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Hydroxyl radical generation and DNA strand scission mediated by natural anticancer and synthetic quinones

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Using ESR and spin-trapping techniques, it was found that synthetic 2-dimethylamino-3-chloro-1,4-naphthoquinone and the natural anticancer quinone daunomycin, when added to a system containing purified NADPH-cytochrome P-450 reductase, NADPH, ferric ions, and oxygen, (i) generated hydroxyl radicals and (ii) caused single-strand scission of supercoiled DNA of the plasmic pBR322. Since these two effects of the quinones were correlated to each other, we propose that potential anticancer quinones can be effectively screened by measuring their ability to form hydroxyl radicals in the above system.

Hydroxyl radical; DNA strand scission; ESR; Spin trapping

1. INTRODUCTION

Any synthetic compound capable of cleaving DNA is considered to be a potential anticancer drug (for review, see e.g. [1]). One rational approach to creating effective anticancer drugs is to synthesize structural analogues of naturally available anticancer drugs in the hope of obtaining less toxicity and lower production cost [2]. Another approach is to design functional analogues on the basis of mechanistic studies of action of well-known effective drugs. This latter approach has not yet been sufficiently employed for anthracycline antibiotics (the most effective modern drugs in cancer chemotherapy) because their mechanism of action has not been fully understood. Recently, however, studies have suggested that their therapeutic and side effects are mediated by hydroxyl radicals [3–5]. We have previously studied the generation of hydroxyl radicals in liver microsomes in the presence of anthracycline antibiotics and some synthetic quinones, and suggested a general scheme for their redox transformations in the microsomal electron-transfer chain [6,7].

Using this scheme we attempted in this study to compare the efficiency of daunomycin, a natural anticancer quinone, and synthetic 2-dimethylamino-3-chloro-1,4-naphthoquinone (DCNQ) to generate hydroxyl radicals in the presence of NADPH-cytochrome P-450 reductase, NADPH, ferric ions, and molecular oxygen and their ability to cause strand scission of pBR322 DNA in the same system. It was thus found that the hydroxyl radical formation and the DNA cleavage are correlated to each other.

2. MATERIALS AND METHODS

Plasmid pBR322 was isolated as described in [8]. NADPH-cytochrome P-450 reductase, purified from rat liver microsomes according to [9], was kindly supplied by V.V. Lyakhovich. The reductase activity was determined by measuring the rate of cytochrome *c* reduction [10]. The generation of hydroxyl radicals was determined from ESR spectra of the spin

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Abbreviations: DCNQ, 2-dimethylamino-3-chloro-1,4-naphthoquinone; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide

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trap 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) in the presence of 5% dimethylsulfoxide [11]. The ESR spectra were measured in a Bruker ER 200 D-SRC spectrometer using a flat sealed cell (volume, 200 μl). The experimental conditions were as follows: field, 3474 G; sweep width, 100 G; microwave power, 19.8 mV; and modulation amplitude, 1 G. The DNA scission was detected from the transformation of supercoiled pBR322 DNA into the relaxed form. The reaction mixture (10 μl) contained 2.3×10^{-8} M DNA (1×10^{-4} M nucleotide pairs), 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10^{-4}–10^{-6} M quinone, 10^{-5}–10^{-7} M Fe^{3+}-EDTA, 10^{-3} M NADPH and 0.15 unit/ml of the reductase. The reaction was run at 37°C for 30–60 min with subsequent addition of 2–3 μl of 0.1% bromophenol blue in 70% glycerol. The mixture was subjected to electrophoresis in 1% agarose gel in a horizontal slab gel apparatus (5 V/cm) using 40 mM Tris-acetate (pH 7.8) containing 20 mM sodium acetate, 1.8 mM EDTA and 1 μg/ml of ethidium bromide as solvent. The DNA bands were detected and photographed using a long-wavelength ultraviolet light source. The supercoiled and relaxed DNAs have different mobilities in this electrophoresis and could be distinguished from each other.

DCNQ was synthesized as described in [12]. Daunomycin was obtained from Minmedprom (USSR), NADPH from Reanal (Hungary), and superoxide dismutase and catalase from Serva (FRG). The spin trap DMPO was purchased from Sigma (USA) and purified as described in [13].

