Cancer Chemother Pharmacol (1992) 30: 451–458

Cancer Chemotherapy and Pharmacology

Lack of involvement of reactive oxygen in the cytotoxicity of mitoxantrone, CI941 and ametantrone in MCF-7 cells: comparison with doxorubicin*

Geoffrey R. Fisher and Laurence H. Patterson

Department of Pharmacy, Leicester Polytechnic, Po Box 143, Leicester LE1 9BH, U. K.

Received 20 December 1991/Accepted 27 March 1992

Summary. The MCF-7 cell S9 fraction and whole MCF-7 cells can mediate one-electron-redox cycling of doxorubicin, giving rise to concomitant oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH), formation of a drug semiquinone free radical, consumption of molecular oxygen and formation of superoxide anions and hydroxyl radicals. Doxorubicin redox cycling was consistent with DNA strand breakage and cell kill in MCF-7 cells. In contrast, no evidence for redox cycling was found for mitoxantrone (MIT), CI941 or ametantrone (AMET) in MCF-7 cells. Despite the absence of redox cycling, the CI941, MIT, and AMET concentrations resulting in 50% mortality (LC_{50}; 1.5×10^{-10}, 5.2×10^{-9} and 1.2×10^{-6} M, respectively) of MCF-7 cells were lower than that of DOX (3.0×10^{-6} M). Furthermore, the higher cytotoxicity of MIT and CI941 as compared with AMET or DOX was associated with greater efficiency in inducing DNA strand breakage in MCF-7 cells as determined by alkaline elution. Since MIT and CI941 proved to be the most potent DNA-damaging and cytotoxic agents in this study, the ability of DOX to undergo redox cycling does not appear to confer increased cytotoxic potential on this agent. The present study revealed several important aspects with regards to the structural modification of anthraquinone antitumour agents. Firstly, the C1 and C4 postitioning of the hydroxyethylamino side chains on MIT, CI941 and AMET is associated with a lack of flavin reductase-mediated activation of these agents. Secondly, the possession of a C5 or C8 aromatic hydroxyl group appears to be intimately involved in the enhanced DNA strand breakage and cytotoxic potency of MIT and CI941, since AMET does not possess these groups. These findings indicate that future development of quinone antitumour agents should concentrate on compounds that do not undergo redox cycling but do possess aromatic hydroxyl groups, since the latter appear to be responsible for the enhanced cytotoxicity of MIT and CI941.

Introduction

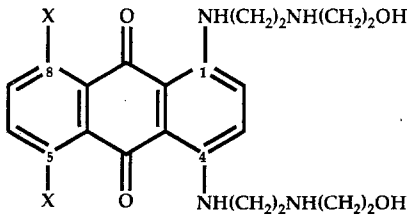
Mitoxantrone (MIT), ametantrone (AMET) and the anthrapyrazole CI941 are structurally similar antitumours agents that are broadly based on doxorubicin (DOX; see Fig. 1). All three agents possess a basic hydroxyethylamino side chain in the CI and C4 position, but only MIT and CI941 possess aromatic hydroxyl groups at the C5 and/or C8 position of the chromophore. MIT and AMET were developed with the intention of maintaining the DNA-complexing ability of DOX but removing the systemic side effects, notably cardiotoxicity [36, 46]. MIT is used in the treatment of advanced breast cancer, non-Hodgkin's lymphoma, and several types of leukaemia, and its cardiotoxicity is lower than that of DOX [18, 56]. However, MIT does not have the same spectrum of activity as DOX. Further attempts to produce a non-cardiotoxic anthraquinone with a wider spectrum of activity resulted in the development of the anthrapyrazoles, including CI941 [57]. The rationale behind the development of anthrapyrazoles was to render the electron-deficient quinone chromophore more resistant to enzymic reduction by forming a quasi-iminoquinone whilst maintaining the planar chromophore and basic side chains essential for DNA binding and antitumour activity. CI941, the most active of the anthrapyrazoles, is currently undergoing phase I/II clinical trials [1, 38].

One proposal for the mechanism underlying the cytotoxicity and cardiotoxicity of DOX involves the formation of damaging reactive oxygen species via flavin reductase-mediated redox cycling ([3], reviewed in [29]). The lower cardiotoxicity of MIT and CI941 has been proposed to be

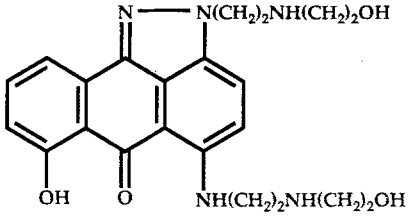
* This work was funded by the Cancer Research Campaign (UK)

Offprint requests to: G. R. Fisher

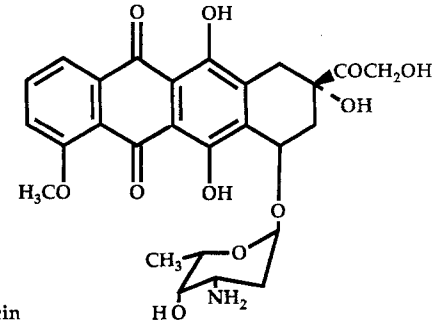
Abbreviations: DOX, doxorubicin; MIT, mitoxantrone; AMET, ametantrone; SOD, superoxide dismutase; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; ESR, electron spin resonance; SSF, strand scission factor



I X = H = ametantrone
II X = OH = mitoxantrone



III CI-941



IV doxorubicin

Fig. 1. Structures of AMET, MIT, CI941 and DOX. It should be noted that the IUPAC nomenclature for CI941 and MIT indicates that the C1 and C4 positions of MIT correspond to the C3 and C6 positions of CI941 and the C5 and C8 positions of MIT correspond to the C7 and C10 positions of CI941. However, for ease of comparison, we apply the nomenclature for MIT to both agents

due to the reduced propensity of these agents to undergo redox cycling. Indeed, extensive evidence exists that free radical formation by MIT as compared with DOX is greatly reduced in the presence of rabbit heart microsomes, purified P450 reductase, reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase [40–42], human liver microsomes and rat-liver and -heart microsomes [4, 5]. Similar observations have been made for AMET using rabbit- and mouse-liver microsomes [40–42] and for CI941 using rat-liver microsomes and purified rat-liver cytochrome P450 reductase [30]. The above studies showed that MIT and AMET were capable of forming free radicals, albeit to a lesser degree than DOX, in various non-tumour cell fractions. However, no systematic study has been conducted to determine whether redox cycling plays a role in the antitumour activity of these agents. The aim of the present study was to investigate the role of redox cycling of MIT, AMET and CI941 in the DNA strand breakage and cytotoxicity induced by these agents in a tumour cell line, namely, MCF-7 human breast-cancer cells. We [20] and other groups [59, 65] have shown in previous investigations that DOX undergoes redox cycling in this cell line. The reactive oxygen formation mediated by DOX was involved in MCF-7 cell kill [13, 14], reduced reactive oxygen formation being detected in DOX-resistant cells [58]. In the present study, we compared the redox activity of MIT, AMET and CI941 with that of DOX in MCF-7 cells. Some of these data have previously been presented in abstract form [21, 24, 26].

