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Microsomal lipid peroxidation induced by Adriamycin, epirubicin, daunorubicin and mitoxantrone: a comparative study

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Summary. Rat-liver microsomes and NADPH could reduce Adriamycin, epirubicin and daunorubicin to their free radical forms, which enhanced peroxidation of microsomal lipids less than 2-fold in air but 3- to 5-fold at a pO_2 of 4 mmHg. Mitoxantrone was not reduced by microsomes and had no effect on microsomal peroxidation. Daunorubicin caused more lipid peroxidation than similar concentrations of either Adriamycin or epirubicin, which were equally efficient. In each case peroxidation was iron-dependent and could be catalysed by ferritin. The antioxidants β-carotene and α-tocopherol inhibited lipid peroxidation at low or high pO_2. The dose-for-dose difference in the cardiotoxicity of epirubicin compared with Adriamycin is not explained by its effect on microsomal lipid peroxidation. However, the lower incidence of cardiotoxicity with mitoxantrone may be a consequence of its inability to form free radical species and promote lipid peroxidation.

Introduction

A number of anthracycline and related compounds have been developed with the aim of obtaining an anticancer drug that is as effective as Adriamycin or daunorubicin but less cardiotoxic; epirubicin and mitoxantrone have shown some promise clinically [12, 30]. There is mounting evidence that free radical reactions are involved in the cardiotoxicity of Adriamycin and daunorubicin. Both are reduced by heart microsomal cytochrome P-450 reductase [6, 10] and by mitochondrial enzymes [18] to semiquinone radicals that are thought to give rise to cell-damaging processes such as lipid peroxidation [10, 11]. Lipid peroxidation has been measured in rats following Adriamycin administration [19], and this drug promotes the peroxidation of microsomal membrane lipids in vitro [9, 10, 23]. This process is iron-dependent and is maximal at O_2 partial pressures of 4–8 mmHg [23], the levels that are observed in functioning muscle cells [2]. Ferritin can provide the iron catalyst for the reaction [23], and lipid peroxidation under optimal conditions is inhibited by β-carotene and, to a lesser extent, by α-tocopherol [25].

This study was undertaken to determine whether Adriamycin, daunorubicin, epirubicin and mitoxantrone also promote ferritin-catalysed microsomal lipid peroxidation with maximal effect at low pO_2 and whether the reportedly low cardiotoxicities of epirubicin and mitoxantrone relate to their abilities to induce peroxidation. The ability of α-tocopherol and β-carotene to inhibit drug-induced peroxidation was also investigated. The study was carried out using rat-liver microsomes. A previous investigation using Adriamycin [23] has shown that the peroxidation of heart microsomes occurs under conditions similar to those used here, and by a similar mechanism.

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Materials and methods

Microsomes were prepared from the livers of Wistar rats [3] and protein content was determined according to the method of Lowry et al. [8]. All procedures were carried out in acid-washed glassware, and levels of adventitious iron were decreased by treating solutions with chelex resin (BioRad Laboratories, Calif). All biochemicals were obtained from Sigma (St Louis, Mo) except Adriamycin and epirubicin (from Farmitalia; Carlo Erba, Italy), daunorubicin (from May and Baker; London, UK) and mitoxantrone (from Lederle; Pearl River, NY).

Cytochrome c reduction. Cytochrome P-450 reductase activity was measured by incubating microsomes (0.5 mg protein) and NADPH (100 μ*M*) with 100 μ*M* cytochrome c in 1 ml 10 m*M* phosphate buffer (pH 7.4). Reduction of cytochrome c was monitored at 550 nm ($\Delta\varepsilon_{\text{reduced-oxidised}} = 21{,}100\ m^{-1}cm^{-1}$).

