5 and Table 1. The absorption intensity was gradually increased with the increase of the concentration of all the four bases. Affinity to G, C and T is greater than that to A. The results indicate that AV-153-Na can interact with the four types of bases, with somewhat different affinities. In order to evaluate the role of ionic and hydrogen bonds in AV-153-Na interactions with bases, titration was performed in solutions of 1M NaCl and 8M urea. In these, media affinity of AV-153-Na to bases was weakened, especially for G; the shape of spectra was also changed. However, interactions were not abolished.

3.6 Intracellular localization of AV-153-Na and protein binding. As the capability of AV-153-Na to bind DNA in vivo was proven, we needed evidence whether the compound could reach cell nucleus in the cells where it could interact with DNA. We have tried to answer this question by profiting of the intrinsic fluorescence of the compound with the aid of laser confocal scanning microscopy. Results are presented in Figure 6, left panel. AV-153-Na heavily stains the cytoplasm; however, some fluorescence is visible also in the nucleus, mainly in the nucleolus. Staining of cytoplasmic structures raised the question about capability of the compound to bind proteins. Fluorescence titration of AV-153-Na with human serum albumin gave a positive answer to the question - fluorescence of the compound increased in the presence of HSA. Results of CD confirmed the ability to bind a protein (Figure 6, left panel A and B). The addition of the compound to the HSA solution decreased ICD signals in both minimal bands (208 and 222 nm) in the presence of AV-153-Na, which suggests binding with HSA, causing protein conformational changes due to a slight protein unfolding. (Wang et al. 2008).

3.7 Decomposition of peroxynitrite in the presence of the AV-153-Na. Published data indicate the ability of some 1,4-DHP to scavenge peroxynitrite chemically (Lopez-Alarcon et al. 2004). We also tested the ability of the AV-153-Na salts to degrade peroxynitrite chemically by studying the kinetics of decomposition of peroxynitrite in the presence of DHP followed by means of spectrophotometry.

The curves are presented in Figure 7. The average rate of decomposition of peroxynitrite at concentration 0.38 mM was $0.0157 \mu\text{mol}/\mu\text{l} \cdot \text{min}$. AV-153-Na did not affect the time of decomposition of peroxynitrite, AV-153-Na; it remained $0.0157 \mu\text{mol}/\mu\text{l} \cdot \text{min}$.

3.8 Radical scavenging - ESR measurements. The ability of the AV-153-Na and one other 1,4-DHP to scavenge free radicals, namely OH radical produced in Fenton reaction, was tested by the ESR method. We have tested AV-153-Na compared to a weak DNA binder AV-154-Na at 1000 μM concentration. The signals of the second component of the EPR spectra were measured on the 3rd min ($I_3$) and the 5th min ($I_5$) and the difference between $I_3$ and $I_5$ was calculated (Figure 8 A). Scavengers of OH radicals should increase the difference between $I_3$ and $I_5$. Representative kinetics of the decrease of EPR signal intensity is shown in Figure 8 B. AV-154
does not interfere with the rate of the reaction, and the impact of AV-153-Na is minimal. Similar results were obtained for other AV-153 salts. Thus, correlation between radical scavenging and DNA-binding capacities was not observed.

3.9 Protection of living cells against peroxynitrite-induced damage. The DNA-protecting action of AV-153 salts against peroxynitrite-induced damage was tested in living cells. Results of the comet assay experiments performed on HeLa cell treated with peroxynitrite alone or in the presence of AV-153-Na are presented in Figure 9. Treatment with peroxynitrite drastically increased the levels of DNA damage. AV-153-Na reduced the extent of DNA damage produced by peroxynitrite. Pre-incubation with the compound at concentrations 50 nM for 45 min appeared to produce significant effects (Figure 9A). When administered simultaneously with peroxynitrite, the compound produced a much weaker protective effect. The reference compound AV-154-Na, which does not bind DNA did not protect it against DNA damage either (Figure 9B).

3.10 Effects of AV-153-Na on the activity of DNA repair enzymes. These were tested using Glyco-SPOT and ExSy-SPOT assays. Longer pre-incubation times were chosen to reveal possible changes in protein expression.

