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THE EFFECT OF THE ANTHRAPYRAZOLE ANTITUMOUR AGENT CI941 ON RAT LIVER MICROSOME AND CYTOCHROME P-450 REDUCTASE MEDIATED FREE RADICAL PROCESSES

INHIBITION OF DOXORUBICIN ACTIVATION *IN VITRO**

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Abstract—The anthrapyrazole CI941 is one of a new series of DNA complexing drugs which displays high level broad spectrum antitumour activity in mice. In view of the proposed role of drug free radical formation, superoxide generation and lipid peroxidation in anthracycline and anthraquinone induced toxicities, the redox biochemistry of CI941 has been investigated. Studies have been performed *in vitro* using rat liver microsomes and purified cytochrome P-450 reductase. In addition, the ability of CI941 to undergo chemical reduction has been examined. Pulse radiolysis of CI941 demonstrated that the drug can undergo chemical reduction with a one electron reduction potential of $E_7^1 = -538 \pm 10$ mV. However, electron spin resonance (ESR) spectroscopy studies using either NADPH fortified microsomes or cytochrome P-450 reductase, failed to detect a drug free radical signal. Unlike doxorubicin, CI941 (150 μM) inhibited basal rate microsomal NADPH consumption by 45%. Furthermore, CI941 (50–200 μM) antagonised doxorubicin stimulated (1.8-fold) NADPH oxidation by over 50%. CI941 also antagonised the formation of a doxorubicin free radical ESR signal in a concentration dependent manner. CI941 induced minimal superoxide generation in the presence of either microsomes or cytochrome P-450 reductase and inhibited doxorubicin induced (50 μM) superoxide formation by up to 80% (50–200 μM CI941). Importantly, CI941 inhibits both basal rate and doxorubicin (100 μM) stimulated lipid peroxidation (52% inhibition at 5 μM CI941). These data suggest that CI941 is unlikely to induce free radical mediated tissue damage *in vivo*. On the contrary, CI941 may have a protective role if used in combination with doxorubicin.

CI941 (Fig. 1) is one of a new series of 5-[(amino-alkyl)amino]-substituted anthra(1,9-*cd*)pyrazol-6(2H)ones (anthrapyrazoles), which have been shown to possess excellent experimental anti-tumour activity in mice [1, 2]. These preclinical studies demonstrated that a number of anthrapyrazoles have activity similar to doxorubicin and superior to mitozantrone in terms of potency and spectrum of activity. Structurally the anthrapyrazoles are similar to the anthraquinones differing primarily in the central quinone moiety, by virtue of a fourth ring, to form a modified quinoneimine. The rationale behind this modification was to design a drug which maintained a high affinity for DNA yet possessed diminished or absent cardiotoxicity as a result of a reduced tendency for semiquinone free radical formation [3, 4]. The planar chromophore and cationic nature of doxorubicin are essential for its high affinity intercalative binding to DNA and presumably its associated effects on DNA and RNA synthesis [5, 6]. These structural features are maintained in the anthrapyrazoles and in this respect they have been shown to bind to DNA and induce single and double strand breaks [7]. However, whereas doxorubicin and mitozantrone inhibit whole cell DNA and RNA synthesis equally, the anthrapyrazoles have a more potent effect on DNA synthesis than on RNA synthesis [4, 7] an observation which may have clinical relevance.

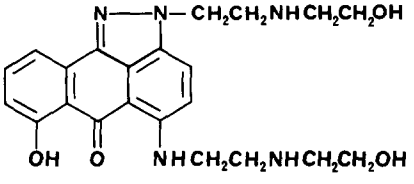

Fig. 1. The anthrapyrazole CI941.

