MECHANISMS OF PROBUCOL PROTECTION AGAINST DEVELOPMENT OF ADRIAMYCIN-INDUCED CARDIOMYOPATHY

Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfilment of the requirements for the Degree of:

DOCTOR OF PHILOSOPHY

BY

NATASHA ILISKOVIC

Department of Physiology
Faculty of Medicine
1997

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Dedicated to my parents

Aleksandar and Nadežda Ilišković

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LIST OF ABBREVIATIONS

ADP	-	Aortic Diastolic Pressure
ADR	-	Adriamycin
ASP	-	Aortic Systolic Pressure
FFA	-	Free Fatty Acids
GSH	-	Reduced Glutathione
GSHPx	-	Glutathione Peroxidase
GSSG	-	Oxidized Glutathione
HDL	-	High Density Lipoproteins
LDL	-	Low Density Lipoproteins
LOV	-	Lovastatin
LVEDP	-	Left Ventricular End-Diastolic Pressure
LVPSP	-	Left Ventricular Peak Systolic Pressure
PROB	-	Probucol
SOD	-	Superoxide Dismutase
TBARS	-	Thiobarbituric Acid Reactive Substance
TRO	-	Trolox

ABSTRACT

Adriamycin (doxorubicin) is an antitumor drug but its potential usefulness is restricted because of the cardiotoxic side effects. Research on the mechanisms of adriamycin-induced myocardial dysfunction has resulted in several postulates including interaction of adriamycin with deoxyribonucleic acid and inhibition of nucleic acid as well as protein synthesis, increased free radical formation and oxidative stress, release of vasoactive amines, changes in adrenergic functions, abnormalities in the mitochondria, lysosomal alterations, alterations in sarcolemma and membrane bound enzymes, imbalance of myocardial electrolytes and occurrence of Ca^{2+} overload. Although adriamycin-induced injury appears to be multifactorial and complex, one mechanism common to most of these postulates is an increase in oxidative stress leading to myocardial dysfunction. An evidence in support of this oxidative stress hypothesis was provided by earlier research from our laboratory. We also demonstrated protection against adriamycin-cardiomyopathy by probucol – a well known lipid-lowering drug with antioxidant properties. Since probucol is : I) a lipid lowering drug; II) a strong antioxidant; and III) shown by us to increase superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) activities, the protection may be coming from any or all of these three possibilities. Thus the objective of this thesis research was to further define the mechanism for the protective effect of probucol. Another important objective of this research was to test the antitumor efficacy of adriamycin in the proposed adjunct therapy.

Among the three possibilities listed above, improvement in the antioxidant reserve appears to be an important factor. Thus the hypothesis tested was that probucol protection against adriamycin-induced cardiomyopathy is through an improvement in antioxidant defence

both by a direct antioxidant effect and an increase in endogenous antioxidants with or without a lowering of the plasma and cardiac lipids. Rationale for such a thinking was that probucol contains two phenolic groups which confer strong antioxidant property to this drug and we have earlier shown that probucol protection was accompanied by an increase in important endogenous antioxidant enzyme activities.

Our approach was to induce adriamycin cardiomyopathy and heart failure in rats by a procedure established in our laboratory. In order to delineate the mechanism of protection by probucol, beneficial effects of lipid-lowering as well as of an increase in myocardial antioxidants were analysed by comparing effects of probucol with another lipid-lowering drug (lovastatin) as well as another antioxidant (trolox). In all experiments, we monitored hemodynamic function; cardiomyopathic changes; myocardial antioxidant enzyme activities, glutathione [reduced (GSH) and oxidized (GSSG)] and lipid peroxidation. Plasma lipids, plasma albumin levels, serum free fatty acids as well as cardiac lipids were also analysed. Another important question whether probucol modifies antitumor property of adriamycin was also answered by comparing the effects of adjunct therapy with that of adriamycin alone in tumor bearing mice.

Male Sprague-Dawley rats weighing 250 ± 25 g were divided into different groups: CONT (control), ADR (adriamycin), PROB (probucol), PRO + ADR (probucol + adriamycin), LOV (lovastatin), LOV + ADR (lovastatin + adriamycin), TRO (trolox), TRO + ADR (trolox + adriamycin). Adriamycin was administered i.p. in six equal injections over a period of two weeks for a total cumulative dose of 15 mg/kg body weight to ADR, PROB + ADR, LOV + ADR and TRO + ADR groups. Probucol (cumulative dose 120 mg/kg body

weight), lovastatin (48 mg/kg cumulative dose) and trolox (48 mg/kg, cumulative dose) were also administered i.p. in twelve equal injections over a period of 4 weeks: two weeks prior to treatment with adriamycin and two weeks alternating with adriamycin. Both treated and control animals were monitored daily for 3 weeks after the last injection for general condition and body weight. At the end of 3 week post-treatment duration, hemodynamic function [left ventricular systolic pressure (LVSP), left ventricular end diastolic pressure (LVEDP), aortic systolic (ASP) and aortic diastolic (ADP) pressure] in animals was assessed. Myocardial tissue was studied with respect to antioxidant enzyme activities [superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and catalase], glutathione (reduced and oxidized), lipid peroxidation, triglycerides and total cholesterol. Plasma lipids including total cholesterol, low density lipoproteins (LDL), high density lipoproteins (HDL) and triglycerides were analysed by using standard kit from Sigma. Serum free fatty acids (FFA) were determined by standard kit from WAKO (NEFA C) and serum albumin was determined by Sigma albumin reagent kit (#625-2). FFA/albumin ratio, known to influence heart function, was calculated. Tumor regression was studied in syngeneic DBA/2 mice (inoculated with L5178Y-F9 lymphoma cells) that received both adriamycin and probucol treatment. Appropriate statistical tests were applied for analysis of the data.

After 3 weeks of post-treatment period, rats in the ADR group showed reduced weight gain and 45% mortality. Development of congestive heart failure was established by clinical signs such as: ascites, congested liver and depressed cardiac function manifested in decreased LVSP, ASP and ADP, as well as increased LVEDP. Adriamycin treatment decreased GSHPx activity and increased lipid peroxidation. SOD and catalase activities did

not show any change. GSH was decreased and GSSG was increased causing a significant decrease in GSH/GSSG ratio. Adriamycin increased plasma triglycerides, total cholesterol, HDL and LDL. Myocardial triglycerides and total cholesterol were also increased. Serum free fatty acids (FFA) were increased and plasma albumin was decreased, resulting in an increased FFA/albumin ratio.

In the PROB + ADR group, probucol treatment completely prevented development of congestive heart failure and normalized all parameters that were pathologically changed due to adriamycin administration. There was no mortality and ascites were insignificant. All hemodynamic parameters were maintained at control levels. GSHPx and SOD activities were increased compared to controls and lipid peroxidation was returned to normal . In the PROB group, higher levels of SOD compared to controls were also observed. GSH/GSSG ratio was improved mainly due to decrease in GSSG. Probucol normalized myocardial and plasma triglycerides and total cholesterol, and significantly decreased plasma HDL and LDL levels. FFA were normalized, while albumin remained at its decreased levels. However, the result was a normalization of FFA/albumin ratio, compared to ADR group.

In the LOV + ADR group, lovastatin significantly attenuated but did not completely prevent cardiomyopathic changes due to adriamycin. Mortality and ascites formation were decreased. Hemodynamic parameters were improved, though LVEDP remained above control levels and ASP was still significantly less, compared to controls. Lovastatin had no effect on adriamycin-induced increase in lipid peroxidation and decrease in GSHPx activity, as well as on changes in the glutathione system. Plasma total cholesterol and LDL levels as well as myocardial triglycerides and total cholesterol were decreased by lovastatin treatment.

However, plasma triglycerides and HDL levels were still higher than control levels in LOV + ADR group. FFA levels were decreased and albumin levels remained the same, having the same end result as observed in PRO + ADR group: normalization of FFA/albumin ratio.

In the TRO + ADR group, trolox treatment significantly improved but did not completely prevent development of adriamycin-induced cardiomyopathy as was the case with lovastatin. Ascites formation was quantitatively comparable to that observed in LOV + ADR group, while mortality data could not be obtained due to changed Animal Care Committee regulations where mortality as the end point was no longer allowed. All hemodynamic parameters, except LVEDP, were normalized. Adriamycin-induced increase in lipid peroxidation was prevented by trolox treatment, while decrease in GSHPx activity as well as decreased GSH/GSSG ratio were maintained at lower, adriamycin-induced levels. Trolox did not have any influence on adriamycin-induced changes in plasma and myocardial lipids, as well as on FFA and albumin levels.

Experiments, done on tumor inoculated mice, showed comparable decrease in tumor size in both ADR and PROB + ADR groups of mice, indicating that probucol does not effect antitumor properties of adriamycin.

In conclusion, data show that ADR cardiomyopathy as well as heart failure are associated with an antioxidant deficit, lipid peroxidation, increase in myocardial and plasma lipids as well as increase in FFA/albumin ratio. Improved cardiac function and zero mortality with probucol in ADR treated animals may be related to the maintenance of the antioxidant status, improved redox ratio, and prevention of lipid peroxidation in the heart along with the normalization of the myocardial and plasma lipids as well as FFA/albumin ratio. Since

lovastatin or trolox improved either lipid profile or prevented lipid peroxidation but never both simultaneously, there was only a partial improvement in cardiac function with these drugs. These results emphasize the significance of multiple sites and modes of action of a therapeutic agent for a complete prevention of the cardiomyopathic changes. Probucol, with its unique combination of lipid lowering properties, innate antioxidant activity (in the lipid phase), as well as promotion of endogenous antioxidants (cytosolic antioxidant defences) appears to have that optimal combination of different characteristics. Clinical potential of these findings needs to be tested.

I. INTRODUCTION

Adriamycin (also known as doxorubicin) is a potent anti-tumor antibiotic used for the treatment of a variety of soft and solid human malignancies. However, the treatment may be complicated by the acute and chronic side-effects. The acute side-effects such as myelosuppression, nausea, vomiting and arrhythmias are reversible and/or clinically manageable (Lefrak *et al.*, 1973; Arena *et al.*, 1975). One major chronic side-effect is the development of cardiomyopathy and ultimately congestive heart failure (Buja *et al.*, 1973; Lefrak *et al.*, 1973). In some patients the first signs of chronic adriamycin-induced cardiomyopathy appeared 4 to 20 years after discontinuation of the treatment (Steinhertz *et al.*, 1991). Thus, the risk of developing heart failure remains a lifelong threat.

Since the early reports of adriamycin-induced cardiomyopathy in the mid-1970s, clinical as well as basic research efforts have focused on understanding the pathophysiology of adriamycin-induced congestive heart failure. Several different mechanisms have been suggested to explain the development of adriamycin-induced cardiomyopathy, including the inhibition of nucleic acid and protein synthesis (Buja *et al.*, 1973; Arena *et al.*, 1974), release of vasoactive amines (Bristow *et al.*, 1980), changes in adrenergic function (Tong *et al.*, 1991), abnormalities in the mitochondria (Gosalvez *et al.*, 1979), lysosomal alterations (Singal *et al.*, 1985), altered sarcolemmal Ca^{2+} transport (Singal and Pierce, 1986), changes in adenylate cyclase, Na^{+}-K^{+} ATPase and Ca^{2+} ATPase (Singal and Panagia, 1984), imbalance in myocardial electrolytes (Olson *et al.*, 1974), free radical formation (Kalyanaraman *et al.*, 1980; Doroshow, 1983; Singal *et al.*, 1987), reduction in myocardial antioxidant enzyme activities (Revis and Marusic, 1978; Siveski-Iliskovic *et al.*, 1994a), lipid peroxidation (Myers

et al.,1 977; Singal *et al.*, 1985; Singal et al, 1987) and depletion of non-protein tissue sulfhydryl compounds (Doroshow et al, 1979; Olson *et al.*, 1980; Odom *et al.*,1992). Cardiomyopathic changes in the heart found after chronic treatment with adriamycin include dilation of the heart, focal degeneration, atrophy of myocytes and fibrosis. Typical morphological changes of cardiac cells include cytoplasmic vacuolization due to distention of the sarcoplasmic reticulum, loss of myofibrils, disruption of sarcomeres, swelling and lysis of mitochondria, margination of chromatin along the nuclear membrane and intracellular edema. The cause of adriamycin-induced cardiomyopathy is probably multifactorial and complex, but free oxygen radicals and lipid peroxidation appear to play an important role (Myers *et al.*, 1977; Doroshow,1983; Singal *et al.*, 1987; Kaul *et al.*,1993; Siveski-Iliskovic *et al.*,1994a). Accordingly, different free oxygen radical scavengers and antioxidants have been used to prevent or mitigate these adverse effects.

Probucol, an antioxidant as well as lipid-lowering drug, has been reported in this laboratory to completely prevent the adriamycin-induced cardiomyopathy in rats (Siveski-Iliskovic *et al.*, 1995). The protection was also seen with respect to mortality, ultrastructural changes, hemodynamic function and oxidative stress (Siveski-Iliskovic *et al.*, 1995). As adriamycin depressed myocardial antioxidants and probucol countered this effect, observed protection was suggested to be due to the enhancement of endogenous antioxidants (Siveski-Iliskovic *et al.*, 1994a). However, knowing that probucol acts as a lipid-lowering drug, is an antioxidant and a promoter of endogenous antioxidants, distinction between these possible mechanisms of action is necessary.

In the present study, we examined the contribution of lipid-lowering in the probucol protection by comparing its effects with another lipid-lowering drug with no known antioxidant properties (lovastatin). The question about role of antioxidant properties of probucol, as well as its promotion of endogenous antioxidants, was approached by comparing antioxidant effects of probucol against adriamycin-induced cardiomyopathy with another antioxidant, trolox, an analog of vitamin E with no known lipid-lowering properties. Thus, using an already established animal model of adriamycin-induced cardiomyopathy (Siveski-Iliskovic *et al.*, 1994 a,b), effects of lovastatin and trolox were compared with that of probucol with respect to adriamycin-induced changes in plasma and myocardial lipids, serum free fatty acids and albumin levels and ratio, myocardial glutathione peroxidase, superoxide dismutase, catalase, reduced and oxidized glutathione, lipid peroxidation and hemodynamics.

Another question that remained to be resolved was whether combination of probucol with the antitumor drug had any effect on the anticancer activity of adriamycin. We approached this problem by testing the antitumor efficacy of adriamycin given in combination therapy with probucol in a tumor-bearing mice animal model.

Findings described in this study advance our understanding of the pathogenesis of adriamycin-induced cardiomyopathy as well as the mechanism of its prevention with probucol. Overall, our investigation strengthens the rationale for undertaking clinical trials for testing the efficacy of combination therapy with probucol and adriamycin.

II. LITERATURE REVIEW

A. GENERAL BACKGROUND

In Italy, in the early 1960's, some strains of a fungus *Streptomyces peucetius* were found to contain daunomycin (daunorubicin), an antibiotic with powerful antitumor activity (Arcamone *et al.*, 1964). A similar drug, doxorubicin (adriamycin), was isolated from a mutant of *Streptomyces peucetius* and found to be a more potent cytostatic than daunorubicin. In a number of experimental as well as clinical studies, adriamycin showed good results against different neoplasms (Di Marco, 1969; Gilladoga, 1976; Lefrak *et al.*, 1973).

However, in the early seventies, chronic use of adriamycin was observed to cause serious cardiotoxic side effects. In a retrospective study of 366 patient records, it was shown that congestive heart failure developed in about 30% of the patients when the total cumulative dose of adriamycin administered was over 550 mg/m^2 body surface area (Lefrak *et al.*, 1973). Adriamycin cardiomyopathy and heart failure were further confirmed to be dose-dependent phenomena by other studies (Praga *et al.*, 1979). Hence, an empirical dose of 550 mg/m^2 of adriamycin was considered the upper limit in its clinical application (Lefrak *et al.*, 1973).

B. ADRIAMYCIN AND PHARMACOKINETICS

Adriamycin is a tetracyclic aglycone to which an amino sugar is attached through a glycosidic bond (Figure 1). In addition to its aerobic fermentation from *Streptomyces peucetius*, adriamycin can be synthesized from daunomycin (Arcamone, 1972). The drug is available in Canada as Adriamycin hydrochloride. Adriamycin is readily soluble in water, physiological saline and alcohols, but is only slightly soluble or insoluble in less polar solvents

(Arcamone, 1972). In its lyophilized form adriamycin is stable and active at room temperature for years. Even in solution (pH 7.0), it is stable for more than a month. Adriamycin is rapidly cleared from the plasma and accumulated in different tissues including the heart within minutes, while it is excreted slowly (Kimura, 1972; Yesair, 1972). Its half life in myocardial tissue is about 48 h. A good portion (40-50%) of the drug is metabolized in the liver and excreted in bile, while smaller amounts are excreted via the kidneys in urine (Benjamin, 1975; Bachur *et al.*, 1974; Bachur, 1979). Adriamycin is not absorbed by the gastrointestinal tract (Arena *et al.*, 1972).

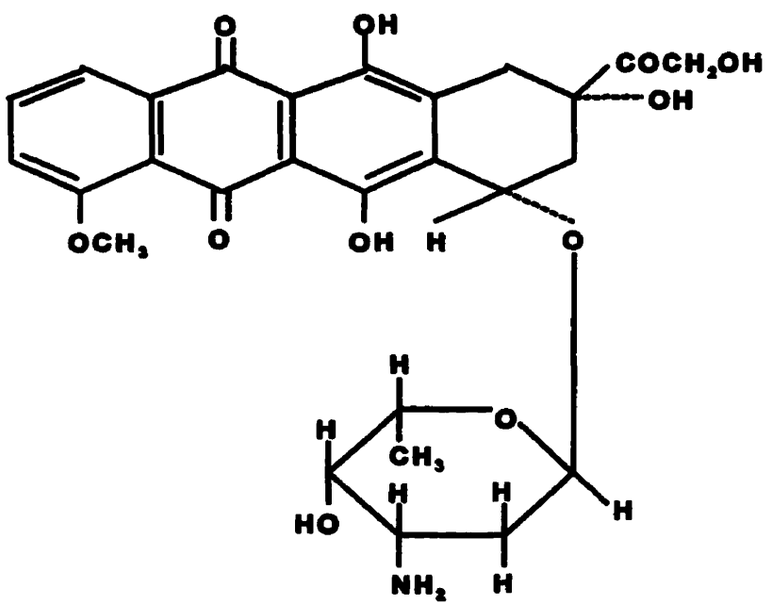


Figure 1: Chemical structure of adriamycin.

C. ADRIAMYCIN DISTRIBUTION AND METABOLISM

Adriamycin is very histophillic and penetrates rapidly into the cells and nuclei (Buja *et al.*, 1973; Egorin, 1974). The mechanism of adriamycin transport is complex and not completely understood, possibly because the drug can permeate the membrane in several different ways including both carrier-mediated transport and simple diffusion (Skovsgaard and Nissen, 1982). Antitumor action of adriamycin is a consequence of its actions in the nuclei and is described in detail later in this section.

During its passage through the liver, adriamycin is metabolized into adriamycinol. Adriamycinol has a more toxic effect on the heart than adriamycin. As compared to adriamycin, adriamycinol causes a greater decrease in cardiac contractility, as well as in calcium and Na^+/K^+ sarcolemmal pump and Mg-dependent ATP-ase activity in the submitochondrial vesicles (Boucek *et al.*, 1987). The same study demonstrated that adriamycin could be converted to adriamycinol in cardiac tissue *in vitro*. It has been suggested that an accumulation of this potent cardiotoxic adriamycin metabolite, adriamycinol, in the myocardium might be largely responsible for the toxicity (Boucek et al., 1987). Pharmacokinetic and tissue distribution of adriamycin and adriamycinol can be affected by different pathological conditions: In this regard, a longer maintenance of adriamycin in the plasma as well as higher quantities of adriamycin accumulated in different tissues, including hearts of diabetic rats were described (Lee *et al.*, 1995).

