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Protective Effect of Butylated Hydroxyanisole on Adriamycin-induced Cardiotoxicity

JAYESH VORA, BAN-AN KHAW*, JAGAT NARULA* AND MEHDI BOROUJERDI

*Department of Pharmaceutical Sciences, Northeastern University, Boston, MA 02115 and *Centre for Drug Targeting and Analysis, Northeastern University, Boston, MA 02115, USA*

Abstract

Adriamycin has a wide spectrum of antitumour activity with dose-related cardiotoxicity as a major side effect. This cardiotoxicity has been suggested to result from the generation of oxygen free radicals. The objective of the present study was to investigate the influence of the antioxidant, butylated hydroxyanisole co-therapy on the cardiotoxicity of adriamycin, in-vivo.

The aqueous solubility of butylated hydroxyanisole was enhanced by inclusion complex formation with hydroxypropyl-β-cyclodextrin. The extent of drug-induced myocardial damage in rats was assessed using intravenous ^{111}In-labelled antimyosin Fab and chronological changes in serum creatine kinase levels. There was a dose-related increase in myocardial antimyosin uptake in rats, which reached a plateau at an adriamycin dose of 10 mg kg^{-1}. The antimyosin uptake at this dose (% dose g^{-1} = 0·1942 ± 0·0150, n = 8) was significantly reduced by co-administration of butylated hydroxyanisole with adriamycin (10 mg kg^{-1} of each) to 0·1462 ± 0·0116 (n = 5, $P < 0.05$).

Assessment of cardiotoxicity in the rats was also performed by measuring serial changes in serum creatine kinase levels. Increasing doses of adriamycin caused an increase in serum creatine kinase levels with peak values obtained between 2 and 8 h after dosing. These values decreased upon co-administration of butylated hydroxyanisole with adriamycin at 10 mg kg^{-1}, each and 30 mg kg^{-1}, each by 29 and 41%, respectively. On the other hand, butylated hydroxyanisole did not inhibit the tumouricidal activity of adriamycin as investigated in-vitro using the NMU rat mammary adenocarcinoma cell-line.

The significant reduction in anthracycline cardiotoxicity by butylated hydroxyanisole coadministration may result from its scavenging action on adriamycin-mediated free-radical formation or its enhancement of activity of enzymes involved in the metabolism of adriamycin.

Adriamycin is an anthracycline antibiotic with a wide spectrum of antitumour activity (Calabresi et al 1985) but its use is limited predominantly by its cardiotoxicity. The drug is converted to a semiquinone free radical by NADPH-cytochrome P450 which could then lead to generation of superoxide and hydroxyl radicals, causing membrane-lipid peroxidation, DNA strand breaks, and enzyme inactivation possibly leading to cell death (Powis & Hacker 1991; Myers 1992). It is likely that the cardiotoxicity is a direct result of this drug-induced free radical formation. Several antioxidants have been shown to reduce adriamycin-induced cardiomyopathy (Doroshow et al 1981; Geetha et al 1990). However, the common food antioxidants, butylated hydroxyanisole and butylated hydroxytoluene, have not yet been evaluated. Since these antioxidants form a part of the regular diet, it would be beneficial to examine their effect on anthracycline cardiotoxicity.

Materials and Methods

Preparation of butylated hydroxyanisole-hydroxypropyl-β-cyclodextrin complex

The aqueous solubility of butylated hydroxyanisole (Sigma Chemical Co., St Louis, MO) was enhanced via formation of an inclusion complex with hydroxypropyl-β-cyclodextrin (Pharmatec, Inc., Alachua, FL). The butylated hydroxyanisole-hydroxypropyl-β-cyclodextrin inclusion complexes were prepared by mixing equimolar amounts of the antioxidant and the cyclodextrin in water at about 40°C for six days. The resulting clear solution after lyophilization formed a powder which dissolved instantaneously in water. The complex was characterized using phase-solubility analysis, X-ray diffraction and infra-red spectroscopy (Vora & Boroujerdi 1995).

Correspondence: M. Boroujerdi, Department of Pharmaceutical Sciences, Northeastern University, Boston, MA 02115, USA.

