

Interactions of the anti-tumor sesquiterpene hydroquinone avarol with DNA *in vitro*

MIROSLAVA VUJČIĆ^{1#}, SRĐAN TUFEGDŽIĆ^{1#}, ZORAN VUJČIĆ^{2#},
MIROSLAV J. GAŠIĆ^{1#} and DUŠAN SLADIĆ^{2*#}

¹*Institute of Chemistry, Technology and Metallurgy, Department of Chemistry, Njegoševa 12, 11000 Belgrade and* ²*Faculty of Chemistry, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia*

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Abstract: Changes in electrophoresis pattern after interaction of supercoiled plasmid pBR322 DNA with avarol was studied at a micromolar concentration of reactants under mild reaction conditions. Interactions of avarol with linear high-molecular CT-DNA at millimolar concentrations were analyzed by electrophoresis and UV spectrophotometry. It was observed that avarol is capable of quenching ethidium bromide fluorescence in DNA bands. An increase in the absorbance of DNA was detected. The results indicate the binding of avarol to DNA and/or modification of nucleotide bases.

Keywords: avarol, hydroquinone, anti-tumor activity, pBR322 DNA, calf thymus DNA.

INTRODUCTION

DNA is the presumed intracellular target for a variety of quite structurally diverse low molecular weight ligands. A number of DNA-reactive synthetic and natural products or their derivatives have chemical, biological and medical significance as potential artificial gene regulators or clinically important chemotherapeutic agents. Low molecular weight ligands recognize and interact with DNA in different ways, including 1) DNA strand cleavage, 2) non-covalent association with the minor groove of DNA, 3) intercalation between DNA base pairs, 4) alkylation of a component nucleotide and 5) a combination of various effects.¹ Avarol is a marine sesquiterpenoid hydroquinone, isolated from the marine sponge *Dysidea avara*.² It was shown that avarol (Fig. 1) and the corresponding quinone avarone possess interesting activities towards DNA *in vivo*.^{3–6} The aim of this study was to investigate the interactions of avarol with DNA *in vitro*. The possibility of avarol to induce DNA cleavage was measured by determining the

Serbian Chemical Society member.

* Corresponding author. E-mail: dsladic@chem.bg.ac.yu

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ability of the hydroquinone to transform the naturally occurring, covalently closed circular supercoiled form of plasmid pBR322 DNA to the open circular, relaxed form. The efficiency of the interaction of avarol with plasmid DNA was monitored by agarose gel electrophoresis experiments. Furthermore, the possibility of avarol of intercalating DNA molecules was investigated by UV spectral measurements of calf thymus DNA in presence of avarol. The obtained results might shed light on the mechanism of the anti-tumor activity of avarol.

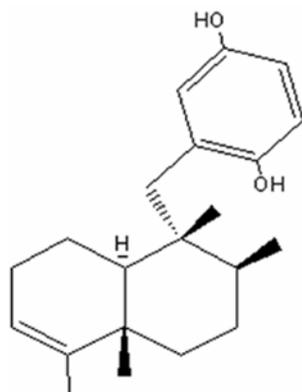


Fig. 1. Structure of avarol.

EXPERIMENTAL

Chemicals

Avarol was isolated from the sponge *Dysidea avara*, collected in the Bay of Kotor (Montenegro), as previously described.² Plasmid pBR322 from *Escherichia coli* RRI (containing more than 80 % supercoiled DNA) was purchased from Sigma–Aldrich, USA. Calf thymus DNA (lyophilized, highly polymerized) was obtained from Serva, Heidelberg. Agarose was purchased from Pharmacia-Biotech (GE Healthcare). All buffer solutions were prepared in deionized water and filtered through 0.2 μm filters (Nalgene, USA).

Interaction of avarol with plasmid pBR322

Avarol was dissolved in DMSO (0.01 g ml⁻¹). Plasmid DNA (0.5 μg) was mixed with various amounts of avarol, so that the final concentrations of avarol were 10 μM , 20 μM and 30 μM , in 20 μl of the reaction mixture containing TAE buffer pH 8.24 (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), or in 20 μl of the reaction mixture containing bicarbonate buffer (40 mM, pH 8.4). The contents were immediately analyzed by electrophoresis or incubated for 2 h at 37 °C with vortexing from time to time. The reaction was terminated by adding 5 μl of loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF and 30 % glycerol in water). The samples were subjected to electrophoresis on 1 % agarose gel prepared in TAE buffer. The electrophoresis was performed at a constant voltage (80 V) for about 1.5 h (until bromophenol blue had passed through 75 % of the gel) in TAE buffer. After electrophoresis, the gel was stained for 30 min by soaking in an aqueous ethidium bromide solution (2 μl of 10 % (w/v) ethidium bromide in 40 ml of water). The gel was then destained for 5 min by keeping it in sterile distilled water. The stained gel was illuminated under a UV transilluminator Vilber–Lourmat (France) at 312 nm and photographed with a Panasonic DMC-LZ5 Lumix Digital Camera.

