Biochemical Pharmacology, Vol. 40, No. 9, pp. 2069–2078, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

MITOXANTRONE–DNA BINDING AND THE INDUCTION OF TOPOISOMERASE II ASSOCIATED DNA DAMAGE IN MULTI-DRUG RESISTANT SMALL CELL LUNG CANCER CELLS

PAUL J. SMITH,* SALLY A. MORGAN, MARY E. FOX and JAMES V. WATSON
MRC Clinical Oncology and Radiotherapeutics Unit, MRC Centre, Cambridge CB2 2QH, U.K.

(*Received* 17 *May* 1990; *accepted* 29 *June* 1990)

Abstract—The cytotoxicity anti-tumour intercalating agents such as the anthraquinone mitoxantrone is thought to relate to DNA binding and the trapping of DNA topoisomerase II complexes on cellular DNA. We have studied the uptake, nuclear location, DNA binding mode and DNA damaging capacity of mitoxantrone in a small cell lung carcinoma cell line (NCI-H69) compared with an *in vitro*-derived variant subline (NCI-H69/LX4) that exhibits "classical" multi-drug resistance (MDR). Variant cells maintained under doxorubicin selection showed reduced RNA levels that returned to control values within 7 days of growth under non-selective conditions. Variant cells released from selection stress showed resistance to DNA cleavage by doxorubicin, mitoxantrone, 4′-epidoxorubicin, 4′-deoxy-doxorubicin but reduced resistance to aclacinomycin A and a 9-alkyl substituted anthracycline in broad agreement with the cross-resistance patterns for cytotoxicity. Mitoxantrone treated NCI-H69 cells were found to accumulate DNA–protein crosslinks during a 4 hr post-treatment incubation period whereas variant cells maintained depressed levels of crosslinking. There was no apparent abnormality in the availability or drug sensitivity of topoisomerase II assayed in crude nuclear extracts of NCI-H69/LX4 cells. Whole cell uptake of radiolabelled mitoxantrone was depressed (50%) in NCI-H69/LX4 compared with NCI-H69, whereas assessment of nuclear-bound drug in individual cells by a fluorescence quenching technique showed at least a 10-fold greater level of target protection. The quenching results provide evidence of a high affinity, saturable mode of drug binding, favoured at low drug concentrations, that correlated with DNA cleavage capacity. We propose that the cytotoxic action of mitoxantrone is dependent upon a restricted and persistent form of binding to DNA that favours the long-term or progressive trapping of topoisomerase II complexes.

DNA intercalating agents [1] have gained widespread use as anti-tumour agents where cytotoxicity is thought to relate to the induction of DNA damage or the disturbance of nucleic acid metabolism [2, 3]. The bis(alkylamino)anthraquinone mitoxantrone is related structurally to the DNA intercalating anthracycline doxorubicin (Adriamycin®) and shows activity against advanced breast cancer and the acute leukaemias [4]. Mitoxantrone displays an ability to bind to DNA by at least partial intercalation between stacked bases with evidence of a secondary mode of binding involving association with the anionic exterior of the helix [5, 6]. The mode of binding to DNA is probably an important factor in the ability of intercalators to distort the helix and disturb DNA–protein interactions [7].

Recently, it has been recognized that several classes of anti-tumour DNA intercalating agents, including the anthracyclines and the anthraquinones, have the capacity to stabilize "cleavable complexes" between DNA and the nuclear enzyme DNA topoisomerase II [3, 8, 9]. Although there is increasing evidence that complex formation is responsible for the anti-tumour activity of these drugs [3, 9], cytotoxicity may additionally involve membrane effects [10] and free-radical damage to DNA [11]. Nuclear location of the drug [12] and DNA strand cleavage [13], dictated at least in part by intracellular availability, appear to be critical features in determining cytotoxicity of the anthracene anti-cancer drugs.

Mammalian cells can actively modify the availability of drugs, such as mitoxantrone and doxorubicin, for interaction with nuclear targets by the expression of the multi-drug resistance (MDR) phenotype. "Classical" MDR involves the enhanced efflux of drug molecules via a P-glycoprotein-dependent pump located in the plasma membrane [14, 15]. Rapid drug efflux in MDR reduces the intracellular concentration of drug molecules, effectively protecting sensitive targets such as nuclear DNA and DNA topoisomerases from drug attack. However, the degree of resistance does not always correlate with the extent to which intracellular accumulation of drug is depressed [16]. The availability [17] or intrinsic drug sensitivity [9] of a nuclear target such as topoisomerase II may be an additional factor in determining the sensitivity of a cell to an anti-tumour DNA intercalating agent. Here we have studied the effects of the MDR phenotype on the DNA binding and damaging potential of mitoxantrone in intact human cells. To monitor the mode of DNA–mitoxantrone interaction with cellular DNA we have utilized the ability of intercalating agents to compete

* To whom correspondence should be addressed.

with the UV-excitable DNA-specific fluorochrome Hoechst 33342 for binding sites on nuclear DNA [18]. A novel feature of this study is the application of spectral analysis [19–22] of Hoechst 33342 fluorescence in single cells, as monitored by flow cytometry, to provide preliminary information on the nuclear binding characteristics of mitoxantrone.

The study compares the sensitivity to intercalator-induced DNA damage of a classical small cell lung carcinoma (SCLC) cell line with that of an *in vitro* derived multi-drug resistant variant [16, 23] with particular reference to the role of mitoxantrone-DNA binding.

MATERIALS AND METHODS

Cell culture. The four established (SCLC) classical cell lines used in this study were: NCI-H69 (referred to as H69; kindly donated by Dr D. N. Carney, NDI, Bethesda, MD, U.S.A.), and three lines (POC, FRE and MAR) generously donated by Dr M. Ellison (Ludwig Institute, Sutton, U.K.). The doxorubicin-resistant variant of NCI-H69 (designated NCI-H69/LX4 and referred to as LX4) was maintained under selective conditions in the presence of 0.4 μg doxorubicin/mL. The variant cell line exhibits the classical multidrug resistance phenotype including overexpression of P-glycoprotein [16, 20, 23].