Antimyosin antibody and labelling with ^{111}In

The labelling procedure involved addition of ^{111}indium chloride (1 mCi)(Amersham, Arlington, IL) in citrate (1 M, pH 5·5) in a solution of diethylenetriamine pentaacetic acid-derivatized antimyosin Fab (250 μg)(Centocor, PA). The reaction mixture was vortex mixed and incubated at room temperature for 1 h. The radiolabelled antibody was isolated by size-exclusion gel filtration on a Sephadex G25 (Sigma) column (10 mL) using saline as the eluent.

Animals and experimental protocol

Male Sprague-Dawley rats, 150-200 g, were used (Charles River, Cambridge, MA) (Table 1). The drug (adriamycin or adriamycin with butylated hydroxyanisole-hydroxypropyl-β-cyclodextrin complex) was administered intravenously into the caudal vein as a solution in physiological saline. Negative-control animals received physiological saline or only butylated hydroxyanisole-hydroxypropyl-β-cyclodextrin complex in saline by tail vein injection. Positive-control animals received subcutaneous isoprenaline overdose (85 mg kg^{-1}) which is known to result in significant myocyte necrosis (Benjamin et al 1989; Yunge et al 1989). In each case, the total volume of injection was approximately 1 mL. After drug dosing, ^{111}In-

Table 1. Dose groups of the animals used in this study.

Drugs (mg kg^{-1})		Myocardial antimyosin uptake (% injected dose g^{-1})	Serum creatine kinase[b] (units L^{-1})
Adriamycin	Butylated hydroxyanisole[a]		
0[c]	0	10	20
	10	5	5
	30	-[d]	5
4	0	8	5
	0	12	5
10	2·5	4	-
	5	4	-
	10	5	5
20	0	7	5
30	0	4	5
	30	-	5
Isoprenaline	0	4	-

[a]Butylated hydroxyanisole-hydroxypropyl-β-cyclodextrin complex. [b]Serum creatine kinase measurements were made in a crossover design, each rat receiving saline treatment 5-6 days before receiving adriamycin or adriamycin-butylated hydroxyanisole. [c]Control groups which did not receive adriamycin were administered the same volume of saline or butylated hydroxyanisole in saline intravenously. [d]Not determined.

labelled antimyosin antibody (40-50 μCi) was administered intravenously via the caudal vein.

Dosing schedule for adriamycin and antimyosin

The "24-24" dosing schedule consisted of intravenous administration of adriamycin at time zero, antimyosin at 24 h, with the animals being killed at 48 h. For the "48-48" dosing schedule the rats received adriamycin at time zero, antimyosin at 48 h, with the animals being killed at 96 h. The "6 day-24 h" (6d–24) dosing schedule consisted of dosing of animals with adriamycin at time zero, antimyosin at 6 days, with the animals being killed on the 7th day.

The influence of butylated hydroxyanisole (as butylated hydroxyanisole-hydroxypropyl-β-cyclodextrin complex) on adriamycin cardiotoxicity was investigated according to the 24–24 dosing schedule. Increasing doses of antioxidant (2·5, 5 and 10 mg kg^{-1}) were co-administered with adriamycin (10 mg kg^{-1}) to distinct groups of animals at time zero and antimyosin at 24 h, the animals being killed at 48 h.

Quantitative antimyosin uptake in the myocardium and non-target organs

Heart, lungs, liver, spleen, kidneys, tail and a sample of blood and skeletal muscle from the abdominal region were quickly excised, washed with saline, weighed, and counted in an automatic gamma counter (LKB Compugamma).

Caudal vein cannulation for serum creatine kinase assay

Serial changes in serum creatine kinase levels during the experimental period were evaluated in a crossover design in rats following saline injection on day 1 and adriamycin or adriamycin-butylated hydroxyanisole administration after a washout period of 5–6 days. For either treatment, rats were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}) and the caudal vein was cannulated with polyethylene tubing (PE-10) using a 19-gauge needle as a trocar. Saline or drug solution was administered approximately 1 h after cannulation when serum creatine kinase levels were found to have subsided to basal values. Blood samples (60–80 μL) were collected in non-heparinized capillary tubes at 0, 2, 4, 6, 8, 24, and 48 h following dosing. Serum was separated by centrifugation at 10 000 *g* for 4 min and stored at −20°C until analysis. Creatine kinase was assayed using a commercially available reagent based on the modified Szasz method.