D. MECHANISMS OF ADRIAMYCIN ANTITUMOR ACTIONS

Several different mechanisms have been proposed to explain the antitumor action of adriamycin. Most of these are suggested to involve the production of free radicals. However,

non-free radical dependent mechanisms have also been shown to explain the antitumor effects of the drug. Some of the studies supporting for or arguing against one or more of these mechanisms are also listed in Table 1 and following is a brief analysis of different studies.

D.1. Free Radical Based Cytotoxicity:

D. 1. a. Evidence For

A free radical is a molecule or atom that contains one or more unpaired electrons and is highly reactive. It can act as an oxidizing and/or reducing agent depending upon the substrate. Production of free radicals generally has a local effect. However, secondary radicals, formed from them, can also have distant biological effects via chain reactions. The specific chemical structure of adriamycin (Figure 1) suggested a free radical-dependent mechanism for its antitumor action. In the presence of oxygen, redox cycling of adriamycin-derived quinone-semiquinone yields superoxide radicals. Superoxide dismutase, an endogenous antioxidant enzyme, catalyses the formation of hydrogen peroxide from superoxide radicals, with a subsequent formation of hydroxyl radicals (Kaul *et al.*, 1993). It is now well established that free radicals cause DNA damage and different mechanisms for radical-induced DNA strand breaks have been analysed in detail in a recent review (Breen and Murphy, 1995).

Increased levels of oxygen species due to adriamycin have been confirmed by direct measurements as well as by indirect approaches. Hydroxyl radical formation was detected by a spin-trapping technique in MCF-7 human breast cancer cells exposed to high concentrations of adriamycin (Sinha *et al.*, 1987 a,b). Addition of NADPH in the extracellular medium stimulated, while superoxide dismutase and catalase inhibited the

Table 1: A listing of mechanisms proposed for the antitumor action of adriamycin*

Mechanism		Dose of Adriamycin	Expt. Condition	Source
1) Free radical induced DNA breakage	*Supportive:*	500 µM	in vitro	Bachur *et al.*, 1982
		0.4 mM	in vitro	Berlin & Haseltine, 1981
		1 mg/ml	in vitro	Gutteridge & Toeg, 1982
		1 mg/ml	in vitro	Sinha & Gregory, 1981
		10^{-6} - 10^{-8} M	in vitro	Sinha *et al.*, 1987 a,b
		0.1 - 1 µM	in vitro	Winterbourn *et al.*,1985
		0.4 - 0.75 µM	in vitro	Doroshow, 1986
		2.8 x 10^{-6} - 10^{-4} M	in vitro	Potmesil *et al.*, 1984
		10 - 30 µg/ml	in vitro	Panneerselvam *et al.*, 1987
		160 µg	in vitro	Rowley & Halliwell, 1983
	Not Supportive:	250 - 300 µg/ml	in vitro	Sato *et al.*, 1977
		0.1-1 µM sensitive cells 10-100µM resistant cells	in vitro	Cervantes *et al.*, 1988
		0 - 5 µM	in vitro	Keizer *et al.*, 1989
		15 mg/kg	in vivo	Myers *et al.*, 1977
		10 mg/kg	in vivo	Freeman *et al.*, 1980
		20 mg/kg	in vivo	Yoda *et al.*, 1986
		2 -15 mg/kg	in vivo	Shimpo *et al.*, 1991
		15 mg/kg	in vivo	Siveski-Iliskovic *et al.*,1995
2) Adriamycin-iron complex and oxidative destruction of DNA	*Supportive:*	5 nmol	in vitro	Eliot *et al.*, 1984
		5 mM; 30 µM	in vitro	Muindi *et al.*, 1984, 1985
		10^{-3} - 10^{-9} M	in vitro	Ramu *et al.*, 1984
	Not Supportive:	0.1 - 2.0 µM	in vitro	Hamilton *et al.*, 1985
		20 mg/kg	in vivo	Doroshow *et al.*, 1981
		0.1 µg/ml	in vitro	Wadler *et al.*, 1986
3) Intercalation of adriamycin into double-stranded DNA		1 mg/ml	in vitro	Sinha and Chignell, 1979
		1.3 - 55 µM	in vitro	Graves and Krugh, 1983
4) Inhibition of topoisomerase II		0.5 - 1.25 µg/ml	in vitro	Tewey *et al.*, 1984
		0 - 10 µM	in vitro	Deffie *et al.*,1 989
		A direct correlation between topoisomerase II activity and adriamycin cytotoxicity has also been reported		Capranico *et al.*, 1989 Potmesil, 1988 Potmesil *et al.*, 1988
5) Induction of Apoptosis		0.1 - 10 µM	in vitro	Gartenhaus *et al.*, 1996
		EC90 and 10 X EC90	in vitro	Skladanowski and Konopa, 1993

*) For further details on species and dosage, please see reference list.

formation of hydroxyl radicals, suggesting that some hydroxyl radicals were generated outside the cells (Sinha *et al.*, 1987 a,b). The role of hydroxyl radicals in adriamycin cytotoxicity has also been suggested by other studies (Potmesil *et al.*, 1984; Panneerselvam *et al.*, 1987). Furthermore, in the nuclei isolated from rat liver, heart and kidney, free radical species of adriamycin and daunorubicin were also detected by electron paramagnetic resonance spectrometry (Bachur *et al.*, 1982). These observations indicated that some cytotoxic xenobiotics can be activated to a free radical state at the site where damage to nuclear DNA may result (Bachur *et al.*, 1982).

In the study where end-labelled DNA was included with NADPH, NADPH cytochrome P-450 reductase and adriamycin, extensive DNA strand scission was observed (Berlin and Haseltine, 1981). It was suggested that DNA breakage caused by enzymatically derived adriamycin-free radicals is mediated by molecular oxygen, probably by hydroxyl radicals and hydroxyl ions (Berlin and Haseltine, 1981). Adriamycin possesses iron binding affinity and the adriamycin-iron complex catalyses hydroxyl radical formation which occurs in the vicinity of DNA and has the potential to significantly damage DNA (Muindi *et al.*, 1984; 1985). Deoxyribose breakdown was also found in a system containing deoxyribose, adriamycin semiquinone radicals and iron (Gutteridge and Toeg, 1982). Conversion of adriamycin to its C7-deoxyaglycone metabolite is increased in the absence of oxygen (Averbuch *et al.*, 1985). Though C7-deoxyaglycone is pharmacologically inactive, its intermediate C7 free radical can bind covalently to DNA (Sinha and Gregory, 1981). At a low partial pressure of oxygen, the reaction between adriamycin and H_2O_2 , catalysed by very

low concentrations of iron caused breakdown of deoxyribose (Bates and Winterbourn, 1982; Winterbourn *et al.*, 1985).

Significant reduction in cell killing by adriamycin was found with the addition of superoxide dismutase and catalase, while only iron-chelating agents capable of penetrating the cell plasma membrane also had similar results in the tumor cell line, MCF-7 (Doroshow,1986). Since superoxide dismutase and catalase molecules can not cross the plasma membrane, superoxide and hydroxyl radicals generated at the outer surface of cells might contribute to the cytotoxicity of adriamycin in MCF-7 tumor cells. In this regard, demonstration of the cytotoxic action of adriamycin without its entry into the tumor cell provided strong evidence for the transmembrane transfer of injury (Tritton and Yee, 1982). Reduction of cytotoxicity with iron-chelating agents capable of entering tumor cells suggested that intracellular iron or iron-proteins may also contribute to the antineoplastic activity of adriamycin (Doroshow, 1986).

Production of free radicals in the presence of adriamycin is well established and the fact that free radicals can cause DNA damage is also established. However, in almost all of these *in vitro* studies, extremely high concentrations of adriamycin were used as compared to the therapeutic doses, suggesting that free radical mechanism may not be important in *in vivo* clinical conditions (Table 1) (Singal *et al.*,1995).

D. 1. b. <u>Evidence Against</u>

Effects of free radical scavengers on the antitumor properties of adriamycin have been studied in a number of different *in vivo* animal models. There was no impact of tocopherol on the suppression of DNA synthesis in the P388 ascites tumor after adriamycin

administration (Myers *et al.*, 1977). Treatment with N-acetylcysteine (Freeman *et al.*, 1980) and glutathione (Yoda *et al.*, 1986) in combination with adriamycin had no significant effect on adriamycin cytotoxicity. Administration of ascorbic acid, a strong antioxidant, to mice inoculated with leukaemia L1210 or Ehrlich ascites had no effect on the antitumor activity of adriamycin, but significantly prolonged life of animals due to reduction of cardiotoxicity (Shimpo *et al.*, 1991). Probucol, another potent antioxidant, had no effect on antitumor activity of adriamycin in a L5178Y-F9 lymphoma bearing mice model (Siveski-Iliskovic *et al.*, 1995). All of these treatments reduced cardiotoxicity in different animal models. Although free radical injury is suggested to be the main cause of adriamycin cardiotoxicity, it may not be playing a critical role in the *in vivo* antitumor activity of adriamycin.

Furthermore, application of intracellular superoxide dismutase and catalase did not protect A2780 and A2780AD cells against adriamycin cytotoxicity (Cervantes *et al.*, 1988; Keizer *et al.*,1988). Murine S180 cells were not protected against adriamycin by the iron-chelating agent ICRF 187 (Wadler *et al.*, 1986). However, iron chelators were protective against adriamycin in the case of MCF-7 and Ehrlich ascites cells (Doroshow, 1986; Alegria *et al.*, 1989). These discrepancies may also suggest that there may be different mechanisms of adriamycin-induced cytotoxicity in different types of tumor cells (Keizer *et al.*, 1990).

D. 2. Other Mechanisms of Cytotoxicity:

D. 2. a. Non-Free Radical Dependent

In addition to the free radical induced DNA strand break, antitumor action of adriamycin may also involve binding of adriamycin-iron complex to DNA, intercalation of

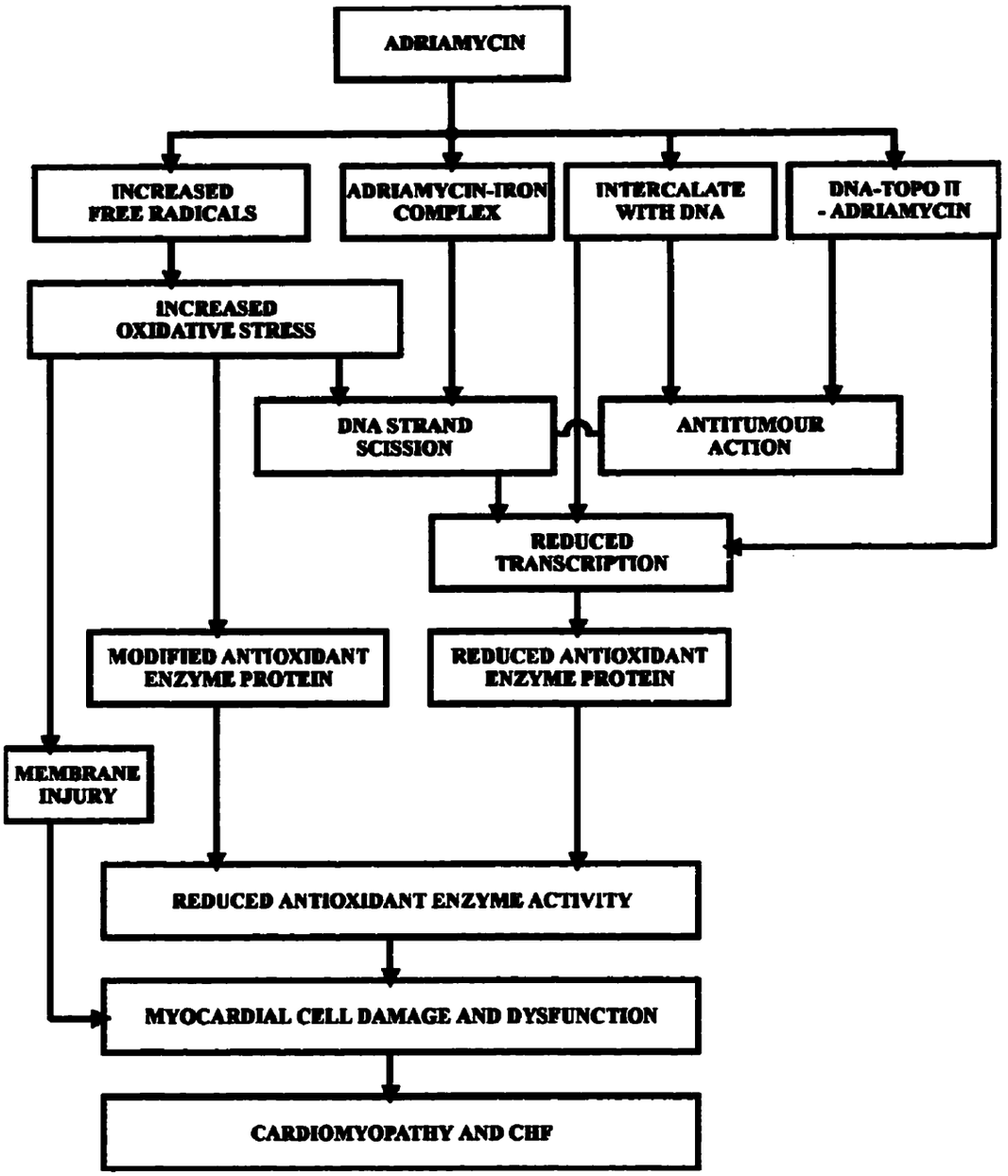


Figure 2. **A proposed scheme for the antitumor as well as cardiotoxic effects of adriamycin.**

adriamycin between base pairs in the DNA and inhibition of the topoisomerase II as proposed in Figure 2.

When discussing literature dealing with possible role of adriamycin-induced free radical generation in the adriamycin cytotoxicity, one has to consider the fact that most of the studies described above were done in isolated systems mixing adriamycin *in vitro* with different subcellular fractions such as mitochondria, nuclei, sarcoplasmic reticulum, DNA, etc. The situation in intact cells might be different due to many factors such as DNA binding of adriamycin, the presence of endogenous antioxidants and diffusion limitations, etc. In this regard in some cell types, 99.8% of adriamycin was found to be present in DNA-bound form (Gigli *et al.*, 1988) and double stranded DNA-adriamycin complex has been shown to prevent redox-cycling of adriamycin (Berlin and Haseltine, 1981; Rowley and Halliwell, 1983; Sato *et al.*, 1977). Redox activity of adriamycin described in intact nuclei may be due to extremely high concentrations (>500 μM) of adriamycin (Bachur *et al.*, 1982). Semiquinone radical, formed in the cytoplasm, has a diffusion radius of 0.6 μm under anaerobic and 0.1 μM under aerobic conditions (Svingen and Powis, 1981). An average cell has a diameter in the range of 5-20 μM, suggesting that only a minor amount of the formed semiquinone radicals produced in the cytoplasm could reach the nucleus (Keizer *et al.*, 1990). Inhibitors of flavoprotein-mediated reduction of drugs did not protect SW-1573 human lung tumor cells against cytotoxicity of adriamycin, although flavoprotein dependent cytotoxicity of mitomycin C was inhibited (Keizer *et al.*, 1989).

Adriamycin-iron complex, as such, has also been shown to bind DNA and cause damage (Eliot *et al.*,1 984). This type of adriamycin binding is different from its intercalation

as the drug-metal complex bound to DNA caused spectral changes different from those seen with adriamycin intercalation. Furthermore, adriamycin intercalated with DNA is not available for iron binding (Eliot *et al.*, 1984). Although adriamycin-iron complex bound to DNA can be reduced by thiols to produce hydroxyl radicals (Keizer *et al.*, 1990), the significance of this reaction with respect to cytotoxicity is not clear. In this regard, thiol depleted tumor cells exposed to adriamycin did not show decreased cytotoxicity to the drug (Ramu *et al.*, 1984; Hamilton *et al.*, 1985) and conversely an increase in thiol content did not show potentiation of adriamycin cytotoxicity (Doroshow *et al.*, 1981).

More recently, studies have focussed on DNA topoisomerase II as a primary target of anthracyclines. This enzyme is important in regulating the three-dimensional structure (or topology) of double-stranded DNA, as the DNA strands need to open or be "nicked" to permit such topological changes (Booser and Hortobagyi, 1994). Adriamycin binds to the binary DNA-topo II complex, forming an irreversible ternary complex, thus preventing the broken DNA from re-establishing continuity and functional integrity (Capranico *et al.*, 1989). It is suggested that topoisomerase II acts specifically during mitosis (Holm *et al.*, 1989a). Drug-stabilized topo II-DNA complex interferes with movement of DNA replication forks, leading to cell death (Holm *et al.*, 1989b). Different types of tumor cells have different levels of topoisomerase II (Potmesil, 1988; Potmesil *et al.*, 1988). Under normal conditions, the highest level of this enzyme is found in the spleen and thymus. Neoplasms with the highest topoisomerase II levels are those that clinically behave aggressively and have a high proliferative status (Holden *et al.*, 1990). Since topoisomerase II plays a critical role in DNA replication, hypersensitivity to adriamycin was found in CHO mutant ADR-1 cells, which have

increased levels of topoisomerase II (Davies *et al.*, 1988). P388 leukemic cells sensitive to adriamycin contained not only higher topoisomerase II enzyme levels but also higher enzyme activity compared to its adriamycin-resistant counterparts (Deffie *et al.*, 1988; Deffie *et al.*,1989).

Adriamycin caused protein-associated DNA double strand breaks (Deffie *et al.*,1988) which are thought to be mediated by DNA topoisomerase II. These DNA topoisomerase II-mediated strand breaks were shown to be associated with adriamycin-mediated cytotoxicity in P388 leukemic cells. Adriamycin actions on L1210 cells have been shown to cause two types of DNA strand breaks in a concentration-dependent manner. With drug concentrations of up to 2.8 μM, only protein-associated strand breaks occurred which are thought to be DNA topoisomerase II-mediated (Deffie *et al.*,1989). At this concentration (2.8 μM), adriamycin killed more than 99.99% of the cells (Ross and Smith, 1982). At higher concentrations protein-associated strand breaks decreased and direct strand breaks increased (Potmesil *et al.*,1984). These direct strand breaks, unassociated with protein, may be mediated by mechanisms other than topoisomerase II.

D. 2. b. <u>Apoptosis</u>

Programmed cell death, or apoptosis, is a phenomenon that has attracted a lot of attention in recent years. Adriamycin-induced apoptosis was observed in tumor cells (Skladanowski and Konopa, 1993; Anand *et al.*, 1995). Alopecia, or loss of hair due to chemotherapy, was suggested to be the result of adriamycin-induced apoptosis of hair follicle cells (Cece *et al.*, 1996). Renal and intestinal epithelial cells from adriamycin-treated spontaneously hypertensive rats showed apoptosis while none was observed in

cardiomyocytes (Zhang *et al.*, 1996). Adriamycin was also shown to cause apoptotic response in human T-cell leukemia virus type I-transformed lymphoid cell line (Gartenhaus *et al.*,1996). Further studies dealing with the possible role of apoptosis in adriamycin cardiotoxicity are needed before ruling out its role in the loss of myocardial function.

It needs to be reiterated that for adriamycin concentrations achievable clinically, free radical mediated DNA injury may not be important. A close scrutiny of the studies listed in Table 1 will show that adriamycin-induced free radical mediated DNA damage is readily demonstrable only in *in vitro* studies using higher concentrations, while most of the *in vivo* studies are not supportive of the role of free radicals in the antitumor action of adriamycin.