The Glyco-SPOT assay revealed a specific and significant decrease of Tg (thymine glycol) repair by AV-153-Na, which manifested itself when an extract with a higher concentration of protein (15 µg/ml) was used in the assay, and a trend for inhibition of enzymes involved in U-G and U-A repair (Figure 10). Other glycosylases/AP endonucleases activities were not affected.

Results with ExSy-SPOT assay appear to be more interesting in this sense. AV-153-Na stimulates the excision/synthesis repair of lesions repaired by Base Excision Repair (8-oxoG, abasic sites and alkylated bases [Figure 11]). As this stimulating effect is not detected with the Glyco-SPOT assay, it involves either the synthesis step of the repair process or alternative repair pathways able to handle oxidative lesions.

4. Discussion

In the present study, we have reproduced formerly obtained data about the ability of AV-153-Na to interact with DNA; the effect was reproduced using DNA from a different source as well as different methodical approaches (Buraka et al. 2014). Former results on rat liver and plasmid DNA obtained by means of UV/VIS and infrared spectroscopy were reproduced by means of novel assays using ct-DNA. The fluorescence titration confirmed the data obtained formerly by UV/VIS and infrared spectroscopy (Buraka et al. 2014), indicating the fact of the direct interaction between the compound and DNA. A similar increase in the fluorescence of a compound after binding to DNA was reported for numerous compounds (Jana et al. 2012;
Shamsuzzaman et al. 2013), indicating a decrease of the fluorescence-quenching effect of solvent molecules after penetration of the molecule in a hydrophobic environment (Shamsuzzaman et al. 2013). Similarly, in cyclic voltammetry experiments, the shift in the peak potential indicated intercalation of the compound to DNA double-helix (Sirajuddin et al. 2013). The increase in the peak current in the presence of DNA is due to an increase of the apparent diffusion coefficient according to Randles-Sevcik equation (Shah et al. 2010). The ability of the AV-153 salts to intercalate DNA molecule was again confirmed by EtBr displacement assay, using 260 nm excitation light this time, and circular dichroism spectroscopy. The presence of a negative induced circular dichroism band increasing with every added DNA portion with a red shift again indicates an intercalative binding mode (Garbett et al. 2007; Thimmaiah et al. 2015). The intercalating activity of AV-153-Na, which is considered to be mutagenic, might seem to be in contradiction with its reported antimutagenic and DNA-protecting activities (Goncharova et al. 2001; Ryabokon et al. 2009a; Ryabokon et al. 2008; Ryabokon et al. 2005; Ryabokon et al. 2009b). However, analysis of literature data reveals the coexistence of DNA-binding activity with antimutagenic effects. Natural polyphenols provide a good illustration of this statement: many of them effectively bind DNA through intercalation; however, most of these compounds are considered to be antimutagenic. The latter activity is attributed to antioxidant properties of this class of compounds (Janjua et al. 2009; Zhang et al. 2011). It appears that the DNA-damaging and DNA-protecting activities cohabitate in the molecules of flavonoids. It seems that 1,4-DHP molecules can also unite potentially different activities. 1,4-DHP are able to scavenge different reactive oxygen and nitrogen species themselves; the reactions can be observed in vitro (Pacheco et al. 2013; Vijesh et al. 2011). In order to test if the antimutagenic effects of the AV-153-Na are due to its capability to scavenge free radicals and peroxynitrite, we have studied these effects using in vitro systems. Unexpectedly, it turned out that the compound does not react with peroxynitrite, and ability to scavenge hydroxyl radical turned out to be modest. However, data of comet assay when the AV-153-Na was tested for ability to modify level of DNA breakage in HeLa cells exposed to peroxynitrite are much more convincing. Pre-incubation with AV-153-Na significantly decreased the DNA damage. Perhaps a higher efficiency of low concentrations of AV-153-Na reflects a shift of the equilibrium between DNA damage being a consequence of intercalation and DNA protection towards DNA protection. The necessity for pre-incubation and lower efficiency of simultaneous administration with peroxynitrite indicates that AV-153-Na induces some changes in the cells favouring protection of DNA or DNA repair, as the compound does not interact directly with the peroxynitrite. Moreover, the good DNA binder AV-153-Na was an effective DNA protecting agent, while AV-154-Na, which does not interact with DNA at all, did not protect it against peroxinitrite. It seems that data on the impact
of AV-153-Na on the activity of the excision repair enzymes makes understanding of the mechanism of action of the compound possible. AV-153-Na activates enzymes involved in the excision repair pathway. The observed decrease in Tg removal produced by AV 153-Na apparently contradicts data about DNA-protecting effects of the compound. However, it should be taken into account that in mammals two bifunctional glycosylases, NTH1 and NEIL1, show overlapping activities aimed on the removal of Tg (Sampath 2014). Our data do not permit us to determine which enzyme was inhibited. Although this finding cannot explain the DNA-protecting effects of AV-153-Na, it appears to be interesting.