3. RESULTS AND DISCUSSION

Both daunomycin and DCNQ were reduced by NADPH in the presence of purified NADPH-cytochrome P-450 reductase to semiquinones and hydroquinones, which could be readily reoxidized by atmospheric oxygen. Under anaerobic conditions, the formation of daunomycin and DCNQ semiquinones were detectable by ESR measurements (not shown). In a system containing quinone, NADPH, and the reductase, aerobic oxidation of NADPH took place with $K_m = 2.5 \times 10^{-4}$ M for daunomycin and $K_m = 7.7 \times 10^{-5}$ M for DCNQ. It has been reported that upon addition of ferric ions to this system the following reactions leading to the formation of hydroxyl radicals occur [14].

$$Q + e \longrightarrow Q^{\cdot -} \quad (1)$$

$$Q^{\cdot -} + O_2 \longrightarrow Q + O_2^{\cdot -} \quad (2)$$

$$O_2^{\cdot -} + O_2^{\cdot -} \xrightarrow{2H^+} H_2O_2 + O_2 \quad (3)$$

$$Q^{\cdot -} + Fe^{3+} \longrightarrow Q + Fe^{2+} \quad (4)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\cdot} \quad (5)$$

where Q denotes quinone. The formation of hydroxyl radicals in this system could be detected by observing the ESR spectrum of $CH_3^{\cdot}$-DMPO spin adduct (fig.1a). The kinetics of hydroxyl radical formation were measured from the intensity of the sextet low-field component of the ESR spectrum and are shown in fig.1b. As can be seen, both daunomycin and DCNQ showed practically the same ability to form hydroxyl radicals in the presence of NADPH and the reductase. The addition of catalase completely inhibited both daunomycin- and DCNQ-mediated hydroxyl radical generation, whereas superoxide dismutase had practically no effects. In the absence of quinone the spin adduct formation was not detected. As reported previously [6,7], the addition of Fe^{3+}-EDTA increased the hydroxyl radical formation mediated by both quinones.

We next examined DCNQ-mediated cleavage of pBR322 DNA in the presence of NADPH, the reductase, and Fe^{3+}-EDTA as described in section 2 and the results are shown in fig.2. Only supercoiled pBR322 (form I) was detected in lane 1, where the plasmid was incubated without additions. When the complete system was incubated, a large amount of the open circular form of DNA (form II) was observed in addition to form I (lane 4), indicating that DCNQ-mediated scission of the plasmid DNA had occurred. Omission of either

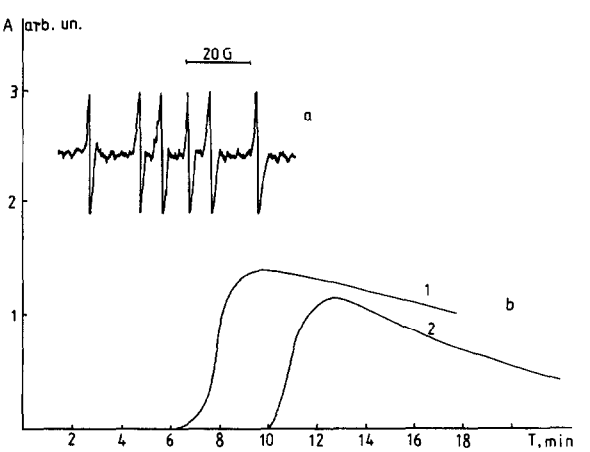

Fig.1. (a) ESR spectrum of $CH_3^{\cdot}$-DMPO spin adduct formed from DMPO and hydroxyl radicals. (b) Kinetics of the $CH_3^{\cdot}$-DMPO spin adduct accumulation measured from the intensity of the sextet low-field component. The system contained 10^{-3} M NADPH, 0.15 unit/ml of the reductase, 0.1 M DMPO, 5% dimethylsulfoxide, 2.5×10^{-4} M daunomycin (1) or 2.5×10^{-4} M DCNQ (2) in 0.1 M potassium phosphate buffer (pH 7.6).

