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REDOX CYCLING IN MCF-7 HUMAN BREAST CANCER CELLS BY ANTITUMOR AGENTS BASED ON MITOZANTRONE

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In a series of hydroxyethylaminoalkylaminoanthraquinones (AQ's) based on mitozantrone, 1-AQ (340%) and 1,8-AQ (137%) stimulated basal rate NADPH oxidation (72 + 18 pmol min^{-1} mg S9 $protein^{-1}$) whilst 1,4-AQ, 1,5-AQ and mitozantrone had no effect. A similar trend was observed for $O_2^{\cdot-}$ generation (measured as nmol acet. cyt c reduction min^{-1} mg $protein^{-1}$) by these compounds in MCF-7 S9 fraction: 1-AQ (9.5) and 1,8-AQ (7.9), whilst 1,5-AQ, 1,4-AQ and mitozantrone showed no significant effect. All the AQ's including mitozantrone were cytotoxic to MCF-7 cells in a dose dependent manner with EC_{50} values as follows: 1-AQ (0.01 μm) > doxorubicin (0.4 μM) > mitozantrone (0.6 μM) > 1,8-AQ (2.0 μM) > 1,5-AQ (4.0 μM) > 1,4-AQ (8,0 μM). Thus the redox active AQ's were also the most cytotoxic. Mitozantrone however was not redox active but was more cytotoxic than all but 1-AQ hence it would appear that factors other than free radical generation contribute to the antitumor activity of this group of compounds.

KEY WORDS: Redox-cycle, anthraquinones, MCF-7 cells, superoxide anions, doxorubicin, cytotoxicity

INTRODUCTION

Mitozantrone is an alkylaminoanthraquinone with antitumor activity against a variety of solid tumours[1] leukaemias[2] and non-Hodgkin's lymphoma.[3] Mitozantrone is broadly based on the anthracycline doxorubicin, the anthraquinone moiety of both

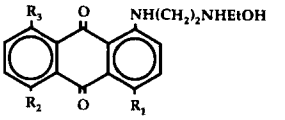

TABLE I
Alkylaminoanthraquinones used in this study

compound	R^1	R^2	R^3
mitozantrone	$NH(Me)_2NHEtOH$	OH	OH
1-AQ	H	H	H
1,4-AQ	$NH(Me)_2NHEtOH$	H	H
1,5-AQ	H	$NH(Me)_2NHEtOH$	H
1,8-AQ	H	H	$NH(Me)_2NHEtOH$

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agents being implicated in their mechanism of cytotoxicity. Such a mechanism has been suggested to involve a redox cycle in which the anthraquinone moiety undergoes NADPH-dependent reductive metabolism to a semiquinone free radical which subsequently generates cell damaging reactive oxygen.[4,5] This study investigates the cytotoxic potential of four alkylaminoanthraquinones (table 1) based on mitozantrone and their propensity for free radical generation in tumour cells. MCF-7 human breast cancer derived cells[6] have been used since studies with doxorubicin have indicated this cell line has the metabolic capability to generate free radicals[7,8] and treatment of advanced breast cancer is a clinical use of mitozantrone.[9]

MATERIALS AND METHODS

Mitozantrone dihydrochloride and doxorubicin were gifts from Cyanamid G.B., Gosport, Hants UK and Farmitalia Carlo Erba, Milano, Italy respectively. The hydroxyethylalkylaminoanthraquinones shown in table 1 were synthesized as previously described.[10] Cell culture materials were purchased from Flow Laboratories, Irvine, Scotland and all other reagents and chemicals from Sigma Chemical Co. Poole, Dorset, UK.

Cell Culture and Cytotoxicity Testing.

MCF-7 cells were cultured using RPMI-1640 medium supplemented with foetal bovine serum (10%) and harvested using EDTA (0.1%) in phosphate buffered saline (pH 7.4). MCF-7 S9 fraction was prepared by centrifugation (9000 × g, 30 min.) of sonicated log phase 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 as described above for 24 hr, after which time the anthraquinones were added at concentrations (given in the text) for a further 24 hr. The drug containing medium was then removed, the monolayer washed with ice-cold phosphate buffered saline, and fresh medium added. The cells were allowed to grow for a further 96 hr. Total remaining cells were counted using a ZB Coulter counter.

NADPH Oxidation and Superoxide Anion Generation.

NADPH oxidation was monitored essentially as described previously.[11] NADPH (0.1 mM) was added to 0.5 mg MCF-7 S9 cell fraction in a final volume of 1 ml phosphate buffered saline (pH 7.4, 100 mM) and the rate of NADPH oxidation monitored at 340nm. Superoxide anion formation was measured in MCF-7 S9 cell fraction (0.1 mg protein ml^{-1}) as superoxide dismutase inhibitable reduction of acetylated cytochrome c essentially as described previously.[11] Both assays were carried out at 37°C in the presence and absence of antitumor agents at concentrations given in the results.