We also report evidence of possible binding of the AV-153-Na to cell nucleus. The study further reveals binding to cytoplasmic structures and a high affinity to proteins. It might happen that cytoplasmic proteins retain the main part of AV-153-Na molecules after exposure of the cells to the compound; only a small part of the molecules reaches DNA, where these activate DNA repair systems but do not produce harmful effects due to a very low local concentration in the nucleus.

Summarizing the data, it can be proposed that binding of the compound to DNA is identified by DNA repair systems as DNA lesions, and activity of DNA repair systems is increased. It seems that AV-153-Na per se does not induce mutations; however, it triggers the the activity of DNA repair enzymes, thus making cells less vulnerable by other mutagens.

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Fig 1. Fluorescence titration of AV-153-Na with ct-DNA. Structure of the compound is given on the right. Six spectra out of 18 obtained are shown.
Fig. 2 Cyclic voltammograms of 5 mM AV-153-Na in 0.1 M Tris-HCl buffer (pH 7.4) without DNA (a) and in the presence of 10 μM (b) and 20 μM of DNA.
Fig. 3 Spectrofluorimetric ethidium bromide extrusion assay. A - changes in spectra of EtBr-DNA complex, six spectra out of 25 obtained are shown. B - Stern-Volmer plot

Fig 4. Circular dichroism experiments. A - Circular dichroism spectra of ct- DNA in absence and presence of AV-153-Na. AV-153-Na concentration was increased by 10µM at each step up to 40µM. DNA concentration was 50 µM. Measurements were performed in 10 mM HEPES buffer. B – Induced circular dichroism spectra of AV-153-Na CD spectra (500 µM) in presence of 62.5 µM(a), 125 µM (b); 250 µM (c) and 500 µM of DNA. Measurements were performed in HEPES buffer.
Fig 5. AV-153-Na absorption spectra in absence and presence of bases in different solutions. Concentrations of bases were increased for 10 μM with each titration. A – Adenine, B – Cytosine, C, E, F – Guanine, D – Thymine. A – D – in 5 mM Tris HCl, pH 7.4, 50 mM NaCl; E – 1 M NaCl; F – 8 M urea.
Fig 6. Right panel - images of HeLa cells obtained by laser scanning confocal microscopy. Cells were treated with AV-153-Na, DNA was stained with propidium iodide (PI). Light blue – reflected light; green - distribution of AV-153-Na, red – propidium iodide. The overlay image of all channels is also shown. Pictures of the optical section were taken 3 µm from the cell surface. All the scale bars are in 7.5 µm size. Left panel A - Fluorescence titration of AV-153-Na with human serum albumin (5 µM each time). B - Circular dichroism spectra of HSA in absence and presence of AV-153-Na. AV-153-Na concentration was increased by 1µM at each step up to 12µM. HSA concentration was 300 nM.
Figure 7. Decomposition of peroxynitrite (0.38 mM) in the presence of AV-153-Na added up to 0.16 mM at pH 10. The 1,4-DHP was added also to the control cuvette.