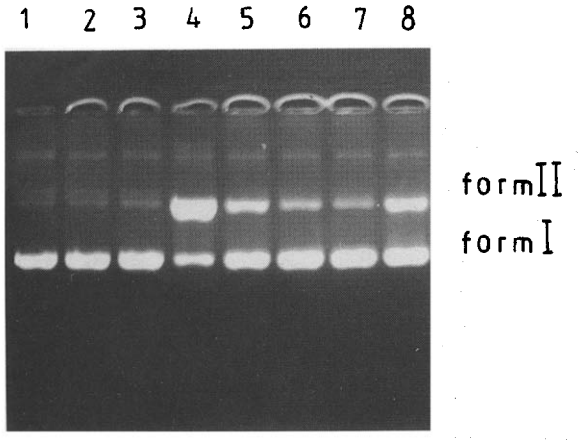

Fig.2. DCNQ-mediated scission of pBR322 DNA. The experimental conditions are described in section 2. Lanes: 1, supercoiled DNA (0.5 mg) alone; 2, DNA and 10^{-4} M DCNQ; 3, DNA, 10^{-4} M DCNQ and 10^{-5} M Fe^{3+}-EDTA; 4, DNA, 10^{-4} M DCNQ, 10^{-5} M Fe^{3+}-EDTA, 10^{-3} M NADPH and 0.15 unit/ml of the reductase (complete system); 5, complete system minus Fe^{3+}-EDTA; 6, complete system minus DCNQ; 7, complete system plus 50 units/ml of catalase; 8, complete system plus 0.05 mg/ml of superoxide dismutase.

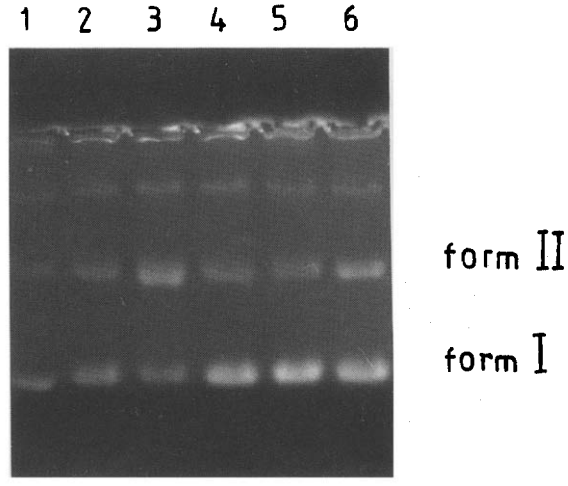

Fig.3. Daunomycin-mediated scission of pBR322 DNA. The experimental conditions are described in section 2. Lanes: 1, supercoiled DNA (0.5 mg) alone; 2, DNA, 10^{-4} M daunomycin, 10^{-5} M Fe^{3+}-EDTA, and 10^{-3} M NADPH; 3, DNA, 10^{-4} M daunomycin, 10^{-5} M Fe^{3+}-EDTA, 10^{-3} M NADPH, and 0.15 unit/ml of the reductase (complete system); 4, complete system minus Fe^{3+}-EDTA; 5, complete system plus 50 units/ml of catalase; 6, complete system plus 0.05 mg/ml of superoxide dismutase.

NADPH or the reductase resulted in the abolition of cleavage (lanes 2 and 3). In the absence of exogenous iron (Fe^{3+}-EDTA) the conversion of form I to form II occurred to a considerably smaller extent (lane 5) than in the complete system (lane 4). Even in the absence of DCNQ, the reduced reductase can be slowly reoxidized by atmospheric oxygen [7] leading to the generation of $O_2^{\cdot-}$ and H_2O_2. If Fe^{3+}-EDTA was included in this system, hydroxyl radicals were produced and thus the DNA scission took place (lane 6), though the formation of form II was much less than in the complete system. Upon addition of catalase to the complete system, the DNA cleavage was strongly inhibited (lane 7), whereas the inhibition was much less pronounced when superoxide dismutase was added (lane 8).

Fig.3 shows the results of similar experiments using daunomycin instead of DCNQ. A comparison of the results obtained when the plasmid DNA alone was incubated (lane 1) with that obtained in the complete system (lane 3) indicates that daunomycin-mediated DNA scission (production of form II) took place. Omission of either the reductase (lane 2) or Fe^{3+}-EDTA (lane 4) from the complete system strongly inhibited cleavage. As in the case of DCNQ, catalase decreased the generation of form II profoundly (lane 6), whereas superoxide dismutase caused only a slight inhibition (lane 6). Fig.4 shows the effects of the concentrations of daunomycin, DCNQ, and Fe^{3+}-EDTA on the generation of the open circular form of DNA. Lane 1 shows the plasmid DNA incubated without additions. Lanes 2 through 5 are the results obtained using decreasing concentrations of daunomycin (10^{-4}–5×10^{-6} M), lanes 6 through 9 with decreasing concentrations of DCNQ (10^{-4} -5×10^{-6} M), and lanes 10 through 13 with decreasing concentrations of Fe^{3+}-EDTA (10^{-5}–5×10^{-7} M) in the presence of 10^{-4} M DCNQ. It is clear that the DNA cleavage was dependent on the concentrations of the quinones and Fe^{3+}-EDTA.