Materials and methods

Materials. Reduced nicotinamide adenine dinucleotide phosphate (NADPH), cytochrome c, superoxide dismutase (SOD), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), deferoxamine mesylate, Hoechst H33258 (bisbenzamide) and tetraethyl ammonium hydroxide were obtained from Sigma Chemical Co. (Dorset, UK). Polycarbonate membrane filters (2.0 μM, 25-mm diameter) were obtained from Nucleopore (USA). Proteinase K was obtained from BDH (Dorset, UK). Cell-culture materials were obtained from Flow Labs (Dorset, UK). MCF-7 S9 fraction was prepared as previously described [18].

Determination of NADPH oxidation and superoxide anion formation. NADPH oxidation was followed spectrophotometrically (37° C, 340 nm) in an incubate of phosphate buffer (pH 7.4, 100 mM), NADPH (0.1 mM), drug and S9 fraction (0.5 mg protein ml^{-1}). Superoxide anion formation was determined spectrophotometrically (37° C, 550 nm) following the superoxide dismutase-inhibitable reduction of acetylated cytochrome c [2] in a system containing KCl (150 mM)-TRIS (50 mM, pH 7.4), acetylated cytochrome c (0.07 mM), NADPH (1.0 mM) and drug in the presence or absence of SOD (300 μg ml^{-1}).

Electron spin resonance spectrometry. Electron spin resonance (ESR) spectrometry was carried out using a Varian E109 X-band ESR spectrometer at room temperature (typically 20° – 23° C). For drug semiquinone detection, the incubate consisted of phosphate buffer (100 mM, pH 7.4), MCF-7 S9 fraction (1.7 mg protein ml^{-1}), NADPH (1.0 mM) and drug. The solution was deaerated with oxygen-free nitrogen (15 min) prior to ESR determinations (ESR parameters: microwave power, 5 mW; microwave frequency, 9.467 GHz; time constant, 1 s; modulation amplitude, 4 Gauss; scan time, 4 min).

ESR spin-trapping studies using DMPO were carried out in an incubate composed of phosphate buffer (200 mM, pH 7.4), MCF-7 S9 fraction (0.68 mg protein ml^{-1}), deferoxamine mesylate (1 mM), DMPO (100 mM), NADPH (0.5 mM) and drug (ESR parameters: microwave power, 10 mW; microwave frequency, 9.467 GHz; modulation amplitude, 2.0 Gauss; time constant, 0.25 s; scan time, 4 min).

Determination of cellular DNA damage by alkaline elution. MCF-7 cells (5×10^6) were incubated with drug (60 min, 37° C) at either the concentration producing 50% cell kill (LC_{50}, see below saline) or 100 μM. The cells were washed free of drug using ice-cold phophate-buffered saline (PBS) and placed on ice. The cells were then subjected to alkaline elution as described by Kohn et al. [43]. The cells were loaded onto 2.0 μM (25-mm diameter) polycarbonate membrane filters and lysed for 1 h with a solution consisting of sodium dodecyl sarkosine (0.2%), disodium ethylenediaminetetraacetic acid (EDTA) (0.04 M), NaCl (2 M; pH 10) supplemented with proteinase K (0.01 Anson units ml^{-1}). Cellular DNA was eluted from the filter using EDTA (free acid, 0.02 M) and tetraethyl ammonium hydroxide (0.1 M, pH 12.3). DNA in collected fractions was quantitated fluorimetrically using Hoechst H33258 dye as described by Stout and Becker [62]. Results were plotted semi-logarithmically as the log of the proportion of DNA remaining on the filter against the cumulative volume eluted (in millilitres). From this graph the strand scission factor (SSF) was derived using the equation SSF = -log (F_x/F_c), where F_x represents the fraction of DNA retained on the filter at the midpoint volume of the total volume eluted from drug-treated DNA and F_c represents the corresponding value for untreated DNA.

Cytotoxicity studies. MCF-7 cells were cultured using RPMI-1640 medium supplemented with glutamine (2 mM) and foetal bovine serum (10%). For cytotoxicity studies, MCF-7 cells were seeded into 12-well

plates (10^4 cells ml^{-1} $well^{-1}$) and incubated for 24 h. The cells were then incubated with a range of drug concentrations ($10^{-5}-10^2$ μM) in fully supplemented RPMI-1640 medium for 1 h. The drug solution was removed, the cells were washed with ice-cold PBS and fresh medium was added. The cells were cultured for a further 6 days, removed from the well plates by treatment with EDTA (0.1%)-PBS (pH 7.4) and counted using a ZB Coulter counter.

Uptake studies. Drug (15 μM) uptake into MCF-7 cells (10^6 ml^{-1}) resuspended in fully supplemented RPMI-1640 medium was determined by following the loss of drug from the medium over a period of 1 h by high-performance liquid chromatography (HPLC) using a reverse-phase C18 column, a mobile phase of 28% acetonitrile and 72% ammonium formate (80 mM, pH 3.25) and a flow rate of 1.0 ml min^{-1}.

Results

Redox cycling of DOX, MIT, CI941 and AMET in the MCF-7 cell S9 fraction

Redox cycling involves flavin reductase-mediated one-electron reduction of a quinone along with concomitant oxidation of NAD(P)H. In the case of anthraquinones, the resultant semiquinone free radical is transient in nature and reacts immediately with molecular oxygen in the presence of the latter to re-form the parent quinone and generate the superoxide anion. Formation of superoxide anions can lead via either spontaneous or SOD-mediated dismutation to the formation of hydrogen peroxide, which can enter the iron-catalysed Haber-Weiss reaction, resulting in hydroxyl radical formation. Thus, NADPH oxidation, semiquinone formation, oxygen consumption and superoxide anion and hydroxyl radical formation are the parameters that need to be determined to show that a drug is undergoing redox cyling.