Fig 8 A EPR spectra of DMPO-OH radicals generated in Fenton reaction in presence
of DMPO. 1 - EPR spectra of DMPO-OH radicals 3 min after mixing the components for Fenton reaction. 2 - EPR spectra of DMPO-OH radicals 5 min after mixing the components for Fenton reaction. I₃ and I₅ - intensities of EPR signals used for quantification of DMPO-OH radicals in corresponding time. 3 – difference between 3 min and 5 min spectra indicating decrease of the signal intensity and lack of generation of other radicals. B – time course of decrease of intensity of DMPO-OH radical spectra. 1 – control mixture; 2 – in presence of AV-154-Na; 3 – in presence of AV-153-Na. Chemical structure of AV-154-Na is given in insertion.
Fig 9 Effects of AV-153-Na and AV-154-Na against peroxynitrite caused DNA damage in HeLa cell line tested by comet assay. A – AV-153-Na. 1 – control (intact cells); 2 – vehicle control (60 µM of NaOH); 3 – peroxynitrite (200 µM); 4 – incubation with the tested 1,4-DHP (100 nm, 45 min), 5-pre-incubation with 10 nm of 1,4-DHP (45 min) and treatment with peroxynitrite; 6 - pre-incubation with 50 nm of 1,4-DHP (45 min) and treatment with peroxynitrite; 7 - pre-incubation with 100 nm of 1,4-DHP (45 min) and treatment with peroxynitrite; 8 – simultaneous treatment with 1,4-DHP (10 nm) and peroxynitrite; 9 - simultaneous treatment with 1,4-DHP (50 nm) and peroxynitrite; 10 - simultaneous treatment with 1,4-DHP (100 nm) and peroxynitrite; B – AV-153-Na. 1 – control (intact cells); 2 – incubation with 50 nm of AV-153-Na for 3 h; 3 – vehicle control (60 µM of NaOH; 3 hours); 3 – peroxynitrite (200 µM); 5-pre-incubation with 50 nm of AV-153-Na (3 hours) and treatment with peroxynitrite; C – AV-154-Na, all designations are as in A.

*** - p<0.001 versus peroxynitrite group, ns – not significant.
Fig. 10. Effect of AV-153-Na on cellular Base Excision Repair activities (GlycoSPOT assay). The test was run with 15 µg/ml of extract prepared from non-treated cells (Control) and cells treated for 3h, 12h and 24h with AV-153-Na as described in Materials and Methods. Results are expressed as cleavage rate for each lesion. U-G and U-A : uracil paired either with G or with A; A-8oxoG: A paired with 8oxoguanine; 8oxoG-C: 8-oxoguanine paired with C; EthA-T ethenoadenine paired with T; Tg-A : thymine glycol paired with A; Hx-T : hypoxanthine paired with T; THF-A : abasic sites analogue paired with A. *p<0.05, *** p<0.001 versus Tg-A control.
**Excision/Synthesis Repair - Mean of the 3 Series**

Fluorescence Intensity (A.U.)

- **Control**
- **AV-153 3h**
- **AV-153 12h**
- **AV-153 24h**

**Treated / Non Treated Signals - Mean of the 3 Series**

- **AV-153 3h**
- **AV-153 12h**
- **AV-153 24h**
Fig. 11. Effect of AV-153-Na on cellular Excision/Synthesis Repair (ExSy-SPOT assay) of major base lesions. The repair reaction was conducted with nuclear extracts prepared from non-treated cells and cells treated for 3h, 12h and 24h with AV-153-Na (see Materials and Methods). For each lesion, we calculated the ratio of the fluorescence intensity obtained with the treated cells over the fluorescence intensity obtained with the control cells. The values > 1 reflect an induction of the Excision/Synthesis Repair activities. **p<0.01 versus 8oxoG control; ####p<0.0001 versus AbaS control; $$p <0.01 versus AlkB control.
Table 1

Affinity of bases to AV-153-Na

<table>
<thead>
<tr>
<th>Base</th>
<th>Binding constants of the AV-153 salts in different media</th>
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<tbody>
<tr>
<td></td>
<td>AV-153-Na, 5 mM Tris-HCl, 50 mM NaCl</td>
</tr>
<tr>
<td>Adenine</td>
<td>2.6 x 10^3</td>
</tr>
<tr>
<td>Cytosine</td>
<td>3.6 x 10^3</td>
</tr>
<tr>
<td>Guanine</td>
<td>8.8 x 10^3</td>
</tr>
<tr>
<td>Thymine</td>
<td>3.5 x 10^3</td>
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