Drug preparations and treatments. Hoechst dye No. 33342 (CP Laboratories, Bishop's Stortford, U.K.) solutions were prepared as previously described [19, 20]. Mitoxantrone, (1,4-dihydroxy-5, 8-bis((2-[(2-hydroxyethyl)amino] ethyl)amino))-9, 10-anthracenedione dihydrochloride [Novantrone], was kindly supplied by Dr A. Man (Lederle Laboratories, Gosport, U.K.) as a solid and stored as aqueous stock solutions of 1 mg/mL at −20°. Radiolabelled mitoxantrone was provided by Lederle Laboratories with an estimated specific activity of 7 Ci/mmol. We are grateful for the gifts of the following compounds: 4′-epidoxorubicin and 4′-deoxydoxorubicin from Farmitalia (Milan, Italy), Ro 31-1215 from Roche Products Ltd (Welwyn Garden City, U.K.) and aclacinomycin A from Lundbeck Ltd (Luton, U.K.). All drugs except aclacinomycin A were stored as aqueous stock solutions at −20°. Aclacinomycin A was dissolved in 0.1% propylene glycol. For all drug treatments, cells from exponentially growing cultures were resuspended in fresh growth medium 4 hr prior to drug additions. For acute drug exposures, cells at 5×10^5/mL were treated for 1 hr at 37° prior to washing twice in cold PBS and further manipulation.

Growth curves. Drug toxicity measurements were carried out on exponentially growing cultures. Cells were inoculated into 96-well (flat bottom) cell culture-quality microtitre plates at densities of 1×10^5 cells/mL in growth medium (total volume of 300 μL/well) containing serial dilutions of mitoxantrone. Plates were incubated for 6 days under normal conditions and all densities determined using a Coulter particle counter for protease-disaggregated cell suspensions (1 mg/mL neutral protease and 20 min incubation at 37°).

Radiolabelling studies. Exponentially growing cultures were diluted to 2×10^5 cells/mL in fresh culture medium and 1 mL volumes in Eppendorf vials treated with a range of [^{3}H]mitoxantrone drug concentrations for 1 hr at 37°. Cells were washed three times with cold PBS using short centrifugations (3 sec) in an Eppendorf bench microfuge. Cell pellets were lysed in 1 N NaOH and neutralized with 1 N HCl prior to removing samples for liquid scintillation counting.

DNA damage assay. DNA strand breaks (comprising frank and protein-associated breaks) were measured by an adaptation [24] of the fluorometric method described by Kanter and Schwartz [25] involving the time-dependent partial unwinding of cellular DNA in alkaline solutions. Cells were resuspended in LS buffer (see below; 5×10^5 cells/mL) and distributed in 0.5 mL volumes into glass tubes for the determination of unwinding rates (for a period of 60 min at ice temperature) in quadruplicate as described previously [24]. DNA strand breakage rates were expressed as radiation equivalents (Gy) by calibration with X-irradiated cells (SCLC cells gave 4.25 F units/Gy; assume 2.7 strand breaks/10^{10} daltons mol. wt/Gy; [24]).

Chromatin compactness. This method detects changes in the compactness of residual nuclear structures (i.e. nucleoids) obtained by exposure of cells to non-ionic detergent and high salt conditions. The current version of the technique is essentially that described previously [24]. Briefly, drug-treated or control cells were resuspended in cold PBS (50 μL containing 2.5×10^5 cells) and deposited into 150 μL of lysis buffer [giving a final concentration of 2 mM EDTA, 0.5% (v/v) Triton X-100, 100 mM Tris-(hydroxymethyl)-aminomethane pH 8.0 and 1 M NaCl], over 3.8 mL 15–30% linear sucrose gradients containing 1 mM EDTA and 10 mM Tris-(hydroxymethyl)-aminomethane pH 8.0 and 1 M NaCl. Cells were lysed on top of the gradients for 30 min at room temperature and then centrifuged for 20 min at 25,000 rpm in an MSE Superspeed 65 ultracentrifuge using a 6 × 4.2 mL swing-out rotor. The sucrose gradients contained 1 μM Hoechst 33342 (a non-intercalating DNA-specific fluorochrome) for the direct determination of the relative (vs control) distance sedimented by the nucleoids visualized as a band using near-UV illumination. Factors that affect nucleoid sedimentation rate have been discussed previously [26].

K-SDS precipitation of protein–DNA complexes. The method used was essentially a modification [27] of the method described by Rowe *et al.* [28]. Briefly, cells were labelled with 1.85×10^{-3} MBq/mL [^{14}C]thymidine (sp. act. 1.92 GBq/mmol) for 48 hr, followed by a 4 hr chase period in fresh medium. After a 1 hr exposure to drug, cells were either washed twice with PBS and frozen as centrifuged pellets of 1×10^5 cells/sample or incubated in fresh medium for various recovery periods before freezing. The frozen cells, in samples containing 1.2×10^5 cells, were lysed in SDS prior to precipitation of protein–DNA complexes with KCl. These precipitates were washed three times and their radioactivity expressed as a percentage of the total radioactivity in the lysate.

Filter binding assay for DNA–protein crosslinking. The assay is essentially that of Minford *et al.* [29]

except that linear pBR322 plasmid DNA (3'-recessed ends radiolabelled with [α-^{35}S]dATP) was used as the substrate for drug-dependent DNA–protein crosslinking as described previously [17]. Briefly, crude nuclear protein extracts were prepared from permeabilized cells by treatment with 350 mM NaCl for 20 min. The extracts were used in a filter binding assay in 200 μL reaction volumes containing 15 ng radio-labelled pBR322 DNA and mitoxantrone. Crosslinking was determined by retention of DNA on polyvinyl chloride filters as measured by scintillation counting.

Flow cytometric analysis of cellular DNA and RNA content. Simultaneous measurements of RNA and DNA in unfixed, acid/detergent-treated cells stained with acridine orange was achieved using the flow cytometric method described previously [30]. The red (>630 nm) and green (520 mm) fluorescence emissions from each cell were measured simultaneously to yield RNA and DNA estimations respectively. Light scatter at 90° was also measured for the exclusion of cell debris and the estimation of cell size. For each sample 10^4 cells were analysed.