The MCF-7 S9 cell fraction and whole MCF-7 cells can mediate redox cyling of DOX, giving rise to concomitant stimulation of NADPH oxidation, DOX semiquinone formation, consumption of oxygen, superoxide anion formation and hydroxyl radical formation [20, 23, 24]. These studies indicated that the MCF-7 cell line was a good model system for investigating redox cycling of other agents. For the investigation of redox cycling of MIT, AMET and CI941, the effect of these agents on NADPH oxidation and superoxide anion formation in the MCF-7 cell S9 fraction was determined and compared with the effect of DOX. In addition, ESR studies were performed to determine whether any semiquinone or hydroxyl radical formation could be detected for these agents in the MCF-7 cell S9 fraction.

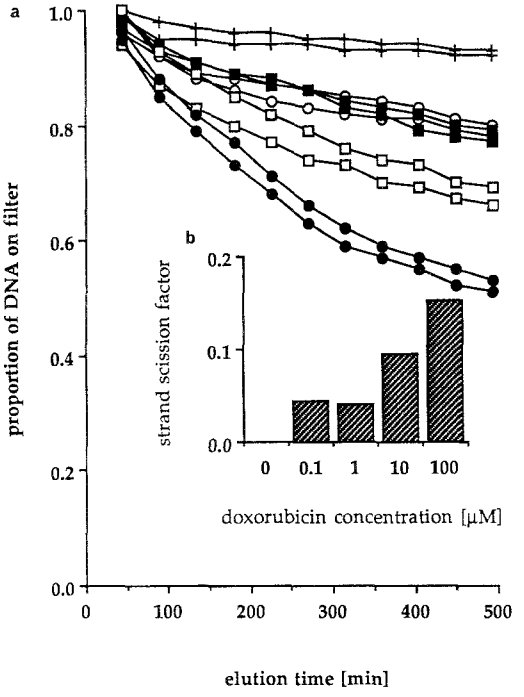


Fig. 2. a Elution profiles of MCF-7 DNA in MCF-7 cells treated for 60 min with DOX at 0.1 (○), 1 (■), 10 (□) and 100 μM (●) and in untreated cells (+). **b** Strand scission factors derived from **a**. Elution was carried out under full deproteinising conditions. Data points represent mean values for 2 determinations

It can be seen from Table 1 that DOX (100 μM) stimulated NADPH oxidation and superoxide anion formation in the MCF-7 S9 fraction. A strong signal for the DOX semiquinone was detected under anaerobic conditions only by ESR spectrometry [20]. DOX-mediated hydroxyl radical formation was detected in both the MCF-7 S9 fraction and intact MCF-7 cells in the presence of air by ESR spin-trapping with DMPO [20, 24].

In contrast to DOX, both MIT and CI941 inhibited base-rate NADPH oxidation (Table 1). AMET appeared to stimulate NADPH oxidation slightly in the MCF-7 S9 fraction, albeit to a much lesser degree than did DOX. This observation is consistent with previous results obtained using these agents in animal tissues [42]. No superoxide

Table 1. Evidence for lack of redox cycling of MIT, AMET and CI941 as compared with doxorubicin in the MCF-7 S9 fraction

Drug	NADPH oxidation[a]	Superoxide formation[b]	Semiquinone formation[c]	Hydroxyl radical formation[d]
DOX	758.74±0.1	7.54±1.9	65.0	120.0
MIT	−22.3 ±2.2[e]	0.98±2.09	ND	ND
CI941	−23.04±2.9[e]	1.71±0.95	ND	ND
AMET	91.0 ±37	ND*	ND	ND

[a] NADPH oxidised (nmol min^{-1} mg $protein^{-1}$) in the presence of 100 μM drug (base rate subtracted)

[b] SOD-inhibitable reduction of acetylated cytochrome c(nmol min^{-1} mg $protein^{-1}$) in the presence of 100 μM drug

[c] Peak-to-peak height of semiquinone spectrum (arbitrary units) after 30 min incubation (drug concentration, 400 μM except for ametantrone)

[d] Peak-to-peak height of DMPO-OH spectrum (low-field line) after 30 min incubation (drug concentration, 400 μM). See Materials and methods for full experimental details

[e] Inhibited base-rate NADPH oxidation

ND, Not detected

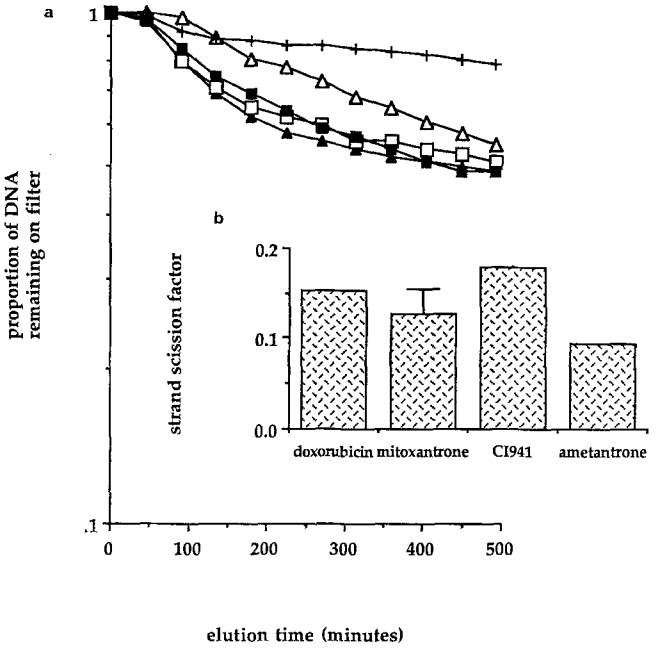


Fig. 3. a Elution profiles of MCF-7 DNA in MCF-7 cells treated for 60 min with 100 μM DOX (■), MIT (□), CI941 (▲) or AMET (△) and in untreated cells (+). **b** Strand scission factors derived from **a**. Elution was carried out under full deproteinising conditions. Data points represent mean values for 4 determinations from 2 independent experiments

