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Original scientific paper

Interactions of cytotoxic amino acid derivatives of *tert*-butylquinone with DNA and lysozyme

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Abstract: The interactions of nine amino acid derivatives of *tert*-butylquinone with biomacromolecules were studied. Sodium dodecyl sulphate (SDS) gel electrophoresis and mass spectrometry confirmed the absence of modifications of lysozyme by any of the synthesized compounds. Spectrophotometric studies demonstrated hyperchromism, *i.e.*, the existence of interactions between the quinones and calf thymus DNA (CT-DNA). Determination of the binding constants by absorption titration indicated weak interactions between the quinone derivatives and CT-DNA. The quenching of fluorescence of the intercalator ethidium bromide (EB) from the EB–CT-DNA system and of the minor groove binder Hoechst 33258 (H) from the H–CT-DNA system by the synthesized derivatives indicated interactions of the compounds and CT-DNA. Circular dichroism (CD) spectra demonstrated a non-intercalative binding mode of the quinone derivatives to CT-DNA. Molecular docking results confirmed binding to the minor groove. The electrophoretic pattern showed no cleavage of the pUC19 plasmid in the presence of any of the synthesized compounds. The ability of the derivatives to scavenge radicals was confirmed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) test. All the presented results suggest that the DNA minor groove binding is the principal mechanism of action of the examined amino acid derivatives.

Keywords: quinone; DNA binding; minor groove; fluorescence.

INTRODUCTION

Understanding the mechanism of interactions of various anticancer drugs with biomacromolecules is a crucial aspect of drug discovery. Investigation of

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the interactions of pharmacologically active quinones with biomacromolecules is a challenging task, because of their complex mechanism of action. Quinones are highly reactive compounds and the general mechanism of their action involves three processes: first, the redox couple quinone/hydroquinone can generate reactive oxygen species that damage biomacromolecules and inhibit electron transport in mitochondria and oxidative phosphorylation; second, quinones can be alkylated by cellular nucleophiles, such as proteins, enzymes and DNA molecules; third, compounds with a quinone moiety can non-covalently interact with DNA molecules.[1–7] Most of the clinically useful quinone alkylating antitumor agents are active only after bioreductive activation. The term bioreductive activation includes a series of mechanisms of reduction of anticancer agents to reactive intermediates that can later undergo nucleophilic addition.[8–11] In this way, some degree of selectivity is attained.

In a previous study, besides very active avarone derivatives with amino acids, nine amino acid derivatives of the avarone mimic *tert*-butylquinone were synthesized.[12] For the derivatisation, amino acids with non-polar aliphatic and aromatic residues including one D-amino acid, were selected. The derivatives showed moderate cytotoxic activity and a good selectivity on a panel of cancer cell lines. In this paper, the mode of action of the synthesized derivatives at the molecular level on linear and circular DNA, and a model protein were investigated.

EXPERIMENTAL

Materials and methods

Lysozyme from hen egg white was obtained from Sigma–Aldrich, USA. An Eppendorf Mini Spin Plus centrifuge was used. Sodium dodecyl sulphate–polyacrylamide (SDS–PA) gel electrophoresis was performed using a Hoefer SE 600 Ruby vertical electrophoresis system. Mass spectra were recorded on a linear ion trap – Orbitrap hybrid mass spectrometer (LTQ OrbiTrap XL) with a heated electrospray ionization probe, HESI (Thermo Fisher Scientific, Bremen, Germany). UV–Vis spectra were collected on a Cintra 40 UV/Visible spectrometer. Fluorescence spectra were recorded using a Lumina fluorescence spectrometer (Thermo Fisher Scientific, Finland) equipped with a 150 W xenon lamp. The splits on the excitation and emission beams were fixed at 10 nm. Calf thymus DNA (CT-DNA) was purchased from Serva, Heidelberg. Agarose was obtained from Amersham Pharmacia-Biotech, Inc, USA. A HE 33 mini submarine electrophoresis unit (Hoefer, USA) with an EPS 300 power supply was used for the agarose gel electrophoresis. The stained gel was illuminated under a UV transilluminator (Vilber Lourmat, France) at 312 nm and photographed with a Nikon Coolpix P340 digital camera through a Deep Yellow 15 filter, Tiffen, USA. The plasmid pUC19 was purchased from Sigma–Aldrich, USA. A Thermo Scientific Appliskan (Thermo Fisher Scientific, Finland) was used for measuring absorbance at 517 nm for determining the DPPH scavenging activity.