Some of the toxicities associated with the clinical use of doxorubicin have been attributed to the metabolic reduction of the drug to a reactive free radical [8–10]. *In vitro* studies using microsomal enzymes and purified cytochrome P-450 reductase have demonstrated that a number of quinone anti-tumour agents can undergo one electron reduction to a semiquinone free radical with the concomitant oxidation of NADPH (Fig. 2) [11–14]. The drug free radical may then react with molecular oxygen to form the superoxide anion ($O_2^{\cdot-}$). Superoxide anions can give rise to other highly reactive species such as the hydroxyl radical (OH), singlet oxygen ($^1O_2$) and

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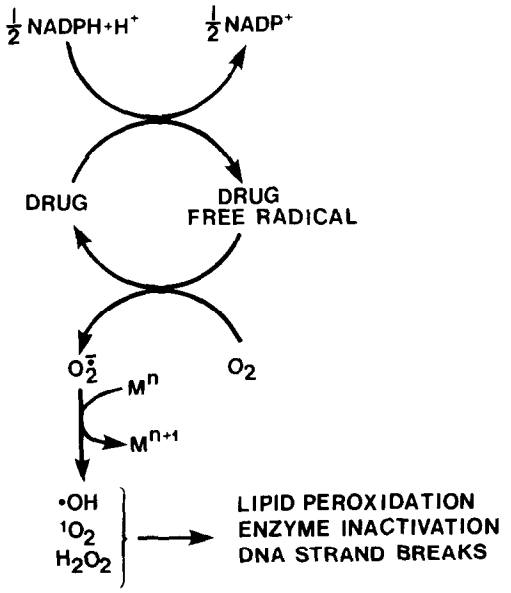

Fig. 2. The redox cycling of quinone based anticancer drugs (M^n = metal ions).

hydrogen peroxide (H_2O_2) [15–17] (Fig. 2). These activated oxygen species can in turn induce destructive cellular events such as enzyme inactivation, DNA strand cleavage and membrane lipid peroxidation [17–20]. It would, therefore, appear that metabolic one electron reduction of anthracycline based antitumour agents to drug free radicals may give rise to the undesirable side effects of these compounds, especially cardiotoxicity [21]. In this respect the anthraquinones mitozantrone and ametantrone and the quinoneimine anthracycline, 5-iminodaunorubicin, have a reduced tendency for free radical formation [22–24], a feature which may account for their reduced cardiac toxicity. CI941, as a modified quinoneimine, is also unlikely to be readily reduced. Hence this study examines the potential chemical and metabolic reduction of CI941 and the effect of the drug on NADPH oxidation, superoxide anion formation and lipid peroxidation. Furthermore, since there may be a shift in the mode of action of CI941 compared to doxorubicin [7], both compounds could be given in combination. Thus the effect of CI941 on doxorubicin free radical formation has also been investigated.

MATERIALS AND METHOD

Drugs and chemicals. CI941 was a generous gift from Warner-Lambert Pharmaceutical Research (Ann Arbor, MI). Doxorubicin hydrochloride, biochemicals and reagents were purchased from either Sigma Chemical Co., (Poole, Dorset, U.K.) or Boehringer Mannheim (Lewes, Sussex, U.K.). Bio-Rad protein assay reagent was purchased from Bio-Rad laboratories (D8000 München, F.R.G.).

Liver microsomes and purified cytochrome P-450 reductase. Liver microsomes were prepared from male Wistar rats (8–10 weeks old) fed diet and water *ad libitum* as described by Gorrod *et al.* [25]. Microsomes were stored as 100,000 *g* pellets at −78° until required and resuspended in sodium phosphate (180 mM) buffer with KCl (225 mM) pH 7.4 immediately prior to use to give an approximate concentration of 1.0 mg microsomal protein/ml. Purified cytochrome P-450 reductase was prepared from phenobarbitone induced rats as described previously [26]. The purified fractions were stored at −78° until required. Cytochrome P-450 reductase and microsomal protein concentrations were determined using the Bio-Rad one step assay kit which uses coomasie blue as a dye reagent with bovine serum albumin as a standard.

Pulse radiolysis of CI941. Pulse radiolysis experiments were conducted using the Paterson Laboratories 10 MeV linear accelerator facility. The optical detection system consisted of a xenon lamp, a Kratos monochromator and an EMI 9558 QA photomultiplier. Microcells with optical pathlengths of 2.5 cm (volume = 0.15 ml) were used. The signals from the photomultiplier were recorded on a Tektronix 7612D programmable digitizer and analysed by a Hewlett-Packard 8836s computer. Free radical decay was followed at 520 nm.