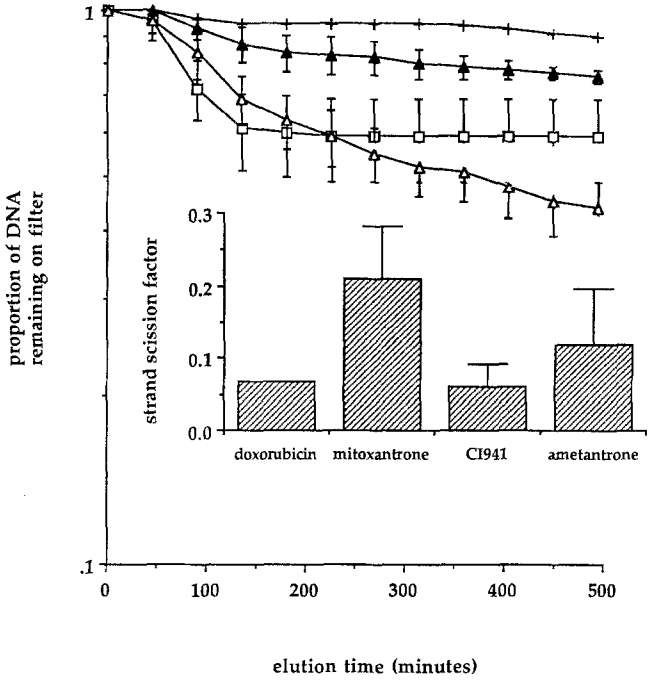


Fig. 4. a Elution profiles of MCF-7 DNA in MCF-7 cells treated for 60 min with LC_{50} concentrations of MIT (5.2×10^{-9} M □), CI941 (1.5×10^{-10} M; ▲) of AMET (1.2×10^{-6} M; △) and in untreated cells (+). **b** Comparison of strand scission factors derived from **a** with that obtained for an LC_{50} concentration of DOX. Elution was carried out under full deproteinising conditions. Data points represent mean values ± SD for 4 determinations from 2 independent experiments

anion formation was detected for MIT or AMET in the MCF-7 S9 fraction, whereas a small amount of superoxide anion formation was found for CI941. Increasing the concentration of these agents above 100 μM did not have any effect (data not shown). Furthermore, no drug semiquinone or hydroxyl radical formation was observed in the MCF-7 S9 fraction for MIT, AMET or CI941 using ESR (Table 1).

Cellular DNA strand breakage induced by DOX, MIT, CI941 and AMET in MCF-7 cells

Figure 2a shows that DOX produced a concentration-dependent increase in the rate of elution of MCF-7 cell DNA. This was reflected by a corresponding drug-concentration-dependent increase in strand scission factor at DOX concentrations of between 1 and 100 μM (Fig. 2b), indicating that DOX induced strand breaks in MCF-7 cell DNA. These strand breaks were revealed following deproteinisation of the drug-treated cellular DNA. As strand breaks revealed under non-deproteinised conditions were not determined, protein-associated strand breakage could not be distinguished from other types under these assay conditions.

Figure 3 shows the elution profile of DNA from MCF-7 cells treated for 1 h with 100 μM DOX, CI941, MIT or AMET. Figure 3b compares the strand scission factors derived from Fig. 3a and indicates that all four compounds produced similar elution profiles and strand scission factors under the conditions used. Figure 4a shows the elution

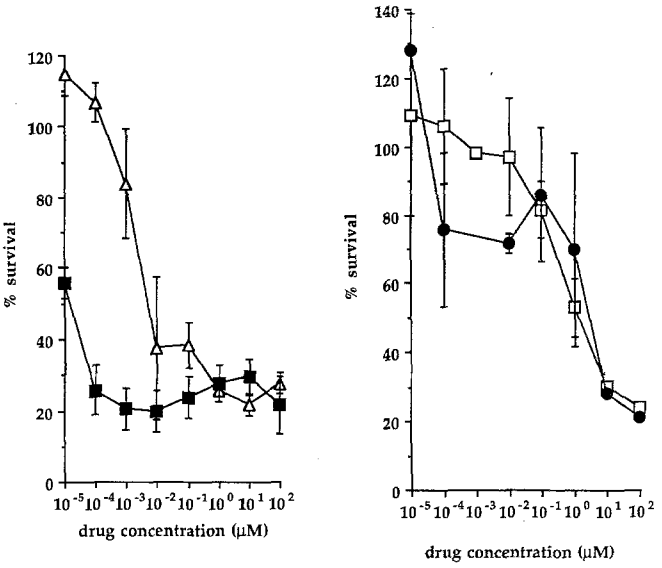


Fig. 5. Cytotoxicity of DOX (●), MIT (△), CI941 (■) and AMET (□) towards MCF-7 cells following a 1-h exposure period and a further 6-day drug-free incubation interval

profile of DNA from MCF-7 cells treated for 1 h with CI941, MIT or AMET at the LC_{50} concentrations derived from Fig. 5. CI941 was observed to have a significantly slower DNA elution profile as compared with MIT and AMET. A comparison of the strand scission factors determined for DOX, CI941, MIT and AMET under these con-

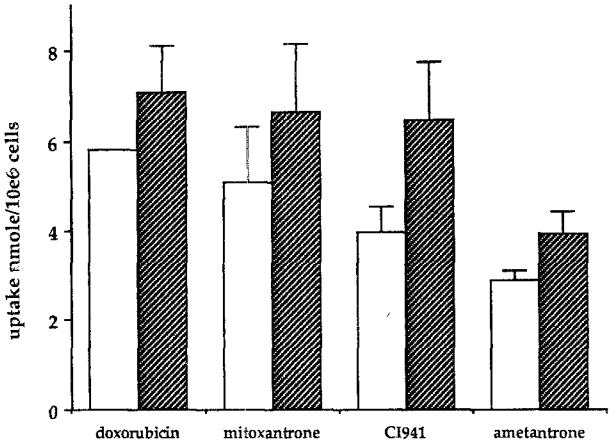


Fig. 6. Uptake of DOX, MIT, CI941 and AMET by MCF-7 cells. Cellular uptake of drug was determined immediately following the addition of drug (15 μM □) or after 60 min incubation with drug (■). Data points represent mean values ± SD for 3 determinations

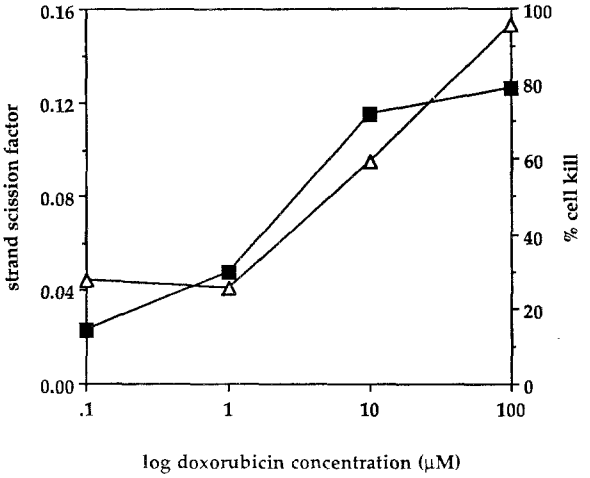


Fig. 7. Correlation between DOX-induced DNA strand breakage (*strand scission factor*, △) and cell kill (■) in MCF-7 cells. DOX cytotoxicity was determined following a 1-h exposure period and a further 6-day drug-free incubation interval. Strand breakage was determined by the alkaline elution assay following a 1-h exposure to drug (see Materials and methods). Data points represent mean values ± SD for 3 determinations

ditions (Fig. 4b) reveals that DNA strand breakage induced by MIT was approximately 3-fold that produced by DOX or CI941. The amount of strand breakage caused by AMET was intermediate between that produced by MIT and that induced by CI941 or DOX.