E. MECHANISMS OF ADRIAMYCIN-INDUCED CARDIOMYOPATHY

Since the early reports of adriamycin-induced cardiomyopathy, several different mechanisms have been suggested to explain the development of adriamycin-induced cardiomyopathy. The list includes an inhibition of nucleic acid and protein synthesis (Buja *et al.*, 1973; Arena *et al.*,1974), release of vasoactive amines (Bristow *et al.*, 1980), changes in adrenergic function (Tong *et al.*, 1991), abnormalities in the mitochondria (Gosalvez *et al.*, 1979), lysosomal alterations (Singal *et al.*, 1985), altered sarcolemmal Ca^{2+} transport (Singal and Pierce, 1986), changes in adenylate cyclase, Na^{+}-K^{+} ATPase and Ca^{2+} ATPase (Singal and Panagia, 1984), imbalance in myocardial electrolytes (Olson *et al.*, 1974), cardiac sympathetic denervation (Takano *et al.*, 1995), free radical formation (Singal *et al.*, 1985; Kalyanaraman *et al.*, 1980; Doroshow , 1983), reduction in myocardial antioxidant enzyme activities (Revis and Marusic, 1978; Siveski-Iliskovic *et al.*, 1994a), lipid peroxidation (Singal *et al.*,1987; Myers *et al.*, 1977; Singal *et al.*, 1985) and depletion of non-protein tissue sulfhydryl

compounds (Doroshow *et al.*, 1979; Olson *et al.*, 1980; Odom *et al.*, 1992). The cause of adriamycin-induced cardiomyopathy is probably multifactorial and complex, but free oxygen radicals and lipid peroxidation appear to play an important role (Figure 2).

A characteristic feature of adriamycin cardiomyopathy is loss of myofibrils which is possibly caused by drug-induced inhibition of nucleic acid and protein biosynthesis (Lefrak *et al.*, 1973). An inhibitory effect of adriamycin on the formation of alpha-actinin/actin tridimensional networks and bundles has been reported (Dalle Donne *et al.*, 1992). Increased lipid peroxidation and release of lysosomal enzymes, as reviewed later, may also contribute to adriamycin cardiotoxicity (Singal *et al.*, 1985). Adriamycin selectively decreased alpha cardiac actin mRNA in the rat heart when compared to other mRNAs examined in the heart and skeletal muscle, and administration of cardioprotective chelating agent ICRF-187 did not modify this effect (Papoian and Lewis, 1990, 1991). This may suggest that the observed decrease in actin mRNA was not related to iron mediated free radical formation, but to some other mechanisms. A novel human RNA polymerase II subunit, down regulated by adriamycin, was recently cloned, and suggested to be a new potential mechanism of adriamycin-induced toxicity (Fancilli *et al.*, 1996). The most significant reduction in this mRNA expression was found in adriamycin treated heart and skeletal muscle (Fancilli *et al.*, 1996).

Another ultrastructural hallmark is vacuolisation of the cytoplasm, due to a swelling of the sarcoplasmic reticulum and T-tubules (Billingham, 1979; Buja *et al.*, 1973; Lefrak *et al.*, 1973; Ferrans, 1978; Van Vleet *et al.*, 1978; Singal, 1983). However, the consequence of cytoplasmic vacuolisation in the myocardial function is still not understood completely.

The sarcoplasmic reticulum is essential for the muscle contraction-relaxation process because it sequesters intracellular calcium. Any changes in this process would affect myocardial contractility. Bellini and Solcia, (1985) demonstrated that zinc iodide-osmium tetroxide (ZI0) reactive material could be seen in the sarcoplasmic reticulum soon after injection of adriamycin, implying damage to the sarcoplasmic reticulum. A dose-related inhibition of Ca^{2+} uptake by adriamycin was described in the rat heart sarcoplasmic reticulum preparation (Harris *et al.*, 1985). Perfusion with adriamycin (0.1 mM) was shown to significantly suppress potentiation of the post-rest contractions in the isolated rat heart (Asayama et al.,1995). Post-rest contractions are believed to be due to the Ca^{2+} release from the sarcoplasmic reticulum (SR), hence, authors suggested that cardiomyopathy might be a consequence of SR injury, which leads to Ca^{2+} overload (Asayama et al., 1995).

Adriamycin has a significant influence on the activity of a number of sarcolemma-bound enzymes. *In vitro* studies demonstrated that low concentrations of adriamycin stimulate adenylate cyclase activity and increase cyclic AMP concentration, while an inhibitory effect is noted at higher drug concentrations (Azuma *et al.*, 1981; Singal and Panagia, 1984). There are conflicting reports about the effect of adriamycin on Na^+/K^+ ATPase activity (Gosalvez *et al.*, 1979; Kim *et al.*, 1980; Komori *et al.*, 1985; Singal and Panagia, 1984). Ca^{2+} pump ATPase (Caroni *et al.*, 1981) and 5'-nucleotidase activity (Singal and Panagia, 1984) in the sarcolemma were not affected. It appears that adriamycin may have selective effects on the functional activities of the sarcolemma.

Sarcolemmal Ca^{2+} bound at the low-affinity sites on the membrane has been associated with cellular Ca^{2+} influx and the binding correlates with the developed force (Bers *et al.*,

1981; Dhalla *et al.*, 1982). In *in vitro* studies, adriamycin was found to stimulate low-affinity Ca^{2+} binding but it depressed contractile function (Singal and Pierce, 1986). Adriamycin *in vitro* also stimulates Ca^{2+}-ATPase activity (Singal and Panagia, 1984). These observations may indicate a permeability change leading to an oversupply of Ca^{2+}. Adriamycin-induced oxidation of protein thiols and inhibition of Ca^{2+}-ATPase activity have also been reported (Vile and Winterbourn, 1990). Another factor in support of permeability change is the increase in LDH, CPK and SGOT enzymes in the serum of patients undergoing adriamycin therapy (Lefrak *et al.*, 1973). The intracellular localization of lanthanum, an extracellular tracer, in the hearts of adriamycin-treated rats also indicated a permeability change (Singal *et al.*, 1985). During 30 min of perfusion of isolated ferret hearts with adriamycin, Ca^{2+} significantly increased compared to a control, with concomitant isovolumic left ventricular pressure decrease. This suggests that Ca^{2+} overload induced by adriamycin is associated with acute contractile failure (Kusuoka *et al.*, 1991).

Leakage of the lysosomal hydrolytic enzymes into the cell may be very important in different disease states. Lysosomal changes due to adriamycin have been demonstrated (Singal *et al.*, 1985). The increased number of lysosomes and activated lysosomal hydrolysis in the myocardium as well as an increase in the lysosomal enzymes in the circulation may indicate that adriamycin has a direct effect on this membrane system. These effects of adriamycin on the membrane systems appear to be the result of free radical formation and lipid peroxidation. Myocardial increase in MDA concentration, which is a product of lipid peroxidation (Siveski-Iliskovic *et al.*,1994a) in adriamycin-treated animals, supports this hypothesis. Superoxide dismutase (SOD) did not offer protection against these membrane

changes, implying that the superoxide radical as such may not be contributing in the lipid peroxidation of lysosomal injury due to adriamycin (Singal and Tong, 1988).

Numerous studies have investigated functional changes in mitochondria. Calcium overload in mitochondria by ATP and respiration dependent mechanism was observed (Miwa *et al.*, 1986). Adriamycin inhibits electron transport and oxidative phosphorylation and decreases mitochondrial ATP-ase activity. Decrease in myocardial high energy phosphates (HEP) due to adriamycin has also been reported (Azuma *et al.*, 1981; Muhamed *et al.*, 1982; Jackson *et al.*, 1983; Pelikan *et al.*, 1986; Singal and Pierce, 1986). Depletion of myocardial ATP stores, can be due to lack of production and/or increased consumption accompanied by stimulation of Ca^{2+}-activated enzyme activities under the influence of intracellular Ca^{2+} overload (Revis and Marusic, 1979; Singal *et al.*, 1985; Dhalla *et al.*, 1985). Hexakis (2-methoxyisobutyl isonitrile) technetium I, a gamma-emitting radiopharmaceutical with myocellular accumulation properties dependent on mitochondrial membrane potential, has been used for monitoring of adriamycin-induced alterations in cardiac energetics, and drug concentrations as low as 0.1 μM inhibited its accumulation within mitochondria (Piwnica-Worm *et al.*, 1993).

A decrease in myocardial mitochondrial respiratory function was observed in mice 48 h after acute exposure to adriamycin (single intraperitoneal injection of 60 mg/kg) (Matsumara *et al.*, 1984). However, another study of mitochondrial lipid peroxidation, membrane fluidity and activities of mitochondrial respiratory chain in isolated mice mitochondria 48 h after a single adriamycin injection did not show any significant acute toxicity. Mitochondrial toxicity was detected after chronic drug administration (Praet and

Ruysschaert, 1993). Specific binding of adriamycin to the phospholipid cardiolipin was found to be related to the inhibition of mitochondrial activity (Nicolay *et al.*, 1987). The low energy state in cardiac tissue was demonstrated by ^{31}P-NMR measurements in the living animal (Nicolay *et al.*, 1987). Adriamycin also caused breakage of the mitochondrial DNA helix and slowed the rate of mitochondrial DNA synthesis as well as the formation of complete DNA molecules (Ellis *et al.*, 1987). A direct correlation was observed between the severity of adriamycin-induced cardiac failure and impairment of mitochondrial function (Bachman *et al.*, 1975; Bachman and Zbinden, 1979).

Another suggested mechanism of adriamycin cardiomyopathy is through adriamycin-induced production of heat-shock proteins, which then stimulate potent T lymphocyte responses that may contribute to cardiac damage (Huber, 1992). In *in vivo* studies, it was demonstrated that adriamycin treated mice developed both cytolytic T lymphocytes and antibodies to drug-treated myocytes (Huber and Moraska, 1992).

F. FREE RADICALS AND LIPID PEROXIDATION

The superoxide radical, hydrogen peroxide and the hydroxyl radical can initiate a chain reaction which results in oxidative damage of the cell membrane. Peroxidation of polyunsaturated fatty acids in the cell membrane leads to severe damage and degradation of the cell membrane as well as causes protein aggregation. The hydroxyl radical formed can react with polyunsaturated fatty acids to form lipid radicals and lipid hydroperoxide. Free oxygen radicals and breakdown products of lipid peroxidation have been detected in hearts exposed to adriamycin (Myers *et al.*, 1977; Alegria *et al.*, 1989; Singal and Pierce, 1986; Thornalley and Dodd, 1985). In the presence of transition metal ions, the chain reaction

continues. It has been suggested that iron plays an important role in adriamycin-induced lipid peroxidation. In the presence of transition metal ions, the chain reaction continues and free iron appears to play a particularly important role in adriamycin-induced lipid peroxidation. Without free iron, lipid peroxidation is minimal and even a low concentration of free iron can lead to a substantial lipid peroxidation (Griffin-Green *et al.*, 1988).

Adriamycin may act by transferring an electron directly to Fe^{3+}, and Fe^{2+} thus produced can reduce oxygen to hydrogen peroxide (Hasinoff *et al.*, 1988), the terminal respiratory enzyme in the mitochondria and inhibit its function. The Fe^{3+}-adriamycin-cytochrome c oxidase complex initiates free radical reaction and $OH^{\cdot}$ could damage the nearby cardiolipin bound to cytochrome c oxidase (Hasinoff *et al.*, 1988). Adriamycin appears to have a high affinity for the negatively charged phospholipid, cardiolipin, which is abundant in the inner membrane of mitochondria (Goormaghtigh *et al.*, 1980; 1986; Nicolay *et al.*, 1984; 1985). Structural damage to the mitochondria in the heart is also frequently reported in human biopsies as well as in experimental studies (Lefrak *et al.*,1973; Ferrans,1978; Singal *et al.*,1987). Thus mitochondria appear to be particularly vulnerable to adriamycin-induced damage. Free radical dependent myocardial DNA lesions, prevented by a spin trapping compound, were found to be involved in the delayed adriamycin cardiotoxicity in a rat animal model (Monti *et al.*, 1995).

Other membrane systems containing polyunsaturated fatty acids are also potential targets for lipid peroxidation. Changes of the sarcotubular membranes have been found in several ultrastructural studies of the biopsy material obtained from patients (Lefrak *et al.*, 1973; Ferrans, 1978) as well as in the myocardium in animal experiments (Tong *et al.*,1991;

Singal *et al.*, 1985). Adriamycin is known to stimulate the production of free radicals in both isolated cardiac sarcoplasmic reticulum and mitochondria (Kalyanaraman *et al.*, 1980; Doroshow, 1983; Doroshow and Reeves, 1981).

In an *in vivo* study, adriamycin caused a significant increase in cardiac lipid peroxides while no lipid peroxidation was observed in the liver (Odom *et al.*, 1992). Increased susceptibility of the myocardium to oxygen injury compared to some other tissues, may be due to the lower myocardial antioxidant reserve (Doroshow *et al.*, 1980). Compared to the heart, liver has high levels of glutathione as well as glutathione peroxidase and is relatively resistant to adriamycin-induced lipid peroxidation and toxicity (Odom *et al.*, 1992). However, non-protein thiol concentrations in the liver were decreased by adriamycin treatment suggesting that peroxidative damage was perhaps mitigated by glutathione (Odom *et al.*, 1992). Reduction in glutathione may also be a reflection of the reported covalent binding of glutathione to adriamycin and its free radical metabolites (Sinha and Gregory, 1981; Julicher *et al.*, 1973).

Glutathione levels have been described to undergo different changes due to adriamycin treatment. A dose of 15 mg/kg of adriamycin in a rat heart caused a small increase in reduced glutathione (GSH) levels at 24 h after injection; a dose of 30 mg/kg caused no change, while 50 mg/kg of adriamycin caused a decrease in cardiac GSH at 6 h, followed by an increase at 24 h (Boor, 1979). In the isolated rat hearts, perfused for 30 min with 35 μM of adriamycin, the GSH levels were almost halved due to the drug perfusion , while the amount of oxidized glutathione (GSSG) was not significantly changed (Julicher *et al.*, 1 986). A single injection of 15 mg/kg of adriamycin was found to significantly lower levels of GSH in the liver, heart

and erythrocytes of Swiss ICR-HA mice. However, GSH levels returned back to control values in erythrocytes, and even above control levels in liver and heart tissue 24 h after adriamycin administration (Olson *et al.*,1 980). Also, when endogenous levels of GSH were depleted by diethyl maleate, adriamycin lethality was increased (Olson *et al.*, 1980).

In acute studies, where rabbits received up to 10 mg/kg total dose of adriamycin and were sacrificed 3-72 h after the treatment, an increase of up to 50% in total and reduced glutathione, and no change in oxidized glutathione, was described (Jackson *et al.*, 1984). In chronic studies, in which 22 mg/kg of adriamycin was given over a period of 10 weeks and rabbits were sacrificed 24 h after the last injection, an increase of 23-36% in the total and reduced glutathione, and also no change in oxidized glutathione, was found (Jackson *et al.*, 1984). Decrease in cellular ATP and total glutathione, due to adriamycin treatment, in rat cardiac myocytes in culture was described to be time and concentration- dependent (Singh *et al.*, 1989). Rats treated subcutaneously with 19.5 to 21.0 mg/kg of adriamycin over a period of 14 weeks, sacrificed 1-3 weeks after the last injection have shown a 50% increase in glutathione levels in kidney, 20% increase in heart, and a 20% decrease in liver, as well as increased cardiac glutathione peroxidase activity about 30% (Thayer, 1988).

Although the details of the processes of lipid peroxidation are not clear, the measurement of peroxidation end products such as propane, ethane and pentane can also provide an indication of the formation of lipid peroxide (Hunter et al, 1964). The fatty acid content of the membrane is also used to determine the extent of lipid peroxidation (Griffin-Green *et al.*, 1988). Some investigators have calculated that 1 mole of MDA is generated for

every 14-21 moles of unsaturated fatty acids consumed (Baker *et al.*, 1966; Reiter *et al.*, 1987; May *et al.*, 1968).

Increased MDA levels were reported in both *in vitro* and *in vivo* studies studying the myocardial effects of adriamycin (Singal and Pierce, 1986; Siveski-Iliskovic *et al.*, 1994a; 1995; Myers *et al.*, 1977). Reduction of adriamycin cardiotoxicity by the use of different antioxidants, which will be discussed later in this review, provides support to the hypothesis that adriamycin cardiomyopathy may be mediated by increased oxidative stress (Pascoe *et al.*, 1987; Tanigawa *et al.*, 1986; Myers *et al.*, 1977; Siveski-Iliskovic *et al.*, 1995). In addition to the indirect approach that consists of the measurement of end products of lipid peroxidation, increased levels of oxygen species due to adriamycin have also been detected directly, by electron spin resonance spectroscopy (Costa *et al.*, 1988; Thornalley *et al.*, 1985; Alegria *et al.*, 1989). Hence, free radicals and lipid peroxidation are recognized as one important mechanism of adriamycin cardiotoxicity.

G. RISK FACTORS

While the majority of patients should not receive more than 550 mg/m^2 of adriamycin in order to avoid its cardiotoxic side effects, there are patients who could tolerate higher doses of the drug with no apparent side effects. On the other hand, presence of other risk factors can potentiate the cardiotoxic effects of the drug at a cumulative dose much below the suggested upper limit (Bristow *et al.*, 1978). Examination and determination of different risk factors for development of adriamycin-induced cardiomyopathy has also been done (Minow *et al.*, 1977). Some of the proposed risk factors are as follow:

G. 1. Radiation Therapy

Radiation therapy, previous and concomitant, is thought to potentiate adriamycin-induced cardiac damage. In this regard, latent radiation-induced cardiac changes were potentiated by adriamycin treatment (Billingham *et al.*, 1977). Echocardiography in children and adolescents, treated with adriamycin and radiotherapy, had shown abnormal left ventricular contractility in 50% of the patients treated with both adriamycin and irradiation, compared to 32% of patients treated with adriamycin only and 8% treated with radiotherapy only (Pihkalo *et al.*, 1996). Previous mediastinal radiation therapy has also been shown to add to development of adriamycin cardiomyopathy at a dose below the recommended dosage limit of 550 mg/m^2 body surface area (Fajardo *et al.*, 1976; Minow *et al.*, 1977; Billingham *et al.*, 1979). A synergistic effect of adriamycin and mediastinal radiation was also reported (Fajardo *et al.*, 1976). It was observed that animals receiving both adriamycin and radiation treatment developed more extensive cardiac lesions than animals receiving only one or the other of the treatments. Patients with previously completed or concomitant radiation therapy should be monitored closely for the possible development of cardiomyopathy and for them adriamycin total dose should be adjusted to well below 550 mg/m^2.

G.2. Liver disease

The highest concentrations of adriamycin and its metabolites are detected in the lung, kidney, spleen and liver (Bachur, 1975). Because adriamycin is mainly metabolized in the liver, biliary excretion has a very important role. If the biliary excretion is compromised, adriamycin will accumulate in the body which may enhance drug toxicity. A study of patients with severe liver disease receiving adriamycin revealed elevated plasma levels of adriamycin

(Benjamin *et al.*, 1974). These patients were prone to fatal and life-threatening conditions. Thus a decrease in the adriamycin dose in patients with liver disease was suggested (Benjamin, 1975).

G. 3. Combination Chemotherapy

During chemotherapy, adriamycin is very often used in combination with other antitumor drugs such as cyclophosphamide, vincristine and bleomycin. Adriamycin-cyclophosphamide treatment potentiates adriamycin cardiotoxicity even if the dosage of adriamycin is below the limit of 550 mg/m^2 (Minow *et al.*, 1977). Opposite findings were reported by Bristow *et al.*, (1978) as these authors could not find an increase in the risk of adriamycin-induced cardiomyopathy due to concurrent cyclophosphamide-adriamycin therapy. In any case, for the patients receiving cyclophosphamide-adriamycin combination therapy, reduction of adriamycin cumulative dose of 550 mg/m^2 body surface area is advisable. Combination therapy with bleomycin and vincristine was also found to be associated with higher incidence of a refractory adriamycin cardiomyopathy (Praga *et al.*, 1979).

