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1,4-BIS{2-[(2-HYDROXYETHYL)AMINO]ETHYLAMINO}-9,10-ANTHRACENEDIONE, AN ANTHRAQUINONE ANTITUMOUR AGENT THAT DOES NOT CAUSE LIPID PEROXIDATION *IN VIVO*; COMPARISON WITH DAUNORUBICIN

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Daunorubicin administration to mice produces a marked stimulation of lipid peroxidation in both liver and heart 48 hours following administration. In direct contrast 1,4-Bis{2-[(2-hydroxyethyl)amino]ethylamino}-9,10-anthracenedione (HAQ) does not induce lipid peroxidation in the liver and actually inhibits this event in the heart. In addition, neither daunorubicin nor HAQ deplete reduced glutathione in liver or heart 48 hours after drug administration. Daunorubicin induced glutathione (GSH) depletion was observed 2.5 hours following administration. These results correlate with daunorubicin increased microsomal oxygen consumption whilst HAQ produced no measurable effect on the rate of microsomal oxygen utilisation. It would appear that redox cycling to produce free radical oxygen involved in lipid peroxidation and GSH depletion, an established action of daunorubicin, does not occur with HAQ. This apparent lack of HAQ reactivity may help explain the relatively low cardiotoxicity of this novel antitumour agent.

1,4-Bis{2-[2-hydroxyethyl)amino]ethylamino}-9,10-anthracenedione (HAQ) has been shown to possess antitumour activity against a wide range of experimental tumours and cell lines alone (1-4) and in combination with clinically useful agents (5). HAQ bears a broad structural resemblance to the anthracyclines such as doxorubicin (adriamycin) and daunorubicin in that both types of antitumour agent have a planar anthraquinone structure and ionisable side-chain moieties. Such features are generally accepted as being responsible for the high affinity of intercalative binding of doxorubicin and daunorubicin to DNA. The dose-limiting severe cardiotoxic side effect of these anthracyclines is well established (6). This problem has prompted several

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Abbreviations: HAQ - 1,4-Bis{2-[(2-hydroxyethyl)amino]ethylamino}-9,10-anthracenedione dihydrochloride; GSH - reduced glutathione.

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studies to evaluate the cardiotoxic potential of the anthraquinones and HAQ is shown to be generally less cardiotoxic than doxorubicin (1,7). The interest in HAQ is therefore partly because of its potential as an alternative antitumour agent to the anthracyclines. There is some evidence that relates the cardiotoxicity of daunorubicin to the generation of reactive oxygen species in heart tissue (8) and with the resultant lipid peroxidation (9). Reductive metabolism to produce the anthracycline semiquinone is implicated in these events (10). Reduced glutathione (GSH) is suggested to protect against anthracycline mediated heart and liver damage since depletion of this cofactor excacerbates toxicity (11) whilst supplementation by sulphydryl compounds ameliorates toxicity (11,12).

HAQ is a candidate for metabolic reduction and associated consequences since it contains the anthraquinone moiety of the anthracyclines. In view of this we report here the effects of HAQ on lipid peroxidation *in vivo* and relate this to GSH content of liver and heart and to microsomal oxygen consumption. The effect of daunorubicin on these events has also been investigated to enable a direct comparison of the two types of antitumour agent.

METHODS

Daunorubicin hydrochloride (Cerubidin) was purchased from May and Baker, UK. HAQ was synthesised essentially as described in (13) as the dihydrochloride salt. Both drugs were diluted in isotonic sterile saline and administered as a single dose intraperitoneally to F1 generation Swiss male mice (25 ± 2g) using a dose volume of 4 ml Kg^{-1}. Control mice were dosed at the same time with isotonic saline. Animals were sacrificed and liver and heart subcellular fractions were prepared as described in (14). Protein was determined using the Bio-rad one-step assay employing bovine serum albumin as a calibration standard. Data was analysed using students t test.

Reduced glutathione (GSH) was determined in 9000 x g liver fractions using 5,5'-dithiobis-2-nitrobenzoic acid (Ellman reagent) and measuring the resultant yellow solution at 412 nm as described in (15). GSH in heart homogenate was measured fluorimetrically at an excitation wavelength of 330 nm and emission wavelength 425 nm after derivatisation with o-phthalic dicarboxaldehyde (16).