Efficacy assessment of tumouricidal activity of adriamycin-butylated hydroxyanisole

To exclude the possible inhibitory effect of butylated hydroxyanisole on tumouricidal activity of adriamycin, NMU cells (derived from *N*-nitrosomethylurea-induced rat mammary adenocarcinoma (Cohen 1982)) were grown at 37°C in Dulbecco's modified minimum essential medium (Eagle) with Earle's balanced salt solution, 10% foetal bovine serum, gentamicin sulphate (50 g mL^{-1}) and 0·6 g L^{-1} L-glutamine under a humid atmosphere of 5% CO_2 : 95% air. Cell survival following exposure to adriamycin, adriamycin-butylated hydroxyanisole or saline control was determined by a standard in-vitro clonogenic assay.

Statistical analyses

The data are presented as mean ± standard deviation of replicate experiments. Antimyosin uptake by heart and other organs was expressed as percent of injected dose per gram of organ. Serum creatine kinase levels were presented as units L^{-1} at 30°C. Comparisons between two means were accomplished by Student's *t*-test. Multiple comparisons were done using one-way analysis of variance.

Results

Relationship between myocardial antimyosin uptake and dose of adriamycin

The myocardial antibody uptake increased with increasing doses of adriamycin administered (Table 2). The maximum damage occurred at a dose of 10 mg kg^{-1} in the rats with 24-24 and 48–48 dosing regimens. No further increase was observed in the myocardial antimyosin uptake at doses of

Table 2. Influence of dosing regimen on the myocardial antimyosin uptake in rats.

Adriamycin (mg kg^{-1})	Dosing regimen	
	24–24	48–48
0	0·1154 ± 0·0127	0·1113 ± 0·0071
4	0·1531 ± 0·0109[a]	0·1379 ± 0·0116[a]
10	0·1942 ± 0·0150[a]	0·1666 ± 0·0246[a]
20	0·1825 ± 0·0238[a]	0·1459 ± 0·0221[a]
30	0·2025 ± 0·0337[a]	–[b]

Mean ± s.d. [a]$P < 0·05$ vs respective control. [b]Not determined

Table 3. Influence of butylated hydroxyanisole[a] on the adriamycin-mediated myocardial antimyosin uptake.

Adriamycin (mg kg^{-1})	Butylated hydroxyanisole (mg kg^{-1})	Dosing regimen 24–24
0	0	0·1154 ± 0·0127
0	10	0·0934 ± 0·0025)
10	0	0·1942 ± 0·0150[b]
10	2·5	0·1907 ± 0·0226[b]
10	5	0·1673 ± 0·0129[b,c]
10	10	0·1462 ± 0·0116[b,c]

Mean ± s.d.
[a]As hydroxypropyl-β-cyclodextrin complex. [b]$P < 0·05$ compared with saline control. [c]$P < 0·05$ compared with adriamycin 10 mg kg^{-1}, ontylated hydroxyamisole 0 mg kg^{-1}.

Table 4. Biodistribution of ^{111}In-antimyosin in various non-target organs in rats with 24–24 dosing schedule.

Tissue	Treatment Saline	Adriamycin (10 mg kg^{-1})	Adriamycin-butylated hydroxyanisole[a] (10 mg kg^{-1} each)
Lungs	0·156 ± 0·045	0·190 ± 0·022	0·145 ± 0·024
Liver	0·886 ± 0·204	1·120 ± 0·070	1·095 ± 0·159
Spleen	0·402 ± 0·069	0·493 ± 0·084	0·239 ± 0·019
Kidneys	1·984 ± 0·243	2·228 ± 0·186	2·476 ± 0·322
Skeletal muscle	0·162 ± 0·067	0·101 ± 0·053	0·0536 ± 0·006

Mean ± s.d. [a]As hydroxypropyl-β-cyclodextrin complex.

20 mg kg^{-1}. The extent of adriamycin-induced myocyte necrosis remained unchanged with 6d–24 dosing regimen at 10 mg kg^{-1} adriamycin. The myocardial antimyosin uptake in isoprenaline-induced myocyte damage was comparable with the antimyosin uptake in animals receiving high doses of adriamycin.

The rats in 24–24 dosing regimen exhibited maximal antimyosin uptake at 10 mg kg^{-1}; therefore, this protocol was used in evaluating the influence of butylated hydroxyanisole on myocardial antimyosin uptake (Table 3). A butylated hydroxyanisole dose-dependent reduction in adriamycin-induced myocardial damage was observed upon co-administration of the antioxidant with the anthracycline. The decrease in antimyosin uptake was not significant when 2·5 mg kg^{-1} butylated hydroxyanisole was co-administered with adriamycin. However, the decline in myocardial antimyosin uptake became significant as the dose of butylated hydroxyanisole increased to 5 or 10 mg kg^{-1} at a constant dose of adriamycin.