Lipid peroxidation. Microsomes (0.5 mg/ml) were incubated with 100 μ*M* NADPH and either 1 μ*M* $FeCl_3$ or 50 μg/ml ferritin in phosphate buffer (pH 7.4) with drug as required. The pO_2 was manipulated by carrying out reactions in rubber-stoppered glass tubes and bubbling the solutions with O_2-free N_2, then adding air with a gas-tight syringe. Lipid peroxidation was measured as the formation of thiobarbituric acid-reactive products at 532 nm (A_{532}) [29]. β-carotene or α-tocopherol was added in 10 μl chloroform to a pellet of microsomes by gentle homogenisation followed by resuspension in buffer as previously described [24]. Cytochrome P-450 reductase activity was standardised such that a 50 μl preparation reduced cytochrome c in air at a rate of 3 μ*M*/min when incubated with 100 μ*M* NADPH in 1 ml buffer.

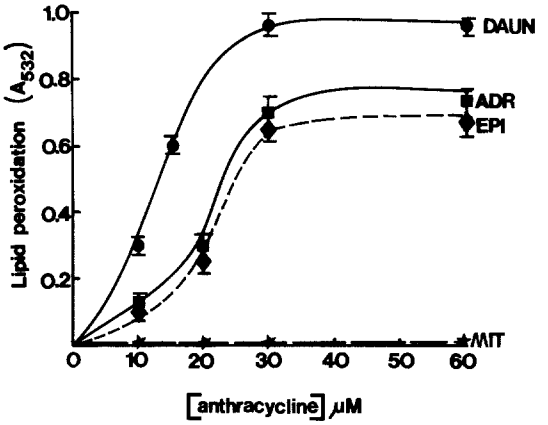

Fig. 1. Effect of anthracycline concentration on the peroxidation of rat-liver microsomes. Incubations included microsomes, NADPH, ferritin and either daunorubicin (●), Adriamycin (■), epirubicin (◆), or mitoxantrone (★). Reactions were carried out at a pO_2 of 4 mmHg

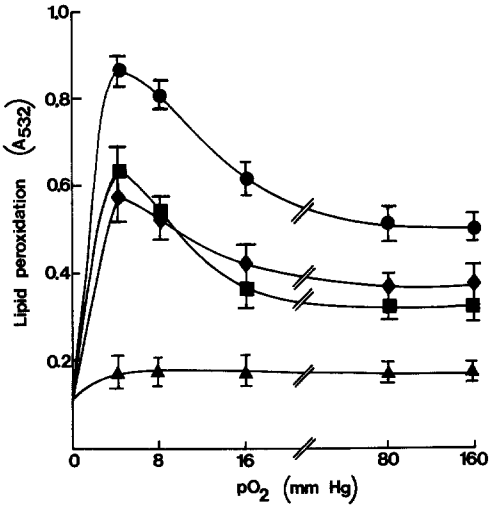

Fig. 2. Dependence of anthracycline-promoted lipid peroxidation on pO_2. Microsomes were incubated with NADPH, ferritin and either 30 μ*M* daunorubicin (●), 30 μ*M* Adriamycin (■), 30 μ*M* epirubicin (◆), or no addition (▲) as described in *Materials and methods.* Each point represents the mean ±SD for two sets of duplicates

Results

Adriamycin, daunorubicin and epirubicin at a concentration of 30 μ*M* approximately doubled microsomal reduction of cytochrome c; however, even at a concentration of 100 μ*M*, mitoxantrone had no effect (Table 1). Correspondingly, all three anthracyclines promoted lipid peroxidation in the presence of ferritin at a pO_2 of 4 mmHg in a concentration-dependent manner (Fig. 1); mitoxantrone did not promote peroxidation. As previously observed with Adriamycin [23], lipid peroxidation induced by epirubicin and daunorubicin was dependent on iron (Table 2).