Cytotoxicity studies

The cytotoxicity of these agents was determined following a 1-h drug-exposure period and continued growth of the cells for 6 days. Figure 5 shows that CI941 and MIT were considerably more cytotoxic than either DOX or AMET under these conditions, the rank order of cytotoxicity (LC_{50}) being CI941 (1.5×10^{-10} M) >MIT (5.2×10^{-9} M) >AMET (1.2×10^{-6} M) >DOX

Table 2. One-electron-reduction potentials and DNA binding constant of DOX, MIT, CI941 and AMET

Drug	Reduction potential (e1/7 at pH 7.0)	DNA affinity constant Kapp M^{-1})
DOX	328 ± 5 mV[a]	6.5×10^{6} [e]
MIT	527 ± 5 mV[b]	2.6×10^{8} [f]
CI941	538 ± 10 mV[c]	1.0×10^{8} [g]
AMET	520 ± 8 mV[d]	2.83×10^{6} [h]

[a, b] From Basra et al. [5]
[c] From Graham et al. [30]
[d] From Butler (personal communication)
[e] From Capranico et al. [8]
[f, g] From Hartley et al. [31]
[h] From Gandecha [28]

(3.0×10^{-6} M). The LC_{50} of CI941 was approximately 34 times lower than that of MIT, 8×10^{3} times lower than that of AMET and 2×10^{4} times lower than that of DOX. Similarly, the LC_{50} of MIT was approximately 230 times lower than that of AMET and 576 times lower than that of DOX. These results are consistent with previous findings that MIT and CI941 are considerably more cytotoxic than DOX and AMET in vitro [7, 30, 47, 49]. Cell-uptake studies were carried out to determine whether these differences in cytotoxicity could be explained by differences in the cellular accumulation of these agents by MCF-7 cells. Figure 6 shows that uptake of each of these agents is associated with a rapid extracellular binding phase during the first few seconds of the incubation. This is consistent with the findings previously reported for DOX [35, 48, 60], MIT and AMET [10, 47, 66, 68]. The level of uptake of MIT, CI941 and DOX (15 μM) following 1 h incubation with MCF-7 cells was similar (Fig. 6). The uptake of AMET after 1 h incubation was approximately 40% lower than that of MIT, CI941 or DOX. However, it is unlikely that this difference in uptake explains the large difference found in the cytotoxicity of this agent as compared with CI941 and MIT (LC_{50}, 230 and 8×10^{3} times lower, respectively).

Discussion

This study represent the first systematic investigation of the role of redox cycling in the mechanism underlying the antitumour action of MIT, AMET and CI941. We have previously determined that DOX undergoes reductase-mediated redox cycling in the MCF-7 cell S9 fraction and whole MCF-7 cells [20, 23, 24]. In contrast, MIT, AMET and CI941 show little propensity to undergo redox cycling in MCF-7 cells as compared with DOX (see Table 1). The absence of enzymic reduction of MIT and CI941 determined in the present study may be explained by several factors. Firstly, as MIT, AMET and CI941 have more negative one-electron-reduction potential (Table 2) than does DOX, the reduction of these agents by flavoprotein reductases is not facilitated. Secondly, due to the steric hindrance produced by the 1,4-substituted hydroxyethylamino side chains, these agents may be poorer substrates for reductases than is DOX. Other structural analogues of

MIT and AMET whose hydroxyethylamino side chains have been substituted at the (1)-, (1,5)- and (1,8)-position on the anthraquinone chromophore have been found to undergo redox cycling in MCF-7 cells [23, 25]. The site of electron transfer from the flavoprotein to the anthraquinone is not known but is presumed to involve the flavin co-enzyme FMN [41]. Agents such as MIT and AMET, which are poorer substrates for cytochrome P450 reductase than is DOX, have been found to bind to FMN with higher avidity than DOX [41]. This has been proposed to hinder the rate of electron transfer to these agents [41]. Thirdly, MCF-7 cells contain a much lower level of cytochrome P450 reductase (cytochrome c reduction, 1.19 nmol min^{-1} mg $protein^{-1}$) [19] as compared with the human liver (166.1 ± 49.4 nmol min^{-1} mg $protein^{-1}$) and mouse liver (222.3 ± 4 nmol min^{-1} mg $protein^{-1}$) tissue used in previous studies [5]. However, the quantity of S9 protein used in the present study was sufficient to produce measurable redox cycling of DOX.

Reductase-mediated redox cycling of DOX in MCF-7 cells was consistent with a DOX-concentration-dependent induction of strand breakage in cellular DNA (Fig. 2). DOX-dependent induction of DNA strand breakage correlated with increasing MCF-7 cell kill at equivalent DOX concentrations (Fig. 7). It is widely believed that DOX-induced DNA strand breakage and resultant cell kill is at least in part mediated by reactive oxygen species formed during redox cyling of DOX in MCF-7 cells. Previously, DOX MCF-7 cell kill has been shown to involve reactive oxygen formation [13, 52], and MCF-7 cells with increased levels of antioxidant enzyme systems have been shown to be resistant to DOX (14, 58]. Lower levels of DOX-mediated reactive oxygen formation were detected in the resistant cells as compared with the wild-type cells [58].

Furthermore, in other cell lines, DOX has been shown to produce frank DNA strand breakage that is likely to be generated by free radical formation [34, 37, 51, 52]. However, despite the good correlation found between DOX free-radical formation, DNA strand scission and cytotoxicity in the present study, the presence of protein-associated DNA strand breaks cannot be ruled out, since we could not discriminate between protein-associated and non-protein-associated strand breaks. Protein-associated strand breaks are considered to be formed by drug-mediated trapping of a cleavable topoisomerase II-DNA complex, which, on denaturation with sodium dodecyl sulphate or proteinase K, results in DNA double-strand breaks [67]. DOX has previously been shown to inhibit purified DNA topoisomerase II and mediate protein-associated strand breaks by this enzyme [64]. Furthermore, DOX has been found to generate protein-associated DNA strand breaks in vitro [6, 8, 44, 69], and variation in topoisomerase II activity has been shown to contribute to multidrug resistance in DOX-resistant cells [11, 12]. The relative contributation and importance of reactive oxygen and topoisomerase-mediated DNA strand breakage in doxorubicin-treated MCF-7 cells has yet to be discerned.