RESULTS

The basal level of NADPH oxidation in MCF-7 S9 cell fraction was stimulated significantly by 1-AQ and 1,8-AQ but not by mitozantrone, 1,5-AQ and 1,4-AQ as

shown in figure 1. The most potent stimulator of NADPH oxidation was 1-AQ (about 5-fold). However by comparison doxorubicin stimulated NADPH oxidation some 12-fold. In this same cell fraction 1-AQ, 1,8-AQ and doxorubicin all stimulated significantly superoxide anion generation whilst mitozantrone, 1,5-AQ, and 1,4-AQ were unable to generate superoxide anions (see Figure 2). The marked stimulation of both NADPH and superoxide anions in MCF-7 S9 fraction by 1-AQ was shown to be dose dependent (Figure 3a & b).

The survival of MCF-7 cells in the presence of increasing concentrations of alkylaminoanthraquinones, mitozantrone and doxorubicin is shown in figure 4a & b. These results show that all the drugs tested were cytotoxic in a dose dependent manner. Using the EC_{50} (effective concentration that kills 50% of cells) as a comparative measure of activity then the decreasing order of cytotoxicity was 1-AQ (0.01 μM) > doxorubicin (0.4 μM > mitozantrone (0.6 μM) > 1,8-AQ (2.0 μM) > 1,5-AQ (4.0 μM) > 1,4-AQ (8.0 μM).

DISCUSSION

The stimulation of NADPH oxidation and concomitant superoxide anion radical formation by doxorubicin provides evidence that this agent can redox cycle in the MCF-7 cell line. This supports previous studies[12] and further implicates reactive oxygen formation in the MCF-7 cell kill activity of doxorubicin.[7,8] Mitozantrone, on the other hand, did not redox cycle in MCF-7 cell fraction. However the ability of

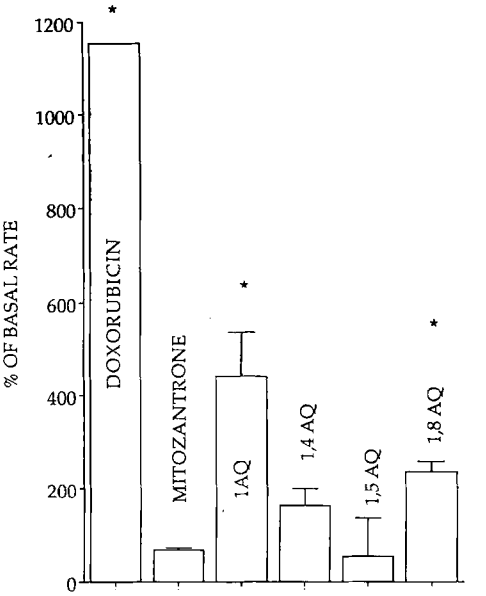

FIGURE 1 Effect of alkylaminoanthraquinones on NADPH oxidation mediated by the S9 fraction prepared from MCF-7 cells.
Results are the percent change over basal rate NADPH oxidation (72 + 18 pmol min^{-1} mg $protein^{-1}$). Error bars represent standard deviation of 3–5 observations. *$p < 0.01$).

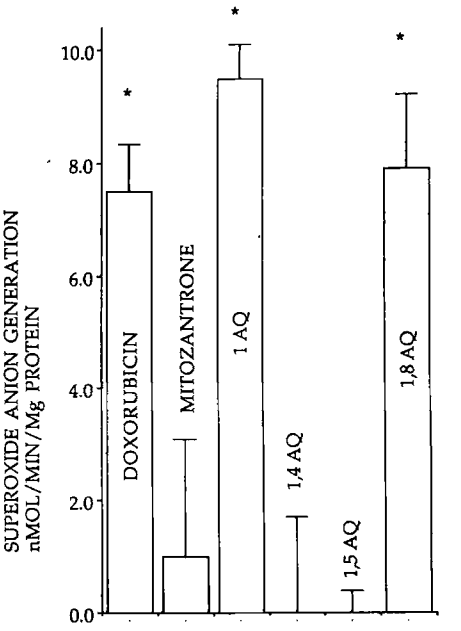

FIGURE 2 Stimulation of superoxide anion radical generation by alkylaminoanthraquinones in S9 fractions prepared from MCF-7 cells.
Superoxide anion radical formation was determined as superoxide dismutase inhibitable reduction of acetylated cytochrome c as described in Methods. Error bars represent standard deviation of 3–5 observations.