Table 1. Effects of Adriamycin, epirubicin, daunorubicin and mitoxantrone on microsomal cytochrome c reduction[a]

Addition	Rate of cytochrome c reduction (μ*M*/min)
None	3.02
Adriamycin (30 μ*M*)	5.53
Epirubicin (30 μ*M*)	5.87
Daunorubicin (30 μ*M*)	5.79
Mitoxantrone (30 μ*M*)	3.00
Mitoxantrone (100 μ*M*)	3.04

[a] Microsomes were incubated in air with NADPH, cytochrome c and drug as indicated. Each result represents the mean of three experiments that agreed within 5%

A_{532} with no added iron was probably a result of adventitious transition metals catalysing peroxidation. Ferritin (50 μg/ml containing 1.04 nmol Fe/μg) was as efficient as 1 μ*M* iron, and iron release from ferritin was required since desferrioxamine inhibited peroxidation (Table 2). Daunorubicin and epirubicin, like Adriamycin, showed the same pO_2 maximum at 4 mmHg (Fig. 2). Lipid peroxidation promoted by daunorubicin was significantly greater than that promoted by either Adriamycin or epirubicin (Figs. 1 and 2).

At concentrations that have previously been shown to inhibit Adriamycin-promoted lipid peroxidation maximally [25], the antioxidants β-carotene and α-tocopherol were efficient inhibitors of peroxidation promoted by epirubicin and daunorubicin (Table 3). In each case, β-carotene was more effective than α-tocopherol at low pO_2, whereas α-tocopherol was better under aerobic conditions.

Discussion

Adriamycin has been shown to promote lipid peroxidation in liver and heart microsomes, with a maximal effect at a low pO_2 [23]. Peroxidation depends on the reduction of Adriamycin to its semiquinone by cytochrome P450 reduc-

Table 2. Effect of iron on anthracycline-dependent peroxidation of microsomal lipids[a]

Addition	Lipid peroxidation (A_{532})				
	None	Adriamycin (30 μ*M*)	Epirubicin (30 μ*M*)	Daunorubicin (30 μ*M*)	Mitoxantrone (30 μ*M*)
None	0.058	0.105	0.110	0.138	ND
1 μ*M* $FeCl_3$	0.174	0.632	0.587	0.872	0.014
50 μg ferritin	0.071	0.742	0.707	0.984	0.012
50 μg ferritin + 100 μ*M* desferrioxamine	0.064	0.082	0.089	0.096	ND

[a] Microsomes were incubated with NADPH and drug as indicated at a pO_2 of 4 mmHg. A_{532} values were read against a no-NADPH blank, which has been subtracted. Each result represents the mean of four experiments that agreed within 5%. ND, not done

Table 3. Effect of β-carotene and α-tocopherol on anthracycline-promoted lipid peroxidation at high and low pO_2[a]

Addition	% Inhibition of lipid peroxidation (A_{532})			
	pO_2 = 4 mmHg		pO_2 = 162 mmHg	
	β-carotene	α-tocopherol	β-carotene	α-tocopherol
Adriamycin (30 μ*M*)	65	51	50	62
Epirubicin (30 μ*M*)	67	53	42	64
Daunorubicin (30 μ*M*)	64	50	38	69

[a] Microsomes, ferritin, NADPH and anthracycline were incubated with and without antioxidant (50 nmol/mg microsomal protein). Blanks due to antioxidant and anthracycline and microsomes incubated with no NADPH were subtracted and the percent ages of inhibition were calculated. Each value represents the mean of three experiments that agreed within 5%

tase and the subsequent reduction of an iron catalyst. Both reduction steps are inhibited by O_2 [24]; however, O_2, is required for propagation of the peroxidative chain, and these opposing effects of O_2 give the low pO_2 maximum for the process [23]. The present study has shown that, like Adriamycin [23], daunorubicin and epirubicin promote microsomal lipid peroxidation with maximal efficiency at a low pO_2. In agreement with other studies [4, 7, 13], we demonstrated that mitoxantrone was not reduced by microsomes and did not promote peroxidation of microsomal lipids. In fact, our results support those of Kharasch and Novak [7], who have shown that mitoxantrone inhibits peroxidation. Although mitoxantrone contains a potentially reducible quinone group, its redox potential is −0.45 mV [16], compared with −0.32 mV for Adriamycin, daunorubicin and epirubicin [15], and is probably too low to enable its reduction by the microsomal reductase.