Flow cytometric analysis of mitoxantrone–DNA interaction by Ho33342–DNA. After the treatment of cultures with mitoxantrone, washed cells were permeabilized by freeze-thawing using the method described previously [19]. Cells were resuspended in LS buffer (10 mM Tris–HCl, pH 8; 100 mM NaCl; 10 mM EDTA; 1 mg/mL bovine serum albumin) and diluted to 2.5×10^5 cells/mL containing 5 μM Ho33342 prior to flow cytometric analyses. Samples were analysed using a flow cytometer [19], incorporating an Innova 3000K krypton laser (Coherent Corp., Palo Alto, CA, U.S.A.) tuned to the 337 line at a light power of 200 mW to excite Ho33342–DNA fluorescence. The emission spectrum of Ho33342 stained DNA extends over a range of 400 to 600 nm. The optical analysis system at 90° to the intersection of the laser beam with the cell stream included a series of dichroic mirrors and filters to analyse fluorescence at 490 and 600 nm (each ± 5 nm) wavelengths representing violet and red light, respectively. Forward and 90° scatter were also analysed for the identification of cell debris. Four parameters namely, 90° and forward scatter pulse width, and pulse area for the two fluorescence channels were then written to disc as a fully cross-correlated data file for subsequent display and analysis. Median fluorescence values were calculated for specified population distributions.

RESULTS

Cross-resistance to DNA strand cleavage by intercalating agents

Previous studies [16, 31] have demonstrated that H69 cells accumulate doxorubicin (1 hr exposure) in a dose-dependent manner for drug concentrations of up to 12 μg/mL. Table 1 shows that within this high dose range, doxorubicin-induced DNA strand cleavage is also dose-dependent and that the multi-drug resistant variant LX4 shows reduced cleavage consistent with the previously observed reduction in whole cell uptake of doxorubicin [16, 31].

We have also determined whether sensitivity to strand cleavage correlates with cross resistance for other related drugs (Table 1; Fig. 1; [32, 33]). The drugs ranked in the order of doxorubicin < 4'-epi-doxorubicin < 4' deoxy-doxorubicin < aclacinomycin A < Ro 31-1215 in terms of the ability of high doses to induce strand cleavage in parental cells. These differences can be expected due to variations in the rate of cellular uptake in addition to any intrinsic differences in the capacity to induce DNA damage. The LX4 cell line, known to show cross-resistance to the cytotoxic effects of 4'-epi-doxorubicin and 4'-deoxy-doxorubicin, also demonstrated resistance at the level of drug-induced strand cleavage (Table 1). Furthermore, LX4 was found to be less resistant to strand cleavage induced by the related compounds aclacinomycin A and Ro 31-1215 (Fig. 1) in keeping with the reduced cross-resistance for cytotoxicity. Some variability was noted in the levels of drug-induced DNA damage detected on different occasions although the relative patterns, for doxorubicin vs analogue or H69 vs variant, were consistent. However, there was no clear correlation between the magnitude of resistance measured by cytotoxicity, measured at low doses, and the initial levels of DNA strand cleavage measured at high doses of drugs typically used in cellular uptake experiments. Thus although the general patterns for intercalator-induced DNA strand cleavage were in broad agreement with the cross-resistance patterns observed for cytotoxicity [33] it is clear that additional factors may determine the lethality of an intercalator such as the dose dependency for nuclear drug accumulation/damage induction, the nature of the drug–DNA interaction, the form of genomic lesions induced and their persistence. We have exploited this *in vitro* human biological system to examine the roles of the above factors in the cytotoxic action of the anthraquinine drug mitoxantrone.

Characteristics of mitoxantrone resistance

The ability of mitoxantrone to induce lesions detected as DNA strand breaks in H69 cells was found to be dose dependent (Fig. 2) with an initial highly sensitive response (up to approx. 1 μg/mL × 1 hr), an extensive plateau and evidence of inhibition of DNA lesion induction at high doses (>5 μg/mL × 1 hr). The initial, low dose reponse of H69 cells to mitoxantrone was observed with other SCLC cell lines (Fig. 2).

LX4 cells assayed 24 hr after removal from selective conditions (growth in 0.4 mg doxorubicin/mL) showed a high degree of resistance to mitoxantrone induced DNA damage (e.g. >7.5 and <0.5 Gy X-radiation equivalents at 0.5 μg/mL for H69 and LX4 cells, respectively). Resistance to mitoxantrone-induced DNA damage was reduced by at least 50% following adaptation to growth in non-selective medium for 7 days (Fig. 2). Figure 2 demonstrates that culture adaptation was accompanied by changes in RNA content of cells, suggesting that cells maintained under selection are metabolically stressed. The contour plots (Fig. 3) represent flow cytometric analyses of relative DNA vs RNA contents of H69 and LX4 cells and their light scatter characteristics (cell size). DNA contents, as a function of cell cycle position and cell size, were found to be similar for

Table 1. DNA intercalator-induced DNA damage in SCLC cell lines

Drug	Drug-induced DNA strand cleavage (X-radiation equivalents; Gy)* H69 2.5 μg/mL‡	H69 5 μg/mL‡	LX4 5 μg/mL‡	RF† cytotoxicity
Doxorubicin (DOX)	1.76 ± 0.73	3.22 ± 0.87	1.95 ± 0.56	97
4'Epi-DOX	3.32 ± 0.66	3.91 ± 0.75	0.89 ± 0.16	528
4'-Deoxy-DOX	5.13 ± 1.41	6.87 ± 1.62	0.94 ± 0.21	64
Aclacinomycin	5.69 ± 1.62	10.61 ± 1.13	7.36 ± 1.08	6
Ro 31-1215	8.31 ± 1.72	10.75 ± 0.78	9.95 ± 2.31	12

* See Materials and Methods for details; LX4 cultures removed from doxorubicin selection for 7 days.

† RF = resistance factor; quoted in Ref. 33; calculated as the ratio (i.e. LX4/H69) of 50% inhibitory doses for the cell lines determined by a tetrazolium (MTT) dye assay for cytotoxity.

‡ One hour exposure.

Data represent arithmetic means (±SE) of values from four independent experiments.

