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INVOLVEMENT OF HYDROXYL RADICAL FORMATION AND DNA STRAND BREAKAGE IN THE CYTOTOXICITY OF ANTHRAQUINONE ANTITUMOUR AGENTS

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Four 9,10-anthraquinones (AQ) mono- or bis-substituted with the $-NH(CH_2)_2NH(CH_2)_2OH$ group were studied. 1-AQ, 1,5-AQ and 1,8-AQ but not 1,4-AQ (100 μM) generated pBR322 plasmid DNA single strand breaks in the presence of purified NADPH dependent cytochrome P450 reductase. 1-AQ, 1,5-AQ and 1,8-AQ (at 100 μM) stimulated hydroxyl radical formation in MCF-7 S9 cell fraction (as measured by dimethyl pyrolline N-oxide spin trapping) and MCF-7 DNA strand breaks as measured by alkaline filter elution. In contrast 1,4-AQ did not stimulate hydroxyl radical formation and produced considerably less strand breaks in MCF-7 cells compared to the other AQ's. It would appear that the position of the $-NH(CH_2)_2NH(CH_2)_2OH$ groups on the chromophore is an important determinant in the metabolic activation of cytotoxic anthraquinones. This may contribute to the cytotoxicity (ID_{50} values) of 1-AQ (0.06 μM), 1-8-AQ (0.5 μM) and 1,5-AQ (12.3 μM) but not the 1,4-AQ (1.2 μM).

KEY WORDS: Hydroxyl radicals, anthraquinones, DNA strand breaks, MCF-7 cells, cytotoxicity.

INTRODUCTION

Cytotoxic anthraquinones are a group of synthetic agents in which the central anthraquinone chromophore is substituted with one or more alkylamino side chains bearing terminal amino functional groups which are protonated at physiological pH. The planar structure and cationic charge of this type of compound is responsible for the observed high binding affinity with DNA and hence is likely to be associated with their mode of action.[1,2] Cytotoxic anthraquinones are being investigated as compounds that are more simple derivatives of the biosynthetic anthracyclines, notably doxorubicin, but with improved broad spectrum antitumour activity and low or absent cardiotoxicity.[3] Free radical generation, in particular reactive oxygen, has been implicated in both the cytotoxic and cardiotoxic action of the anthracyclines.[4] Some anthraquinone antitumour agents including mitoxantrone have also been shown to generate free radicals in biological systems [5,6] and NAD(P)H-dependent flavoprotein reductases have been implicated in this metabolic activation [7]. Redox cycling by 1-AQ and 1,8-AQ in MCF-7 human breast cancer cells has previously been demonstrated.[8] We investigate here the relationship between reactive oxygen generation, notably hydroxyl radical formation, DNA strand breakage and cytotoxicity of 1-AQ, 1,8-AQ, 1,5-AQ and 1,4-AQ in MCF-7 cells human breast cancer derived cells.

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MATERIALS AND METHODS

Drugs, Biological Materials and Reagents

The anthraquinones used in this study were synthesised as described previously.[1] Biochemicals and reagents were purchased from Sigma Chemical Co., Poole, Dorset, UK and cell culture material from Flow Labs, Irving (Dorset, UK). The pBR322 plasmid DNA was isolated from *E. coli* essentially as described previously.[10] NADPH dependent cytochrome P450 reductase from rat liver was prepared as described previously.[11]

MCF-7 Cell Culture and Preparation of Cell Fractions

MCF-7 cells were cultured at 37°C in RPMI 1640 medium supplemented with glutamine (2 mM), foetal calf serum (10%) and gassed with 10% CO_2. Confluent cells were harvested using EDTA (0.1%) in phosphate-buffered saline (PBS, pH 7.4). MCF-7 S9 fraction was prepared by centrifugation (9000 × g, 30 min.) of sonicated cells (10^7 ml^{-1}) and collecting the supernatant which was stored at 70°K until required. To test for cytotoxicity, MCF-7 cells (10^5 ml^{-1}) were cultured at 37°C as described above for 24 hr after which time the anthraquinones were added at various concentrations in the range 0.1 nM-100 μM for one hour. The drug containing medium was then removed, the monolayer washed with ice-cold PBS and fresh medium added. The cells were allowed to grow for a further 6 days. Total remaining cells were counted using a ZB Coulter counter and the concentration of drug that inhibited 50% cell growth (ID_{50}) determined from the plot of percentage cell survival versus drug concentration.