Details related to mass spectrometry, UV–Vis spectroscopic and fluorescence measurements are given in Supplementary material to this paper.

Preparation of the quinones

The amino acid derivatives of *tert*-butylquinone were synthesized as described previously.[12]

Modification of lysozyme

Modifications of lysozyme were performed in 20 % dimethyl sulfoxide (DMSO), 20 % methanol and distilled water, respectively. The quinones were dissolved in 240 μL of DMSO, methanol and water, respectively (final concentration 2 mg mL^{-1}) and lysozyme was dissolved in 960 μL of water (final concentration 5 mg mL^{-1}). The reaction mixtures contained 50 mM $NaHCO_3$ and the pH value of the mixture was 8.5. The reaction was performed at r.t. with stirring for 48 h. During the reaction, the formation of a precipitate was observed when DMSO and methanol were used. The precipitate did not contain free quinones. The mixture was centrifuged at 14000 rpm and the supernatant was desalted by dialysis against distilled water. Concerning the precipitate, three volumes of water were added and after centrifugation, the resulting solution was dialysed against distilled water.

Modifications of lysozyme were also performed by the same procedure without $NaHCO_3$, in aqueous buffer solutions (100 mM phosphate buffer pH 7.4, 100 mM acetate buffer pH 4.5 or 100 mM ammonia buffer pH 10). Under these conditions, no precipitate was formed.

The formation of a possible conjugate was checked using SDS electrophoresis and mass spectrometry.

SDS PA gel electrophoresis

SDS polyacrylamide gels were made by a standard procedure.[13] The concentration of the running gel was 10 % and of the stacking gel, 4 %. Each sample (25 μL) was applied to the gel. The voltage was held constant (80 V) until the samples entered the running gel. For separation through the running gel, the voltage was raised to 150 V.

DNA binding experiments

All buffer solutions were prepared in deionised water and filtered through 0.2 μm filters, Nalgene, USA. Stock solutions of the tested compounds were prepared in DMSO at a concentration of 10 mM. Calf thymus DNA (CT-DNA, lyophilized, highly polymerized, purchased from Serva, Heidelberg, Germany) was dissolved in Tris buffer (20 mM Tris–HCl, pH 7.5) overnight at 4 °C. This solution was stored at the same temperature and was stable for several days. The ratio of the UV absorbance at 260 and 280 nm (A_{260}/A_{280} of 1.94) indicated that the aqueous solution of CT-DNA was sufficiently free of protein. The concentration of CT-DNA (3.12 mg mL^{-1}) was determined from the UV absorbance at 260 nm using an extinction coefficient ε_{260} = 6600 M^{-1} cm^{-1}.[14]

CD studies

The CD spectra of CT-DNA (0.5 mM) alone and with two concentrations of the derivatives (0.25 and 0.50 mM) were recorded using a Jasco J-815 circular dichroism spectrometer. The temperature of the sample was kept constant at 25 °C. All CD spectra were recorded in the range from 220 to 310 nm at a scan speed of 200 nm min^{-1} with a spectral bandwidth of 0.1 nm. The average of three scans was taken in all the experiments. The background spectrum of the bicarbonate solution (40 mM, pH 8.4) was subtracted from the spectra of DNA and DNA–derivative complexes.

DNA cleavage experiments

The plasmid pUC19 (2686 bp) used for the DNA cleavage experiments was prepared by its transformation in chemically competent cells of *Escherichia coli* strain XL1 blue. Amplification of the clone was realised according to the protocol for growing *E. coli* culture overnight in LB medium at 37 °C.[15] Purification was performed using Qiagen Plasmid plus Maxi kit. DNA was eluted in 10 mM Tris–HCl buffer (pH 7.9) and stored at –20 °C. The concentration of plasmid DNA (460 ng μL^{-1}) was determined by measuring the absorbance of the DNA-containing solution at 260 nm. One optical unit corresponds to 50 μg mL^{-1} of double-stranded DNA.

The cleavage reaction of supercoiled pUC19 by the different compounds (final concentration 1 mM) was investigated by incubation of 460 ng of plasmid in a 20 μL reaction mixture in 40 mM bicarbonate solution (pH 8.4) at 37 °C for 90 min. The reaction mixture was vortexed from time to time. The reaction was terminated by short centrifugation at 10000 rpm and the addition of 5 μL of loading buffer consisting of 0.25 % bromophenol blue, 0.25 % xylene cyanol FF and 30 % glycerol in TAE buffer (40 mM Tris–acetate, 1 mM EDTA, pH 8.24).