Interaction of avarol with calf thymus DNA

Calf thymus DNA (CT-DNA) was dissolved in 20 mM Tris-HCl pH 7.5/20 mM NaCl overnight at 4 °C. DNA concentration was adjusted with buffer to 5 mg ml⁻¹. This stock solution was stored at 4 °C and was stable for several days. A solution of calf thymus DNA in water gave a ratio of UV absorbance at 260 and 280 nm, A_{260}/A_{280} of 1.89–2.01, indicating that DNA was sufficiently free of protein. The concentration of DNA was measured by measuring the absorbance of DNA-containing solution at 260 nm. One optical unit corresponds to 50 µg ml⁻¹ of double stranded DNA (based on the known molar absorption coefficient value of 6600 M⁻¹cm⁻¹).⁷ 2.5 µl of stock solution of CT-DNA in 497.5 µl of TAE or bicarbonate buffer were incubated at 37 °C for 2 h, vortexing from time to time, with 10, 20 and 30 µl of an ethanolic solution of avarol (175 mg ml⁻¹), corresponding to final avarol concentrations of 11, 22 and 33 mM, respectively). Subsequently, the samples were cooled to 4 °C and centrifuged at 12000 rpm. 400 µl of the supernatant were used for measurements of absorbance on a UV Cintra 40 UV/Visible spectrometer. 10 µl of the supernatants were analyzed by electrophoresis on a 1 % agarose gel as described above.

RESULTS AND DISCUSSION

It was previously shown that incubation of pBR322 DNA with avarol caused a conversion of fully supercoiled DNA to nicked, circular molecules only in an oxygen atmosphere.³ In this work pBR322 was incubated with avarol in air (milder reaction conditions). The electrophoresis pattern of the interaction of different concentrations of avarol with plasmid pBR322 DNA is shown in Fig. 2. No effects were observed immediately upon addition of avarol to the plasmid, regardless of the concentrations of avarol, as shown in Fig. 2A, lanes 1–4. After 2 h of reaction at 37 °C, no relaxation of supercoiled DNA was detected (Fig. 2A, B). These results differ from the previous ones obtained in an oxygen atmosphere but are in accordance with those obtained in nitrogen atmosphere.³ It is likely that the amount of oxygen in air is insufficient for the process of DNA nicking by oxygen radicals to be detected. Some hydroquinone antioxidants also did not show damage to DNA unless the generation of semiquinone and hydroxyl radicals was additionally stimulated.⁸ However, a dose-dependent quenching of the fluorescence of ethidium bromide was observed, as shown in Fig. 2B, lanes 1–4. The intensity of the bands diminished with increasing amount of avarol, indicating changes in the DNA molecules. This capability of avarol to interfere with the intercalation of ethidium bromide in supercoiled DNA was more pronounced in the bicarbonate than in the TAE buffer system (Fig. 2C).

Analysis of interaction of avarol with linear, high-molecular calf thymus DNA (CT-DNA) resulted in almost complete quenching of fluorescence in both buffer systems (Fig. 3). The nature of the observed DNA damage by avarol was investigated by DNA absorbance measurements. The absorption spectrum of CT-DNA was recorded in the absence and presence of increasing amounts of avarol. Typical sets of absorption spectra obtained in TAE and in bicarbonate buffers are shown in Fig. 4. An increasing quantity of avarol induced a hypochromic effect. The effect was again more pronounced in bicarbonate buffer. This hypochro-

mism can be ascribed to intercalative binding of avarol to the DNA helix.⁹ Since avarol has only one planar ring, this type of binding should be weak, but the binding could be enhanced by positioning the sesquiterpene part of the molecule in the minor groove. Another possible cause of hypochromism could be modification of guanine and thymine residues, as was shown previously for some hydroquinones.¹⁰