Spin Trapping of Hydroxyl Radicals in MCF-7 S9 Cell Fraction

The formation of hydroxyl radicals by anthraquinones in MCF-7 S9 cell fraction was investigated using a Varian E109 X-band electron spin resonance (esr) spectrometer. at room temp. Anthraquinones were incubated with MCF-7 S9 fraction (1.5 mg) supplemented with NADPH (0.5 mM), desferrioxamine (1 mM) and 100 mM 5,5-dimethyl-1-pyrolline N-oxide (DMPO). The reaction mixture was diluted to a final volume of 1.0 ml using 10 mM Tris-HCl buffer, pH 7.4. transfered to a quartz flat cell and fitted into the cavity of the esr spectrometer at room temperature. Hydroxyl radical formation by the cytotoxic anthraquinones was compared by measuring the signal intensity of the mid-field line of the DMPO-OH adduct spectrum.[12]

Assay for pBR322 Plasmid DNA Strand Breakage

Anthraquinones were incubated in the dark with pBR322 plasmid DNA (0.3 μg) in the presence of NADPH (1 mM) cytochrome P-450 reductase (23 U) and Tris-HCl buffer pH 8.0 in a final volume of 100 μl. The reaction was initiated by the addition of plasmid DNA to the other components, the reactants were then centrifuged for a few seconds to ensure thorough mixing and incubated at 37°C for 30 min. Each reaction was terminated by the addition of NaCl (5.0 M, 100 μl) to facilitate extraction. The drugs were extracted with water saturated n-butanol (200 μl, 3–5 extractions) until no colour could be observed in the aqueous phase. The DNA was precipitated by the addition of 400 μl ethanol (-20°C) and left for 1 h at -20°C. The samples were then centrifuged for 10 min at 12,000 × g in a microfuge to pellet the

DNA and the ethanol supernatant disgarded. Residual ethanol was removed using a glass capillary followed by placing the samples under vacuum for 5 min. The DNA was then reconstituted with 5 μl Tris-EDTA buffer pH 8.0, centrifuging (3 sec) to ensure thorough mixing and incubation at 37°C for 15 min to facilitate dissolution. Drug mediated DNA single strand and double strand breaks were identified by separating the supercoiled plasmid DNA (Form I) from open cicular (form II) and linear (Form III) using submerged agarose gel (1%, in Tris base (24.2%)/glacial acetic acid (5.7%)/EDTA (0.05 M) buffer, TAE) electrophoresis (150 V, 1 h). DNA was visualised by staining with ethidium bromide (2 μg/ml), transilluminating under uv light (300 nm) and the gels photographed (Pelicula Tmax 100 4052 professional film, Kodak) using two orange filters (Wratten orange filter No. 23a) to enhance contrast. The negatives were scanned at 636 nm using a laser densitometer (LKB 2202) linked to an Apple II microcomputer system and LKB "Gel Scan" dedicated software for data capture and analysis of non-Gaussian peaks.

Alkaline Filter Elution of MCF-7 Cell DNA

MCF-7 cells (5×10^6 ml^{-1}) in RPMI-1640 growth medium were incubated at 37°C with the anthraquinones for one hour following which the cells were washed free of drug using ice-cold PBS and placed on ice. The cells were then lysed and the cell DNA subjected to alkaline elution using the procedure described by Kohn (17) using a 0.8 μm (925 mm diameter) polycarbonate membrane filter (Nucleopore, USA) and single stranded DNA eluting solution of 0.02 M EDTA free acid/tetraethylammonium hydroxide (0.1 M), pH 12.3 with a flow rate of 0.035 ml min^{-1}. DNA was quantitated by fluorimetry using Hoechst H33258 (bisbenzamide) dye (excitation 340 nm emission 440 nm).

RESULTS

DNA Damage by Cytotoxic Anthraquinones

The chromophore of the four 9,10-anthraquinones (AQ) mono- or bis- substituted with the -$NH(CH_2)_2NH(CH_2)_2OH$ group in the 1 position (1-AQ), 1 and 4 positions (1,4-AQ), 1 and 5 positions (1,5-AQ), and 1 and 8 positions (1,8-AQ) is shown as an insert to Figure 1. The effect of these anthraquinones on plasmid DNA when activated with NADPH cytochrome P450 reductase is shown in Figure 1. The results demonstrate that 1-AQ, 1,5-AQ and 1,8-AQ at 100 μm produced at least 30% increase in DNA single strand breaks (Form II) in the presence of NADPH P450 reductase compared to a control where the anthraquinones were absent. In comparison 1,4-AQ (100 μM) produced a 10% increase in DNA single strand breaks. In the absence of NADPH P450 reductase all of the anthraquinones produced a 10% increase in plasmid DNA single strand breaks (results not shown) consistent with low-level damage as a result of the binding of these anthraquinones to DNA.[1]