Electron spin resonance (ESR) studies. ESR studies were carried out at room temperature (21°) with a Varian E3 x-band spectrometer at a microwave frequency of 9.5 GHz. The incubation mixture consisted of CI941 (100 μM–5 mM) and/or doxorubicin (concentrations given in text), liver microsomal protein (approx. 6 mg) and NADPH (5 mM) in 200 mM phosphate buffer (total volume 1 ml) rendered essentially anaerobic by purging with nitrogen. For the studies with cytochrome P-450 reductase the 1 ml incubation mixture consisted of 200 mM phosphate buffer, 1 mM NADPH and 5 μg cytochrome P-450 reductase (equivalent to 200 units of enzyme activity).

NADPH utilisation. The effect of CI941 and doxorubicin on the rate of liver microsomal NADPH oxidation was measured over 5 min by following the decrease in NADPH absorbance at 340 nm (CI941 does not absorb strongly at this wavelength, Fig. 3). The incubation mixture (1 ml) comprised sodium phosphate buffer (180 mM, pH 7.4)/KCl (225 mM) and rat liver microsomes (140 μg protein) with or without CI941 (50–200 μM) and/or doxorubicin (50 μM). The rate of NADPH oxidation was quantitated using an absorption coefficient of 6.22 mM^{-1} cm^{-1} [27]. Spectrophotometric determinations were performed using a Perkin-Elmer 552 spectrophotometer operating in dual beam mode. The temperature was maintained at 37° by means of a Perkin-Elmer C570 0710 temperature controller.

Superoxide anion formation. Superoxide anion formation was determined at 37° by two methods.

1. The difference in the rate of acetylated cytochrome *c* reduction observed before and after the addition of superoxide dismutase (320 μg/ml). The 1 ml incubation mixture contained 150 mM KCl/Tris (50 mM) buffer pH 7.4, acetylated cytochrome *c* (0.07 mM). NADPH (1 mM) and 0.2 μg cytochrome P-450 reductase. Superoxide anion formation was determined using the absorption coefficient

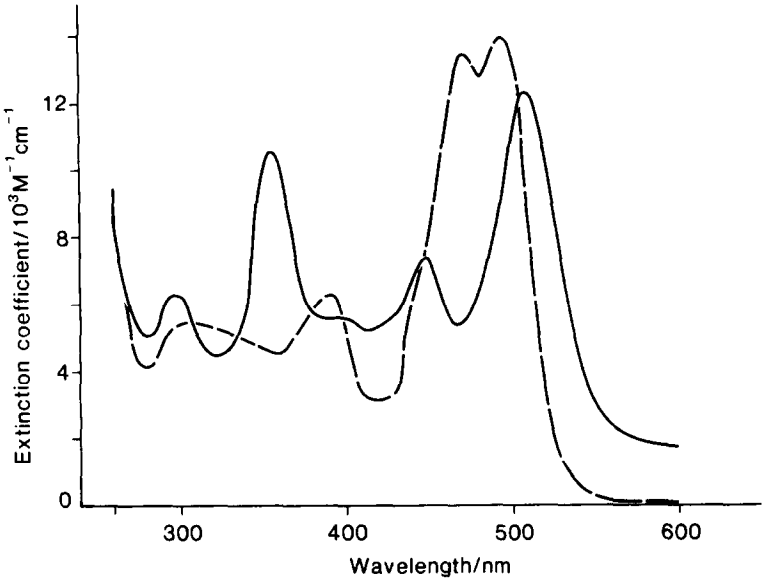

Fig. 3. Pulse radiolysis of CI941. U.v./visible spectra of CI941 (dotted line) and the CI941 radical (solid line) following a 108 μsec pulse in 10 mM phosphate buffer (pH 7.0).

21 $mM^{-1} cm^{-1}$. Acetylated cytochrome *c* was prepared according to published methods [28, 29].