Despite the lack of redox cycling of MIT, CI941 and AMET, these agents induced cellular DNA strand breakage equivalent to that produced by DOX at a concentration of 100 μM (Fig. 3). The LC_{50} values determined for CI941, MIT and AMET in MCF-7 cells were 2×10^4, 576 and 2.5 times lower, respectively, than that found for DOX (Fig. 5). The higher cytotoxicity of MIT and CI941 was associated with greater efficiency in inducing DNA strand breakage in MCF-7 cells at the LC_{50} of these agents (Fig. 4). This observation is consistent with previous reports that the efficiency of MIT in producing protein-associated DNA strand breaks was 250 times greater than that of AMET and that the former drug was much more cytotoxic than the latter in vitro [15, 45, 47, 49]. Furthermore, AMET-induced strand breaks were repaired more efficiently than those caused by MIT [45], and MIT has been shown to condense nucleic acids much more potently than does AMET [39].

The lack of reactive oxygen species generated by MIT, AMET and CI941 in MCF-7 cells suggests that the DNA strand breaks detected for these agents in the present study are likely to represent protein-associated strand breaks mediated by DNA topoisomerases. In this respect, MIT has previously been shown to inhibit MCF-7 topoisomerase II [9] and to produce DNA-topoisomerase II-mediated protein-associated strand breaks in vitro [17, 32]. The enhanced cytotoxicity of MIT and CI941 as compared with DOX and AMET towards MCF-7 cells may be related to the relative propensity of these agents to induce topoisomerase II-mediated DNA cleavage.

Another significant contribution to this enhanced cytotoxicity may involve the persistence of MIT and CI941 in the cell due to their stronger affinity for cellular macromolecules such as membranes [63] and DNA as compared with that of DOX and AMET (see Table 2). Consistent with this hypothesis, MIT has been found to persist at much higher levels than daunorubicin in hepatoma cells following the removal of cells from exposure to these agents [16]. The finding of MIT bound to postmortem tissue at 272 days following administration of the drug illustrates the binding tenacity of this agent [18]. MIT has been demonstrated to bind persistently to soluble cytoplasmic proteins and nucleic acids [55]. Furthermore, following the removal of cells from exposure to MIT, the continued depression of DNA synthesis and the persistence of MIT-induced topoisomerase II -DNA complexes [27] and strand breaks [33] have been observed. It has been proposed that the persistent binding of MIT to DNA favours long-term trapping of such topoisomerase complexes [61]. Consistent with this proposal, MIT-resistant P388 cells showing a low cellular accumulation of MIT have been found to contain a reduced level of tightly bound or non-exchangeable drug [50]. As the properties of CI941 are comparable with those of MIT, including DNA binding (Table 2), cytotoxicity (Fig. 5) and DNA strand breakage (Fig. 4), such a mechanism involving intracellular persistence and topoisomerase II inhibition would seem likely for this agent as well.

An alternative mechanism of action for MIT that has recently been reported involves the oxidative activation of this agent by peroxidase [53] to a species that can covalently bind to DNA [54] and cause DNA strand breakage [22]. However, no evidence for the occurrence of this mechanism in vitro has yet been presented.

Despite the related anthraquinone-based structure of the agents used in this study, it is apparent that they possess different propensities to undergo reductase-mediated redox cycling, induce cellular DNA strand breakage and produce MCF-7 cell kill. The differences in the mechanism(s) of action of these agents appear to be intimately related to the structural differences between them. The most important structural modification with respect to mechanism revealed in the present study appears to be the C1 and C4 positioning of the hydroxyethylamino side chains on the chromophore of MIT, CI941 and AMET. This structural feature is associated with the absence of detectable reductase-mediated metabolic activation of these agents as compared with anthraquinones possessing identical side chains that are located at different positions on the chromophore [23, 25]. Furthermore, the presence of aromatic hydroxyl groups at the C5 and/or C8 position on MIT and CI941 is associated with an increase in their efficiency in producing cellular DNA strand breaks, enhanced DNA binding (Table 2) and higher cytotoxicity (Fig. 5) as compared with AMET, which does not possess these groups (see Fig. 1).

Since the non-redox-cycling agents MIT and CI941 proved to be the most potent cytotoxic agents in this study, the ability to undergo redox cycling does not appear to confer increased cytotoxic potential on agents such as DOX or other alkylaminoanthraquinones [25]. This observation indicates that future development of quinone antitumour agents should concentrate on compounds that do not undergo redox cycling. Furthermore, these agents should also possess aromatic hydroxyl groups, since these appear to be responsible for the enhanced cytotoxicity of MIT and CI941 as compared with AMET.

Acknowledgements. We would like to thank Dr. J. Butler, Patterson Laboratories for Cancer Research, Christie Hospital and Holt Radium Institute, Manchester, for determining the reduction potential of ametantrone by pulse radiolysis for this study.