The antimyosin uptake profile by the various non-target organs in the CD rat with 24–24 dosing schedule did not demonstrate any significant change in biodistribution compared with saline control animals (Table 4).

Relationship between serum creatine kinase levels and the dose of adriamycin

The myocardial damage resulting from adriamycin administration was further investigated in the rat using serum creatine kinase levels as an indicator of anthracycline cardiotoxicity. Preliminary studies indicated that serum creatine kinase levels reached their basal levels within 1 h of caudal vein cannulation. Furthermore, sodium pentobarbital anaesthesia did not alter the basal creatine kinase levels in rats (Fig. 1). The peak serum creatine kinase concentrations increased as the dose of adriamycin administered increased, reaching a maximum between 2 and 8 h after drug administration. Using this toxicity indicator, co-administration of butylated hydroxyanisole with adriamycin resulted in a significant reduction in anthracycline cardiotoxicity. At 10 mg kg^{-1} each of adriamycin and

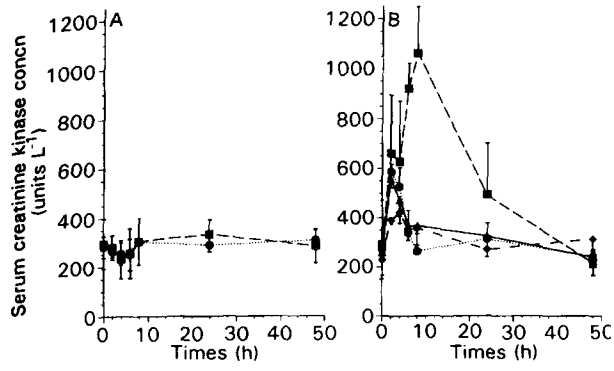

FIG. 1. Profile of serum creatine kinase control (A) and adriamycin-treated (B) rats. A. ■ Anaesthetized rats, ● conscious rats. B. ◆ 4, ▲ 10, ● 20, ■ 30 mg kg^{-1} adriamycin.

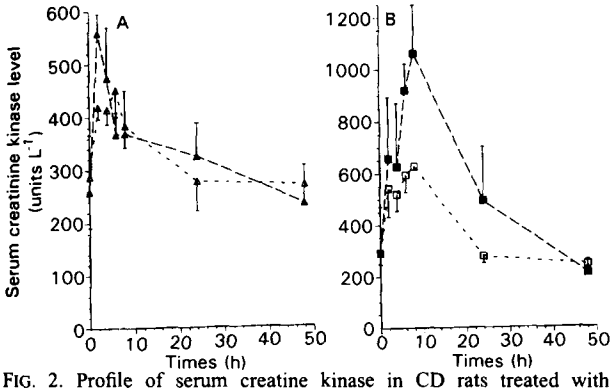

FIG. 2. Profile of serum creatine kinase in CD rats treated with adriamycin (▲, ■) or adriamycin-butylated hydroxyamisole (△, □). A. 10 mg ks^{-1}, B. 30 mg kg^{-1}.

butylated hydroxyanisole, a 29% decline in peak creatine kinase levels was observed (Fig. 2). Similarly, coadministration of butylated hydroxyanisole with adriamycin at 30 mg kg^{-1}, each, resulted in a 41% decrease in peak creatine kinase level.

Tumouricidal activity of Adriamycin-butylated hydroxyanisole

The influence of butylated hydroxyanisole cotherapy on the efficacy of adriamycin was investigated using NMU cell line derived from rat mammary adenocarcinoma (Cohen 1982). This experiment demonstrated that the antioxidant does not alter the efficacy of adriamycin (Table 5). The fraction of cells surviving exposure to adriamycin or adriamycin-butylated hydroxyanisole remained unaltered at each of the concentrations evaluated.