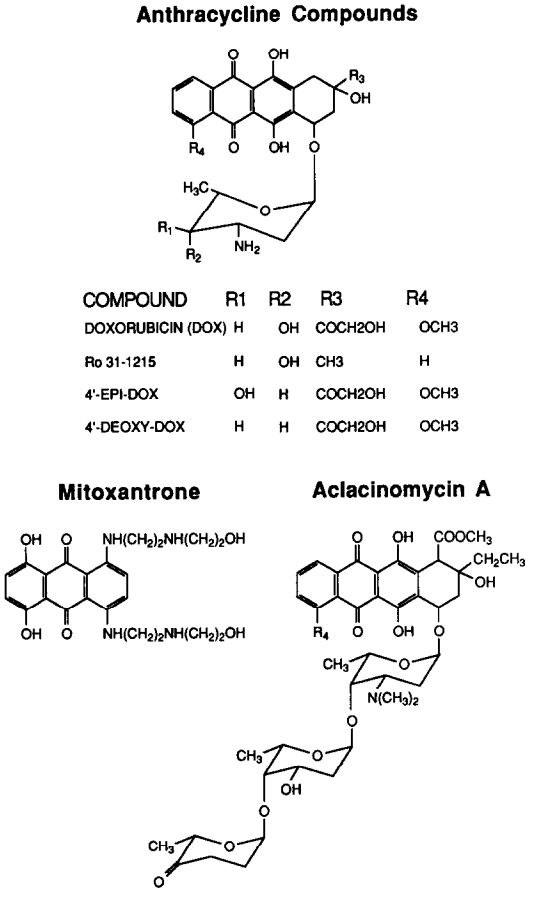

Fig. 1. Structures of anti-tumour DNA intercalators used in study.

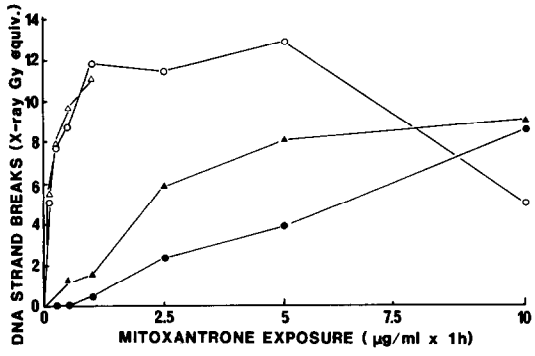

Fig. 2. Induction of DNA strand breakage (assayed under alkaline conditions) in SCLC cells by mitoxantrone. Symbols: (○) H69; (△) mean response of three classical SCLC cell lines (POC, FRE and MAR); (●, ▲) LX4 cells assayed immediately and 7 days after removal from ADM selection, respectively.

H69 and LX4 cells. The LX4 cell line showed a depressed RNA content (approx. 80% of the H69 value) for cells throughout the cell cycle when maintained under selective conditions in doxorubicin. However, growth of LX4 cells for 7 days in normal growth medium results in the establishment of RNA levels similar to H69 cells. Normal culture-adapted LX4 cells retain a high level of resistance to mitoxantrone induced cytotoxicity (Fig. 4: 50% growth inhibition at 0.5 and 50 ng/mL for H69 and LX4 cells, respectively). The removal of variant cells from doxorubicin selection for 7 days was adopted as a standard method of handling cultures prior to experimental manipulation.

DNA–protein crosslinking studies

Use of the K-SDS precipitation method to measure DNA–protein crosslinking (Table 2) showed that acute exposure to mitoxantrone increased the background level of 1.7% crosslinking to 3.8% (at 0.52 mg/mL) in H69-P cells when assayed at the end of the drug treatment period. The absolute levels of crosslinking for a given dose of mitoxantrone are lower in the resistance cell line than in the parental line. Significantly, the levels of complexes induced