Normal heart tissue contains 30–60 μg ferritin/g [1]. Since most cellular iron is restricted within ferritin, the ability of Adriamycin, daunorubicin and epirubicin to release iron from ferritin in a microsomal system [20, 23] suggests that ferritin may be a physiological source of iron that could be available to catalyse reactions such as the peroxidation of lipids. α-Tocopherol has been shown to protect a number of biological systems from Adriamycin-promoted free radical damage in vitro [14, 17], although results in vivo have been equivocal [22, 26]. We have recently shown that β-carotene and α-tocopherol can each inhibit Adriamycin-dependent microsomal lipid peroxidation, with β-carotene producing the greater inhibition at a low pO_2 [25]. The present work demonstrates that peroxidation promoted by epirubicin and daunorubicin is also inhibited by both antioxidants, with β-carotene again being more efficient than α-tocopherol at a low pO_2.

Clinically, epirubicin is less cardiotoxic than an equivalent dose of Adriamycin or daunorubicin, although the therapeutic advantage is minimal when the higher doses of epirubicin are taken into account [12, 28]. We found that epirubicin promoted less microsomal lipid peroxidation than daunorubicin over the concentration range investigated, but no such difference was observed between epirubicin and Adriamycin in our system. These results support and extend those of other studies showing that Adriamycin and epirubicin are equally able to generate superoxide in microsomes [5, 15, 21]. Although mitoxantrone is not totally clinically free of cardiotoxic side effects, it is much less cardiotoxic than Adriamycin [28]. The lack of both free radical formation and promotion of lipid peroxidation by mitoxantrone may be a reason for this. Although lipid peroxidation may be one of the mechanisms by which cardiotoxicity manifests itself, it does not explain the dose-for-dose difference in the cardiotoxicity of Adriamycin and epirubicin. A more significant factor may be the more rapid glucuronidation of epirubicin, which would facilitate urinary excretion of the drug [27].

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References

1. Bezkorovainy A (1980) Chemistry and biology of iron storage. In: Biochemistry of non heme iron. Plenum, New York, p 207
2. Coburn RF, Mayers LB (1971) Myoglobin O_2 tension determined from measurements of carboxymyoglobin in skeletal muscle. Am J Physiol 220: 66–74
3. Ernster L, Siekevitz P, Palade GE (1962) Enzyme-structure relationships in the endoplasmic reticulum of the rat liver. J Cell Biol 15: 541–562
4. Doroshow JH, Davies KJA (1983) Comparative cardiac oxygen radical metabolism by anthracycline antibiotics, mitoxantrone, bisantrene, 4′-(a-acridinylamino)-methanesulfon-m-antisidide, and neocarzinostatin. Biochem Pharmacol 32: 2935–2939
5. Grankvist K, Henricksson R (1987) Doxorubicin and epirubicin iron-induced generation of free radicals in vitro. A comparative study. Biosci Rep 7: 653–657
6. Handa K, Sato S (1976) Stimulation of microsomal NADPH oxidation by quinone group-containing anticancer chemicals. Gann 67: 523–528
7. Kharasch ED, Novak RF (1983) Inhibitory effects of anthracenedione antineoplastic agents on hepatic and cardiac lipid peroxidation. J Pharmacol Exp Ther 226: 500–506
8. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
9. Mimnaugh EG, Trush MA, Ginsburg E, Gram TE (1982) Differential effects of anthracycline drugs on rat heart and liver microsomal reduced nicotinamide adenine dinucleotide phosphate-dependent lipid peroxidation. Cancer Res 42: 3574–3582
10. Mimnaugh EG, Gram TE, Trush MA (1983) Stimulation of mouse heart and liver microsomal lipid peroxidation by anthracycline anticancer drugs: characterization and effects of reactive oxygen scavengers. J Pharmacol Exp Ther 226: 806–816
11. Myers CE, McGuire WP, Liss RH, Ifrim I, Grotzinger K, Young RC (1977) Adriamycin: the role of lipid peroxidation in cardiac toxicity and tumour response. Science 197: 165–167
12. Neri B, Bartalucci S, Cinelli P, De Leonardis V, De Scalzi M, Cappellini M, Pacini P (1988) Clinical aspects of doxorubicin and epirubicin cardiotoxicity in cancerous patients. Current Ther Res 43: 73–81
13. Novak RF, Kharasch ED (1985) Mitoxantrone: propensity for free radical formation and lipid peroxidation – implications for cardiotoxicity. Invest New Drugs 3: 95–99