G. 4. Previous Cardiovascular Disease

A history of previous electrocardiogram abnormalities and hypertension was found to be associated with an increased incidence of adriamycin-induced cardiomyopathy (Praga *et al.*, 1979). A retrospective study of patients' records demonstrated that the probability of developing adriamycin-induced cardiomyopathy was higher in patients with previous cardiac disease or hypertension (Von Hoff *et al.*, 1979). Cardiac hypertrophy due to aortic banding in a rat resulted in a cardiotoxicity and structural damage to the hypertrophied heart at a

much lower cumulative dose (5 mg/kg body weight) of adriamycin (Singal, 1983; Singal *et al.*, 1984) while no damage was observed in a sham control animals given the same dose of the drug (Singal, 1983). These studies indicated an increased risk of adriamycin cardiomyopathy possibly due to an altered metabolic state (Singal, 1985).

G. 5. Calcium Entry Blockers

Effects of myocardial calcium accumulation in the pathogenesis of adriamycin-induced cardiomyopathy have been studied and has already been reviewed earlier in this section. A review report dealing with this topic was published (Rabkin and Godin, 1985). Use of verapamil and nifedipine, the calcium channel blockers, has yielded controversial results in adriamycin-treated animals. In this regard, verapamil has been shown to be protective against adriamycin-induced cardiomyopathy by one group (Daniels *et al.*, 1976) and worsening of adriamycin cardiotoxicity by verapamil was shown by others (Young, 1975). The suggestion that calcium channel blockers verapamil and nifedipine do not prevent but accentuate adriamycin-induced cardiomyopathy was also reported (Klugman *et al.*, 1981). Ca^{2+} overload is possibly prevented by verapamil at high concentrations, while other cardiovascular effects of the Ca^{2+} channel blockers may become the main factor.

G. 6. Age

Correlation of several different factors with the probability of development of adriamycin-induced heart failure at various drug dose levels was done by a large retrospective study of 4 018 patient records (Von Hoff *et al.*, 1979). A steady increase in the probability of development of congestive heart failure was observed with increasing patient age. Another study reported that patients over 70 years-old and with an increase in the dose of

adriamycin appear to concomitantly increase the probability of the development of congestive heart failure (Bristow *et al.*, 1978). However, Praga *et al.*, (1979) found no statistical significance with respect to age as a risk factor in a study of 1273 patients. In a rat model, an increase in mortality as well as myocardial cell damage was demonstrated with an increase in age (Weinberg and Singal, 1986; Deally and Singal, 1990). Experiments done in a mouse animal model have shown that adriamycin is more toxic to the myocardial mitochondrial function in adult mice than in infant mice (Matsumara *et al.*, 1994).

H. CARDIOVASCULAR SIDE EFFECTS

Adriamycin-induced cardiovascular alterations have been observed in patients and experimental animals. They can be divided into acute and chronic effects. The acute effects develop within minutes or hours after the administration of the drug, they are not life threatening and are reversible or clinically manageable. Chronic effects, on the other hand, develop several weeks or months, or even years, after the therapy was completed. The most serious chronic effect is the insidious onset of a dose-dependent cardiomyopathy often leading to a congestive heart failure (Buja *et al.*, 1973; Chalcroft *et al.*, 1973; Lefrak *et al.*, 1973; Jaenke *et al.*, 1974). Development of cardiomyopathy was described even 4-20 years after adriamycin therapy was completed (Steinhertz *et al*, 1991). This chronic cardiotoxic side effect is the major limitation factor in the use of adriamycin.

H. 1. Acute Cardiotoxicity

Acute cardiotoxic side effects of adriamycin include hypotension, tachycardia and different electrocardiographic abnormalities (Herman *et al.*, 1971; Arena *et al.*, 1972; Lefrak *et al.*, 1973; Zbinden and Brandle, 1975). In a rabbit and a dog, tachycardia, inversion of the

T-wave (Arena *et al.*, 1972; Bachmann *et al.*, 1975) and reduction in the amplitude of EKG waves were reported (Herman *et al.*, 1971). Prolongation of ST segment during adriamycin was described in mice (van Acker *et al.*,1996). Addition of some tubulin-binding agents (vinblastin, vincristine, navelbine) to rhythmic, spontaneously pulsating rat cardiac cells immediately reversed adriamycin-induced arrhythmias (Lampidis *et al.*, 1992).

A negative inotropic effect of adriamycin (5 mg/ml) was demonstrated in isolated heart preparations, and was not reversed by propranolol or quinidine (Arena *et al.*, 1972). However, a new cardiotonic agent, enoximone, antagonised adriamycin-induced depression of contractile force in the isolated spontaneously beating, as well as electrically driven, guinea pig atria (Bossa *et al.*, 1996). A decrease in the contractile properties of the hearts of both rats and rabbits treated with adriamycin was found to be dose-related and progressive (Breed *et al.*, 1979). In the *in vitro* studies, low concentrations of adriamycin were found to increase the contractility in guinea pig atria (Villani *et al.*, 1978), chick heart (Azuma *et al.*, 1981) and isolated papillary muscle (Van Boxtel *et al.*, 1978). The effect of a wide range of adriamycin concentrations (1 nM to 1 mM) on rat papillary muscle contractility was examined. Increase in force was seen at lower doses but a clear depressant effect was observed at higher concentrations of the drug (Singal and Pierce, 1986). These *in vitro* studies provided evidence that a positive inotropic effect or no effect is seen at low drug concentrations (Van Boxtel *et al.*, 1978; Von Hoff *et al.*, 1979; Kim *et al.*, 1980), while a negative effect was observed at higher concentrations of the drug (Singal and Pierce, 1986).

Adriamycin administration, as early as 10 min after a single injection of 10 mg/kg, caused nuclear segregation in mouse myocardium (Lambertenghi-Deliliers, 1976). These

nuclear changes have been attributed to the interaction of the drug with nuclear DNA. Although these nuclear changes were reversed within 14 h of treatment (Lambertenghi-Deliliers, 1976), reversibility aspect of these changes is controversial. Another important observation is that only a small percentage of myocardial cells in patients who died from chronic anthracycline toxicity showed nuclear lesions (Buja *et al.*, 1973). However, in rats injected with a higher dose of adriamycin (40 mg/kg) the nuclear changes did not return to normal (Merski *et al.*, 1976). These differences in the nuclear changes may be due to age, species differences and/or the amount of the drug administered.

Hemodynamic changes due to acute cardiac effects of adriamycin were demonstrated in an open chest, perfused dog hearts(Bristow *et al.*, 1980). A dose-dependant decreases in cardiac output, mean blood pressure, left ventricular end diastolic pressure (LVEDP) and peripheral vascular resistance, as well as increase in the heart rate was noted. These hemodynamic changes were also shown to be reproduced by infusion of histamine and prevented by a histaminergic blocker (Bristow *et al.*, 1980). The same authors suggested that histamine, or histamine plus catecholamines or prostaglandins might play a role in the pathogenesis of adriamycin cardiomyopathy. In a chronic adriamycin treatment of rabbits, mean blood pressure (Arnolda *et al.*, 1986; Wanless *et al.*, 1987) and cardiac output dropped significantly, while total peripheral vascular resistance and heart rate had a tremendous increase (Arnolda *et al.*, 1986; Griffin-Green *et al.*, 1988). Slopes of Frank-Starling curves were flatter in the adriamycin- treated rabbits (Wanless *et al.*, 1987). Circulating norepinephrine and renin levels were elevated (Arnolda *et al.*, 1986). It appears that in the early stage of administration of adriamycin, observed hemodynamic changes may be due to

the adriamycin stimulated histamine release, reduction in peripheral resistance as well as a decrease in circulating blood volume. With the development of cardiomyopathy, physiological compensatory mechanisms leading to an increase in peripheral vascular resistance and a shift of blood supply to major body organs, resulting in the low output congestive heart failure.

H. 2. Chronic Cardiotoxicity

Chronic administration of adriamycin produces typical cardiomyopathy. A consequence of cardiomyopathy is congestive heart failure, which does not respond to any therapy and becomes progressively worse (Lefrak *et al.*, 1973; Buja *et al.*, 1973; Cortes *et al.*, 1975; Von Hoff *et al.*, 1979; Bristow *et al.*, 1978). One of the first reports of the adriamycin caused heart failure in patients was reported in a comprehensive study (Lefrak *et al.*, 1973). It was described that this type of heart failure was dose-dependent. The incidence increased rapidly at doses above 550 mg/m^2 body surface area. A total of 399 patients treated for malignant tumors by repeated administration of adriamycin (total cumulative dose 505 to 1004 mg/m^2 body surface area) over a period of several months showed several characteristic cardiovascular changes: marked hypotension (B.P. 70/50 mm Hg), tachycardia (150 beats /min) and a decrease in the QRS voltage on the EKG, cardiac dilation and ventricular failure (Lefrak *et al.*, 1973). A decrease in cardiac ejection fraction was observed in patients even after a low cumulative dose of adriamycin (Narahara *et al.*, 1979).

Heart failure due to adriamycin was characterized by refractoriness to inotropic drugs and mechanical circulatory assistance. Repeated injections of adriamycin caused a depression in cardiac function in animals and blunted response to norepinephrine (2 and 10 mg/kg) or

epinephrine (0.5 and 1 mg/kg)(Zbinden *et al.*, 1978; Tong *et al.*, 1991). Following chronic exposure to adriamycin, the contractile responses of single isolated rabbit cardiac myocytes to calcium and isoprenaline were depressed, while relaxation velocity remained unaltered (Jones *et al.*, 1990). Inotropic response to isoprenaline administration in rats treated with adriamycin (2 mg/kg once a week for 10 weeks) was reported to be lacking (Gorodetskaya *et al.*, 1990). Reduced responsiveness to epinephrine (x 10^{-5} M) as well as Ca^{2+} was found in 1 1/2 and 6 months old rats treated with adriamycin (Weinberg and Singal, 1986). In an *in vivo* dose-response study, epinephrine-induced cardiovascular response was significantly attenuated in adriamycin-treated rats (Tong *et al.*, 1991). Changes in the diastolic function occur early in the development of cardiomyopathy, prior to any significant fall in the ejection fraction, stroke volume and cardiac index (Tomlinson, 1987). A significant correlation between the change of the left ventricular shape and decrease in the ejection fraction, stroke volume and cardiac index was noticed (Tomlinson, 1987).

I. OTHER MAJOR ADRIAMYCIN SIDE EFFECTS

Besides cardiotoxicity, which is the most serious side effect of adriamycin therapy , a number of other tissues, such as neuronal, renal, hepatic, hematopoetic, etc., are known to be affected.

I. 1. Nephrotoxicity

A nephrotoxic syndrome was reported after the injection of 20 mg/kg i.v. of daunorubicin (Sternberg and Philips, 1967). Another study reported that 7.5 mg/kg of adriamycin (i.v.) caused injury to the glomerulus and made it more permeable to plasma proteins (Bizzi *et al.*, 1983). Typical clinical symptoms of nephrotic syndrome, including

proteinuria, peripheral edema, massive hyperlipidemia, lipiduria and hypoalbuminemia, are seen to be due to adriamycin nephrotoxicity (Joles *et al.*,1993; Washio *et al.*,1994). Alpha-tocopherol was found to decrease hyperlipidemia and the extent of glomerusclerosis in rats with adriamycin-induced progressive heart failure (Washio *et al.*, 1994). Ultrastructural studies revealed vacuolisation of the cytoplasm and thickening of the basal membrane in glomeruli (Sternberg, 1970; Young, 1975). Development of chronic glomerulonephritis due to the nephrotoxicity of anthracyclins in rats as well as the presence of cytomegalic cells in renal tubular epithelium have also been reported (Sternberg *et al.*, 1972). Radioimmunoassay techniques detected the highest concentration of adriamycin equivalents 24 h after injection in rabbits in the kidney tissue (Van Vunakis *et al.*, 1974).

After a single low dose of adriamycin (3 mg/kg), proteinuria was observed after 3 weeks and hypercholesterolemia after 6 weeks (Joles *et al.*,1993). Nephrotic syndrome was even more expressed in rats fed a high-cholesterol diet (Kunitomo *et al.*,1985). Interestingly, the same group of authors described that cholesterol feeding had a lowering effect on the lipid peroxide levels in the serum of adriamycin-treated rats (Kunitomo *et al.*,1985). The model of adriamycin-induced nephrotic syndrome due to focal and segmental glomerulosclerosis is commonly used in nephrology research (Gretz and Strauch, 1993).

I. 2. <u>Hematotoxicity</u>

Adriamycin (i.v.) in a dosage from 0.64 to 6.4 mg/kg/week for up to 20 weeks in pigs resulted in marked hypoplasia in bone marrow and lymphoid tissue, with frequent terminal haemorrhagic diathesis and septicemia (Van Vleet *et al.*, 1979). Associated alterations in peripheral blood included leucopenia, anemia and thrombocytopenia (Van Vleet *et al.* 1979).

In the study in which effectiveness of a weekly regimen of adriamycin administration was tested in 364 patients, increase in leukopenia, was observed in 64% of patients receiving a loading dose of 0.6 mg/kg of adriamycin followed by an increase in the total dose, while only 5% had severe thrombocytopenia (Weiss *et al.*, 1976).

I. 3. Neurotoxicity

A neurotoxicity was reported in a number of experimental and clinical studies. Toxicity of central nervous system as well as peripheral neuropathies were described due to adriamycin administration (Kondo *et al.*, 1987; Gill *et al.*, 1990). Neuropathological changes due to the adriamycin toxicity as early as four days following the treatment were seen (Kondo *et al.*, 1987). The neurons in the cerebral cortex and nucleus caudatus-putamen showed focal clearing of the nuclear chromatin, increase in dense bodies in the cytoplasm and dilatation of the cisternae of the rough endoplasmic reticulum and Golgi apparatus, and after ten days, the cytoplasm of many neurons was vacuolated (Kondo *et al.*, 1987). During clinical studies, incidence of peripheral neuropathy was found to be up to 80% (Willemse *et al.*, 1990).

J. RATS AS AN ANIMAL MODEL

Characteristic myocardial ultrastructural changes associated with adriamycin-induced cardiomyopathy in patients include the loss of myofibrils, cytoplasmic vacuolisation, swelling of mitochondria and increased number of lysosomes (Buja *et al.*, 1973; Lefrak *et al.*, 1973; Jaenke and Fajardo, 1977; Bristow *et al.*,1978). These structural alterations have also been noticed in a variety of animals such as rabbits (Jaenke, 1974; Olson *et al.*,1974), mice (Rosenhoff *et al.*, 1975; Lambertinghi-Deliliers *et al.*,1 976) and rats (Chalcroft *et al.*, 1973; Singal *et al.*, 1985). Loss of myofibrils and the appearance of membrane vesicles have been

used as ultrastructural markers for adriamycin-induced cardiomyopathy. Another important feature of adriamycin-induced cardiomyopathy is that patients are generally refractory to therapies for heart failure (Lefrak *et al.*, 1973). Rats seem to mimic not only myocardial structural changes but also the functional refractoriness of adriamycin-induced cardiomyopathy observed in humans (Lefrak *et al.*,1973; Weinberg and Singal, 1987; Deally and Singal, 1990; Tong *et al.*, 1991). Thus, the use of rat as an animal model has provided valuable information for understanding the pathogenesis of this form of cardiomyopathy (Mettler *et al.*, 1977; Olson and Capen, 1977; Zbinden *et al.*,1978; Siveski-Iliskovic *et al.*,1994b). Furthermore, the development of cardiomyopathy and eventual heart failure in rats due to adriamycin is well characterized and is reproducible (Siveski-Iliskovic *et al.*, 1994b). A simple procedure for inducing adriamycin-induced cardiomyopathy in male rats has been previously described and it involves the administration of 15 mg/kg (i.p.) of the drug, in six equal injections, over 2 weeks (Weinberg and Singal, 1987). Since 1 mg/kg of adriamycin roughly corresponds to 37 mg/m^2 (Freireich *et al.*, 1966), use of 15 mg/kg of adriamycin translates into 555 mg/m^2. Sex-related differences in adriamycin cardiomyopathy were not observed in patients (Von Hoff *et al.*, 1979). Thus, in the rat animal studies, generally male rats have been used.

K. ANTIOXIDANTS AND ADRIAMYCIN-INDUCED CARDIOMYOPATHY

In addition to defining the subcellular mechanisms of adriamycin-induced cardiomyopathy, two contending approaches have been applied to mitigate this side-effect of adriamycin: (i) synthesize a "less toxic" analogue of adriamycin, which would keep the anti-tumor properties of the original drug but would not lead to cardiac failure; and (ii) administer

adriamycin in combination with some other substance to counteract the cardiotoxic effects of adriamycin without interfering with its anti-tumor properties. Although an improved therapeutic index has been accomplished with newer analogues, some cardiotoxicity is always present with these agents (Booser and Hortobagyi, 1994). There is a very fine balance between cellular systems that generate various free radicals and those responsible for the maintenance of antioxidant defense mechanisms (Singal and Kirshenbaum, 1990). It seems that adriamycin tips this balance in favour of oxidants both by increasing the formation of free radicals (Kalyanaraman *et al.*,1980) and by reducing endogenous antioxidant (Doroshow *et al.*,1980; Siveski-Iliskovic *et al.*,1994a). Endogenous antioxidants, like superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSHPx) are no longer able to cope with this increase in free radical production and adriamycin cardiotoxicity gets expressed.

In a study done on mice, it was reported that a bolus injection of vitamin E given 24 h prior to adriamycin administration prevented cardiomyopathic changes (Myers *et al.*, 1977). In this regard, a vitamin E deficient diet in rats resulted in significantly higher mortality compared to those on a normal diet; the experimental animals suffered more ultrastructural myocardial damage due to adriamycin (Singal and Tong, 1988). Further studies demonstrated that vitamin E was effective only against acute adriamycin-induced cardiotoxicity in mice (up to 15 days after adriamycin administration), while it offered no significant protection against lethal toxicity after 5 weeks of adriamycin administration in this species (Mimnaugh *et al.*, 1979). Vitamin E and /or selenium had no significant protective effect against adriamycin-induced mortality in mice (Hermansen and Wassermann, 1985). In a study using the dog model, neither vitamin E nor selenium offered any cardioprotection

against chronic adriamycin-induced cardiomyopathy (Van Vleet *et al.*,1980). Similarly, chronic adriamycin-induced cardiotoxicity was not prevented by vitamin E when this therapy was applied to rabbits (Breed *et al.*, 1980). It is likely that vitamin E may delay the toxic effects of adriamycin and it is unlikely that it completely prevents cardiomyopathy. However, oral selenium supplementation was shown to be beneficial against cardiac toxicity in rats treated with 15 mg/kg of adriamycin over a period of 3 weeks, resulting in increased cardiac mitochondrial glutathione peroxidase activity, reduced myocardial malondialdehyde levels and improved resistance to myocardial ischemia and reperfusion injury (Boucher *et al.*, 1995).

Administration of ascorbic acid (vitamin C), a strong antioxidant, to mice which were inoculated with leukaemia L1210 or Ehrlich ascites carcinoma, was found to have no effect on the anti-tumor activity of adriamycin but nevertheless, the antioxidant significantly prolonged the life of animals treated with adriamycin (Shimpo *et al.*, 1991). Myocardial ultrastructural changes due to adriamycin in these animals were significantly reduced (Shimpo *et al.*, 1991). Vitamin E is effective in interrupting free radical chain reaction in the lipid phase while vitamin C seems to be effective in the cytosolic compartment (Kaul *et al.*,1993). In addition, it is known that vitamin C is important for the regeneration of vitamin E from its radical form thus maintaining the antioxidant reserve in the membrane phase. However, further carefully designed studies are needed to test the efficacy of combinations of vitamins E and C in preventing adriamycin-induced cardiomyopathy.