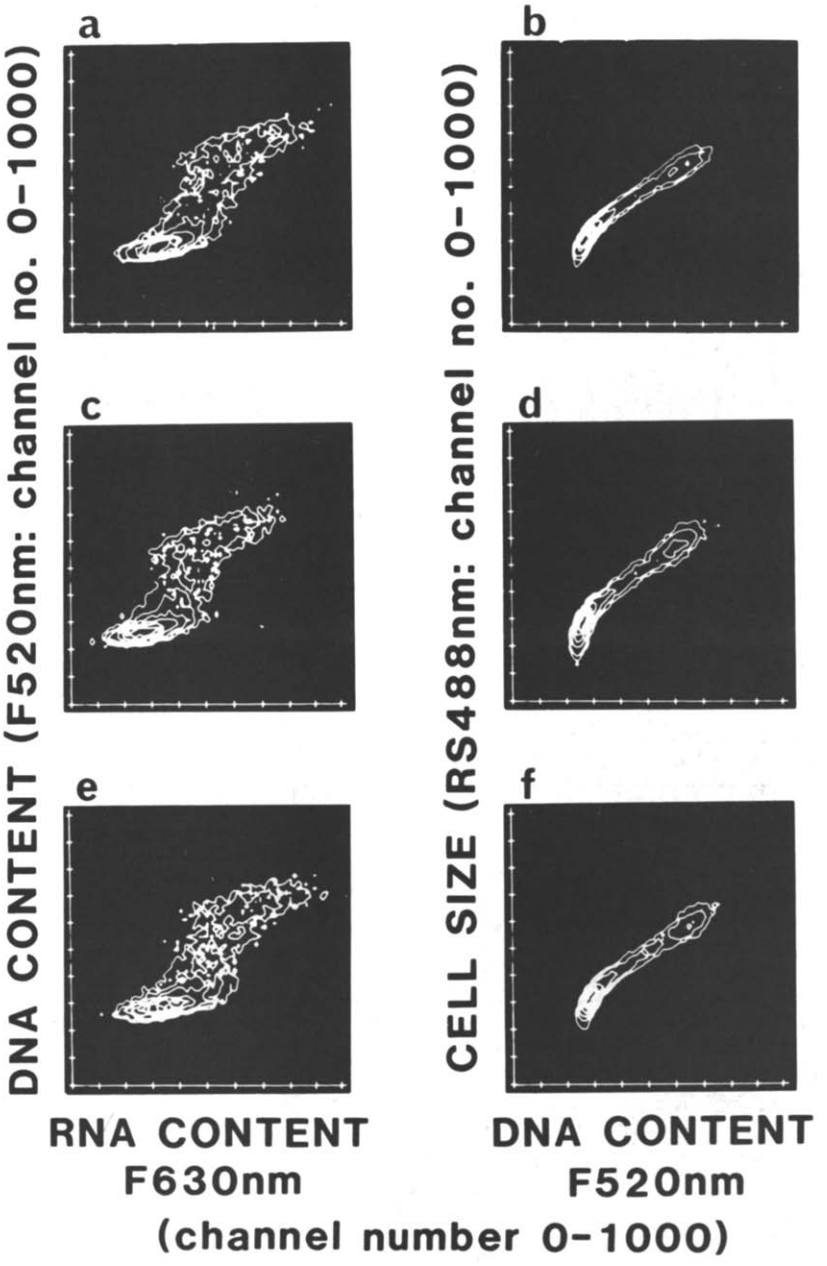

Fig. 3. Flow cytometric analysis of DNA vs RNA contents (panels a, c and e) and DNA vs cell size (panels b, d and f) for SCLC cells. Contour plots represent: H69 cells (panels a and b); LX4 cells maintained under ADM selection (panels c and d); LX4 cells grown in non-selective medium for 7 days (panels c and f).

in the parental cell line (H69-P) increased up to two-fold during a 4 hr post-treatment period whereas the levels in the resistant line decreased slightly.

Reduced levels of mitoxantrone induced DNA damage could be associated with reduced levels or abnormal drug sensitivity of DNA topoisomerase II in the LX4 cells. However, crude nuclear extracts from H69-P and LX4 cells showed similar levels of mitoxantrone-dependent crosslinking activities (Fig. 5). Furthermore, expected inhibition of crosslinking at high drug concentrations was observed for both sources of extracts. We conclude that LX4 cells show normal expression of the target enzyme, reduced intranuclear protein trapping by mitoxantrone and a distinct lack of progressive accumulation of complexes during post treatment incubation.

Nuclear binding studies

The neutral nucleoid sedimentation method monitors changes in compaction of partially de-proteinized chromatin bodies following DNA strand breakage (decreased nucleoid sedimentation) or topological changes in DNA [26]. This method does not detect topoisomerase–DNA crosslinking [34] and

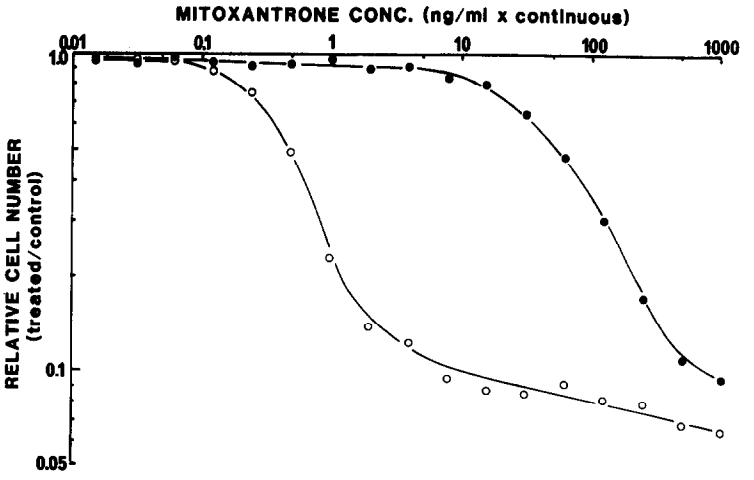

Fig. 4. Relative inhibition of SCLC cell growth by mitoxantrone (6 day exposure). Symbols: (○) H69; (●) LX4.

Table 2. Persistence of mitoxantrone induced DNA–protein crosslinks in SCLC cells

Mitoxantrone concn. (μg/mL × 1 hr)	Post-treatment incubation period (hr)	DNA-protein crosslinking (% total DNA)*	
		H69	LX4
0	0	1.70 ± 0.28	1.16 ± 0.15
0.26	0	2.44 ± 0.10	1.30 ± 0.13
0.52	0	3.83 ± 0.26	2.59 ± 0.17
0	2	1.57 ± 0.14	1.19 ± 0.07
0.52	2	4.82 ± 0.23	2.39 ± 0.17
0	4	1.78 ± 0.12	1.38 ± 0.10
0.52	4	7.72 ± 0.12	2.51 ± 0.11

* Measurement of [^{14}C]DNA complexes as per cent total [^{14}C]DNA precipitated in a potassium/SDS containing solution (see Materials and Methods). Values are means of six determinations (±SE).

Table 3. Velocity sedimentation characteristics of nucleoids from drug treated SCLC cells

Cell line	Relative distance sedimented following 1 hr exposure to 5 μg drug/mL		
	Control	Doxorubicin	Mitoxantrone
H69	Set at 1.0	1.38	2.04
LX4	1.15	1.06	1.0

typical data shown in Table 3 indicate that doxorubicin or mitoxantrone treatment of H69 cells does not generate significant levels of frank DNA breaks, indeed nucleoid sedimentation was increased, suggesting a direct effect of drug binding on nucleoid compaction. LX4 cells were essentially resistant to the effects of either agent indicative of reduced DNA–drug interaction.

The DNA damage and nucleoid studies suggest that LX4 shows a reduced potential for drug–DNA interaction consistent with reduced drug uptake. Whole cell measurements for mitoxantrone uptake (Fig. 6) show that the differences between LX4 and H69 cells are not significant at doses >2.5 μg/mL. A maximum depression of drug uptake in LX4 cells, compared with H69 cells, of 50% was observed in the low dose range of 0.1 to 1 μg/mL. A flow cytometric technique was used to monitor the levels of DNA–

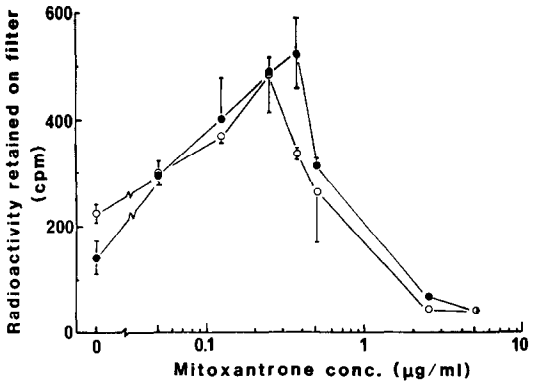

Fig. 5. Mitoxantrone concentration dependence of DNA–protein crosslinking activities in nuclear protein extracts derived from H69 (○) or LX4 (●) cultures.