2. The second method determined the O_2^--dependent reduction of adrenaline to adrenochrome by measuring the increase in absorbance at 480 nm. Adrenochrome formation was quantified using the absorption coefficient 4.02 $mM^{-1} cm^{-1}$ [30]. The incubation mixture (1 ml) consisted of potassium phosphate buffer (100 mM pH 7.7), EDTA (1 mM), NADPH (1 mM), adrenaline bitartrate (2 mM) and either 0.2 μg cytochrome P-450 reductase or microsomal protein (40 μg). Superoxide dismutase (320 μg) totally abolished the basal rate and any drug stimulated O_2^- formation.

Lipid peroxidation. Microsomal lipid peroxidation was measured as thiobarbituric acid reactive material by monitoring the difference in absorbance at two wavelengths (533 nm and 580 nm) and using an absorption coefficient of 153 $mM^{-1} cm^{-1}$ [18]. The open flask incubation mixtures (1.5 ml, 37°) consisted of 150 mM KCl/Tris HCl (50 mM) buffer (pH 7.4), NADPH (1 mM), microsomal protein (1.5 mg), with or without CI941 (5–200 μM) and/or doxorubicin (100 μM). The reaction was terminated after 60 min by the addition of 10% (w/v) trichloroacetic acid (1 ml) and heated to 95° for 30 min with 0.5 ml 1% (w/v) thiobarbituric acid (dissolved in 0.1 M NaOH). Control samples containing all the above components were kept at 4° during the incubation of the samples and used to correct for absorbance due to the drug.

RESULTS

Pulse radiolysis

The one electron reduction of CI941 was carried out by pulse radiolysis of a nitrous oxide saturated phósphate buffer solution (pH 7.0, 10 mM) containing CI941 (50 μM) and sodium formate (0.1 M). The principle of this system is well established [31] and the following reactions are considered to occur:

$$H_2O \xrightarrow{\wedge\!\vee} e^- aq, \dot{O}H, \dot{H}, H_2O_2, H_2 \quad (1)$$

$$e^- aq + N_2O \xrightarrow{H_2O} \dot{O}H + OH^- + N_2 \quad (2)$$

$$\dot{O}H\ (\dot{H}) + HCOO^- \longrightarrow H_2O\ (H_2) + CO_2^- \quad (3)$$

$$CO_2^- + Drug \longrightarrow Drug^- + CO_2 \quad (4)$$

$$Drug^- + Drug^- + 2H^+ \longrightarrow Drug\text{-}H_2 + Drug \quad (5)$$

$$Drug^- + O_2 \longrightarrow Drug + O_2^- \quad (6)$$

Figure 3 shows the u.v./visible absorbance spectra for CI941 and the CI941 free radical. The CI941 free radical decays in the absence of oxygen by disproportionation ($2K_5 = 1.2 \pm 0.7 \times 10^9\ M^{-1} sec^{-1}$]). When air is present the decay of the CI941 radical is presumably by electron transfer to oxygen ($K_6 = 1.8 \pm 0.1 \times 10^9\ M^{-1} sec^{-1}$). The one electron reduction potential was calculated to be $E_7^1 = -538 \pm 10$ mV. This was derived from the equilibrium constants produced by generating drug free radical in the presence and absence of methylviologen by previously established methods [32].

ESR studies and the effect of CI941 *on NADPH oxidation, superoxide anion formation, and lipid peroxidation*

A CI941 free radical signal could not be detected by ESR, following a 60 min anaerobic incubation of the drug (100 μM or 5 mM) in the presence of either NADPH-fortified rat liver microsomes or purified cytochrome P-450 reductase. However, CI941 did have an effect on NADPH utilising processes. Unlike