Discussion

There was an adriamycin dose-related increase in the quantitative myocardial uptake of antimyosin. The uptake appeared to be the most pronounced with 24–24 dosing regimen in the rats, with maximal myocardial damage occurring for 10 mg kg^{-1} adriamycin . The level of myocardial necrosis remained unaltered after higher doses. The antimyosin uptake by the heart following 10 mg kg^{-1} adriamycin was comparable with the myocardial damage resulting from animals treated with isoprenaline overdose. Dose-related increase in antimyosin uptake was confirmed by serial monitoring of serum creatine kinase levels, where an adriamycin dose-related increase in peak serum creatine kinase level was observed. However, this increase in creatine kinase level continued to occur at an adriamycin dose of 30 mg kg^{-1}. The reason for the difference in the quantitation of myocardial damage at higher adriamycin doses between these two indicators is unclear.

Since maximal damage occurred at a dose of 10 mg kg^{-1} adriamycin, this dose under 24-24 dosing schedule was chosen to investigate the influence of butylated hydroxyanisole on the myocardial antimyosin uptake. It is worth noting that this dose corresponds to the routinely used clinical dose of approximately 500 mg m^{-2} for a 75-kg, 1·73-m^2 man. At a constant dose of adriamycin, the myocardial antimyosin uptake decreased with increasing doses of butylated hydroxyanisole. At a dose of 10 mg kg^{-1} each of adriamycin and butylated hydroxyanisole, a 50% reduction in myocardial antimyosin uptake was noted as compared with that obtained from adriamycin administration alone. The degree of reduction in peak creatine kinase serum levels at these doses of adriamycin and butylated hydroxyanisole was 29%; however, at doses of 30 mg kg^{-1} each of adriamycin and butylated hydroxyanisole, the protection conferred by the antioxidant was 41% as measured by peak creatine kinase levels. Since there was no inhibition of adriamycin tumouricidal activity by coadministration of butylated hydroxyanisole, the selective reduction of adriamycin cardiotoxicity by butylated hydroxyanisole may be a clinical advantage.

Even though it was not intended to be a mechanistic study of anthracycline cardiotoxicity, the present findings may indirectly support the association of an oxygen or semiquinone free-radical species in the cardiotoxic mechanisms. The bene-

Table 5. Percentage of NMU cells surviving exposure to various concentrations of adriamycin and adriamycin-butylated hydroxyanisole.

Concn	Cells surviving (%)	
(μg mL^{-1})	Adriamycin	Adriamycin-butylated hydroxyanisole[a]
0	100 ± 0·00	100 ± 0·00
1	42·54 ± 0·44	41·49 ± 3·58
2	33·37 ± 5·30	35·32 ± 3·72
3	16·07 ± 0·52	16·39 ± 1·81
4	14·17 ± 2·09	15·28 ± 2·10
5	14·08 ± 0·88	14·93 ± 0·88

Mean ± s.d. [a]As hydroxypropyl-β-cyclodextrin complex.

ficial effect of the antioxidant on adriamycin cardiotoxicity may be a simple consequence of its antioxidant properties. However, it is equally likely that butylated hydroxyanisole may alter the disposition of adriamycin. Butylated hydroxyanisole has been shown to increase the biliary excretion of paracetamol conjugates (glucuronides and sulphates) in pretreated rats (McLaughlin & Boroujerdi 1987) and dietary butylated hydroxyanisole has been shown to protect mice from paracetamol-induced hepatotoxicity (Rosenbaum et al 1984). Dietary administration of butylated hydroxyanisole to mice has also been shown to increase the specific activity of hepatic glutathione-*S*-transferase (Demkowicz-Dobrazanski et al 1984) and cytochrome P450 in microsomal liver fractions (Benson et al 1978). Butylated hydroxyanisole inhibits DNA adduct formation between benzo(a)pyrene metabolites and DNA in mice (Anderson et al 1981). The beneficial effects of co-administration of butylated hydroxyanisole are consistent with alleviation of adriamycin-mediated cardiotoxicity by other antioxidants (e.g. *N*-acetylcysteine, α-tocopherol, coenzyme Q10, ascorbic acid). The administration of butylated hydroxyanisole as a water-soluble complex with hydroxypropyl-β-cyclodextrin ensures complete bioavailability of the antioxidant. Such inclusion complexes would be expected to dissociate upon dilution in-vivo releasing the antioxidant, which being relatively water-insoluble is likely to be retained by the myocyte longer than other more water-soluble antioxidants such as ascorbic acid and is therefore likely to be more effective. However, since there was an incomplete abolition of cardiotoxicity by co-administration of butylated hydroxyanisole, it is likely that other (non-free radical-dependent) mechanisms may also be involved.

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