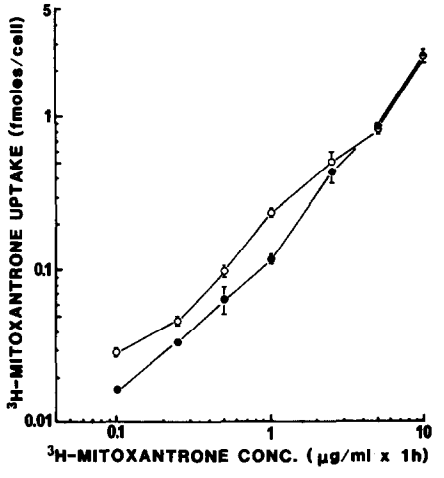

Fig. 6. Cellular uptake of radiolabelled mitoxantrone by H69 (○) and LX4 (●).

drug interaction by the quenching of fluorescence of the DNA specific ligand Ho33342. Since multi-drug resistant cells (including LX4; [20]) are resistant to the uptake of Ho33342 as a vital stain, the ligand was administered to freeze–thaw permeabilized cells derived from control or mitoxantrone treated cultures. Fluorescence quenching was determined for violet and red regions of the emission spectrum. Microscopy indicated that fluorescence was nuclear located. The kinetics of staining and rate of spectral shift were consistent with the nuclei being freely accessible to and in equilibrium with the Ho33342 dye (data not shown). The use of permeabilized cells reduces any potential interference of the nuclear fluorescence signals by cytoplasmic pools of ligand. The results (Fig. 7a and b) show that the degree of quenching in H69 nuclei was dependent on the mitoxantrone dose and the wavelength monitored, with a saturation of quenching potential occurring for the longer wavelength emissions (Fig. 7b). LX4 cells showed significant resistance for fluorescence quenching with the maximal effect being observed for the violet region of the emission spectrum (Fig. 7a). For mitoxantrone doses of up to 1 μg/mL, the dose modification factors for generating similar levels of fluorescence quenching in the two cell types were approx. 6 and >25 for the violet and red regions respectively.

DISCUSSION

Previous studies have indicated that the doxorubicin resistant LX4 cell line demonstrates the cellular and molecular hallmarks of classical MDR and here we show that there is no indication of an abnormality in the nuclear target enzyme DNA topoisomerase II. The present study also reveals that cross-resistance of LX4 to mitoxantrone and other DNA intercalators is seen at the level of DNA damage induction. Importantly, the MDR phenotype appears to limit the nuclear binding of mitoxantrone to an extent which is greater than would be predicted from whole cell uptake estimates suggesting an additional degree of nuclear target protection afforded by the expression of MDR. This protection is commensurate with the high level of resistance to DNA strand cleavage and cytotoxicity. The induction of nuclear DNA cleavage (detected under alkaline conditions) by mitoxantrone closely followed the dose-dependent formation of a mode of DNA binding that could efficiently quench the red region of the fluorescence emission spectrum of bound Ho33342 reporter molecules. We propose that mitoxantrone induces biologically important DNA damage by a persistent and perhaps progressive ability to trap topoisomerase II on cellular DNA and that a saturable mode of drug binding may participate in complex trapping.

Maintenance of the variant cell cultures under doxorubicin selection reduced cellular RNA levels suggesting that the cells were under metabolic stress. Experimental manipulations were carried out on cultures that had been allowed to recover normal RNA levels so that any potential differences between the two cell lines could not be attributed to differences in capacity to elaborate potential protein targets. The re-establishment of control levels of RNA in LX4 was accompanied by at least a two-fold increase in sensitivity to mitoxantrone-induced DNA cleavage. Culture-adapted LX4 cells showed similar cellular characteristics (cell size, growth rate, DNA and gross RNA contents) as those of the parental cell line but retained a high level of cross-resistance to the cytotoxic and DNA damaging action of mitoxantrone.

Increased nucleoid sedimentation was observed for doxorubicin or mitoxantrone treated H69 cells indicating a predominant effect on chromatin compaction. Compaction may relate to the retention of nuclear proteins in mitoxantrone treated cells [7]. In a previous study [13], nucleoids of mouse L1210 cells did not show mitoxantrone-induced changes in compaction suggesting that cell type differences may exist. The compactness of LX4 nucleoids was not affected by mitoxantrone treatment of intact cells indicating reduced drug–DNA interaction. The neutral nucleoid sedimentation technique is sensitive to overt strand scissions at levels equivalent to less than 0.25 Gy X-radiation ([24]; PJS unpublished data)

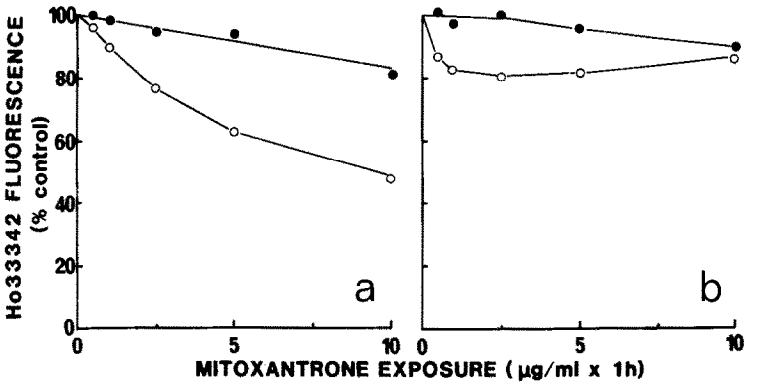

Fig. 7. Quenching of Ho33342–DNA fluorescence as a function of mitoxantrone treatment of H69 (○) or LX4 (●) SCLC cells. Panels (a) and (b) show data for fluorescence emissions monitored at 490 or 630 nm wavelengths, respectively.

whereas mitoxantrone (at 5 μg/mL) induced strand cleavage in LX4 cells at levels greater than 7 Gy X-radiation equivalents. We conclude that the majority of lesions detected in the unwinding assay are alkali-labile and presumably represent cleaved DNA–protein complexes [34].