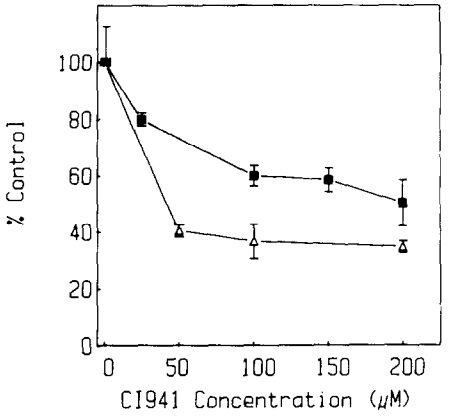

Fig. 4. The inhibition of basal rate NADPH oxidation (29 ± 3.2 nmol min^{-1} mg^{-1} ■—■) and lipid peroxidation (5.3 ± 0.8 nmole mg^{-1} $60\ min^{-1}$ △—△) by CI941 (25–200 μM) in rat liver microsomes. Error bars represent the standard deviation of 3–5 observations.

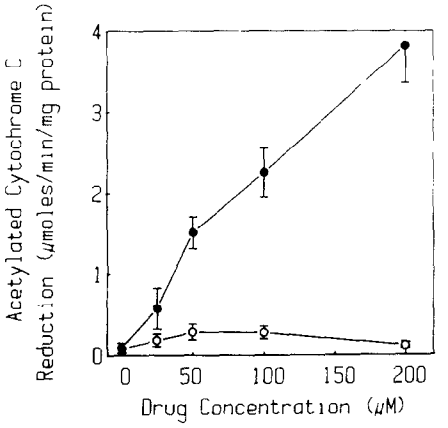

Fig. 6. Superoxide anion formation by purified cytochrome P-450 reductase as determined by the reduction of acetylated cytochrome *c*. Doxorubicin (●—●), CI941 (○—○). Error bars represent the standard deviation of 3–5 observations.

doxorubicin, CI941 (25–200 μM) partially inhibited basal rate NADPH oxidation (29.0 ± 3.2 nmole min^{-1} mg^{-1} protein) by up to 45% at 200 μM (Fig. 4). However, basal rate NADPH oxidation could not be completely abolished.

Superoxide formation was evaluated by two independent assays using either rat liver microsomes or cytochrome P-450 reductase. In both systems, doxorubicin (25–200 μM) resulted in a dose dependent stimulation of superoxide formation as determined by either adrenochrome formation (microsomes, Fig. 5) or by the reduction of acetylated cytochrome *c* (cytochrome P-450 reductase, Fig. 6). In contrast, CI941 (25–200 μM) induced only minimal superoxide formation in liver microsomes (Fig. 5) whilst in the presence of cytochrome P-450 reductase the superoxide levels observed were at the limits of detection of the assay (Fig. 6). An investigation of the effect of the drug on lipid peroxidation revealed that CI941 inhibited basal rate lipid peroxidation (5.35 ± 0.8 nmole mg^{-1} $60\ min^{-1}$) by 66% at 200 μM (Fig. 4). Doxorubicin (100 μM), in comparison, stimulated lipid peroxidation 2.1-fold (data not known).

The effect of CI941 *on doxorubicin induced free radical formation, NADPH oxidation, superoxide generation and lipid peroxidation*

Co-incubation of CI941 and doxorubicin with rat liver microsomes resulted in a concentration dependent decrease in the doxorubicin free radical signal intensity (Fig. 7), as determined by ESR, with 50% inhibition at a doxorubicin/CI941 ratio of 2:1.

Doxorubicin (50 μM) stimulated NADPH oxidation in the presence of microsomes and purified cytochrome P-450 reductase (doxorubicin stimulated rate in microsomes = 53.1 ± 1.3 nmole min^{-1} mg^{-1} and with cytochrome P-450 reductase 18.8 ± 3.2 μmol min^{-1} mg^{-1}). CI941 (25–150 μM)

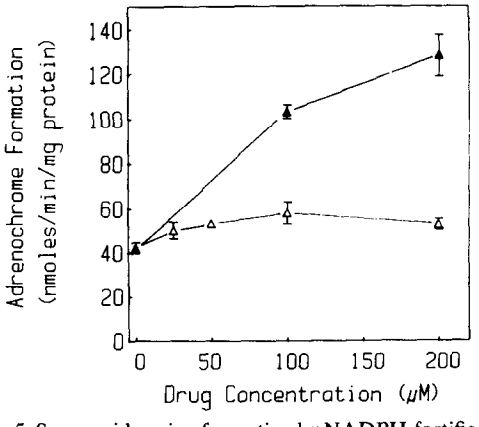

Fig. 5. Superoxide anion formation by NADPH-fortified rat liver microsomes as determined by superoxide dismutase inhibitable adrenochrome formation. Doxorubicin (▲—▲). CI941 (△—△). Error bars represent the standard deviation of 3–5 observations.