The samples (20 μL) were analysed by electrophoresis on 1 % agarose gel prepared in TAE buffer, pH 8.24. The electrophoresis was performed at a constant voltage (80 V) until the bromophenol blue had passed through 75 % of the gel. After electrophoresis, the gel was stained for 30 min by soaking it in an aqueous ethidium bromide solution (0.5 μg mL^{-1}).

Molecular docking

For DNA docking simulations, the crystal structure of dodecamer $d(CGCGAATTCGCG)_2$, PDB ID: 3U2N,[16] was obtained from the Protein Data Bank (http://www.rcsb.org/pdb). All water molecules and ions, as well as ligands were removed using Autodock Tools 1.5.6.[17,18]

Quinone molecules were prepared using Maestro 10.4 from the Schrodinger Suite 2015-4.[19] All possible tautomer structures at pH 7.00±2.00 were generated using Epik 3.4 from the Schrodinger Suite 2015-4[20] and their charges were determined in Jaguar 9.0[21] using the HF/6-31g* method. These charges were used further in docking simulations. The docking simulations were realised in Autodock Vina 1.1.2.[22] The grid box size was set to 25×28×45 $Å^3$ and exhaustiveness to 500. Discovery Studio Visualizer 4.5 was used for visualization of the interactions.[23]

DPPH radical scavenging activity

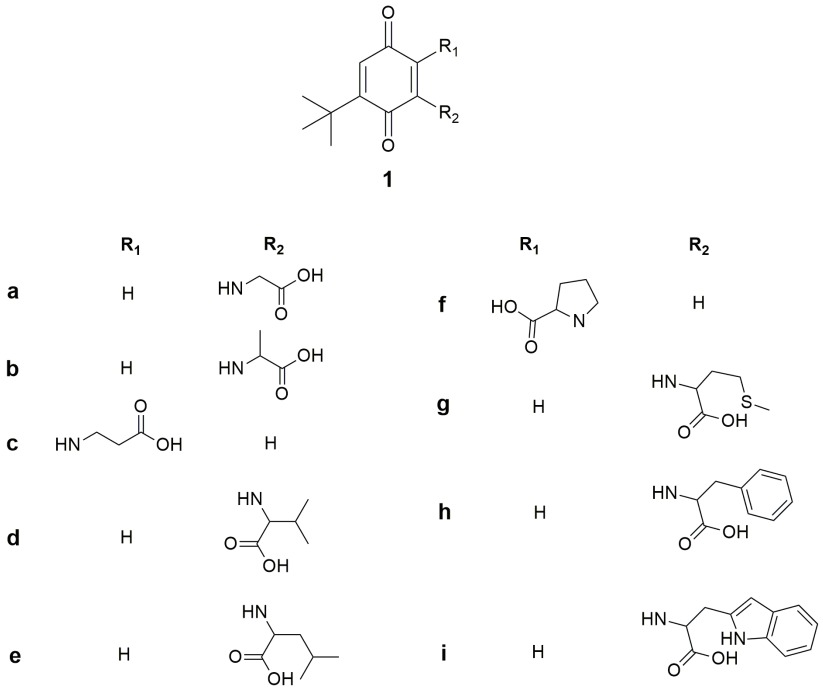
Details of the DPPH radical scavenging activity test are given in the Supplementary material.

RESULTS AND DISCUSSION

The amino acid derivatives of *tert*-butylquinone (Scheme 1) were obtained by nucleophilic addition of an amino acid to the quinone, as previously described.[12]

For investigation of the interactions of quinones with proteins, lysozyme was chosen in a previous study as the enzyme model, since the amino groups of lysine in lysozyme were shown to react by Michael addition with the quinone moiety of similar quinones, including alkylamino derivatives.[24] The modification reaction was monitored by SDS electrophoresis and mass spectrometry. The

results on the SDS electrophoregram (Fig. 1) showed that the synthesized amino acid derivatives did not modify lysozyme. Modification of lysozyme was not observed under any of the applied conditions.



Scheme 1. Structures of the investigated compounds.