Lipid peroxidation (thiobarbituric acid reactive material) was determined as follows. Liver microsomes (0.4 ml) or heart 9000 x g fraction (0.1 ml) were treated with trichloroacetic acid (10%, 1.0 ml) followed by thiobarbituric acid (1%, 1.0 ml) in 0.01 M sodium hydroxide and heated at 95°C for 30 minutes. The resulting solutions were cooled, centrifuged and measured either as the

absorbance difference between 580-532 nm, or fluorimetrically using an excitation wavelength of 515 nm and emission of 550 nm. Aqueous solutions of 1,1',3,3'-tetramethoxypropane added to tissue fractions and treated in an identical manner were found to give linear calibration for the malondialdehyde-thiobarbituric acid product.

Oxygen consumption was measured at 37°C in liver microsomes in the presence of an NADPH generating system prepared as described in (14) and with or without drug, using a Clark type polarographic oxygen electrode. Oxygen consumption was calculated assuming 597 nmole for the total dissolved oxygen content of the reaction mixture.

RESULTS

It is evident from the results in Table 1 that a single dose of daunorubicin significantly stimulated lipid peroxidation in mouse heart by at least two-fold compared to control animals. In contrast, HAQ actually inhibited lipid peroxidation in heart tissue by almost two-fold. Lipid peroxidation in liver was also stimulated by daunorubicin although in this organ HAQ produced no effect compared to non-dosed controls. In all tissues lipid peroxidation was measured 48 hour after ip dosing of daunorubicin or HAQ since a previous study with adriamycin (14-C-hydroxydaunorubicin) showed a similar time lag between drug administration and lipid peroxidation (17). Neither daunorubicin or HAQ induced lipid peroxidation when measured 2.5 hours after dosing (results not shown). GSH levels in heart and liver measured 48 hours following daunorubicin or HAQ administration are also described in Table 1. These results show that neither tissue was depleted

Table 1

Lipid peroxidation and reduced glutathione levels in mouse heart and liver 48h after intraperitoneal dosing with HAQ or daunorubicin

Treatment	Dose	Lipid peroxidation[a] (malondialdehyde equivalents in pmole mg^{-1})		reduced glutathione[a] $\mu g\ g^{-1}$ tissue	
		Liver	Heart	Liver	Heart
Control	-	270 ± 20	168 ± 34	1453 ± 223	115 ± 25
HAQ	20 mg Kg^{-1}	275 ± 10	94 ± 7*	1645 ± 99	154 ± 27
Control	-	200 ± 10	174 ± 20	920 ± 89	81 ± 14
Daunorubicin	15 mg Kg^{-1}	290 ± 30*	398 ± 36*	1018 ± 188	84 ± 15

[a] mean ± S.E. of 4-6 determinations; * $p > 0.05$.

Table 2

Reduced glutathione content of mouse liver 2.5h after ip dosing with HAQ or daunorubicin

Treatment	Dose	reduced glutathione[a] $\mu g\ g^{-1}$ liver
Control	-	2044 ± 188
HAQ	25 mg Kg^{-1}	2040 ± 150
Control	-	1291 ± 140
HAQ	100 mg Kg^{-1}	951 ± 90*
Control	-	1599 ± 62
Daunorubicin	43 mg Kg^{-1}	1069 ± 81*

[a] mean ± S.E. of 4-6 determinations; * $p > 0.02$.

of GSH compared to non-dosed controls over the time period in which lipid peroxidation was evident. The GSH content of liver was, however, significantly depleted (33% less than controls) 2.5 hours following daunorubicin administration. HAQ failed to deplete GSH levels significantly even after a four-fold increase in dose was administered (see Table 2).