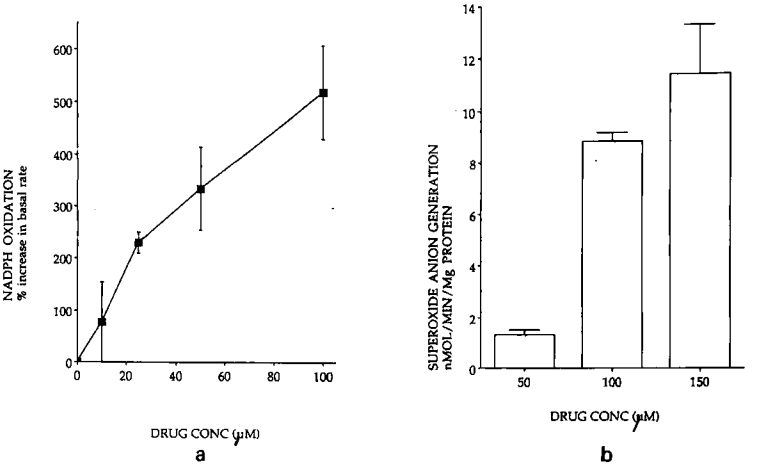

FIGURE 3 Effect of increasing concentration of 1-AQ on (a) NADPH oxidation and (b) superoxide anion radical generation in MCF-7 S9 cell fraction.
Results in (a) are the percent change over basal rate NADPH oxidation (72 + 18 pmol/min/mg protein) and in (b) superoxide anion radical formation was determined as superoxide dismutase inhibitable reduction of acetylated cytochrome c as described in Methods. Error bars represent standard deviation of 3–5 observations.

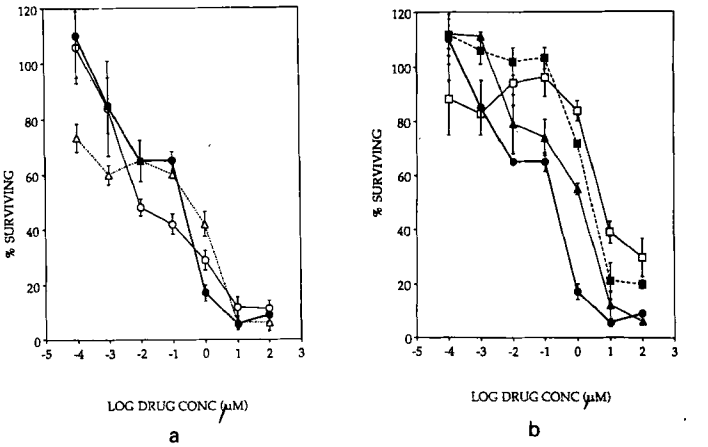

FIGURE 4 Cytotoxicity of alkylaminoanthraquinones to MCF-7 cells. Log phase cells were treated with alkylaminoanthraquinones for 24 hours, washed and allowed to grow for a further 96 hours. Remaining monolayer cells were then counted.
Figure 4a compares 1-AQ (○); mitozantrone (△) and doxorubicin (●). Figure 4b compares 1,4-AQ (□); 1,5-AQ (■); 1,8-AQ (▲) and doxorubicin (●).

mitozantrone to generate low levels of superoxide anions has been shown in human liver[5] which contains a rich source of NAD(P)H dependent enzymes responsible for the metabolic reduction of doxorubicin and mitozantrone.[13] By comparison reductases in MCF-7 cells, as measured by the basal rate of NADPH oxidation, is 10-100 fold less than human liver. This may explain the absence of detectable redox cycling by mitozantrone, a drug which is already known to be a poor substrate for flavin based enzymes.[13]

The 1-AQ and 1,8-AQ both stimulated NADPH oxidation and superoxide anion formation in the MCF-7 cell fraction indicating that these compounds were redox active. In contrast 1,4-AQ and 1,5-AQ did not redox cycle. The one electron reduction potential at pH 7.0 (E_7) of the alkylaminoanthraquinones is between -469 and -497 mV and -527 mV for mitozantrone.[14] Thus, thermodynamically there is no substantial difference in the ability of these agents to form free radical intermediates. In addition, the reduction potentials for all these compounds are sufficiently negative to ensure reduction of oxygen ($E_7 = -0.155$ mV, for 1M oxygen) to the superoxide anion radical especially at the low drug concentrations likely to be present in cell systems. It is likely, therefore, that this marked difference in redox activity is a reflection of the rate of interaction of these compounds with flavin based reductases responsible for one electron reduction of quinones. In this way the positions of the alkylamino-side groups of 1,4-AQ, 1,5-AQ and mitozantrone may sterically hinder the interaction of the anthraquinone chromophore with the flavin component of reductases in a way that does not occur with 1-AQ and 1,8-AQ. Such an interaction has been suggested as important in the transfer of electrons from reductase to doxorubicin.[15]

The alkylaminoanthraquinones including mitozantrone were all shown to be cytotoxic to MCF-7 cells in a dose dependent manner. In particular 1-AQ and 1,8-AQ were more cytotoxic (as measured by EC_{50} determination) than 1,5-AQ and 1,4-AQ. This indicates that redox cycling may contribute to the cytotoxicity of some alkyl-

aminoanthraquinones. However other factors such as DNA binding[10] are likely to contribute to the cytotoxicity observed since mitozantrone, which was shown not to redox cycle, was more active than all but 1-AQ.

Acknowledgements

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