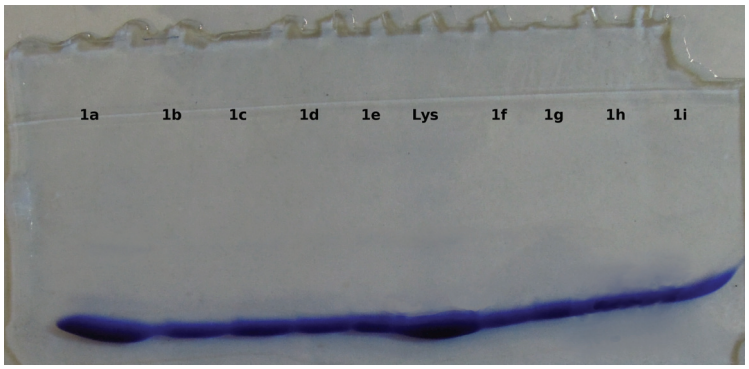


Fig. 1. SDS–PAGE of lysozyme (Lys) and modifications of Lys with derivatives **1a**–**i** in 20 % DMSO.

In the mass spectra obtained for both the supernatants and precipitates only the peak of unmodified lysozyme at m/z = 14305 (*e.g.* 1431.499 for z = 10) was

observed. The obtained results showed that synthesized derivatives of *tert*-butylquinone did not modify lysozyme (Fig. S-1 of the Supplementary material to this paper). Explanation for this behaviour is the steric bulk of *tert*-butylquinone, as well as the rapid establishment of tautomeric equilibrium, and consequently, the absence of the stable quinone structure[12] that is necessary for attack of protein nucleophiles.[24–26] It could be concluded that these compounds cannot be alkylated by the nucleophilic groups of the model protein.

DNA is one of the principal targets of many different classes of drugs that are in clinical use, from anticancer and antiviral drugs to antibiotics. There are two main types of interactions between DNA and low molecular weight molecules, covalent and non-covalent interactions. Due to the rapid interconversion of the tautomeric forms and the assumed difficulty of formation of covalent bonds between the derivatives and biomacromolecules, as evidenced with lysozyme, which contains several highly nucleophilic lysine residues, the focus of this work was on the three most important non-covalent modes of drug–DNA binding: intercalation, groove binding and external binding.

UV–Visible absorption spectroscopy is one of the simplest, most common techniques for studying the stability of DNA and its interactions with small molecules. When a small molecule interacts with DNA, a complex is formed, with changes in the absorbance and/or the positions of peaks.

Representative UV absorption spectra of derivative **1h** at different concentrations, both in the absence and presence of a fixed concentration of CT-DNA, are shown in Fig. 2.

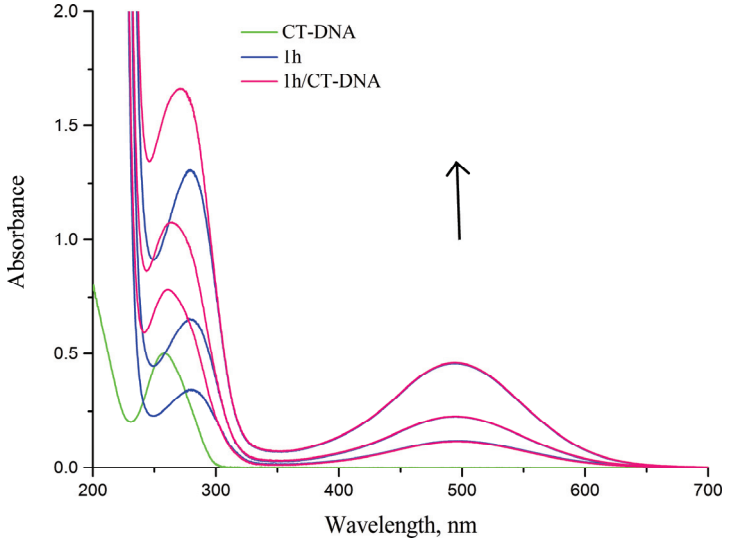


Fig. 2. UV–Vis absorption spectra of different concentrations (50, 100 and 200 μM, from bottom to top) of **1h** before and after interaction with CT-DNA.

In the absence of DNA, the absorption spectra of the derivatives showed absorbance maxima in the range of 276–283 nm. Hyperchromism was observed upon addition of DNA. These results indicated an interaction between the derivatives and DNA molecules. In comparison with the spectrum of CT-DNA, in all absorption spectra, (the exception is the proline derivative **1f**), a bathochromic shift was noted. As small red shifts were followed by hyperchromism with all derivatives (Table I), it could be concluded that the examined amino acid derivatives bind to the double-stranded CT-DNA through non-covalent interactions,[27] probably by groove binding and/or electrostatic interactions rather than intercalation.