Chelation of transition metals may also reduce adriamycin-induced cardiotoxicity by slowing the free radical chain reaction. In this regard, the spin trapping compound alpha-phenyl-tert-butyl nitrone (PBN), when given concurrently with adriamycin, reduced

electrocardiographic abnormalities and also prevented the myelotoxic effects of adriamycin (Paracchini *et al.*,1993). Furthermore, no negative effects on adriamycin-cytotoxicity, evaluated *in vitro*, were observed (Paracchini *et al.*,1993). In a different study, abnormalities associated with adriamycin were reduced in the hearts of dogs pretreated with ICRF-187 (Herman and Ferrans, 1981). The agent ICRF-187 reacts directly with iron and increases its excretion and thereby reduces free oxygen radical production through the Fenton reaction. The cardioprotective action of ICRF-187 during adriamycin treatment may also be a result of its hydrolysis to the d isomer (ICRF-198) which inhibits reduction of Fe^{3+}, and subsequently reduces its role in tissue-damaging free radical reactions (Vile and Winterbourn, 1990). Clinical trials in which ICRF-187 has been used in combination therapy with adriamycin have already been conducted (Seifert *et al.*, 1994). Unfortunately, the addition of ICRF-187 as a combination therapy with adriamycin treatment is known to be associated with an increase in haematological toxicity, thus limiting the clinical application of ICRF-187 (Von Hoff *et al.*,1981).

Oleanolic acid and ursolic acid are compounds with free radical scavenging potential and have been shown to protect against adriamycin-induced lipid peroxidation in liver and heart microsomes *in vitro* (Balanehru and Nagarajan, 1992) while *in vivo* effects remain to be described. Use of the novel selenoorganic compound PZ51 (Ebselen) was seen to offer some protection against adriamycin-induced lipid peroxidation in the heart and the liver tissue (Pristos *et al.*,1992). Though adriamycin-induced toxicity, as measured by total serum creatine kinase activity, was reduced, cardiac hemodynamic function was not examined. The free radical scavenger N-acetylcysteine (NAC) has also been shown to offer some protection

against adriamycin-induced cardiomyopathy in mice (Myers et al 1977; Doroshow *et al.*, 1981). However, treatment with NAC has been found to be ineffective in reducing chronic adriamycin-induced cardiotoxicity in humans (Myers *et al.*, 1983) and dogs (Unverferth *et al.*, 1983; Herman *et al.*, 1985). Catechin, an antioxidant and an iron-chelating agent, offered some protection against adriamycin-induced EKG changes and myocardial contractility of isolated atria (Kozluca *et al.*, 1996). Ambroxol, an expectorant drug and scavenger of hydroxyl radicals, injected 30 min prior to adriamycin, completely prevented the rise in conjugated diens and malondialdehyde levels in the mouse heart (Nowak *et al.*, 1995). Beneficial effects of flavonoids against adriamycin cardiotoxicity have also been described (Husken *et al.*, 1995; van Acker *et al.*, 1995). Most of these studies remain isolated observations waiting further confirmation.

In a recent study, transgenic mice that overexpress different quantities of catalase, were exposed to adriamycin treatment (Kang *et al.*, 1996). Interestingly, a one hundred-fold increase in catalase offered protection against adriamycin cardiotoxicity, two hundred-fold increase offered no protection and five hundred-fold increase actually enhanced adriamycin toxicity (Kang *et al.*, 1996). Parameters measured were cardiac lipid peroxidation, serum creatine phosphokinase and functional changes in the isolated atrium (Kang *et al.*, 1996). The study makes an important point of optimization of the dose of antioxidant used. Another transgenic mice model, overexpressing human manganase superoxide dismutase, have shown resilience against adriamycin-induced ultrastructural mitochondrial damage, and changes in serum creatine kinase and lactate dehydrogenase levels (Yen *et al.*, 1996). Thus, applications of all these substances, although confirming the role of free radicals in the pathogenesis of

adriamycin cardiomyopathy, have fallen short of expectation as ideal agents for protection against adriamycin-induced cardiotoxicity.

L. PROBUCOL

In two recent studies, we have provided evidence that administration of probucol with adriamycin may be a truly efficacious combination therapy (Siveski-Iliskovic *et al.*,1994a; 1995). Probucol was first introduced in the early 1970s as a lipid lowering agent. Although it was used because of its LDL-cholesterol-lowering properties, it was soon noted that the drug lowered HDL-cholesterol to a greater extent than it lowered LDL-cholesterol (Zimetbaum *et al.*, 1990). Probucol treatment in patients with heterozygous familial hypercholesterolemia caused regression of xanthomas which did not correlate with the level of cholesterol reduction (Yamamoto *et al.*,1986). Both probucol and cholestyramine, which is another cholesterol-lowering drug, sharply lowered serum cholesterol levels in the non-human primate experimental model while only probucol led to a regression of atherosclerotic lesions in these animals (Wissler and Vesselinovich, 1983). Susceptibility of low density lipoproteins (LDL), isolated from hypercholesterolemic patients participating in Probucol Quantitative Regression Swedish Trial (PQRST), to oxidation *in vitro* has shown that LDL from patients on a control diet only, or cholesteramine only, had TBARS content increased twenty-fold, while addition of probucol to treatment halved TBARS levels of LDL (Regnstrom *et al.*, 1990). These observations point to the possibility that the beneficial effects of probucol may be independent of its cholesterol-lowering effect.

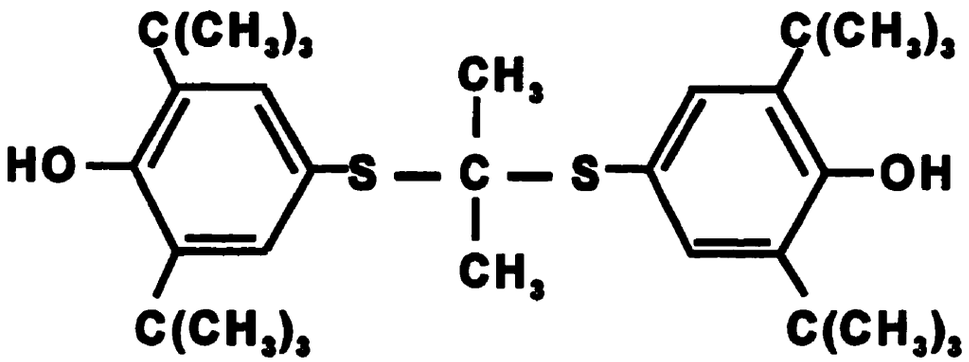


Figure 3. Chemical structure of probucol.

Probucol has no apparent structural similarity to other agents which actively lower cholesterol levels. It is a bis-phenol (Figure 3) which resembles vitamin E, another well-known antioxidant but with only one phenolic group. The vitamin E radical produced during the radical quenching process is reoxidized by vitamin C. This way vitamin E and C compliment each other. However, similar reaction for probucol has not been reported thus far. As it has two phenolic groups in its molecular structure, probucol has been reported to be a strong antioxidant (Parthasarathy *et al.*, 1986; Pryor *et al.*,1988; Mao *et al.*,1991) and this realisation has opened a whole new perspective for the use of this drug.

Probucol was found to be effective in the prevention of restenosis following balloon angioplasty in rabbits (Miyauchi *et al.*, 1993), as well as in preservation of endothelium-dependent relaxations of hypercholesterolmic rabbit aorta without reducing plasma cholesterol (Simon *et al.*,1993). Female domestic swine that were treated with 2 g/day of probucol had significantly reduced neointimal formation following coronary artery balloon injury (Schneider *et al.* 1993).

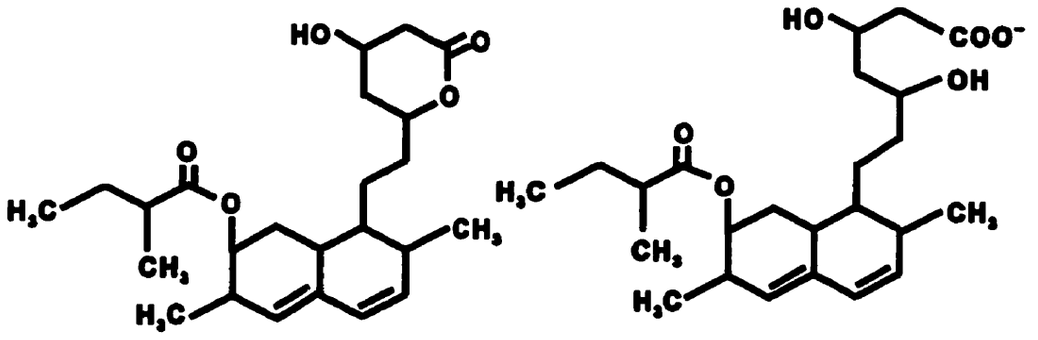
Effects of probucol on renal function in rats with bilateral ureteral obstruction (BUO) were examined (Modi *et al.*, 1990). BUO rats, treated with probucol had greater inulin and para-amino-lypuric (PAH) clearance as well as higher levels of reduced glutathione and lower levels of MDA in the renal cortex compared to BUO rats that did not receive probucol (Modi *et al.*,1990). In the same study, authors compared effects of probucol and lovastatin, and despite significant lipid lowering (observed with probucol as well) lovastatin treatment did not modify renal function in rats with BUO (Modi *et al.* 1990).

Ex vivo susceptibility of lipoproteins isolated from hyperlipidemic patients to oxidation was examined and compared with effects of vitamin E (Dujovne *et al.*,1994). Probucol lowered lipoprotein oxidation susceptibility by 95% while vitamin E decreased it by only 24% (Dujovne *et al.*, 1994). In that same study, oxidation inhibition due to probucol was found to be maximized within 2 weeks of the beginning of probucol treatment and returned to baseline 4 to 6 weeks after discontinuation of probucol (Dujovne *et al.*,1994). The antioxidant activity of probucol was suggested to be directly proportional to its concentration in LDL particles (Mao *et al.*, 1990).

Besides gastrointestinal discomfort and diarrhea, that are relatively common side effects of probucol therapy, some recent literature has described prolongation of QT interval in the EKG and development of tachyarrythmia (Reinoehl *et al.*, 1995). A review dealing in particular with the side effects of probucol, incidence of prolonged QT's was about 10%, and tachyarrythmia (which was described as *torsades de pointes*) was found in only 11 out of 399 patients reviewed (Reinoehl *et al.*, 1995).

M. LOVASTATIN

Lovastatin is a lipid-lowering drug and a member of 3 hydroxy-3 methylglutharyl Coenzyme A (HMG-CoA) reductase inhibitors. Its chemical structure in its active form does



LOVASTATIN **LOVASTATIN active form**

Figure 4. Chemical structure of lovastatin.

not have a phenolic ring (Figure 4). HMG-CoA reductase inhibitors are the most promising and most commonly used lipid-lowering drugs in contemporary medicine. Clinical trials have shown that lovastatin slows the progression of coronary atherosclerosis and inhibits the development of new coronary lesions (Waters et al., 1994). Thus major lovastatin action is reduction in LDL, VLDL and total cholesterol levels (Grundy, 1991). One of the main results of inhibition of cholesterol synthesis is a stimulation of LDL receptor synthesis (Goldstein and Brown, 1979), and that further enhances lovastatin-induced decrease in LDL levels. Lovastatin was also described to rise HDL cholesterol levels (Grundy and Vego, 1985). Lovastatin has no known antioxidant properties. In fact, in a number of studies it has been used as a control for lipid lowering properties of probucol, which is both a lipid lowering drug and an antioxidant. In a study with the rats exposed to bilateral ureteral obstruction, lovastatin did not improve excretory functions of the kidneys, nor did it improve glutathione content in the renal cortex, like probucol did (Modi *et al.*, 1990). Progression of fatty streak lesions in Watanabe heritable hyperlipidemic rabbits maintained at equal total plasma cholesterol levels with probucol or lovastatin treatment, was significantly slower in probucol-treated rabbits (Steinberg et al., 1988). Authors suggested that the antioxidant property of probucol was responsible for that observed difference (Steinberg et al.,1988).

N. TROLOX

Trolox is a water soluble form of vitamin E. Its chemical structure also has a phenolic ring (Figure 5). Trolox has been shown before to prevent cumene hydroperoxide-induced lipid peroxidation in cultured neonatal rat heart cells (Le et al., 1992). In a canine model of

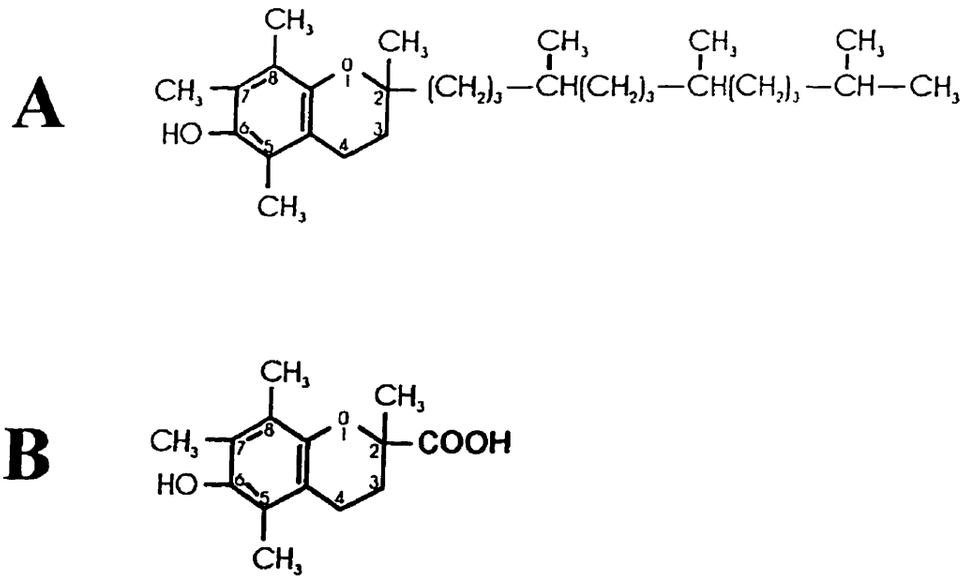


Figure 5. Chemical structure of (A) vitamin E and (B) trolox.

coronary artery occlusion, infusion of trolox and ascorbic acid into the ascending aorta 30 seconds before and 4 minutes after reperfusion was able to significantly reduce the area of infarction within the area at risk (Mickle et al., 1989). Also, trolox was more effective than superoxide dismutase or catalase in the protection of myocyte cell cultures from the free radical damage (Mickle et al., 1989). In myocytes exposed to free radicals, trolox was found to actually accelerate GSHPx reaction and its utilization, leading to lowering of reduced glutathione levels as well as reduced/oxidized glutathione ratio (Le et al., 1995). At the same time, the same group of authors have described less MDA formation in those same trolox exposed myocytes (Le et al., 1995).

Another study compared effects of five different antioxidants, including magnesium-pyridoxal-5'-phosphate glutamate (MPPG), pyridoxal-5'-phosphate (PP), α-tocopherol, trolox and probucol, on copper-induced oxidation of human low density lipoprotein (LDL) (Kogl et al.,1994). The antioxidant efficiency of these drugs was found to be: probucol > MPPG > trolox > α-tocopherol > PP (Kogl et al., 1994). Probucol inhibited copper-induced LDL oxidation for over 24 hours while trolox offered protection for only 3 hours (Kogl et al., 1994).

Perfusion with trolox decreased myocardial injury caused by stop-flow ischemia and reperfusion in an isolated rabbit heart model (Rubinstein et al.,1992). Apoptosis (programmed cell death) that was induced *in vitro*, in mouse thymocytes exposed to H_2O_2, was completely prevented by pre- or post-treatment of cells with trolox (Forrest et al., 1994).

O. HYPERLIPIDEMIA AND HEART FAILURE

First observations of negative influence that altered lipid metabolism had on the development of atherosclerosis were made by Virchow in the second half of the last century (Katz and Messineo, 1981). However, quite extensive evidence has emerged indicating that alterations in lipid metabolism can change cardiac function that would manifest in decreased myocardial contractility, arrhythmia and cell death (Katz and Messineo, 1981). Free fatty acids (FFA), which are mostly a result of enzymatic hydrolysis of triglycerides by a hormone-sensitive lipase are an important negative factor in the heart function. High FFA levels, by detergent-like actions, were found to impair permeability of the cell membranes (Helenius and Simons, 1975). Another important observation was that fatty acids could increase cardiac

oxygen consumption for any level of cardiac function (Challoner and Steinberg, 1966; Mjos, 1971). Electrophysiological changes, mainly shorter duration of cardiac action potentials, were described due to the action of fatty acids (Cowan and Vaughan Williams, 1980). Negative inotropic effects of fatty acids on myocardial function have been described in a number of studies (Opie, 1970; Sevareid et al., 1969) and these effects are further accentuated in the case of ischemic heart (Kjekshus and Mjos, 1972). However, negative studies, where free fatty acids had a positive inotropic effect on the functioning of the ischemic heart have also been reported (Most et al., 1972). Increased fat content in the myocardium could lead to conduction abnormalities (Balsaver et al., 1967) with consequent arrhythmias (Gupta et al., 1969; Sobel et al., 1978). Different phosphodiesterase isoforms found in cytosolic compartment of rat heart were shown to be inhibited by free fatty acids (Dubois et al.,1993). It was suggested that inhibition of phosphodiesterase by free fatty acids might have positive inotropic effect (Dubois et al., 1993). Altered lipid metabolism in the failing heart of cardiomyopathic hamsters (UM-X7.1) and increased phospholipid to protein ratio were described in atria of cardiomyopathic animals, compared to age-matched controls (Vecchini et al.,1995). Higher cholesterol to phospholipid ratio was also found in all regions of cardiomyopathic heart (Vecchini et al., 1995).

Perfusion of isolated rat hearts with solutions containing different free fatty acids/albumin ratios showed that not only high concentrations of free fatty acids have deleterious effects on cardiac contractility but the study also suggested that an increase in the free fatty acids/albumin ratio was an important factor (Willebrands et al., 1973). A reported

decrease in carnitine levels in the heart due to adriamycin (Senekowitsch et al., 1985) may also promote the formation of free fatty acids. In this regard, carnitine plays a central role in the transfer of long-chain fatty acids into the mitochondrial matrix where β-oxidation takes place (Bremer, 1983). Abnormalities in lipid metabolism due to adriamycin-induced nephrotic syndrome have been described earlier in this section. Adriamycin-induced hyperlipidemia (Kunitomo et al.,1985; Washio et al., 1994) not only increases free fatty acids concentration but it is also accompanied by hypoalbuminemia (Joles et al.,1993; Skutelsky et al.,1995). Thus, adriamycin treatment increases the free fatty acids/albumin ratio by affecting both components adversely.

From the literature review, it is apparent that there is a large volume of information available on adriamycin-induced cardiomyopathy in patients as well as in experimental models. It is also clear that we need a better understanding of the pathogenesis of adriamycin-induced cardiomyopathy as well as the mechanism of its prevention by antioxidants particularly probucol. Both of these aspects are the main focus of the present thesis research.

III. MATERIALS AND METHODS

A. ANIMAL MODEL AND TREATMENT PROTOCOLS

Male Sprague-Dawley rats, body weight 250 ± 25 g, were maintained on a normal rat chow and a regular light and dark cycle. Animals were given water and food *ad libitum* and divided into eight groups: CONT (control), ADR (adriamycin-treated), PROB (probucol-treated), PROB + ADR (probucol + adriamycin-treated), LOV (lovastatin-treated), LOV + ADR (lovastatin + adriamycin-treated), TRO (trolox-treated) and TRO + ADR (trolox + adriamycin-treated). A schematic presentation of treatment protocol in different groups is shown in Figure 6.

Adriamycin (doxorubicin hydrochloride) was administered intraperitoneally (Monday, Wednesday and Friday) in six equal injections (each containing 2.5 mg/kg ADR) to animals in ADR, PROB + ADR, LOV + ADR and TRO + ADR groups over a period of 2 weeks for a cumulative dose of 15 mg/kg body weight. Probucol (cumulative dose, 120 mg/kg body weight) was also administered intraperitoneally to PROB and PROB + ADR groups in twelve equal injections (each treatment containing 10 mg/kg) over a period of 4 weeks, two weeks prior and two weeks alternating with adriamycin injections (Tuesday, Thursday and Saturday). CONT animals were injected with the vehicle alone (lactose, 75 mg/kg in saline) in the same regimen as ADR.