The effects described above, especially the effects of catalase, superoxide dismutase, and Fe^{3+}-EDTA, are consistent with the idea that the DNA scission is mediated by hydroxyl radicals [15], and suggest that there is a correlation between the ability of quinones to generate hydroxyl radicals and their efficiency to cleave DNA. A comparison of these two effects of the natural anticancer and synthetic quinones, as first performed

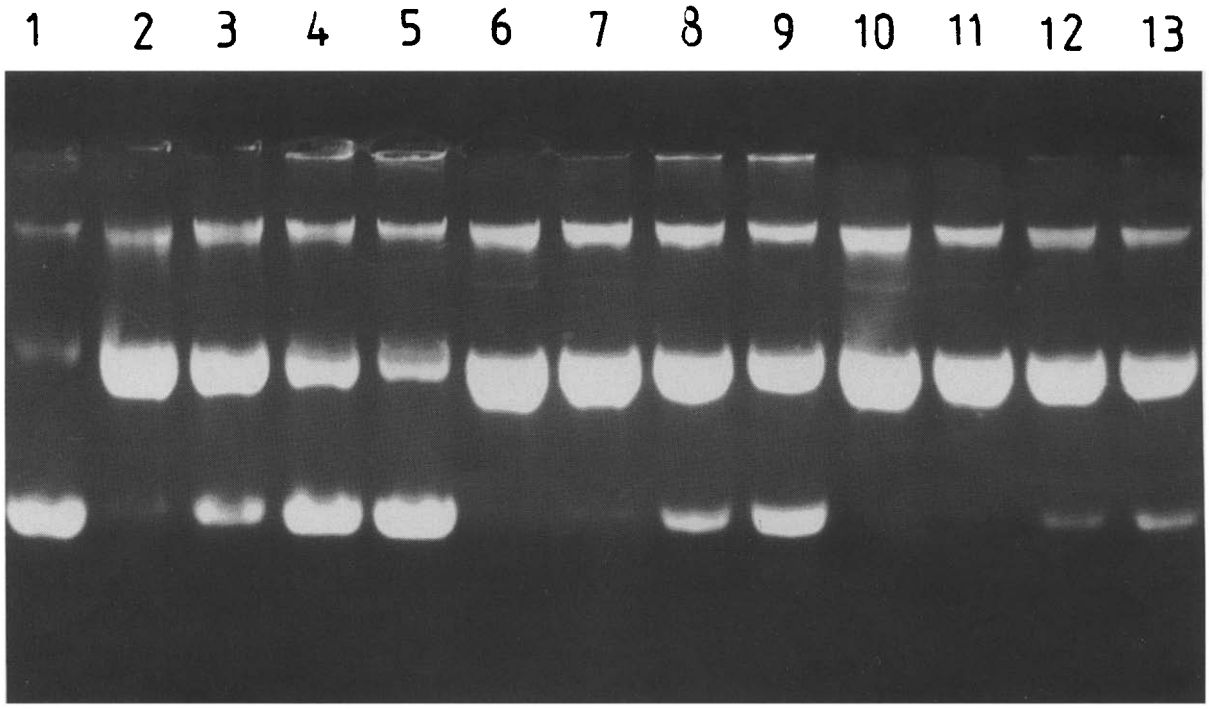

Fig.4. Effects of concentrations of daunomycin, DCNQ and Fe^{3+}-EDTA on scission of pBR322 DNA. The experimental conditions are described in section 2. Lanes: 1, supercoiled DNA (0.5 mg) alone; 2–5 show the results with complete system (lane 3 in fig.3) except that daunomycin concentrations were 10^{-4}, 5×10^{-5}, 10^{-5} and 5×10^{-6} M, respectively; 6–9 show the results with complete system (lane 4 in fig.2) except that DCNQ concentrations were 10^{-4}, 5×10^{-5}, 10^{-5} and 5×10^{-6} M, respectively; 10–13 show the results with complete system (lane 4 in fig.2) except that Fe^{3+}-EDTA concentrations were 10^{-5}, 5×10^{-6}, 10^{-6} and 5×10^{-7} M, respectively.

in this study, provides a basis for a novel, efficient method for screening potentially anticancer quinones. Since any synthetic compounds that cleave DNA are potentially anticancer drugs, as mentioned earlier, such quinones can be selected by measuring their activity to generate hydroxyl radicals in the system containing NADPH, the reductase, ferric ions and molecular oxygen. The finding that synthetic DCNQ can cause DNA strand scission may help to remove the shroud of mystery that natural anticancer quinones possess 'special' properties, which are absent in their synthetic analogues.

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