TABLE I. Hyperchromism induced by the synthesized derivatives **1a**–**i** in 50 μM solution. The induction in % was calculated using expression $100(A_{DNA+D} - A_{DNA} - A_D) / A_{DNA+D}$, where A_{DNA}, A_D and A_{DNA+D} refer to the absorbance of DNA, derivatives in 50 μM solution and complex DNA–derivative, respectively, at 260 nm

Compound	**1a**	**1b**	**1c**	**1d**	**1e**	**1f**	**1g**	**1h**	**1i**
%	26.71	8.55	5.18	8.04	1.12	27.68	16.37	2.20	7.03

In order to obtain information on the stability of the derivative/CT-DNA complex, spectroscopic titrations of solutions of CT-DNA were performed (the results are given in Fig. S-2 of the Supplementary material). The obtained values of binding constants (Table S-I of the Supplementary material) are notably lower than those reported for intercalators, which indicates non-intercalative binding interactions between the quinone derivatives and CT-DNA.[28]

Various well-known DNA binding dyes are used to establish the type of drug–DNA interactions. Ethidium bromide is extensively used as a fluorescence probe that binds to DNA in an intercalative fashion. In case of groove binders, competitive displacement studies are done using a Hoechst dye that binds to the minor groove of double-stranded B-DNA and is highly specific for AT-rich sequences.

In order to investigate further the binding mode of *tert*-butylquinone derivatives to CT-DNA, fluorescence analyses were performed with ethidium bromide (EB) and Hoechst 33258 (H). The results of the competitive fluorescence study of the representative derivative **1h** with the intercalator EB are given in Fig. 3.

The fluorescence intensity of the band at 600 nm of the EB–CT-DNA system decreased significantly with the increasing concentration of all the quinone compounds (Table II). The greatest decreases in the fluorescence intensity were observed for the alanine (**1b**), D-valine (**1d**), phenylalanine (**1h**) and tryptophan (**1i**) derivatives at the highest used concentrations. The fluorescence emission spectrum for the proline derivative **1f** revealed the smallest reduction in the fluorescence intensity, which is consistent with a previous study on the effect of pro-

line in displacing DNA-bound ethidium bromide, in which fluorescence emission data of proline revealed a marginal displacement in contrast to other amino acids.[29]

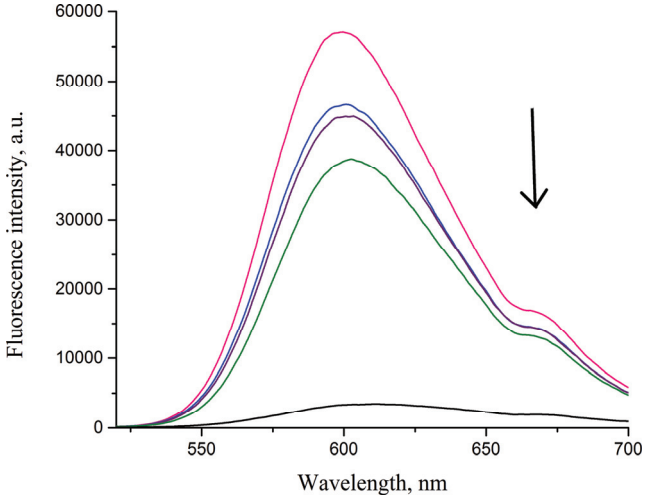


Fig. 3. Displacement of ethidium bromide bound to CT-DNA by derivative **1h**: emission spectra of EB alone (bottom line), EB bound to CT-DNA (top line) and quenching of EB–CT-DNA system by derivative **1h** at increasing concentrations (50, 100 and 200 μM solutions, from top to bottom).