Oxygen consumption in NADPH fortified hepatic microsomes prepared from mice treated with daunorubicin or HAQ 48 hours previously was not significantly different from that measured in control microsomes (Table 3). However, addition of exogenous daunorubicin to these microsomes increased the rate of oxygen consumption three-fold (results not shown). This indicates that the amount of daunorubicin present in liver tissue 48 hours following

Table 3

Effect of daunorubicin and HAQ on oxygen consumption in mouse liver microsomes

Treatment	O_2 consumption (nmole min^{-1} mg^{-1})[a]	
	microsomes from treated mice without additional drug	microsomes from untreated mice ± drug
Control	26.8 ± 3.4	25.3 ± 3.2
HAQ	29.9 ± 3.4	29.7 ± 2.9[b]
Control	34.1 ± 2.8	25.7 ± 3.4
Daunorubicin	35.5 ± 3.4	75.9 ± 3.0[c]

[a] mean ± S.E. 4-6 determinations; [b] 1200 μmole HAQ; [c] 130 μmole daunorubicin.

administration was not sufficient to exert an observable effect *in vitro*. In contrast, additional HAQ had no appreciable effect on the basal rate of oxygen consumption. HAQ was also unable to elicit an increase in the rate of oxygen consumption when added to NADPH fortified liver microsomes from non-treated mice, although under such conditions daunorubicin produced a substantial effect (Table 3). Furthermore, oxygen consumption was not affected by exogenous HAQ even at a concentration approximately nine times that required to give stimulation by daunorubicin.

DISCUSSION

HAQ is a synthetically produced anthraquinone antitumour agent which, although based on the anthracyclines, is significantly less cardiotoxic. This study demonstrates that HAQ does not deplete heart or liver reduced glutathione (GSH) or stimulate lipid peroxidation *in vivo*. These events were associated with daunorubicin, an anthracycline known to be cardiotoxic. This direct comparison suggests that the lack of HAQ cardiotoxicity is due to its inability to stimulate lipid peroxidation, indeed in the heart HAQ actually inhibits this process *in vivo* by some 43%. Daunorubicin stimulated lipid peroxidation is probably mediated by semiquinone and/or free radical oxygen formation. Indirect evidence in support of this is the increase in rate of microsomal oxygen consumption in the presence of daunorubicin. The inability of HAQ to stimulate microsomal oxygen consumption suggests this antitumour agent cannot redox cycle to generate free radical oxygen. In support of this HAQ has been shown to inhibit the normal activity of NADPH dependent cytochrome P450 reductase (18), a microsomal enzyme responsible, in part, for the supply of reducing equivalents during daunorubicin redox cycling. Interruption of electron transfer between NADPH and the reductase or reductase and oxygen by HAQ could explain the lack of increased oxygen consumption and inhibition of lipid peroxidation observed.

Neither HAQ or daunorubicin depleted liver or heart GSH at the time (48 hours) in which lipid peroxidation was observed. This prompted us to measure the effect of both drugs on liver GSH after a shorter time interval following

administration, since doxorubicin has previously been shown to produce a significant but transient effect (less than 12 hours) on the liver and heart GSH pools (19). Daunorubicin did show a significant depletion of GSH 2.5 hours following administration. Whether this transient fall in GSH can be associated with daunorubicin induced lipid peroxidation some 48 hours later requires further investigation. What is clear however is the inability of HAQ to deplete GSH 2.5 hours following administration of either 25 mg Kg^{-1} or 100 mg Kg^{-1}. Of interest is the ability of daunorubicin to deplete cardiac glutathione peroxidase (20), an enzyme which utilises GSH to deactivate lipid peroxides. This could explain the increased heart lipid peroxidation observed without concomittant loss of GSH 48 hours following daunorubicin administration.

Despite the apparent lack of HAQ reactivity we have previously shown that HAQ administration does cause destruction of hepatic cytochrome P450. In the same study cytochrome P450 and glutathione-S-transferase destruction were observed following daunorubicin treatment, concomittant with increased lipid peroxidation (21). Previous reports have described adriamycin mediated cytochrome P450 and other enzyme loss *in vivo* and lipid peroxidation has been cited as a causal effect (22,23).

Clearly, the potential of HAQ to induced cell component damage does not involve lipid peroxidation. Indeed the preliminary evidence suggests that HAQ in the heart might actively protect against lipid peroxidation, a view that has recently been corroborated by HAQ effects *in vitro* (24). Such an action might help explain the relatively low experimental cardiotoxicity observed with HAQ when compared to the anthracyclines. In view of this HAQ warrants further clinical evaluation. In addition, HAQ is a structural variation of the anthracyclines that has proved useful as a lead compound, and we are currently investigating analogues which will intercalate (25) but not exhibit properties that are likely to cause cardiotoxicity.

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