The effect of the anthraquinones on MCF-7 cell DNA was subsequently investigated. Alkaline filter elution of DNA from MCF-7 cells treated with the anthraquinones at 100 μM was used to measure total DNA strand breakage. Figure 2a shows the elution time profile of MCF-7 cell DNA through the filter. This data indicates that a large proportion of the DNA in MCF-7 cells treated with 1-AQ 1,8-AQ and 1,5-AQ is extensively damaged since it is eluted through the filter more rapidly than DNA

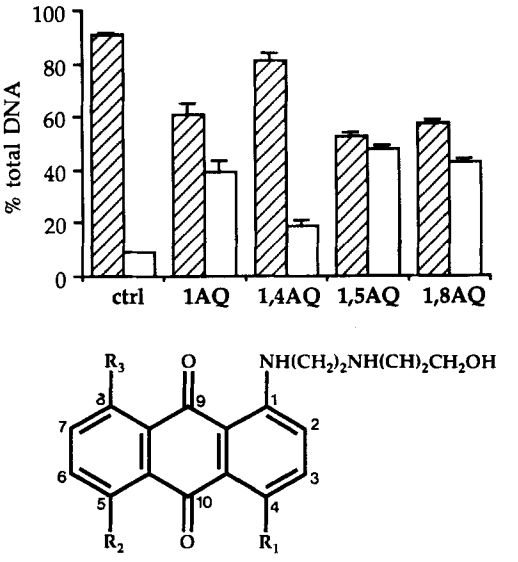

FIGURE 1. Effect of cytotoxic anthraquinones on pBR322 plasmid DNA incubated at 37°C for 30 min. in the presence of NADPH and purified cytochrome P-450 reductase. ctrl = control (without anthraquinones). Results are the mean + s.d. of three determinations. See Methods for experimental detail. -▨- supercoiled DNA (Form I), -□- open circular DNA (single strand break, Form II).

from untreated MCF-7 cells. For cells treated with 1-AQ, 1,8-AQ and 1,5-AQ the majority of the DNA is eluted in the first 200 minutes of the elution profile. In contrast the DNA from MCF-7 cells treated with 1,4-AQ is not significantly different from untreated cells over this same elution time. However the proportion of DNA eluted from the filter for cells treated with 1,4-AQ is greater than for untreated cells over the entire elution time profile (500 minutes). Alkaline filter elution of DNA from MCF-7 cells treated with the anthraquinones at their ID_{50} concentrations is shown in Figure 2b. These data show that at equitoxic concentrations of anthraquinones the DNA elution profiles for all four anthraquinones is very similar. This data also illustrates that the extent of DNA strand breakage at the respective ID_{50} values for 1-AQ (0.06 μM) 1,5-AQ (12.3 μM) and 1,8-AQ (0.5 μM) was significantly less than when measured at 100 μM drug concentration. In contrast DNA strand breakage at the ID_{50} concentration of 1,4-AQ (1.2 μM) was the same as that determined at 100 μM of this drug. All the anthraquinones used in this study had similar uptake profiles into MCF-7 cells (results not shown).

Hydroxyl Radical Formation in MCF-7 Cells

MCF-7 S9 fraction was incubated with the anthraquinones in the presence of DMPO, a hydroxyl radical spin trapping agent. The results in Figure 3 shows an esr spectrum with hyperfine splitting 1:2:2:1 ($A_N = A_H = 14.9$G) characteristic of the DMPO-OH adduct for 1,8-AQ incubated with MCF-7 cell fraction. The addition of catalase, a