TABLE II. The decrease of fluorescence of EB–CT-DNA complex upon addition of **1a**–**i** (200 μM solution). The decrease in % was calculated using the expression $100(I_{\text{FDNA+EB}} - I_{\text{FDNA+EB+D}} - I_{\text{FEB}}) / I_{\text{FDNA+EB}}$, where $I_{\text{FDNA+EB}}$, $I_{\text{FDNA+EB+D}}$ and I_{FEB} refer to the fluorescence intensity of complex EB–DNA, EB–DNA–derivatives and EB, respectively, at 600 nm

Compound	**1a**	**1b**	**1c**	**1d**	**1e**	**1f**	**1g**	**1h**	**1i**
%	21.80	23.87	17.85	29.86	19.74	6.59	20.29	26.79	22.64

According to the results and structural differences between the most active derivatives, it could be concluded that the derivatives did interact with DNA, which led to a decrease in fluorescence intensity, but the results could hardly be considered evidence of their intercalation, especially in view of the UV spectral data that showed hyperchromism and not hypochromism. It is known from the literature that an indirect displacement can occur whereby the compounds induce structural changes in DNA and hence reduce the binding affinity for EB.[30]

Binding of Hoechst 33258 to CT-DNA was followed by excitation at 350 nm with the maximum in fluorescence at 444 nm. The fluorescence intensity at 444 nm of the H–CT-DNA complex decreased with increasing concentration of most of the quinone compounds (Fig. 4, Table III). These results suggest minor groove binding of the amino acid derivatives. It seems that minor groove binding

might be of importance for their action on tumour cells since the three compounds that do not displace H from the complex are among the least active derivatives (IC_{50} never below 30 μM for any tested cell line). These exceptions were **1a**, **1e** and **1f**, which induced an increase in the fluorescence of the H–CT-DNA complex. A possible explanation for the increase in fluorescence is the formation of a ternary complex CT-DNA–dye–quinone. Such behaviour was shown in literature[31] for argininamide with an oligonucleotide aptamer. This external binding of **1a** and **1f** induced a high hyperchromism in the UV–Vis spectra, while binding of leucine derivative **1e** was weak because of its bulky isobutyl group.

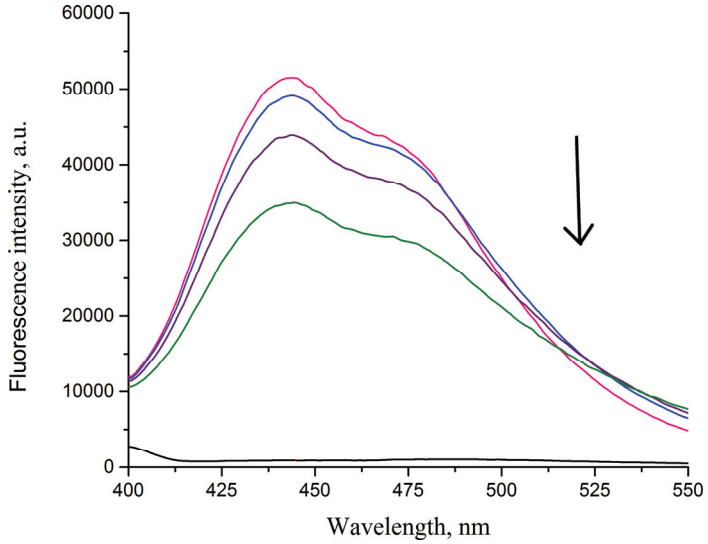


Fig. 4. Displacement of Hoechst 33258 bound to CT-DNA by derivative **1h**: emission spectra of Hoechst 33258 alone (bottom line), H bound to CT-DNA (top line) and quenching of H–CT-DNA system by derivative **1h** at increasing concentrations (50, 100 and 200 μM, from top to bottom).

TABLE III. Decrease in the fluorescence of the H–CT-DNA complex upon addition of **1a**–**i** (200 μM solution). The decrease was calculated using the expression $100(I_{FDNA+H} - I_{FDNA+H+D} - I_{FH}) / I_{FDNA+H}$, where I_{FDNA+H}, $I_{FDNA+H+D}$ and I_{FH} refer to the fluorescence intensity of complex H–DNA, H–DNA–derivatives and H, respectively, at 444 nm

Compound	**1a**	**1b**	**1c**	**1d**	**1e**	**1f**	**1g**	**1h**	**1i**
%	–25.55	19.52	53.83	23.76	–5.50	–37.92	31.16	30.44	33.89

CD spectroscopy is very sensitive to the changes in the secondary structure of biomacromolecules and, therefore, is widely used for detection of changes in secondary structure of DNA upon interactions with small molecules,[32] and non-covalent DNA–drug interactions resulting in altered CD spectral properties.[33,34]

The CD spectrum of CT-DNA displays a positive band around 275 nm due to base stacking and a negative band around 245 nm due to the right-handed helicity of the B-DNA form.[35] Intercalation significantly changes the intensities of both the bands, stabilizing the right-handed B conformation of DNA, while groove binding and electrostatic interactions of small molecules show low or no disturbances.[36] As shown in a representative CD spectrum of **1h** (Fig. 5), after its addition to CT-DNA, there were no significant changes in the CD spectrum. These results provide evidence that the derivatives do not bind to CT-DNA by intercalation, but rather *via* groove binding.