Using the alkaline unwinding technique to monitor strand cleavage, resistance of LX4 was observed for doxorubicin, 4′-epidoxorubicin and 4′-deoxydoxorubicin. Two other analogues, and Ro 31-1215, induced similar levels of DNA damage in H69 and LX4 cells. These findings are in broad agreement with the cross-resistance patterns of the two cell lines to the various drugs screened suggesting that the evaluation of sensitivity to DNA damage-induction provides a reflection of the impact of the MDR phenotype on drug sensitivity. The resistance of LX4 to mitoxantrone was also apparent at the level of strand cleavage and DNA–protein crosslinking. Interestingly, parental cells showed a progressive accumulation of crosslinks during a 4 hr post-treatment incubation period whereas the low levels of crosslinks in LX4 cells remained stable. The progressive accumulation of crosslinks in the parental line is consistent with the persistence of mitoxantrone on nuclear DNA [12]. Drug persistence could contribute to continuous trapping of either newly synthesized topoisomerase molecules or a time-dependent presentation of enzyme to regions of DNA containing bound drug.

Mitoxantrone demonstrates a slight preference for binding to GC sequences but no high degree of sequence specificity [5] and it is likely that some mitoxantrone binding sites overlap with the AT sequences that preferentially bind Ho33342 molecules [21, 22]. The Ho33342–DNA fluorescence quenching results are interpreted here as reflecting competition between the intercalator and the minor groove ligand for binding sites on DNA. However, we note that mitoxantrone-induced changes in DNA compaction may affect the spectral characteristics of nuclear bound Ho33342 molecules. Indeed, this would be consistent with the observations that the fluorescence enhancement observed upon the binding of Ho33342 to DNA is increased in supercoiled preparations [35]. The quenching measurements confirm that LX4 cells show significant depression of the nuclear binding of mitoxantrone. Whole cell uptake measurements of drug reveal a much smaller difference in drug accumulation between the variant and parental cell lines. The results imply that only the levels of nuclear bound drug reflect the capacity to generate DNA damage.

The mitoxantrone dose dependency for fluorescence quenching varies according to the region of the Ho33342 emission spectrum analysed. The violet- or red-biased fluorescence of Hoechst 33342–DNA have been related to the excitation of ligand molecules at sites where the ligand is bound either within or more external to the minor groove of the helix, respectively [36]. The present results can be interpreted as reflecting the presence of high affinity sites for mitoxantrone on nuclear DNA, these sites being saturated at low drug doses (>1 μg/mL) and representing sites at which the competing Ho33342 molecules are attempting to bind more external to the minor groove. It is possible that such high affinity sites may represent successful intercalation of mitoxantrone molecules since this process should predominate at low drug/DNA ratios [5]. The similarity between the mitoxantrone dose dependency for long wavelength fluorescence quenching and the generation of DNA cleavage may indicate that only certain modes of intercalator binding contribute to topoisomerase trapping and this suggestion is consistent with some sequence dependency for cleavable complex generation. Preliminary studies (data not shown) revealed that the wavelength dependency for Ho33342–DNA fluorescence quenching by mitoxantrone could not be reproduced with naked calf thymus DNA in solution suggesting that topologically constrained molecules not in equilibrium with the intercalator may be required to demonstrate the phenomenon.

We have observed that the differential quenching of Ho33342–DNA fluorescence according to the region of the emission spectrum (Fig. 7) can be exploited to separate cell populations with different levels of drug–DNA binding. Bivariate analysis of fluorescence signals of LX4 and H69 cell populations

in artificial 1:1 mixtures of mitoxantrone treated cells, resolved two populations of cells not apparent by the monodimensional analysis of either fluorescence or 90° light scatter signals (data not shown). Such an analysis also revealed that LX4 cells showed similar levels of reduced nuclear binding of mitoxantrone throughout all phases of the cell cycle.

In conclusion we report that MDR in human small cell carcinoma cells can afford a high degree of protection of nuclear DNA from intercalators such as mitoxantrone not predicted from whole cell uptake studies. Mitoxantrone treatment of human cells can lead to a persistence and accumulation of trapped topoisomerase II complexes on DNA, in part explaining the high cytotoxicity of this intercalating agent. Complex persistence may relate to persistent binding of drug molecules at infrequent high affinity drug-binding sites on DNA. Further studies are underway to determine the relative importance of different modes of intercalation in the trapping of potentially cytotoxic topoisomerase II complexes by antitumour drugs.

Acknowledgements—We thank Professor N. M. Bleehen for his encouragement and Catherine O. Anderson for technical assistance.