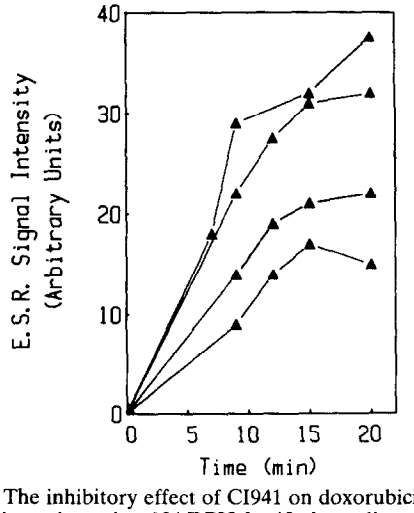

Fig. 7. The inhibitory effect of CI941 on doxorubicin ESR signal intensity using NADPH-fortified rat liver microsomes. Top line doxorubicin alone (160 μM). Lower three lines doxorubicin/CI941 molar ratios of 100:1, 10:1 and 2:1 respectively in descending order.

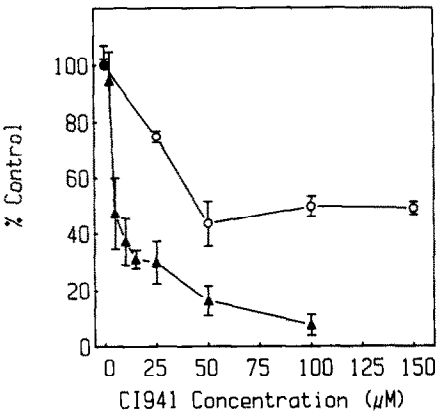

Fig. 8. The effect of CI941 on doxorubicin stimulated microsomal NADPH oxidation and lipid peroxidation. (○—○) NADPH oxidation (50 μM doxorubicin) and lipid-peroxidation (▲—▲) (100 μM doxorubicin). Error bars represent the standard deviation of 3–5 observations.

inhibited doxorubicin stimulated NADPH oxidation by up to 55% with the microsomal preparation (Fig. 8) and by 78% using the purified cytochrome P-450 reductase (Fig. 9). Similarly using cytochrome P-450 reductase a concentration dependent inhibition of doxorubicin (50 μM) stimulated superoxide anion formation was observed with CI941 over the concentration range 50–200 μM with 80% inhibition at 200 μM CI941 (Fig. 9).

Importantly CI941 was a potent inhibitor of doxorubicin stimulated NADPH dependent lipid-peroxidation in microsomes (basal rate = 4.55 ± 0.75 nmols hr^{-1} mg^{-1}; 50 μM doxorubicin stimulated rate = 15.30 ± 1.0 nmoles hr^{-1} mg^{-1}) resulting in 52% inhibition at 5 μM CI941 (Fig. 8).

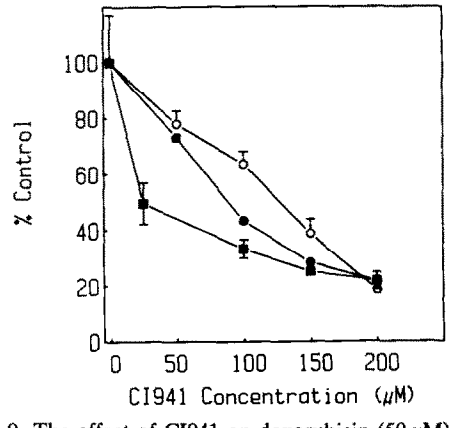

Fig. 9. The effect of CI941 on doxorubicin (50 μM) stimulated superoxide formation and NADPH oxidation using cytochrome P-450 reductase. Superoxide formation as determined by the adrenochrome assay (●—●) and the acetylated cytochrome *c* assay (○—○). NADPH oxidation (■—■). Error bars represent the standard deviation of 3–5 observations.