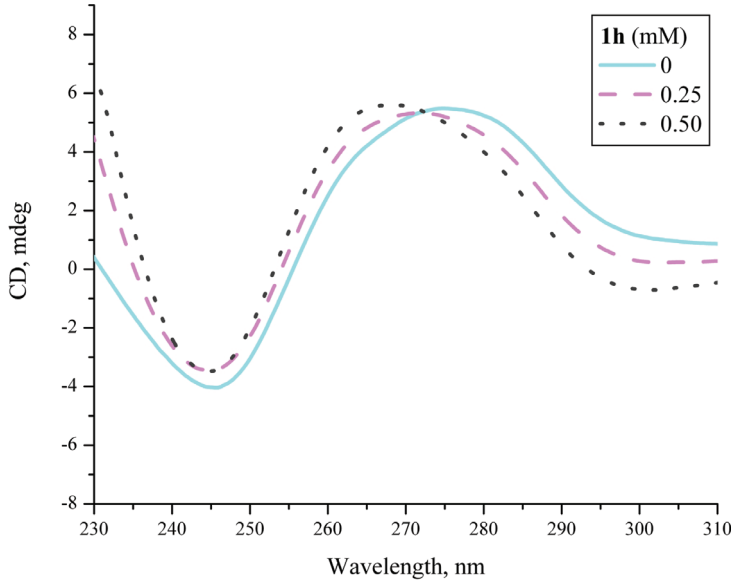


Fig. 5. Effect of **1h** on CD spectra of CT-DNA. CD spectra of CT-DNA (0.50 mM) in bicarbonate solution (40 mM, pH 8.4) with varying concentrations of **1h**. Each spectrum was obtained at 25 °C with 1 mm path length cell.

For the support of the evidence for minor groove binding, molecular docking was performed with the DNA sequence d(CGCGAATTCGCG)$_2$, PDB ID: 3U2N.[16] The calculations suggested binding to the minor groove. No intercalating structures were found. The pattern of binding is presented in Fig. 6. The established interactions between molecules and DNA nucleotides are predominantly hydrogen bonds and some carbon–hydrogen bonding and π–alkyl interactions.

Based on the previous results in which it was shown that several types of avarone derivatives cleaved plasmid DNA,[37] the capability of the synthesized derivatives to cleave pUC19 plasmid was examined using electrophoretic analysis. DMSO did not show any effect on plasmid DNA under the applied experimental

conditions. Electrophoretic pattern showed that there was no cleavage of pUC19 with any of the synthesized compounds under the applied conditions (Fig. 7).

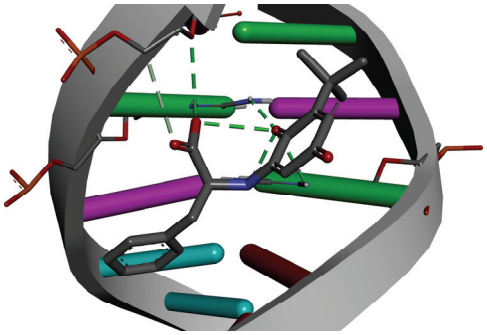


Fig. 6. Binding of compound **1h** to the minor groove.

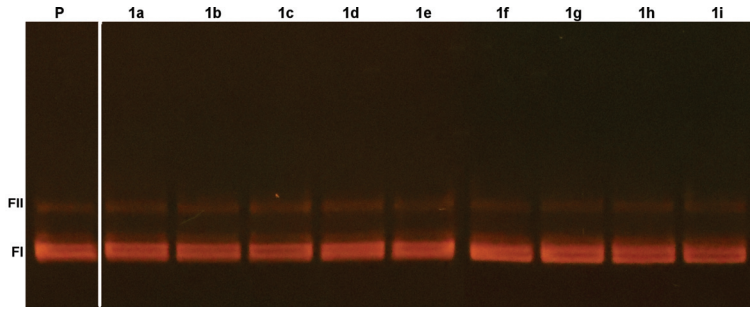


Fig. 7. Agarose gel electrophoretic analysis of plasmid pUC19 without (P) and with derivatives **1a**–**i** (1 mM).