14. Pascoe GA, Reed DJ (1987) Vitamin E protection against chemical-induced cell injury. Arch Biochem Biophys 256: 159–166
15. Peters JH, Gordon GR, Kashiwase D, Lown JW, Yen SF, Plambeck JA (1986) Redox activities of antitumour anthracyclines determined by microsomal oxygen consumption and assays for superoxide and hydroxyl radical generation. Biochem Pharmacol 35: 1309–1323
16. Sinha BK, Motten AG, Hanck KW (1983) The electrochemical reduction of 1,4-*bis*π2-[(2-hydroxyethyl)-amino] ethylamino-anthracenedione and daunomycin: biochemical significance in superoxide formation. Chem Biol Interact 43: 371–377
17. Sonneveld P (1978) Effect of α-tocopherol on the cardiotoxicity of Adriamycin in the rat. Cancer Treat Rep 62: 1033–1036
18. Thayer WS (1977) Adriamycin-stimulated superoxide formation in submitochondrial particles. Chem Biol Interact 19: 265–278
19. Thayer WS (1984) Serum lipid peroxides in rats treated chronically with Adriamycin. Biochem Pharmacol 33: 2259–2263
20. Thomas CE, Aust SD (1986) Release of iron from ferritin by cardiotoxic anthracycline antibiotics. Arch Biochem Biophys 248: 684–689
21. Thornalley PJ, Dodd NJF (1985) Free radical production from normal and Adriamycin-treated rat cardiac sarcosomes. Biochem Pharmacol 34: 669–674
22. Van Vleet JF, Ferrans VJ, Weirich WE (1980) Effect of Vitamin E against Adriamycin-induced toxicity in rabbits. Cancer Res 40: 1022–1027
23. Vile GF, Winterbourn CC (1988) Adriamycin-dependent peroxidation of rat liver and heart microsomes catalysed by iron chelates and ferritin: maximum peroxidation at low oxygen partial pressures. Biochem Pharmacol 37: 2893–2897
24. Vile GF, Winterbourn CC (1988) Microsomal reduction of low molecular weight Fe^{3+}-chelates and ferritin: enhancement by Adriamycin, paraquat, menadione and anthraquinone-6-sulphonate, and inhibition by oxygen. Arch Biochem Biophys 267: 606–613
25. Vile GF, Winterbourn CC (1988) Inhibition of Adriamycin-promoted microsomal lipid peroxidation by β-carotene and α-tocopherol at high and low oxygen partial pressure. FEBS Lett 238: 353–356
26. Wang Y, Madanat FF, Kimball JC, Gleiser CA, Ali MK, Kaufman MW, Van Eys J (1980) Cardiac disease induced by chronic Adriamycin administration in dogs and an evaluation of Vitamin E and selenium as cardioprotectants. Am J Pathol 99: 13–42
27. Weenen H, Van Maanen JMS, Panque MM, Mcvie JG, Pinedo HM (1984) Metabolism of 4′-modified analogs of doxorubicin. Unique glucuronidation pathway for 4′-epidoxorubicin. Eur J Cancer Clin Oncol 20: 919–926
28. Weiss RB, Sarsoy G, Clagett-Carr K, Russo M, Leyland Jones B (1986) Anthracycline analogs: the past, present, and future. Cancer Chemother Pharmacol 18: 185–197
29. Winterbourn CC, Gutteridge JMC, Halliwell B (1985) Doxorubicin-dependent lipid peroxidation at low partial pressures of O_2. J Free Rad Biol Med 1: 43–49
30. Young CW, Raymond V (1986) Clinical assessment of the structure-activity relationship of anthracyclines and related synthetic structures. Cancer Treat Rep 70: 51–63

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