Lovastatin was also given intraperitoneally to the LOV and LOV + ADR groups in 12 equal injections (each injection containing 4 mg/kg of lovastatin, total cumulative dose of 48 mg/kg), over a period of 4 weeks, 2 weeks before adriamycin administration and 2 weeks alternating with adriamycin injections. For determining the appropriate dose of lovastatin,

in a pilot study, three different concentrations (4, 8 and 12 mg/kg) of lovastatin were tested with respect to their lipid-lowering effect. A maximal lipid-lowering response was achieved at the dose of 4 mg/kg (per injection), and therefore this dosage was used.

Trolox was given (i.p.) to the rats in TRO and TRO + ADR groups in 12 equal injections (48 mg/kg total cumulative dose), extrapolated dosage commonly used for vitamin E therapy.

Probucol, lovastatin and trolox were dissolved in coconut oil.

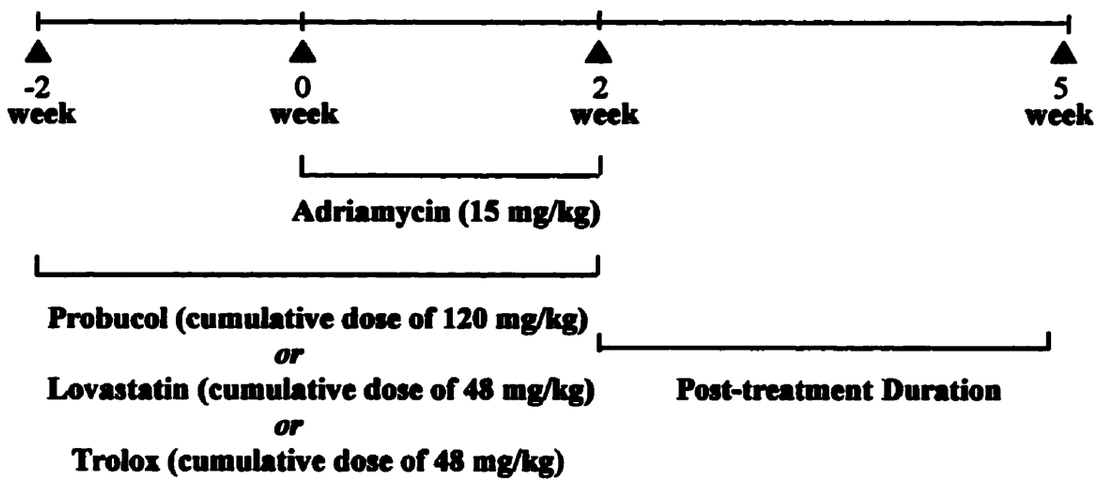


Figure 6: **Schematic representation of treatment protocol used. Male Sprague-Dawley rats (250 ± 25 g body weight) were divided in 8 groups: control, adriamycin, probucol, probucol + adriamycin, lovastatin, lovastatin + adriamycin, trolox, trolox + adriamycin. For details see text.**

All animals were observed for as long as 3 weeks after the last injection for general appearance, behaviour and mortality. At the end of the 3-week post-treatment period,

animals were assessed hemodynamically and sacrificed by decapitation, and blood was collected in heparinized tubes for the assessment of plasma lipids and albumin or in non-heparinized tubes "Vacutainer" brand SST for serum separation for the assessment of free fatty acids (FFA). Atria and other connective tissue from hearts were dissected away, and ventricles were used to assess myocardial lipids, glutathione peroxidase, superoxide dismutase, catalase, glutathione (GSH and GSSG) and lipid peroxidation.

The amount of ascites (fluid in peritoneal cavity) was measured by fluid aspiration with a 60 ml syringe.

Lungs and liver were removed and weighed. For the determination of dry weight, small pieces of lungs and liver were placed in the oven at 65°C until a constant weight was reached. Wet/dry weight ratios were calculated for lungs and liver.

B. HEMODYNAMIC STUDIES

Animals were anesthetized by sodium pentobarbital (50 mg/kg, i.p.). A miniature pressure transducer (Miller Micro-Tip) was inserted into the left ventricle via the right carotid artery. Left ventricular systolic pressure (LVSP) and end-diastolic pressure (LVEDP) as well as aortic systolic (ASP) and diastolic pressures (ADP) were recorded on a Beckman Dynograph recorder and on a computer (on-line analysis, Axotape acquisition data program).

C. BIOCHEMICAL STUDIES

C. 1. Catalase

Ventricles were homogenized in 9 vol. of 0.05M potassium phosphate buffer (pH 7.4) and centrifuged at 40,000 g for 30 min. Supernatant, 50 μL, was added to the cuvette containing 2.95 ml of 19 mM H_2O_2 solution prepared in potassium phosphate buffer

(Claiborne, 1985). The colour was read at 240 nm on a Spectronic 601 spectrophotometer every min for 5 min. Commercially available catalase was used as a standard. Specific activity of the enzyme was expressed as units per milligram tissue protein.

C. 2. Glutathione Peroxidase (GSHPx)

GSHPx activity was expressed as nanomoles of reduced nicotinamide adenine dinucleotide phosphate (NADPH) converted to oxidized nicotinamide adenine dinucleotide phosphate (NADP) per min per milligram protein, with a molar extinction coefficient for NADPH at 340 nm of 6.22×10^6 (Paglia and Valentine, 1967). Cytosolic GSHPx was assayed in a 3 ml cuvette containing 2.0 ml of 75 mM phosphate buffer, pH 7.0. The following solutions were then added: 50 µl of 60 mM glutathione, 100 µl glutathione reductase solution (30 U/ml), 50 µl of 0.12 M Na N_3, 100µl of 15 mM Na_2 EDTA, 100 µl of 3.0 mM NADPH and 100 µl of cytosolic fraction obtained after centrifugation at 20,000 g for 25 min. Water was added to make a total volume of 2.9 ml. The reaction was started by the addition of 100 µl of 7.5 mM H_2O_2, and the conversion of NADPH to NADP was monitored by a continuous recording of the change of absorbance at 340 nm at 1-min intervals for 5 min. Enzyme activity of GSHPx was expressed in terms of milligrams of protein.

C. 3. Superoxide Dismutase (SOD)

Supernatant (20,000 g for 20 min) was assayed for SOD activity by following the inhibition of pyrogallol autooxidation (Marklund, 1985). Pyrogallol (24 mM) was prepared in 10 mM HCl and kept at 4°C before use. Catalase (30 µM stock solution) was prepared in an alkaline buffer (pH 9.0). Aliquots of supernatant (150 µg protein) were added to Tris

HCl buffer containing 25 µl pyrogallol and 10 µl catalase. The final volume of 3 ml was made up with the same buffer. Changes in absorbance at 420 nm were recorded at 1-min intervals for 5 min. SOD activity was determined from a standard curve of percentage inhibition of pyrogallol autooxidation with a known SOD activity. Data were expressed as SOD units per milligram protein as compared with the standard.

C. 4. Thiobarbituric Acid Reactive Substances

Measurement of lipid peroxidation by determining myocardial thiobarbituric acid reactive substances (TBARS) content was performed using a modified thiobarbituric acid (TBA) method (Placer et al., 1966). Hearts were quickly excised and washed in buffered 0.9% KCl (pH 7.4). After removing the atria, extraneous fat and connective tissue, the ventricles were homogenized (in 10% w/v) in the same buffer. The homogenate was incubated for 1 hour at 37°C in a water bath. A 2-ml aliquot was withdrawn from the incubation mixture and pipetted into an 8-ml Pyrex tube. One millilitre of 40% trichloroacetic (TCA) acid and 1 ml of 0.2% TBA were promptly added. To minimize peroxidation during the subsequent assay procedure, 2% butylated hydroxytoluene was added to the TBA reagent mixture (Aust, 1985). Tube contents were vortexed briefly, boiled for 15 min and cooled in a bucket of ice for 5 min. Two millilitres of 70% TCA was then added to all tubes and contents were again vortexed briefly. The tubes were allowed to stand for 20 min. This was followed by a centrifugation of the tubes for 20 min at 3500 rpm. The colour was read at 532 nm on a Spectronic 601 spectrophotometer and compared with a known malondialdehyde standard.

C. 5. Glutathione

Concentration of total glutathione i.e., oxidized (GSSG) + reduced (GSH), was measured in the myocardium by the glutathione reductase/5,5'-dithiobis-(2-nitrobenzoic acid) recycling assay. The rate of dithiobis-nitrobenzoic acid formation is followed at 412 nm and is proportional to the sum of GSH and GSSG present. Myocardial tissue was homogenized in 5% sulfosalicylic acid. The tissue homogenate was centrifuged for 10 minutes at 10,000 x *g*. Supernatant was stored at 4°C until assayed. GSSG alone was measured by treating the sulfosalicylic acid supernatant with 2-vinylpyridine and triethanolamine. The solution was vigorously mixed and the final pH of the solution was checked to be between 6 and 7. After 60 minutes, the derivatized samples were assayed as described above in the glutathione reductase/5,5'-dithiobis-(2-nitrobenzoic acid)-GSSG reductase recycling assay. GSH values were calculated as the difference between total (GSSG plus GSH) and GSSG concentrations. Values are reported in GSH equivalents, micromoles per gram of tissue weight.

C. 6. Lipids, Free Fatty Acids and Serum Albumin Assays

Plasma triglycerides, total cholesterol, high density lipoproteins and low density lipoproteins were determined enzymatically using kits obtained from Sigma Diagnostics (#352, #352-3, #336) and expressed as mg/dl plasma. For cardiac lipids, ventricles (1 g) were homogenized in 10 ml 0.05 potassium phosphate buffer (pH 7.4), centrifuged at 40,000 for 30 min and supernatants were assayed using the same Sigma kits, and expressed as mg/g tissue. Serum free fatty acids (FFA) were determined by standard kit from WAKO (NEFA C), and serum albumin by standard Sigma kit for albumin determination (#625-2).

D. ANTITUMOR EFFECT

In order to assess the effects of probucol on the antitumor efficacy of adriamycin, subcutaneous tumor growth was studied in mice. The L5178Y-F9 lymphoma model, cloned directly from the L5178Y (Wolosin *et al.*, 1979), was chosen because it is one of the standard experimental tumors used to examine chemotherapeutic efficacy of different anticancer drugs, including adriamycin and its derivatives (Leonetti *et al.*, 1993). Tumor cells were injected subcutaneously in the shaved area on the back of the mice.

One week was allowed for the growth of the tumor and then the animals were divided into four groups. In group I, animals received only saline. In group II, adriamycin (6 X 2.5 mg/kg) was injected i.p. on alternate days. In group III, probucol (6 X 10 mg/kg) was injected i.p. on alternate days. In group IV, adriamycin was injected on Monday, Wednesday and Friday while probucol was injected on Tuesday, Thursday and Saturday. During the two weeks of treatment, changes in tumor size were followed daily by measuring the tumor diameters with a verner caliper. For each tumor node, two measurements, at 90° angle from each other were taken and surface area of the tumor was calculated.

E. PROTEINS AND STATISTICAL ANALYSIS

Proteins were determined by the method of Lowry and associates (1951). Data were expressed as mean ± SEM. For a statistical analysis of the data, group means were compared by one-way ANOVA, and Bonferroni's test was used to identify differences between groups. Statistical significance was acceptable to a level of $P<0.05$.

IV. RESULTS

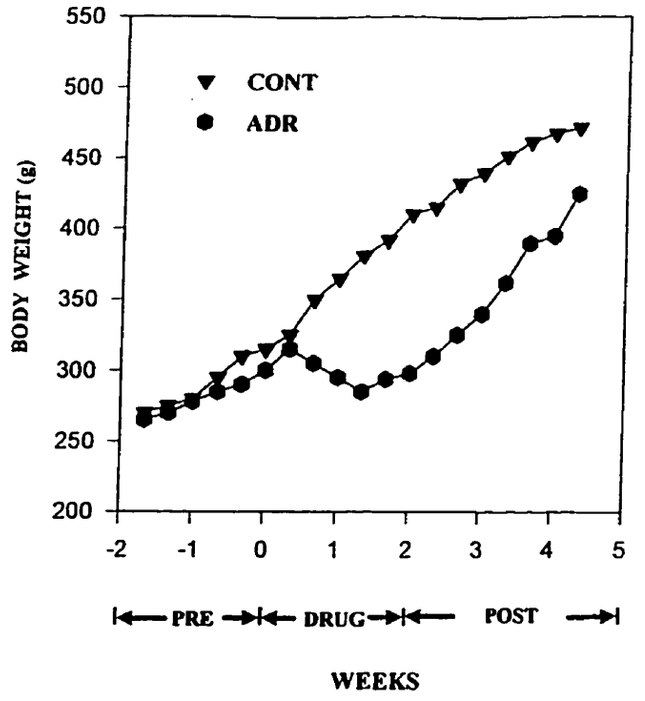
A. GENERAL OBSERVATIONS AND MORTALITY

General appearance of animals in all 8 groups was observed throughout the entire study. Animals in ADR group developed scruffy, yellowish fur and red exudate around the eyes within 1 week of the last adriamycin injection. Similar changes were observed in LOV + ADR group. Animals in PROB + ADR group did not show any of these changes. The most noticeable characteristic of rats in the ADR group was the development of an enlarged abdomen due to accumulation of fluid in peritoneal cavity (ascites). Abdominal distention became apparent within a week after adriamycin treatment was completed. Some abdominal distention due to ascites was noticed in LOV + ADR and TRO + ADR groups, while in PROB + ADR group just 2 out of 10 rats had insignificant amounts of ascites (8 and 15 ml respectively) (Table 2).

There were no deaths in CONT, PROB, LOV and TRO groups (Table 2). Mortality rate was 45% in ADR group. Probucol treatment, in the PRO + ADR group, reduced mortality to zero, while lovastatin in the LOV + ADR group reduced mortality to 20%. Mortality data for TRO + ADR group could not be obtained because animals showing any signs of distress or significant abdominal distention were removed from the study before the observation period was completed in compliance with the new Animal Care regulations. Death as an endpoint is no longer allowed to be studied at this University.

Loss of body weight due to adriamycin treatment was significant in all rats treated with adriamycin with or without the other drugs (Figures 7 and 8). Soon after the second

A



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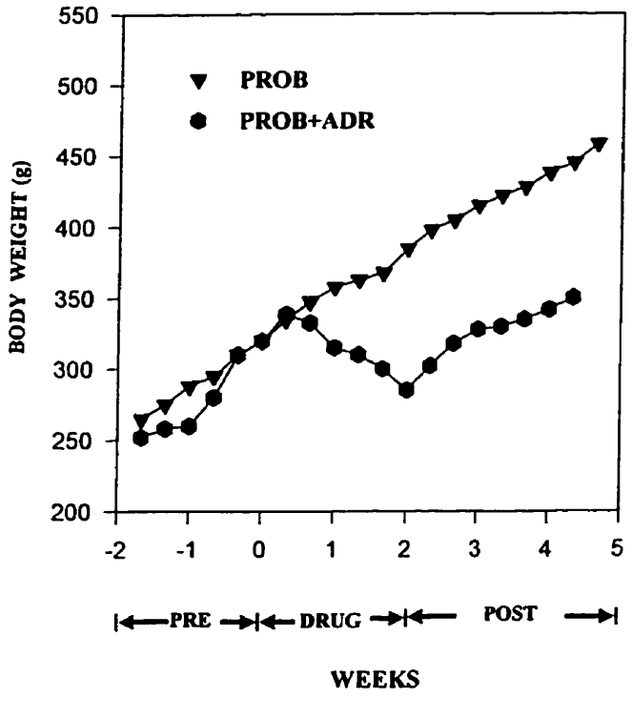
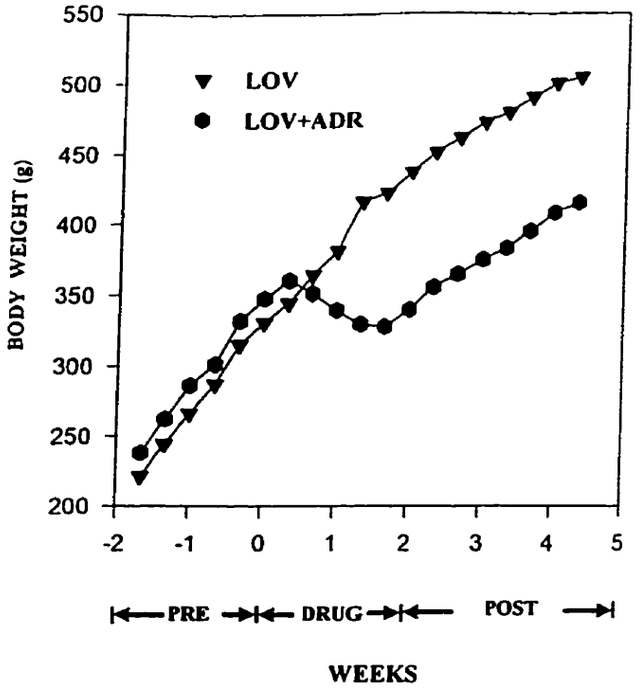


Figure 7 A & B: Effect of adriamycin (ADR) treatment on body weight (panel A). Effects of probucol (PROB) treatment on ADR-induced changes in body weight (panel B). For treatment protocol, please see Material and Methods section. Mean of 25 animals in each group. S.D. not shown for clarity.

A



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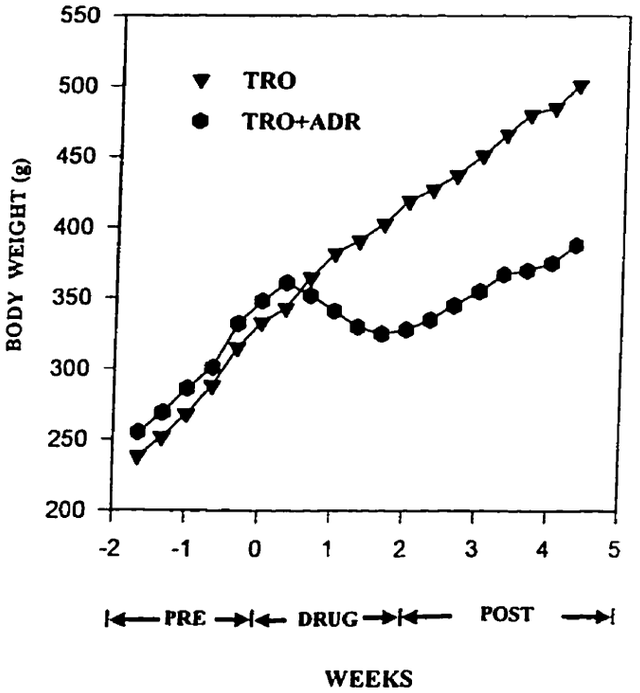

Figure 8 A & B: Effect of lovastatin (LOV) on ADR-induced changes in body weight (panel A). Effect of trolox (TRO) on ADR-induced changes in body weight (panel B). For treatment protocol, please see Material and Methods section. Mean of 25 animals in each group. S.D. not shown for clarity.

injection of adriamycin, difference in body weight between adriamycin-treated and control rats became significant (Figure 7A), and remained significant throughout the post-treatment duration, in spite of some recovery of body weight in adriamycin-treated rats during post-treatment period. Body weight gain was higher in ADR than in PROB + ADR, LOV + ADR and TRO + ADR groups, perhaps due to a larger quantity of ascites accumulated in ADR group (Figures 7 and 8).

Treatment with adriamycin resulted in a significant decrease in the heart weight in the ADR group compared to CONT, PROB, LOV and TRO groups (Table 2). Probucol in the PRO + ADR group completely prevented the loss in heart weight due to adriamycin, while no such protection was seen in the LOV + ADR and TRO + ADR groups. Heart to body weight ratio was significantly decreased only in ADR group, while it was normalized in the PROB + ADR, LOV + ADR and TRO + ADR groups (Table 2).