DISCUSSION

The anthrapyrazole CI941 can be considered as a modified anthraquinoneimine molecule with bis-hydroxyethylaminoalkylamino side chains identical to those found in mitozantrone. The anthraquinone moiety common to both mitozantrone and doxorubicin is central to the metabolic activation of these drugs to free radical species [12, 13, 22]. It has been suggested that doxorubicin free radical generation is involved in the cytotoxicity and cardiotoxicity of this compound (for review see ref. 33). In contrast the involvement of mitozantrone free radical generation in cytotoxicity has still to be evaluated. However, the reduced cardiotoxicity associated with mitozantrone administration compared to doxorubicin [34] may be a consequence of reduced free radical generation in heart tissue [19, 35].

In the present study we have examined the ability of CI941 to become metabolically activated to a drug free radical. Pulse radiolysis of CI941 has shown that the drug can chemically undergo one electron reduction at pH 7. The electron reduction potential (E_7^1 = −538 ± 10 mV) is similar to that previously reported by us for mitozantrone (E_7^1 = −527 mV) [22] indicating that thermodynamically there is no significant difference in the ability of these two drugs to form free radical intermediates. The CI941 free radical species formed by pulse radiolysis in our study was air unstable and like mitozantrone [22] rapidly reduced dioxygen at a rate that was essentially diffusion controlled.

Although a CI941 free radical is feasible from a chemical point of view, metabolically no free radical appeared to be generated by liver microsomes as observed by ESR. Using microsomal enzyme systems we have previously demonstrated mitozantrone free radical formation [22]. Further ESR experiments using a highly purified source of cytochrome P-450 reductase also failed to produce a detectable CI941 free radical. These results indicate that CI941 is not a substrate for one-electron reduction by rat liver microsomal reductases including cytochrome P-450 reductase.

It is possible that the absence of a measurable CI941 free radical by ESR is due to rapid further reduction of this transient intermediate to two-electron and possibly four-electron reduced metabolites. These reduction products have been suggested to occur during polarography of a number of anthrapyrazoles including CI941 [4] although metabolic reductions of this type would not be favoured at the reduction potentials reported. Furthermore CI941 and other anthrapyrazoles were shown to be more resistant to two-electron reduction (−0.983 to −1.085 V) than mitozantrone (−0.775 V) or 5-iminodaunorubicin (−0.625 V) with the addition of hydroxyl groups to the A-ring of the anthrapyrazole chromophore making the molecule progressively harder to reduce [4]. The one-electron reduction potential of CI941 (E_7^1 = −0.538 V) should ensure reduction of oxygen (E_7^1 = −0.155 V, for 1 M oxygen) to the superoxide anion, especially at drug concentrations relevant to biological systems. However, CI941 induces only minimal superoxide anion formation either with microsomes or with

cytochrome P-450 reductase. Indeed the levels measured were barely above the limits of detection of the assays used. CI941 also partially inhibited microsomal NADPH oxidation whereas a stimulation of this process would be expected during NADPH dependent free radical formation. The rate of microsomal NADPH oxidation, despite its inhibition (up to 45%) by CI941, is still sufficient stoichiometrically to account for most of the superoxide produced in the presence of this drug. Therefore on balance the data presented in this study supports the absence of a metabolically generated CI941 free radical. The results with CI941 are in contrast to those shown in this study for doxorubicin and also those reported previously for both doxorubicin and mitozantrone where both drugs were found to stimulate microsomal NADPH oxidation with a concomitant increase in superoxide production by a mechanism that involves a free radical intermediate [12–14, 22].