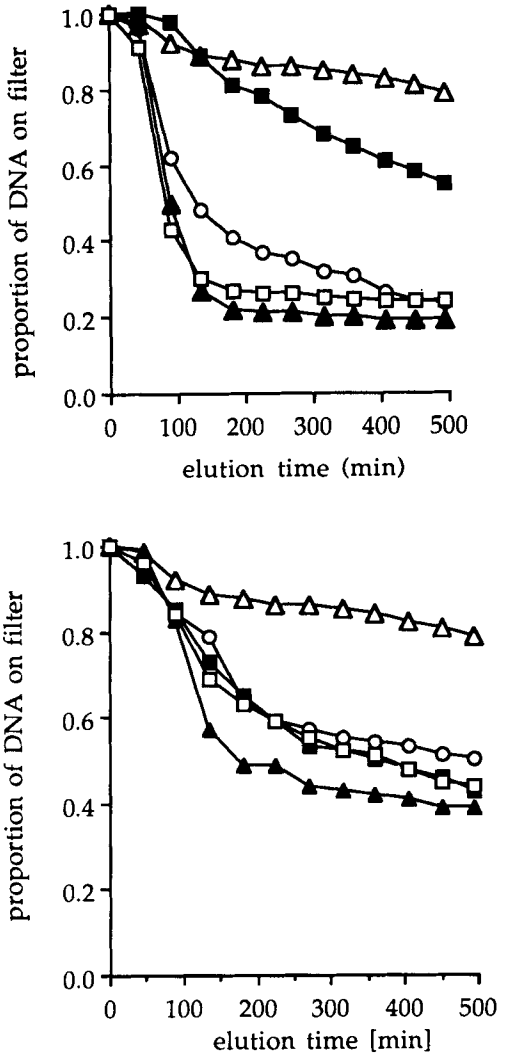

FIGURE 2. Alkaline elution profile of DNA from MCF-7 cells untreated -△-; treated for 1 hour at 37C with 1-AQ -○-; 1,5-AQ -▲-; 1,8-AQ -□- and 1,4-AQ -■- at (a) 100 μM and (b) respective ID_{50} concentrations. See Methods for experimental detail. Results are the mean of three separate determinations.

scavenger of hydrogen peroxide, decreased the intensity of this spectrum whilst the addition of DMSO resulted in an esr spectrum with hyperfine splitting 1:1:1:1:1:1 (A_N = 16.2, A_H = 23.0) consistent with that of DMPO-CH_3. This indicated that the hydroxyl radical was reacting with DMSO to produce a methyl radical that was spin trapped by DMPO. Figure 4 shows the esr signal intensity of one mid-field peak obtained for the DMPO-OH adduct of 1,8-AQ, 1,5-AQ and 1-AQ incubated with MCF-7 S9 fraction. Under these conditions no increase in DMPO-OH was observed in the presence of 1,4-AQ incubated with MCF-7 S9 cell fraction.

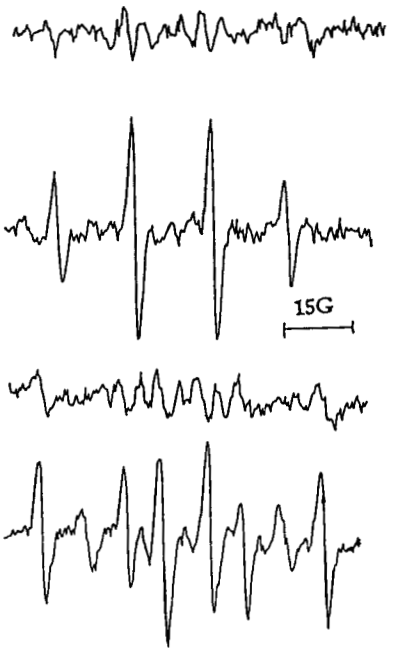

FIGURE 3. Esr spectra of DMPO adducts generated by 1,8-AQ (400 μM) in the presence of NADPH supplemented MCF-7 S9 cell fraction. (A) No drug, (B) DMPO-OH adduct, (C) effect of 300 units of catalase, (D) DMPO-CH_3 adduct in the presence of 200 mM dimethyl sulphoxide. Esr operating conditions were microwave power 10 mW; Magnetic field 9535 G; scan rate 2 min; modulation amplitude 0.5 G; receiver gain 8 $\times$ 10^3; scan range 100 G.

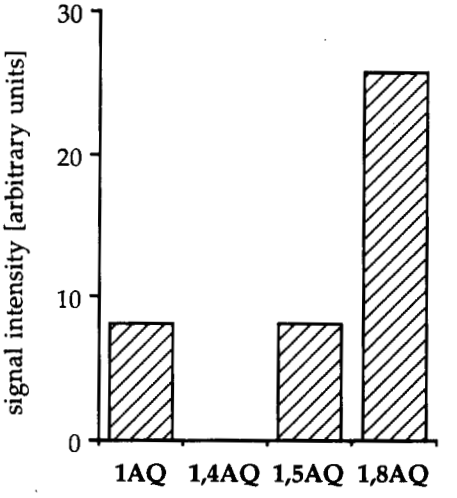

FIGURE 4. Comparison of esr signal intensities of DMPO-OH spin trap generated by cytotoxic anthraquinones incubated with NADPH supplemented MCF-7 S9 cell fraction

DISCUSSION

The anthraquinones used in this study have been prepared in order to investigate the relationship between DNA binding, free radical formation and cytotoxicity in a class of antitumour agent that as exemplified by mitoxantrone has therapeutic potential. The 1,4-AQ most closely resembles mitoxantrone and has undergone clinical evaluation.[13] There is some evidence that the DNA binding affinity of this type of agent is related to their cytotoxicity [1,14] and that such binding is site sequence specific.[2] These anthraquinones have also been shown to be metabolically activated to free radicals by liver microsomes,[5] a rich source of NADPH dependent P-450 reductase. Thus the involvement of free radical mediated DNA damage in the overall cytotoxicity of these anthraquinones was considered.