B. <u>LUNGS AND LIVER WET TO DRY WEIGHT RATIOS</u>

At the time of sacrifice, wet/dry weight ratio for lungs and liver was determined (Table 3). In concert with the signs of congestive heart failure, increase in wet/dry weight ratio for liver was found in ADR and LOV + ADR groups, while the ratio was normalized with probucol and trolox treatment (Table 3). Lungs wet/dry weight ratio was slightly increased in ADR and LOV + ADR groups. However, that differences were not statistically significant (Table 3).

Table 2: **Effects of probucol, lovastatin and trolox on adriamycin-induced changes in heart weight, body weight, mortality and ascites.**

Animal Group	Heart Weight (g)	Heart Weight/ Body Weight Ratio X 10^3	Mortality (%)	Ascites (ml)
CONT	1.63 ± 0.08	2.93 ± 0.07	0	0
ADR	1.10 ± 0.05*	2.34 ± 0.03†	45	110.12 ± 15.6†
PROB	1.52 ± 0.03	2.78 ± 0.09	0	0
PROB + ADR	1.46 ± 0.06	2.66 ± 0.12	0	2.3 ± 1.62
LOV	1.66 ± 0.09	2.89 ± 0.11	0	0
LOV + ADR	1.14 ± 0.02*	2.68 ± 0.05	20	34.28 ± 18.2**
TRO	1.62 ± 0.08	3.05 ± 0.12	0	0
TRO + ADR	1.20 ± 0.04*	3.06 ± 0.06	Not Available	20 ± 4.24**

CONT indicates control; ADR, adriamycin; PROB, probucol; PROB+ADR, probucol + adriamycin; LOV, lovastatin; LOV + ADR, lovastatin + adriamycin; TRO, trolox and TRO + ADR, trolox + adriamycin. Data are mean ± SEM of 8-10 animals for all studies except for mortality. For determining mortality, 20 animals were used in each of the CONT, PROB, PROB + ADR, LOV and TRO groups, and 40 animals each in the ADR and LOV + ADR group. Mortality data were not available for TRO + ADR group (see text for details).

* Significantly different from CONT, PROB, PROB+ADR, TRO and LOV ($P<0.05$).

† Significantly different from CONT, PROB, PROB+ADR, LOV, LOV + ADR, TRO and TRO + ADR ($P<0.05$).

** Significantly different from CONT, ADR, PROB, PROB + ADR, LOV and TRO ($P<0.05$).

Table 3: **Effects of probucol, lovastatin and trolox on adriamycin-induced changes in lungs and liver wet/dry weight ratios.**

Animal Group	Lungs	Liver
CONT	4.08 ± 0.27	2.84 ± 0.03
ADR	4.75 ± 0.21	3.30 ± 0.05*
PROB	4.39 ± 0.18	2.96 ± 0.08
PROB + ADR	4.26 ± 0.19	2.98 ± 0.05
LOV	3.80 ± 0.26	2.99 ± 0.12
LOV + ADR	4.63 ± 0.25	3.36 ± 0.07*
TRO	3.89 ± 0.34	2.68 ± 0.10
TRO + ADR	3.93 ± 0.20	3.16 ± 0.11

Data are expressed as mean ± S.E.M. of 5-7 animals.

* Significantly different from CONT, PROB, LOV, TRO and PROB + ADR groups ($P<0.05$). All abbreviations are the same as in Table 2.

C. HEMODYNAMIC STUDIES

Cardiac function as well as blood pressure readings were taken by placing a catheter with a micro-tip pressure transducer through the right carotid artery first in the aorta and then advancing into the left ventricle. These data on aortic systolic (ASP) and diastolic (ADP), left ventricular peak systolic (LVPSP) and end diastolic (LVEDP) pressures in all groups are shown in Table 4.

There were significant changes in cardiac performance in ADR group (Table 4). LVPSP and ASP were both significantly depressed, while LVEDP was significantly elevated. Probucol treatment in the PROB + ADR group maintained all those parameters at the control levels. Lovastatin treatment had some beneficial effects on adriamycin-induced hemodynamic changes, such that ADP and LVPSP were normalized (Table 4) in the LOV + ADR group. Although there was some improvement in the ASP and LVEDP compared with the ADR group int he LOV + ADR group, these values were still significantly different compared with the CONT group. Trolox treatment in TRO + ADR group returned to the ASP control levels while LVEDP still stayed higher than in CONT, PROB, PROB + ADR, LOV and TRO groups. Although LVEDP in the TRO + ADR group was still higher than CONT, it was one-half than in ADR group (Table 4).

D. ENDOGENOUS ANTIOXIDANT ENZYMES

Endogenous antioxidant enzyme activities, glutathione peroxidase (GSHPx), superoxide dismutase (SOD) and catalase were examined in the hearts from all eight groups and these data are shown in Figures 9, 10 and 11 respectively. Adriamycin treatment caused a significant decrease (about 40%) in GSHPx activity (Figure 9) compared to CONT group,

Table 4: Effects of probucol, lovastatin and trolox on adriamycin-induced hemodynamic changes.

Animal Group	LVPSP mmHg	LVEDP mmHg	ASP mmHg	ADP mmHg
CONT	121.62 ± 2.34	5.23 ± 0.54	110.28 ± 2.88	73.39 ± 1.72
ADR	85.67 ± 1.91**	30.00 ± 2.41*	82.33 ± 2.18*	60.78 ± 0.92*
PROB	115.42 ± 1.9	6.88 ± 3.01	105.12 ± 5.30	70.82 ± 2.12
PROB + ADR	119.16 ± 4.28	8.74 ± 1.9	99.38 ± 6.2	67.22 ± 3.26
LOV	121.68 ± 3.64	5.49 ± 0.92	114.23 ± 4.53	75.38 ± 2.67
LOV + ADR	108.40 ± 5.29	20.08 ± 2.91***	91.31 ± 4.12*	65.86 ± 3.48
TRO	125.12 ± 3.12	6.40 ± 2.90	118.43 ± 4.18	77.44 ± 1.66
TRO + ADR	117.70 ± 2.55	15.32 ± 3.47***	96.18 ± 5.6	69.71 ± 2.88

ASP, aortic systolic pressure; ADP, aortic diastolic pressure; LVPSP, left ventricular peak systolic pressure; LVEDP, left ventricular end-diastolic pressure; CONT, control; ADR, adriamycin; PROB, probucol; LOV, lovastatin; TRO, trolox. Values are mean ± SEM of five to seven experiments. Abbreviations are the same as in Table 2.

* Significantly different from CONT, PROB, PROB + ADR, LOV, TRO and TRO + ADR groups ($P<0.05$).
** Significantly different from all other groups ($P<0.05$).
*** Significantly different from CONT, ADR, PROB, PROB + ADR, LOV and TRO groups ($P<0.05$).

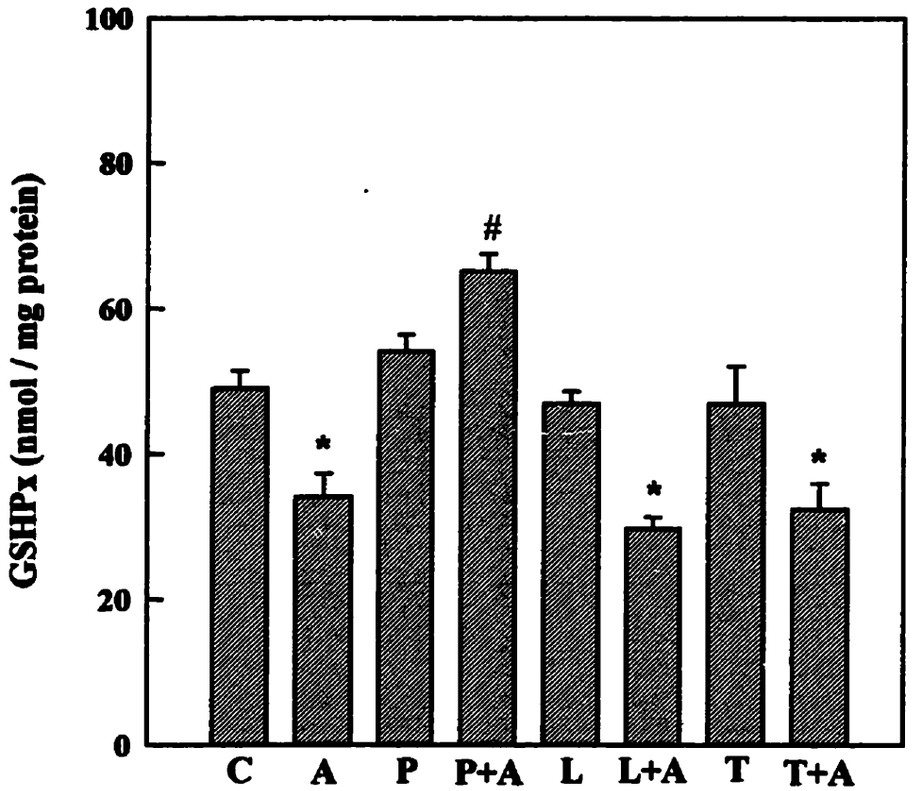

Figure 9:Effects of probucol (P), lovastatin (L) and trolox(T) on adriamycin (A)-induced changes in myocardial glutathione peroxidase (GSHPx) activity. Values are mean ± S.E.M. of 6-8 experiments. *Significantly different from the C, P, P+A, L and T groups (P<0.05). #Significantly different from the C, A, L, L+A, T and T+A groups (P<0.05).

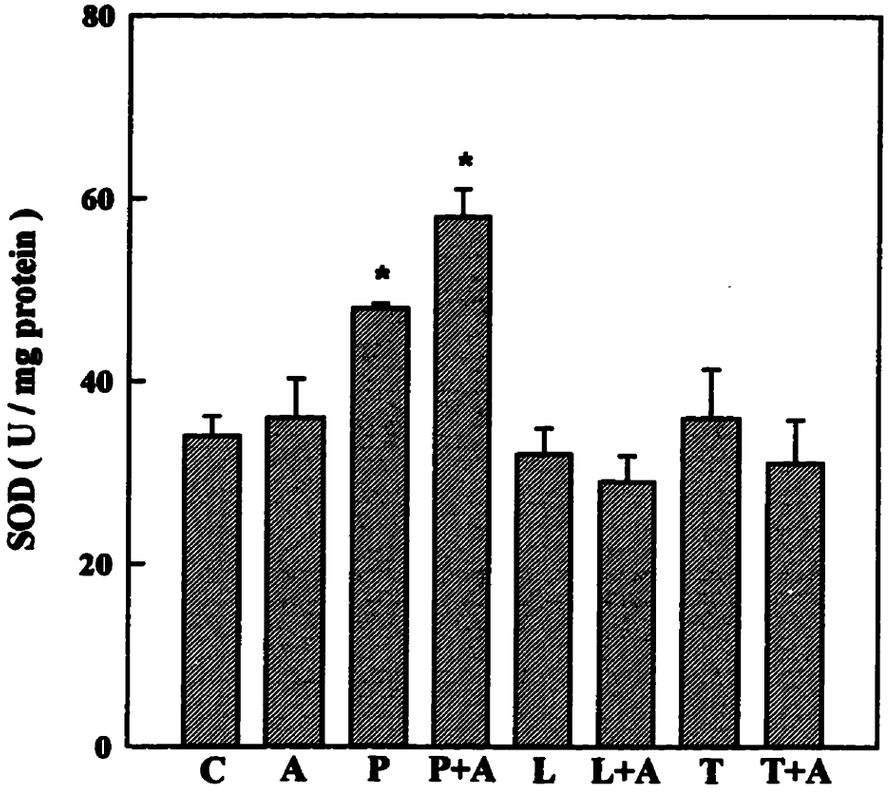

Figure 10: Effects of probucol (P), lovastatin (L) and trolox (T) on adriamycin (A)-induced changes in myocardial superoxide dismutase (SOD) activity. Values are mean ± S.E.M. of 6-8 experiments. *Significantly different from C, A, L, L+A, T and T+A groups (P<0.05).

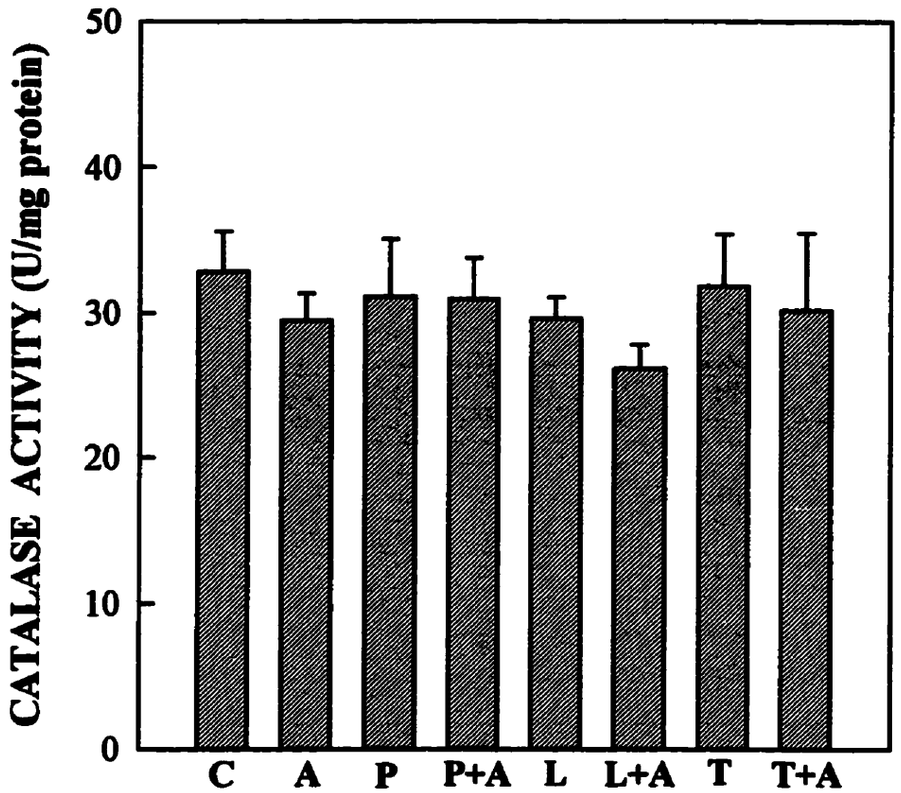

Figure 11: Effects of probucol (P), lovastatin (L) and trolox (T) on adriamycin (A)-induced changes in myocardial catalase activity. Values are mean ± S.E.M. of 6-8 experiments.

while SOD (Figure 10) and catalase (Figure 11) were not changed. Probucol treatment itself caused a significant increase in SOD activity with no change in the GSHPx or catalase activities. In PROB + ADR group, the GSHPx and SOD activities were increased compared to control levels. Catalase activity was unaltered in the PROB + ADR group. Lovastatin or trolox treatment itself did not have any effect on any adriamycin-induced changes in GSHPx activity (Figures 9-11). This activity in the LOV + ADR and TRO + ADR groups was still significantly depressed.

E. GLUTATHIONE

Myocardial GSH and GSSG levels were measured in all groups (Table 5). GSH content was significantly decreased in all groups exposed to adriamycin treatment including ADR, PROB + ADR, LOV + ADR and TRO + ADR groups. Trolox and lovastatin treatment had no effect on adriamycin-induced decrease in GSH levels, while probucol treatment increased GSH levels in PROB + ADR group compared to ADR, TRO + ADR and LOV + ADR groups. However, GSH in the PROB + ADR group still remained significantly decreased compared to CONT, PROB, LOV and TRO groups (Table 5). GSH levels in ADR, TRO + ADR and LOV + ADR groups were decreased about 45% compared to controls, while in PROB + ADR group, that decrease was about 30% compared to control levels (Table 5). There was no difference between GSH levels in CONT, PROB, LOV and TRO groups (Table 5).

GSSG levels were significantly increased in ADR, LOV + ADR and TRO + ADR groups (Table 5). Probucol treatment returned GSSG levels to control values in PROB + ADR group. Lovastatin treatment had no influence on GSSG levels, while trolox treatment

Table 5: Effects of probucol, lovastatin and trolox on adriamycin-induced changes in myocardial reduced (GSH) and oxidized (GSSG) glutathione.

Animal Group	GSH (μmol/g tissue)	GSSG (μmol/g tissue)
CONT	56.16 ± 6.65	10.75 ± 1.03
ADR	26.33 ± 2.48*	16.75 ± 0.55**
PROB	54.00 ± 5.55	10.00 ± 0.93
PROB + ADR	39.00 ± 1.94***	10.40 ± 0.74
LOV	50.00 ± 4.56	8.66 ± 0.80
LOV + ADR	28.20 ± 2.87*	16.40 ± 1.03**
TRO	58.16 ± 4.54	10.28 ± 1.80
TRO + ADR	24.80 ± 1.59*	14.00 ± 0.51**

Abbreviations are the same as in Table 2. Values are mean ± SEM from 6-8 experiments.

* Significantly different from CONT, PROB, LOV and TRO ($P<0.05$).
** Significantly different from CONT, PROB, PROB + ADR, LOV and TRO ($P<0.05$).
*** Significantly different from all other groups ($P<0.05$).

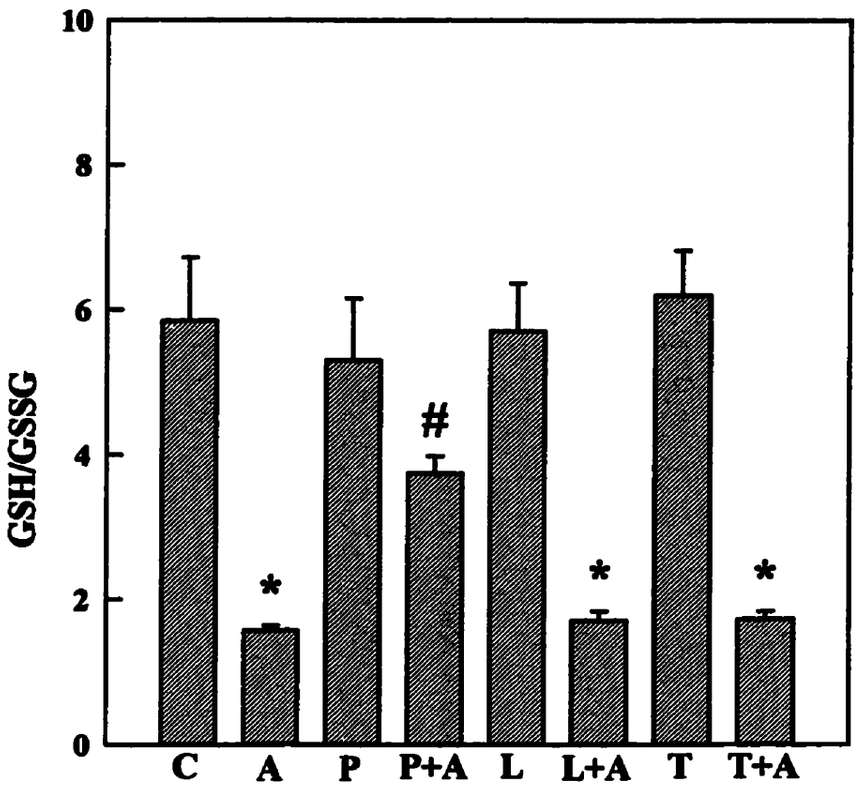

Figure 12: Effects of probucol (P), lovastatin (L) and trolox (T) on adriamycin (A)-induced changes in myocardial ratio of GSH/GSSG (redox state). Values are mean ± S.E.M. of 6-8 experiments. *Significantly different from the C, P, P+A, L and T groups (P<0.05). #Significantly different from the C, A, P, L, L+A, T and T+A groups (P<0.05).

showed trend towards decrease of GSSG, but the difference was not statistically significant from the ADR and LOV + ADR groups (Table 5).