References

1. Allan SG, Cummings J, Evans S, Nicolson M, Stewart ME, Cassidy J, Soukop M, Kaye SB, Smyth JF (1991) Phase I study of the anthrapyrazole biantrazole: clinical results and pharmacology. Cancer Chemother Pharmacol 28: 55
2. Azzi A, Montecucco C, Richter C (1975) The use of acetylated cytochrome c for detection of superoxide radicals produced in biological membranes. Biochem Biophys Res Commun 65: 597
3. Bachur NR, Gordon SL, Gee MV (1978) A general mechanism for microsomal activation of quinone anticancer agents to free radicals. Cancer Res 38: 1745
4. Basra J (1986) A study of free radical formation in liver and heart tissue by alkylaminoanthraquinone antitumour agents based on mitoxantrone. PhD Thesis, Leicester Polytechnic
5. Basra J, Wolf CR, Brown JR, Patterson LH (1985) Evidence for human liver mediated free a radical formation by doxorubicin and mitoxantrone. Anticancer Drug Design 1: 45
6. Bellamy WT, Dorr RT, Dalton WS, Alberts DS (1988) Direct relation of DNA lesions in multidrug-resistant human myeloma cells to intracellular doxorubicin concentration. Cancer Res 48: 6360
7. Capolongo L. Belvedere G, D'Incalci M (1990) DNA damage and cytotoxicity of mitoxantrone and doxorubicin in doxorubicin-sensitive and -resistant human colon carcinoma cells. Cancer Chemother Pharmacol 25: 430
8. Capranico G, De Isabella P, Penco S, Tinelli S, Zunino F (1989) Role of DNA breakage in cytotoxicity of doxorubicin, 9-deoxydoxorubicin and 4-demethyl-6-deoxydoxorubicin in murine leukaemia P388 cells. Cancer Res 49: 2022
9. Crespi MD, Ivanier SE, Genovese J, Baldi A (1986) Mitoxantrone affects topoisomerase activities in human breast cancer cells. Biochim Biophys Acta 136: 521
10. Dalton WS, Cress AE, Alberts DS, Trent JM (1988) Cytogenic and phenotypic analysis of a human colon carcinoma cell line resistant to mitoxantrone. Cancer Res 48: 1882
11. Deffie AM, Batra JK, Goldenberg GJ (1989) Direct correlation between DNA topoisomerase II activity anc cytotoxicity in Adriamycin-sensitive and -resistant P388 leukemia cell lines. Cancer Res 49: 58
12. De Jong S, Zijlstra JG, De Vries EGE, Mulder NH (1990) Reduced DNA topoisomerase II activity and drug-induced DNA cleavage activity in an Adriamycin-resistant human small cell lung carcinoma cell line. Cancer Res 50: 304
13. Doroshow J (1986) Prevention of doxorubicin-induced killing of MCF-7 human breast cancer cell by oxygen radical scavengers and iron chelating agents. Biochem Biophys Res Commun 135: 330
14. Doroshow JH, Akman S, Esworthy S, Chu FF, Burke T (1991) Doxorubicin resistance conferred by selective enhancement of intracellular glutathione peroxidase or superoxide dismutase content in human MCF-7 breast cancer cells. Free Radical Res Commun 12–13: 779
15. Drewinko B, Yang L-Y, Bartogie B, Trujillo JM (1983) Comparative cytotoxicity of bisantrene, mitoxantrone, ametantrone, dihydroxyanthracenedione, dihydroxyanthracenedione diacetate and doxorubicin in human cells in vitro. Cancer Res 43: 2648
16. Ellis AL, Randolph JK, Conway BR, Gewirtz DA (1990) Biochemical lesions associated with the antiproliferative effects of mitoxantrone in the hepatoma cell. Biochem Pharmacol 39: 1549
17. Epstein RJ, Smith PJ (1988) Estrogen-induced potentiation of DNA damage and cytotoxicity in human breast cancer cells treated with topoisomerase II-interactive antitumour drugs. Cancer Res 48: 297
18. Faulds D, Balfour JA, Chrisp P. Langtry HD (1991) Mitoxantrone: a review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in the chemotherapy of cancer. Drugs 41: 400
19. Fisher GR, Gutierrez PL (1991) The reductive metabolism of diaziquone in the S9 fraction of MCF-7 cells; free radical formation and NAD(P)H (quinone-acceptor) oxidoreductase (DT-diaphorase) activity. Free Radicals Biol Med 10: 359
20. Fisher GR, Patterson LH (1988) Free radical formation by doxorubicin in MCF-7 human breast cancer cells. J Pharm Pharmacol 40 [Suppl]: 28
21. Fisher GR, Patterson LH (1989) A comparison of free radical generation by doxorubicin and CI941 in MCF-7 human breast cancer cells. Br J Cancer 60: 476
22. Fisher GR, Patterson LH (1990) DNA strand breakage by peroxidase-activated mitoxantrone. J Pharm Pharmacol 43: 65
23. Fisher GR, Brown JR, Patterson LH (1989) Redox cycling in MCF-7 human breast cancer cells by antitumour agents based on mitozantrone. Free Radical Res Commun 7: 221
24. Fisher GR, Maine JE, Patterson LH (1989) Comparison of free radical generation by doxorubicin and mitoxantrone in MCF-7 human breast cancer cells. J Pharm Pharmacol 41 [Suppl]: 23 P
25. Fisher GR, Brown JR, Patterson LH (1990) Involvement of hydroxyl radical formation and DNA strand breakage in the cytotoxicity of anthraquinone antitumour agents. Free Radical Res Commun 11: 117
26. Fisher GR, Maine JE, Patterson LH (1990) Lack of involvement of reactive oxygen in the cytotoxicity of mitoxantrone and CI941: comparison with doxorubicin. Proc Am Assoc Cancer Res 31: 397
27. Fox ME, Smith PJ (1990) Long term inhibition of DNA synthesis and the persistence of trapped topoisomerase II complexes in determining the toxicity of the antitumour DNA intercalators mAMSA and mitoxantrone. Cancer Res 50: 5813
28. Gandecha BM (1985) Development of potential antitumour agents based on considerations of the mode of action and pharmacokinetics of daunomycin and doxorubicin. PhD Thesis, Leicester Polytechnic