The results of this study show that 1-AQ, 1,8-AQ and 1,5-AQ are substrates for NADPH dependent cytochrome P-450 reductase and that anthraquinone activation by this enzyme produces single strand breakage in plasmid DNA. This enzyme has previously been identified as important in the metabolic activation of anthraquinones.[7] Spin trapping studies using DMPO alone and in combination with DMSO demonstrated the stimulation of hydroxyl radical formation by 1-AQ, 1,5-AQ and 1,8-AQ when incubated with MCF-7 S9 cell fraction. The inhibitory effect of catalase indicates that the hydrogen peroxide is likely to be the precursor of the hydroxyl radicals observed. This is in support of previous evidence which showed that 1-AQ and 1,8-AQ consume NADPH and stimulate superoxide anion formation in MCF-7 cells.[8] This is presumed to be as a result of a redox cycle in which the anthraquinones shuttle electrons from NADPH to molecular oxygen. However 1,5-AQ did not appear to stimulate superoxide anion production[8] and hence this compound may generate hydroxyl radicals directly via hydrogen peroxide. The formation of hydroxyl radicals from superoxide anions or hydrogen peroxide is likely to result from mechanisms involving both the Haber-Weiss cycle and Fenton reaction and can be catalysed by ferrous ions.[9]

Since 1-AQ and 1,8-AQ were previously shown to generate superoxide anions[8] it is likely that absence of DMPO-OOH that results from spin trapping of the superoxide anion is due to the slow rate of reaction ($10\ M^{-1}\ s^{-1}$) of the superoxide anion with DMPO compared with the hydroxyl radical ($2 \times 10^9\ M^{-1}\ s^{-1}$).[15]

The stimulation of hydroxyl radicals in MCF-7 S9 cell fraction by 1-AQ, 1,5-AQ and 1,8-AQ at 100 μM is consistent with the increase in DNA strand breaks observed in MCF-7 cells incubated with the same concentration of anthraquinones and suggests a causative link between the metabolic activation of these agents and DNA damage. The involvement of metabolic activation in the DNA damaging activity of 1-AQ, 1,5-AQ and 1,8-AQ appears to be drug concentration dependent since there was a decrease in MCF-7 cell DNA damage by these agents when measured at their ID_{50} concentrations compared to 100 μM (compare Figure 2a and 2b).

In contrast 1,4-AQ does not appear to stimulate hydroxyl radical formation in MCF-7 S9 cell fraction and was previously shown not to stimulate superoxide anion generation in this cell fraction.[8] This is consistent with the low level of MCF-7 DNA strand breakage in the presence of 1,4-AQ compared to the other anthraquinones used in this study (see Figure 2). The nature of the MCF-7 cell DNA strand breaks generated by 1,4-AQ is not known since the alkaline elution assay used did not discriminate between frank and protein associated DNA strand breaks. The low level of plasmid DNA strand breakage by 1,4-AQ when incubated with purified NADPH

cytochrome P-450 reductase indicates that this compound is a poor candidate for metabolic activation.

Overall it would appear that DNA binding anthraquinones of the type used in this study are also substrates for NADPH dependent cytochrome P-450 reductase and in cellular systems this activation results in hydroxyl radical formation and an elevation in DNA damage. However the 1,4-AQ appears an exception since hydroxyl radical formation and DNA damage is not stimulated in the presence of this compound. All the anthraquinones used in this study have similar one-electron reduction potentials.[16] This suggests that thermodynamically there is no difference in the ability of this series of anthraquinones to undergo free radical formation. Therefore the lack of activation of 1,4-AQ may reflect steric hinderance by this compound inhibiting its interaction with cellular reductases. In support of this the $NH(CH_2)_2NH(CH_2)_2OH$ substitution pattern of 1,4-AQ is found in mitoxantrone an agent that also is not metabolically activated in MCF-7 cells.[8]

The cytotoxic potential of this series of anthraquinones is not directly related to the extent of hydroxyl radical formation and DNA damage since the 1,4-AQ is more cytotoxic than the 1,5-AQ, a compound shown to be metabolically activated. However since 1-AQ is the most redox active agent and is considerably more cytotoxic than 1,4-AQ it is likely that the metabolic activation of those anthraquinones shown to stimulate hydroxyl radical formation will contribute to their overall cytotoxicity.

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