REFERENCES

1. Waring WT, Variation of the supercoils in closed circuit or DNA by binding of antibiotics and drugs: evidence for molecular models involving intercalation. *J Mol Biol* **54**: 247–279, 1970.
2. Denny WA, DNA-intercalating ligands as anti-cancer drugs: prospects for future design. *Anti-Cancer Drug Design* **4**: 241–263, 1989.
3. Liu LF, DNA topoisomerase poisons as anti-tumour drugs. *Annu Rev Biochem* **58**: 351–375, 1989.
4. Alberts DS, Peng YM, Bowden GT, Dalton WS and Mackel C, Pharmacology of mitoxantrone: mode of action and pharmacokinetics. *Invest New Drugs* **3**: 101–107, 1985.
5. Kapusinski J, Darzynkiewicz Z, Traganos F and Melamed MR, Interaction of a new antitumour agent, 1 - 4 - dihydroxy - 5, 8-bis[[2-[(2-hydroxyethyl)amino] - ethyl]amino] - 9, 10 - anthracenedione, with nucleic acids. *Biochem Pharmacol* **30**: 231–240, 1981.
6. Kapuscinski J and Darzynkiewicz Z, Relationship between the pharmacological activity of the antitumour drugs Ametantrone and mitoxantrone and their ability to condense nucleic acids. *Proc Natl Acad Sci USA* **83**: 6302–6306, 1986.
7. Bartowiak J, Kapuscinski J, Melamed MR and Darzynkiewicz Z, Selective displacement of nuclear proteins by antitumor drugs having affinity for nucleic acids. *Proc Natl Acad Sci USA* **86**: 5151–5154, 1989.
8. Wang JC, Recent studies of DNA topoisomerases. *Biochim Biophys Acta* **909**: 1–9, 1987.
9. Glisson BS and Ross WE, DNA topoisomerase II: a primer on the enzyme and its unique role as a multidrug target in cancer chemotherapy. *Pharmacol Ther* **32**: 89–106, 1987.
10. Tritton TR and Yee G, The anticancer agent adriamycin can be actively cytotoxic without entering cells. *Science* **217**: 248–250, 1982.
11. Basra J, Wolf CR, Brown JR and Patterson LH, Evidence for human liver mediated free-radical formation by doxorubicin and mitoxantrone. *Anticancer Drug Design* **1**: 45–52, 1985.
12. Roberts RA, Cress AE and Dalton WS, Persistent intracellular binding of mitoxantrone in a human colon carcinoma cell line. *Biochem Pharmacol* **38**: 4283–4290, 1989.
13. Bowden GT, Roberts R, Alberts DS, Peng Y-M and Garcia D, Comparative molecular pharmacology in leukaemic L1210 cells of the anthracene anticancer drugs mitoxantrone and bisantrene. *Cancer Res* **45**: 4915–4920, 1985.
14. Endicott JA and Ling V, The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu Rev Biochem* **58**: 137–171, 1989.
15. Gerlach JH, Endicott JA, Juranka PF, Henderson G, Sarangi F, Deuchars KL and Ling V, Homology between P-glycoprotein and a bacterial haemolysin transport protein suggests a model for multidrug resistance. *Nature* **324**: 485–489, 1986.
16. Twentyman PR, Fox NE, Wright KA and Bleehen NM, Derivation and preliminary characterisation of adriamycin resistant lines of human lung cancer cells. *Br J Cancer* **53**: 529–537, 1986.
17. Smith PJ and Makinson TA, Cellular consequences of overproduction of DNA topoisomerase II in an ataxia-telangiectasia cell line. *Cancer Res* **49**: 1118–1124, 1989.
18. Preisler HD, Alteration of binding of the supravital dye Hoechst 33342 to human leukemic cells by adriamycin. *Cancer Treat Rep* **62**: 1393–1396, 1978.
19. Smith PJ, Nakeff A and Watson JV, Flow-cytometric detection of changes in the fluorescence emission spectrum of a vital DNA specific dye in human tumour cells. *Exp Cell Res* **159**: 37–46, 1985.
20. Morgan SA, Watson JV, Twentyman PR and Smith PJ, Flow cytometric analysis of Hoechst 33342 uptake as an indicator of multi-drug resistance in human lung cancer. *Br J Cancer* **60**: 282–287, 1989.
21. Zimmer C and Wahnert U, Non-intercalating DNA-binding ligands: specificity of the interaction and their use as tools in biophysical, biochemical and biological investigations of the genetic material. *Prog Biophys Molec Biol* **47**: 31–112, 1986.
22. Latt SA and Stetten G, Spectral studies on 33258 Hoechst and related bisbenzimidazole dyes useful for fluorescent detection of deoxyribonucleic acid synthesis. *J Histochem Cytochem* **24**: 24–33, 1976.
23. Reeve JG, Rabbitts PH and Twentyman PR, Amplification and expression of mdr1 gene in a multidrug resistant variant of small cell lung cancer cell line NCI-H69. *Br J Cancer* **60**: 339–342, 1989.
24. Smith PJ, Ferrous iron-mediated enhancement of DNA damage and recovery potential in bleomycin-treated human cells. *Biochem Pharmacol* **36**: 475–480, 1987.
25. Kanter PM and Schwartz HS, A fluorescence enhancement assay for cellular DNA damage. *Mol Pharmacol* **22**: 145–151, 1982.
26. Smith PJ, Mircheva J and Bleehen NM, Interaction of bleomycin, hyperthermia and a calmodulin inhibitor (trifluoperazine) in mouse tumour cells: II. DNA damage, repair and chromatin changes. *Br J Cancer* **53**: 105–114, 1986.
27. Arundel CA, Vines CM and Tofilon PJ, Chromatin modifications associated with *N*-methylamide-induced radiosensitization of Clone A cells. *Cancer Res* **48**: 5669–5673, 1988.
28. Rowe TC, Chen GL, Hsiang Y-H and Liu LF, DNA damage by anti-tumor acridines mediated by mammalian DNA topisomerase II. *Cancer Res* **46**: 2021–2026, 1986.
29. Minford J, Pommier Y, Flilipski J, Kohn KW, Kerrigan D Mattern M, Michaels S, Schwartz R and Zwelling LA, Isolation of intercalator-dependent protein-linked DNA strand cleavage activity from cell nuclei and identification as topoisomerase II. *Biochemistry* **25**: 9–16, 1986.
30. Smith PJ, Anderson CO and Watson JW, Abnormal

retention of X-irradiated ataxia telangiectasia fibroblasts in G2 phase of the cell cycle: cellular RNA content, chromatin stability and the effects of 3-aminobenzamide. *Int J Radiat Biol* **47**: 701–712, 1985.
31. Coley HM, Twentyman PR and Workman P, Improved cellular accumulation is characteristic of anthracyclines which retain high activity in multidrug resistant cell lines, alone or in combination with verapamil or cyclosporin A. *Biochem Pharmacol* **38**: 4467–4475, 1989.
32. Twentyman PR, Fox NE, Wright KA, Workman P, Broadhurst MJ, Martin JA and Bleehen NM, The *in vitro* effects and cross-resistance patterns of some novel anthracyclines. *Br J Cancer* **53**: 585–594, 1986.
33. Coley HM, Twentyman PR and Workman P, Identification of anthracyclines and related agents that retain preferential activity over Adriamycin in multidrug-resistant cell lines, and further resistance modification by verapamil and cyclosporin A. *Cancer Chemother Pharmacol* **24**: 284–290, 1989.
34. Pommier Y, Mattern MR, Schwartz RE and Zwelling LA, Absence of swivelling at sites of intercalator-induced protein-associated deoxyribonucleic acid strand-breaks in mammalian cell nucleoids. *Biochemistry* **23**: 2922–2927, 1984.
35. Sandhu LC, Warters RL and Dethlefsen LA, Fluorescence studies of Hoechst 33342 with supercoiled and relaxed plasmid pBR322 DNA. *Cytometry* **6**: 191–194, 1985.
36. Bontemps J, Houssier C and Fredericq E, Physicochemical study of the complexes of '33258 Hoechst' with DNA and nucleohistone. *Nuc Acids Res* **2**: 971–984, 1975.