These results differed from previous results with quinone compounds,[37] which showed a significant cleavage of plasmid DNA. The cleavage of DNA molecules by quinones *in vitro* could be explained by the presence of ROS, which leads to single-strand and/or double-strand breaks of the plasmid chain. Quinones have the ability to generate oxygen radicals through semiquinone intermediates formed either after nucleophilic attack on the quinone moiety or after reduction and subsequent reoxidation of hydroquinone or semiquinone into quinone. As the investigated amino acid derivatives interchange rapidly between tautomers and are not susceptible to nucleophilic attack and since the experimental conditions were not reducing, the lack of cleavage could be expected.

Considering the observation that the derivatives do not generate radicals *in vitro*, their ability to scavenge radicals was investigated by the DPPH test. The results of scavenging activity of amino acid derivatives of *tert*-butylquinone are listed in Table IV. All the derivatives showed some antioxidant activity, although weaker than ascorbic acid. The activity could be ascribed to the amino acid moiety. Amino acids were shown to react with DPPH radical by two mechanisms, namely oxidative decarboxylation, initiated by abstraction of hydrogen from the

carboxylic group and oxidative deamination, initiated by hydrogen abstraction from the NH group.[38] Based on the results presented here, which showed that the proline derivative **1f** was the most active compound, and that there are no other clear-cut structure–activity relationships, oxidative decarboxylation is the principal reaction pathway of this series of compounds.

TABLE IV. Antioxidant activity (IC_{50} / mM) of compounds **1a**–**i**

Compound	**1a**	**1b**	**1c**	**1d**	**1e**	**1f**	**1g**	**1h**	**1i**	Ascorbic acid
IC_{50}	1.57	2.87	1.25	8.89	1.27	0.34	1.37	1.02	2.37	0.07

CONCLUSION

In this paper, the mode of biological action of amino acid derivatives of *tert*-butylquinone was investigated at the molecular level. It was observed that the synthesized derivatives, because of the nature of their substituents, have an atypical mode of action for quinones. Neither addition of nucleophilic groups of proteins nor DNA strand nicking was observed. It could be concluded that the preferential mechanism of action of the investigated series of biologically active compounds with the quinone moiety, the structural elements of which allowed rapid interconversion of tautomeric forms, is a non-covalent interaction with DNA molecules, namely minor groove binding. This mechanism of action, with less diverse effects at the molecular level, probably leads to the observed low toxicity of these compounds to the tested normal cell line,[12] making them a promising lead for further anticancer drugs.

SUPPLEMENTARY MATERIAL

Additional experimental details and mass spectra of the unmodified lysozyme and lysozyme after treatment with **1h** are available electronically at the pages of journal website: http://www.shd.org.rs/JSCS/, or from the corresponding authors on request.

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ИЗВОД

ИНТЕРАКЦИЈЕ ЦИТОТОКСИЧНИХ АМИНОКИСЕЛИНСКИХ ДЕРИВАТА *tert*-БУТИЛХИНОНА СА DNA И ЛИЗОЗИМОМ

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Испитане су интеракције девет аминокиселинских деривата *tert*-бутилхинона са биомакромолекулима. SDS гел-електрофорезом и масеном спектрометријом потврђено

је одсуство модификација лизозима било којим од синтетисаних једињења. Спектрофотометријска испитивања су показала појаву хиперхромизма, као последицу постојања интеракција између хинона и CT-DNA. Константе везивања одређене апсорпционом титрацијом указују на слабе интеракције између хинонских деривата и CT-DNA. Синтетисани деривати смањују интензитет флуоресценције етидијум-бромида и боје Hoechst 33258 у комплексу са DNA, што потврђује постојање интеракција са DNA из тимуса говечета (CT-DNA). CD спектри указују на изостанак интеркалације хинона у исту. Резултати молекулског моделовања потврђују везивање деривата у малу бразду. Електрофоретска испитивања су показала да није дошло до цепања плазмида pUC19 у присуству било ког испитиваног деривата. Способност деривата да хватају слободне радикале потврђена је DPPH тестом. Сви приказани резултати указују на то да је механизам дејства аминокиселинских деривата везивање у малу бразду.

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