In addition to its effects on microsomal NADPH consumption CI941 also inhibits NADPH dependent lipid peroxidation. The mechanism of this inhibition is unlikely to be due to disruption of the reactive oxygen initiation reaction of lipid peroxidation since superoxide anion formation would be expected to participate indirectly. The role of superoxide anions in lipid peroxidation is generally believed to via the formation of secondary reactive oxygen species [36]. These are formed through superoxide dismutation to H_2O_2, which in the presence of chelated ferrous ions generates $^{\cdot}OH$. The $^{\cdot}OH$ radical will generate lipid radicals which peroxidise in the presence of dioxygen. However, peroxidised lipids in the form of hydroperoxides can also initiate lipid breakdown via the ferrous ion dependent formation of lipid peroxyradicals and lipid alkoxyradicals [36]. Since CI941 does not inhibit basal rate superoxide anion formation, it seems likely that CI941 inhibits basal rate lipid peroxidation by a mechanism analogous to that described for mitozantrone, namely, inhibition of hydroperoxide initiation and propagation reactions [37]. Inhibition of these processes by CI941 could involve a direct radical scavenging mechanism and/or inactivation of ferrous ion chelates.

The second part of the present study examined the effect of CI941 on doxorubicin activation. ESR studies with doxorubicin in combination with CI941 demonstrated a concentration dependent inhibition of the doxorubicin drug free radical signal with 50% inhibition at a doxorubicin/CI941 ratio of 2:1. This result is consistent with the inhibitory action of CI941 on doxorubicin mediated NADPH oxidation. This antagonism may be the result of an interaction of CI941 with the flavin components (FAD and FMN) of microsomal reductases. Both mitozantrone and doxorubicin are known to bind with high affinity to FMN and FAD [38, 39] an interaction that may be involved in electron transfer from the reductase to those drugs. An interaction by CI941 at this site may prevent the transfer of electrons to doxorubicin thereby inhibiting doxorubicin drug free radical formation. Alternatively a transient CI941 drug free radical species (not detected by ESR) may be formed in preference to a doxorubicin radical. However, the latter seems unlikely in view of the inhibitory effect of CI941 on basal rate NADPH oxidation and its minimal effect on superoxide anion formation when incubated alone with microsomes.

Of interest, is the effect of CI941 on doxorubicin induced superoxide anion formation. Rather than an additive effect, the converse was apparent, namely, CI941 resulted in a concentration dependent inhibition of doxorubicin induced superoxide formation. Presumably the decrease in superoxide anion production is a consequence of the diminished doxorubicin free radical formation alluded to above. An additional result of the combined use of the two agents was a potent inhibitory action of CI941 on doxorubicin stimulated lipid peroxidation. Although antagonism by CI941 at the level of the reductase may be operative this is unlikely to be the principle locus of action, as CI941 is a potent inhibitor of lipid peroxidation (52% inhibition at 5 μM CI941) relative to its inhibitory action on NADPH oxidation (55% inhibition at 150 μM CI941) (Fig. 8). It seems likely therefore that the antagonism of doxorubicin induced lipid peroxidation is by the mechanism proposed for the inhibition of basal rate lipid peroxidation, namely, inhibition of initiation and propagation reactions.

Frank and Novak [40] have recently examined the effects of a number of anthrapyrazoles and aminoacridine derivatives on doxorubicin and iron stimulated lipid peroxidation in phenobarbital induced rabbit liver microsomes. In comparison to our findings, CI941 was not found to be as potent as an inhibitor of lipid peroxidation. However, this may be a reflection of the different systems employed, i.e. phenobarbital induction and/or species variations in the ability to undergo lipid peroxidation.

In conclusion, this study has shown that CI941 has a markedly different redox chemistry to doxorubicin. Rather than producing stimulation, CI941 acts primarily as an inhibitor of free radical mediated processes *in vitro*. In this respect the modified quinoneimine nature of the drug may play an important role in its lack of metabolic activation. Importantly CI941 has been shown to antagonise doxorubicin mediated free radical events such as NADPH oxidation, superoxide anion formation and lipid peroxidation, factors which have been implicated in the toxicities associated with the use of the anthracyclines. Given that CI941 acts as an inhibitor of doxorubicin activation there is a strong rationale for the combined administration of these two antitumour agents.

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