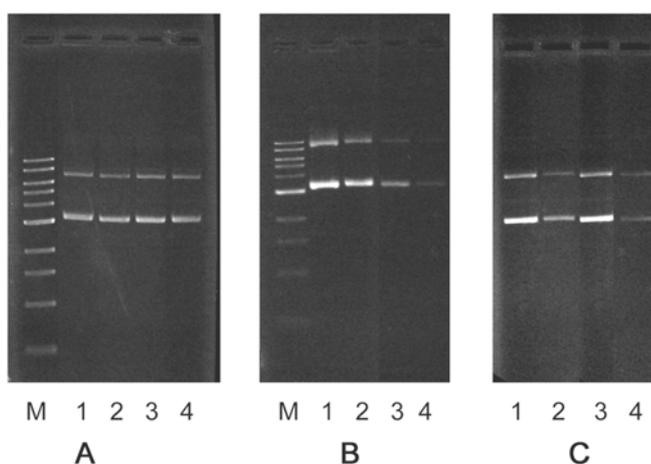


Fig. 2. Agarose gel electrophoresis of pBR322 after treatment with 0, 10, 20 and 30 μM of avarol (lanes 1, 2, 3, 4, respectively) at the beginning of the reaction – Panel A, and after 2 h at 37 $^{\circ}\text{C}$ – panel B. Panel C – Lanes 1 and 3 are pBR322 in TAE and bicarbonate buffer, respectively, without avarol; lanes 2 and 4 are pBR322 after treatment with 10 mM avarol in TAE and bicarbonate buffer, respectively. M – marker DNA.

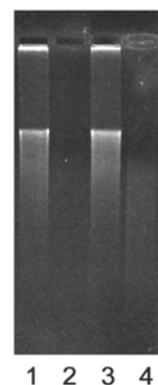


Fig. 3. Agarose gel electrophoresis of CT-DNA in TAE and in bicarbonate buffer without avarol (lanes 1 and 3, respectively) and after treatment with 10 mM avarol in TAE and in bicarbonate buffer (lanes 2 and 4, respectively).

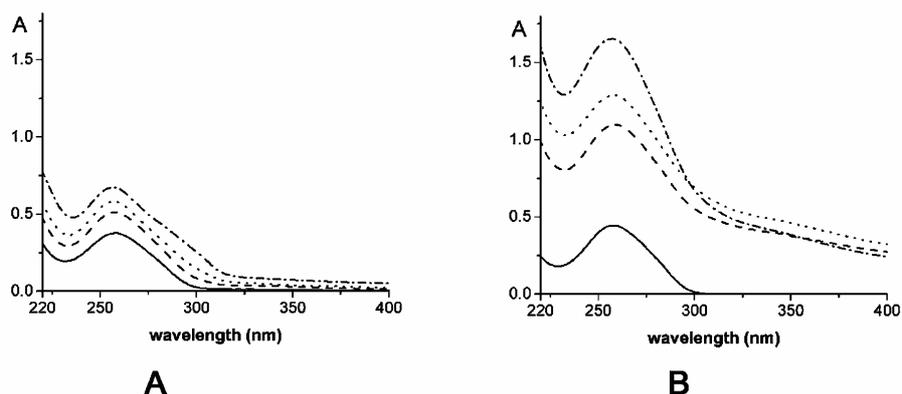


Fig. 4. Absorption spectra of CT-DNA in the absence (—) and presence of 11 (---), 22 (.....), and 33 mM (-·-) avarol. The absorption measurements were performed in TAE (panel A) and bicarbonate buffer (panel B).

CONCLUSIONS

Taken together, these results suggest that avarol possesses some DNA-damaging activity *in vitro*. The absence of changes in the electrophoresis pattern of circular plasmid DNA revealed that micromolar concentrations of the reactants under mild reaction conditions did not induce a relaxation of the supercoiled molecule. However, a quenching of the fluorescence of the ethidium bromide-DNA complex was observed. Interaction of avarol with linear high-molecular CT-DNA at millimolar concentrations gave, in addition to quenching the electrophoresis bands, an increase in the absorbance of DNA. The obtained results suggest that the mechanism of interaction of avarol with DNA under these reaction conditions *in vitro* may be similar, regardless of the source of DNA. These interactions could include binding to DNA as well as damage to nucleotide bases. These effects could be responsible for the pronounced anti-tumor activity of avarol.

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ИЗВОД

ИНТЕРАКЦИЈЕ АНТИТУМОРСКОГ СЕСКВИТЕРПЕНСКОГ
ХИДРОХИНОНА АВАРОЛА СА DNA *IN VITRO*

МИРОСЛАВА ВУЈЧИЋ¹, СРЂАН ТУФЕГЦИЋ¹, ЗОРАН ВУЈЧИЋ², МИРОСЛАВ Ј. ГАШИЋ¹ и ДУШАН СЛАДИЋ²

¹ИХТМ, Центар за хемију, Београд и ²Хемијски факултет, Универзитет у Београду, Београд

Проучаване су промене електрофоретског понашања DNA после интеракција суперхеликоидалног плаزمида pBR322 са аваролом при микроларним концентрацијама реактанта под благим реакционим условима. Интеракције аварола са линеарном високомолекуларном CT-DNA при милиларним концентрацијама анализирани су електрофорезом и UV спектрофотометријом. Уочено је да је аварол у стању да гаси флуоресценцију етидијум-бромида у тракама које потичу од DNA. Детектован је пораст апсорбације DNA. Резултати указују на везивање аварона за DNA и/или модификацију нуклеотидних база.

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