29. Gianni L, Cordon BJ, Myers CE (1983) The biochemical basis of anthracycline toxicity and antitumour activity. Rev Biochem Toxicol 5: 1
30. Graham MA, Newell, DR, Butler J, Hoey B, Patterson LH (1987) The effect of the anthrapyrazole antitumour agent CI941 on rat liver microsome and cytochrome P450 reductase mediated free radical processes. Biochem Pharmacol 36: 3345
31. Hartley JA, Reszka K, Zuo ET, Wilson WD, Morgan AR, Lown JW (1988) Characteristics of the interaction of anthrapyrazole anticancer agents with deoxyribonucleic acids; structural requirements for DNA binding, intercalation and photosensitization. Mol Pharm 33: 265
32. Heinemann V, Murray D, Walters R, Meyn RE, Plunkett W (1988) Mitoxantrone-induced DNA damage in leukaemia cells is enhanced by treatment with high-dose arabinosylcytosine. Cancer Chemother Pharmacol 22: 205
33. Ho AD, Seither E, Ma DDF, Prentice HG (1987) Mitoxantrone-induced toxicity and DNA strand breaks in leukaemic cells. Br J Haematol 65: 51
34. Iliakis, Lazaar W (1987) Effect of sodium chloride concentration on Adriamycin and *N*-trifluoroacetyladriamycin-14-valerate (AD32)-induced cell killing and DNA damage in Chinese hamster ovary cells. Cancer Res 47: 1853
35. Inaba K, Johnson RK (1978) Uptake and retention of Adriamycin and daunorubicin by sensitive and anthracycline resistant sublines of P388 leukaemia. Biochem Pharmacol 27: 2123
36. Johnson RK, Zee-Cheng RK-Y, Lee WW, Acton EM, Henry DW, Cheng CC (1979) Experimental antitumour activity of anthraquinones. Cancer Treat Rep 63: 425
37. Johnston JB, Zwelling LA, Kerrigan D, Lloyd LS, Glazer RI (1983) Comparison of DNA scission and cytotoxicity produced by Adriamycin and 5-iminodaunorubicin in human colon carcinoma cells. Biochem Pharmacol 32: 2253
38. Judson IR (1991) Anthrapyrazoles: true successors to the anthracyclines? Anti-Cancer Drugs 2: 223
39. Kapuscinski J, Darzynkiewicz Z (1986) Relationship between the pharmacological activity of antitumour drugs ametantrone and mitoxantrone (Novantrone) and their ability to condense nucleic acids. Proc Natl Acad Sci USA 83: 6302
40. Kharasch ED, Novak RF (1982) Inhibition of Adriamycin-stimulated microsomal lipid peroxidation by mitoxantrone and ametantrone, two new antineoplastic agents. Biochem Biophys Res Commun 108: 1346
41. Kharasch ED, Novak RF (1983) bis(Alkylamino)anthracenedione antineoplastic agent metabolic activation by NADPH cytochrome P450 reductase and NADH dehydrogenase: diminished activity relative to anthracyclines. Arch Biochem Biophys 224: 682
42. Kharasch ED, Novak RF (1985) Mitoxantrone: propensity for free radical formation and lipid peroxidation – implications for cardiotoxicity. Invest New Drugs 3: 95
43. Kohn KW, Erickson LC, Ewig RAG, Friedman CA (1976) Fractionation of DNA from mammalian cells by alkaline elution. Biochemistry 15: 4629
44. Lawrence TS (1988) Reduction of doxorubicin cytotoxicity by oubain: correlation with topoisomerase-induced DNA strand breakage in human and hamster cells. Cancer Res 48: 725
45. Locher SE, Meyn RE (1983) Relationship between cytotoxicity and DNA damage in mammalian cells treated with anthracenedione derivatives. Chem-Biol Interact 46: 369
46. Murdock KC, Wallace RE, Durr FE, Child FE, Citarella RV, Fabio PF, Angier RB (1979) Antitumour agents: I. 1,4-bis(Aminoalkylamino)-9,10-anthracenediones. J Med Chem 22: 1024
47. Nishio A, Uyeki FM (1983) Cellular uptake and inhibition of DNA synthesis by dihydroxyanthraquinone and 2 analogues. Cancer Res 43: 1951
48. Panneerealvam M, Bredehorst R, Vogel C-W (1987) Resistance of human melanoma cells against the cytotoxic and complement-enhancing activities of doxorubicin. Cancer Res 47: 4607
49. Partridge MBR (1987) A study of the in vitro cytotoxicity of alkylaminoanthraquinone antitumour agents based on mitoxantrone and doxorubicin. PhD Thesis, Leicester Polytechnic
50. Pincus R, Goldman D (1990) Evidence for impaired mitoxantrone and vinblastine binding in P388 murine leukaemia cells with multidrug resistance. Biochem Pharmacol 40: 2625
51. Potmesil M, Kirschenbaum S, Israel M, Levin M, Khetarpal VK, Silber R (1983) Relationship of Adriamycin concentrations to the DNA lesions induced in hypoxic and euoxic L1210 cells. Cancer Res 43: 3528
52. Potmesil M, Israel M, Silber R (1984) Two mechanisms of Adriamycin-DNA interaction in L1210 cells Biochem Pharmacol 33: 3137
53. Reska K, Kolodziejczyk P, Lown JW (1986) Horseradish peroxidase-catalysed oxidation of mitoxantrone: spectrophotometric and electron paramagnetic studies. Free Radicals Biol Med 2: 25
54. Reska K, Hartley JA, Kolodziejczyk P, Lown JW (1989) Interaction of the peroxidase-derived metabolite of mitoxantrone with nucleic acids: evidence for covalent binding of ^{14}C-labeled drug. Biochem Pharamcol 38: 4253–4260
55. Roberts RA, Cress AE, Dalton WS (1989) Persistent intracellular binding of mitoxantrone in a human colon carcinoma cell line. Biochem Pharmacol 38: 4283
56. Shenkenberg TD, Von Hoff DD (1986) Mitoxantrone: a new anticancer drug with significant clinical activity. Annals Internal Medicine 105: 567
57. Showalter HDH, Fry DW, Leopold WR, Lown JW, Plambeck JA, Reszka K (1986) Design, biochemical pharmacology, electrochemistry and tumour biology of antitumour anthrapyrazoles. Anticancer Res 1: 73
58. Sinha BK, Katki AG, Batist G, Cowan KH, Myers CE (1987) Differential formation of hydroxyl radicals by Adriamycin in sensitive and resistant human breast tumour cells: implications for the mechanism of action. Biochemistry 26: 3776
59. Sinha BK, Katki AG, Batist G, Cowan KH, Myers CE (1987) Adriamycin-stimulated hydroxyl radical formation in human breast tumour cells. Biochem Pharmacol 36: 793
60. Skovsgaard T (1978) Carrier mediated transport of daunorubicin, Adriamycin and rubidazone in Ehrlich ascites tumour cells. Biochem Pharmacol 27: 1221
61. Smith PJ, Morgan SA, Fox ME, Watson JV (1990) Mitoxantrone-DNA binding and the induction of topoisomerase II associated DNA damage in multi-drug resistant small cell lung cancer cells. Biochem Pharmacol 40: 2069
62. Stout DL, Becker FF (1982) DNA single strand fluorimetric quantitation after alkaline elution. Anal Biochem 127: 302
63. Taylor RF, Teague LA, Yesair DW (1981) Drug-binding macromolecular lipids from L1210 leukaemia tumours. Cancer Res 41: 4316
64. Tewey KM, Rowe TC, Yang L, Halligan DBD, Liu LF (1984) Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerse II. Science 226: 466
65. Turner MJ III, Everman DB, Ellington SP, Fields CE (1990) Detection of free radicals during the cellular metabolism of Adriamycin. Free Radicals Biol Med 9: 415
66. Wallace RE, Lindh D, Durr FE (1987) Development of resistance and characteristics of a human colon carcinoma subline resistant to mitoxantrone in vitro. Cancer Invest 5: 417
67. Wang JC (1985) DNA topoisomerases. Annu Rev Biochem 54: 665
68. White RJ, Durr FE (1985) Development of mitoxantrone. Invest New Drugs 3: 85
69. Zwelling LA, Michael S, Erickson LC, Ungerleider RS, Nichols M (1981) Protein associated deoxyribonucleic acid strand breaks in L1210 cells treated with the deoxyribonucleic acid intercalating agents 4′-(9-acridinylamino)methanesulfon-*m*-anisidide and Adriamycin. Biochemistry 20: 6553