Redox state was determined by calculating GSH/GSSG ratio (Figure 12). Hearts in ADR group had GSH/GSSG ratio decreased about 60% compared to controls (Figure 12). Lovastatin and trolox treatment did not have any significant influence on the adriamycin-induced decrease in GSH/GSSG ratio. This ratio in LOV + ADR and TRO + ADR groups stayed at depressed levels (Figure 12). However, significant improvement in GSH/GSSG ratio was found in PROB + ADR group. In spite of the improvements in the probucol effect on adriamycin-induced changes in the glutathione system, the GSH/GSSG ratio in PROB + ADR group was still significantly lower than in CONT, PROB, LOV and TRO groups by about 30% (Figure 12). Observed improvement in the redox state of PROB + ADR group hearts was mainly due to a normalization of the GSSG levels (Table 5).

F. LIPID PEROXIDATION

Lipid peroxidation was evaluated by the measurement of thiobarbituric acid reactive substances, and these data are shown in Figure 13. Thiobarbituric acid reactive substance levels were approximately 70% higher in the ADR group. The PROB, PROB + ADR, TRO and TRO + ADR groups did not show any change in the thiobarbituric acid reactive substances. In the LOV + ADR group, there was a significant increase in the thiobarbituric-acid-reactive substances as compared with all other groups except ADR (Figure 13).

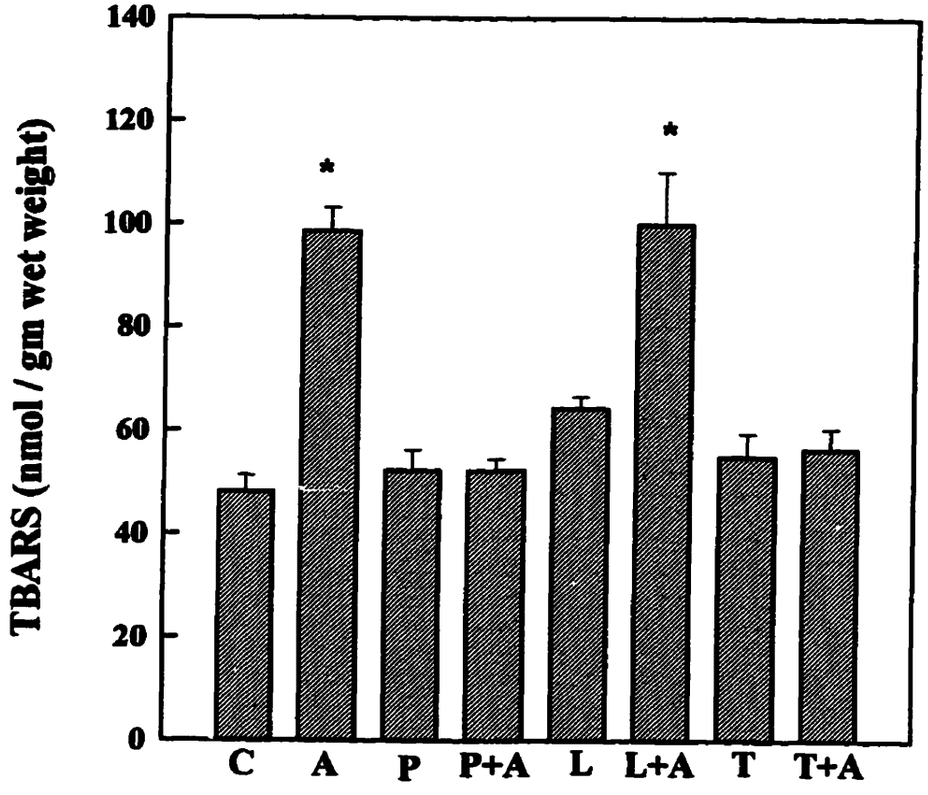

Figure 13: Effects of probucol (P), lovastatin (L) and trolox (T) on adriamycin (A)-induced changes in lipid peroxidation assessed by thiobarbituric-acid-reactive substances (TBARS). Values are mean ± S.E.M. of 6-8 experiments. *Significantly different from C, P, P+A, L, T and T+A groups ($P<0.05$).

G. LIPIDS

Plasma triglycerides, total cholesterol, high-density lipoproteins (HDL), and low-density lipoproteins (LDL) as well as cardiac triglycerides and total cholesterol levels were analyzed in all groups (Tables 6 and 7).

G. 1. Plasma Lipids

Adriamycin treatment caused significant increase in the plasma triglycerides, total cholesterol, HDL and LDL. Although there was a trend toward a decrease in all lipids in the PROB group, the change was significant only in the case of the plasma total cholesterol and HDL as compared to the CONT group. In PROB+ADR group, plasma triglycerides and total cholesterol were normalized towards CONT values. In these animals, plasma HDL were significantly lower compared to the CONT and ADR groups. In the PROB + ADR group, plasma LDL were decreased and were significantly less as compared to the ADR group but still higher than the CONT, PROB and LOV groups (Table 6).

Lovastatin did not influence plasma triglycerides, total cholesterol and LDL. However, plasma HDL were increased in the LOV group as compared to the CONT group. In LOV + ADR group, plasma triglycerides were significantly higher than in CONT and LOV groups but were comparable to the ADR group. Plasma total cholesterol, in this group, was significantly less than the ADR group but was higher than CONT group. Plasma HDL and LDL were higher than the CONT group but were lower than the ADR group.

Trolox treatment did not have any effect on any of measured plasma lipid parameters in the TRO group as compared to the CONT group (Table 6). Trolox treatment in the TRO

Table 6: Effects of probucol, lovastatin and trolox on adriamycin-induced changes in plasma lipids (mg/dL).

Animal Group	Triglycerides	Total Cholesterol	HDL	LDL
CONT	188.00 ± 16.79	88.91 ± 15.18	26.42 ± 5.70	34.68 ± 4.56
ADR	646.23 ± 86.56*	325.29 ± 35.8†	46.05 ± 5.84▲	134.58 ± 17.63†
PROB	146.12 ± 13.72	64.78 ± 4.99††	12.42 ± 0.66▲▲	27.88 ± 3.28
PROB + ADR	231.52 ± 24.52	114.50 ± 11.22	16.85 ± 2.10▲▲	70.48 ± 6.68***
LOV	204.97 ± 58.8	78.33 ± 5.43††	43.61 ± 5.32▲	31.72 ± 2.51
LOV + ADR	588.60 ± 42.76*	170.57 ± 18.32**	54.32 ± 8.12▲	72.17 ± 13.82***
TRO	123.65 ± 26.01	90.95 ± 2.32	34.30 ± 2.83	39.62 ± 6.44
TRO + ADR	822.99 ± 110.28†	353.46 ± 37.51†	52.23 ± 3.79▲	167.54 ± 28.11†

Data are mean ± SEM. of 6-8 animals. Abbreviations are the same as in Table 2.

* Significantly different from CONT, PROB, PROB + ADR, LOV and TRO (P<0.05).
† Significantly different from CONT, PROB, PROB + ADR, LOV, LOV + ADR and TRO (P<0.05).
†† Significantly different from ADR, LOV + ADR and TRO + ADR (P<0.05).
** Significantly different from CONT, ADR, PROB and LOV (P<0.05).
▲ Significantly different from CONT, PROB, PROB + ADR and TRO (P<0.05).
▲▲ Significantly different from CONT, ADR, LOV, LOV + ADR, TRO and TRO + ADR (P<0.05).
*** Significantly different from CONT, ADR, PROB, LOV, TRO and TRO + ADR (P<0.05).

Table 7: Effects of probucol, lovastatin and trolox treatment on adriamycin-induced changes in cardiac lipids (mg/g tissue).

Animal Group	Triglycerides	Total Cholesterol
CONT	27.52 ± 2.09	13.72 ± 1.38
ADR	45.50 ± 3.05*	20.30 ± 1.39*
PROB	24.79 ± 0.99	8.05 ± 0.67†
PROB + ADR	27.86 ± 2.19	11.20 ± 0.92
LOV	28.4 ± 1.59	7.98 ± 0.02†
LOV + ADR	23.9 ± 2.06	10.41 ± 0.01
TRO	30.30 ± 3.61	13.78 ± 1.46
TRO + ADR	40.22 ± 6.24*	25.12 ± 1.47*

Data are mean ± SEM. of 5-7 hearts. Abbreviations are the same as in Table 2.

* Significantly different from CONT, PROB, PROB + ADR, LOV, LOV + ADR and TRO ($P<0.05$).

† Significantly different from CONT, ADR, TRO and TRO + ADR ($P<0.05$).

+ ADR group did not have any effect on the adriamycin-induced changes in plasma lipids seen in ADR group (Table 6).

G. 2. Cardiac Lipids

Adriamycin treatment caused significant increase in the cardiac triglycerides and total cholesterol (Table 7). PROB group had significantly decreased total cholesterol compared to the CONT group, while in PROB + ADR group both cardiac triglycerides and total cholesterol were normalized to control levels. Lovastatin treatment decreased cardiac total cholesterol in the LOV group to the same levels as seen in PROB group. LOV + ADR had cardiac triglycerides and total cholesterol comparable to the control levels (Table 7). Trolox treatment did not have any effect on adriamycin-induced changes in measured cardiac lipids (Table 7).

H. SERUM FREE FATTY ACIDS (FFA), SERUM ALBUMIN AND FFA/ALBUMIN RATIO

Levels of serum FFA were measured in all groups, and data are shown in Table 8. Adriamycin treatment caused a significant increase in FFA levels in ADR group. Probucol, lovastatin and trolox by themselves did not have any effect on the FFA levels in the control animals. Both probucol and lovastatin returned FFA back to control levels in PROB + ADR and LOV + ADR groups, while trolox had no influence. FFA levels in TRO + ADR group were not significantly different from ADR group (Table 8).

Serum albumin levels were significantly and comparably decreased in ADR, PROB + ADR, LOV + ADR and TRO + ADR groups. FFA/albumin ratio was significantly increased in ADR and TRO + ADR groups compared to all other groups (Figure 14). Both

Table 8: **Effects of probucol, lovastatin and trolox on adriamycin-induced changes in serum free fatty acids and serum albumin.**

Animal Group	Serum Free Fatty Acids (mmol/L)	Albumin (mmol/L)
CONT	0.44 ± 0.03	0.55 ± 0.28
ADR	0.79 ± 0.03†	0.45 ± 0.02*
PROB	0.44 ± 0.02	0.53 ± 0.00
PROB + ADR	0.49 ± 0.13	0.45 ± 0.00*
LOV	0.43 ± 0.02	0.54 ± 0.01
LOV + ADR	0.51 ± 0.04	0.46 ± 0.02*
TRO	0.35 ± 0.02	0.49 ± 0.00
TRO + ADR	0.68 ± 0.02†	0.42 ± 0.00*

Data are mean ± SEM of 6-8 hearts. All abbreviations are the same as in Table 2.

* Significantly different from CONT, LOV, PROB and TRO ($P<0.05$).

† Significantly different from CONT, PROB, PROB + ADR, LOV, LOV + ADR and TRO ($P<0.05$).

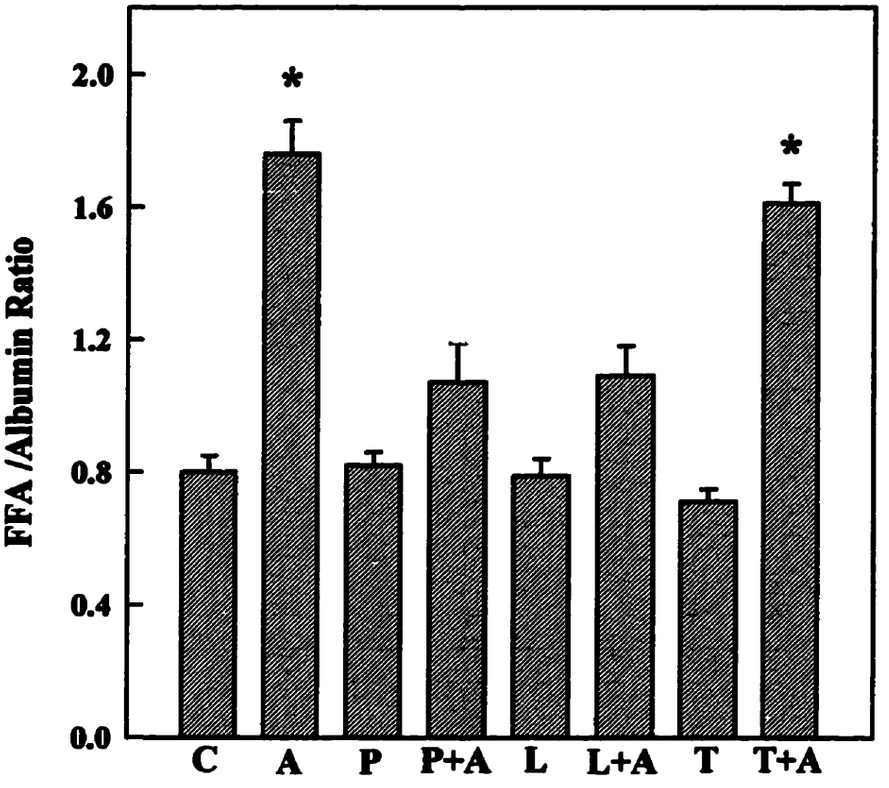

Figure 14: Effects of probucol (P), lovastatin (L) and trolox (T) on adriamycin (A)-induced changes in serum free fatty acid (FFA)/albumin ratio. Values are mean ± S.E.M. of 6-8 experiments. *Significantly different from the C, P, P+A, L, L+A and T groups ($P<0.05$).

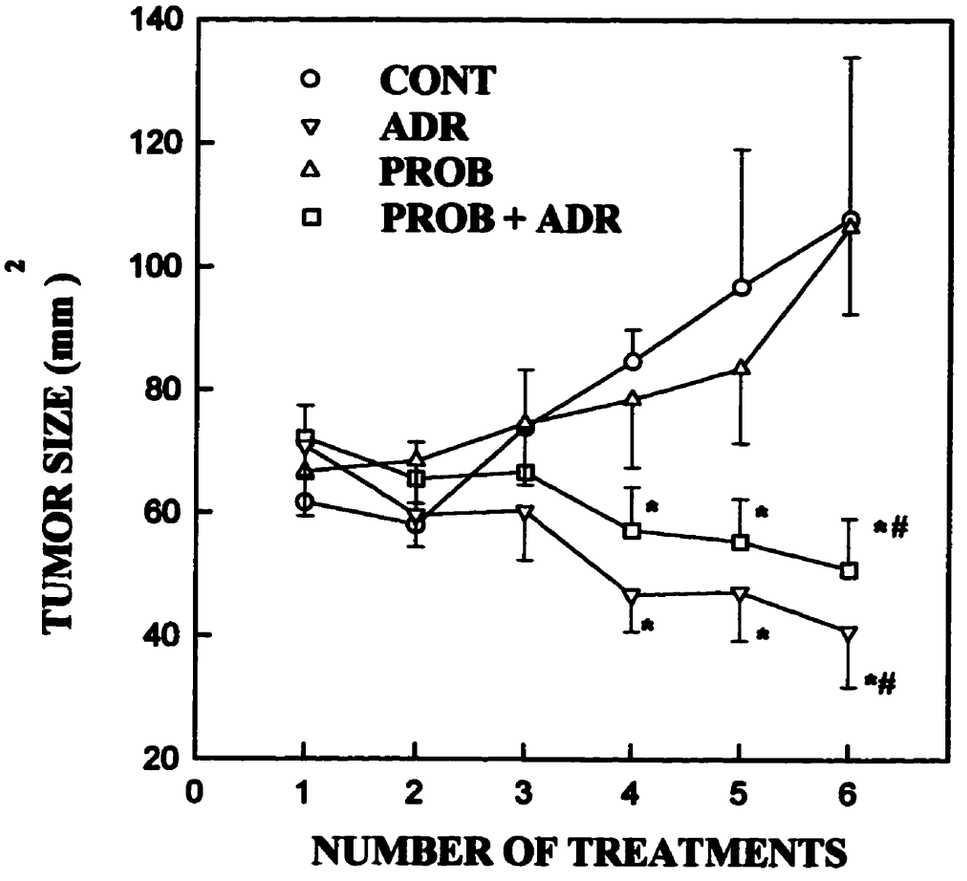

Figure 15: Effects of adriamycin (ADR), probucol (PROB) and probucol + adriamycin (PROB+ADR) on the regression in tumor size in lymphoma-bearing DBA/2 mice. Values are mean ± S.E.M. from 10-12 experiments. *Significantly different from CONT and PROB groups using ANOVA (P<0.05). #Significantly different from CONT and PROB groups using ANOVA and Bonferroni's post-hoc test (P<0.05).

probucol and lovastatin treatments normalized FFA/albumin ratio in PROB + ADR and LOV + ADR groups (Figure 14).

I. ANTITUMOR EFFECT

In order to assess the effects of probucol on the antitumor efficacy of adriamycin, subcutaneous tumor growth was studied in mice (Figure 13). The L5178Y-F9 lymphoma model in mice was chosen because it has been cloned directly form the L5178Y (Wolosin and Greenberg, 1979). Furthermore, it is one of the standard experimental tumors used to examine chemotherapeutic efficacy of different anticancer drugs, including adriamycin and its derivatives (Leonetti et al., 1993). As expected, a significant reduction in the tumor size was seen in the ADR group. Use of probucol as an adjunct therapy in the PROB + ADR group did not interfere with the antitumor property of adriamycin and the regression seen in the PROB + ADR group was comparable to that seen in the ADR group. Thus there was no significant difference in the tumor size between ADR and PROB + ADR groups.

V. DISCUSSION

Adriamycin (doxorubicin) is an excellent anti-tumor antibiotic, very effective against a large number of human malignancies. Potential usefulness of this drug is very limited by the adriamycin cardiotoxicity and development of a dose-related cardiomyopathic process terminating in severe heart failure, refractory to any therapeutic approach (Lefrak *et al.*, 1973; Minow *et al.*, 1975; Singal *et al.*, 1987; Praga *et al.*, 1979). The cardiotoxic effects can be manifested as acute, subacute and chronic. Adriamycin-induced acute cardiac decompensation in patients is rare because of the adequate cardiac reserve (Appelfeld and Egorin, 1984; Singal, 1985). Subacute effects like toxic myocarditis or pericarditis also occur infrequently (Buja *et al.*, 1973). Chronic changes develop in weeks to months or even years after the therapy has been completed (Steinhertz *et al.*, 1991). In order to minimize the incidence of adriamycin-induced cardiomyopathy, a cumulative dose of 550 mg/m^2 body surface area has been suggested as the upper limit. It is possible that some patients may require and may be able to withstand higher dosages for a successful treatment of malignant diseases. On the other hand, due to different risk factors, certain groups of patients may develop cardiomyopathy even if the total cumulative dose of adriamycin is well below 550 mg/m^2. Thus an indiscriminate use of an empirical dose of adriamycin may not be an ideal way of patient management.

Since the time adriamycin cardiotoxicity became evident, scientists have tried different approaches in order to prevent cardiotoxicity and make adriamycin safer for human use. One approach has been to synthesize adriamycin analogs that would have less or no cardiotoxicity. So far, a really better adriamycin analog has not been found (Weiss, 1992). Another line of

research has focussed on finding substances which when given in combination with adriamycin would mitigate cardiotoxicity (Pristos *et al.*, 1992; Von Hoff *et al.*, 1981; Herman and Ferrans, 1981; Siveski-Iliskovic *et al.*, 1994; 1995). First promising findings were obtained with the iron-chelator drug ICRF-187. Because of a serious bone marrow depression with consequent hematological abnormalities, this drug is not optimal for clinical use (Von Hoff *et al.*, 1981). Combination therapy with probucol established by our laboratory has offered complete protection against development of adriamycin cardiomyopathy in rats (Siveski-Iliskovic *et al.*, 1995). The chronic administration of adriamycin in the present study produced typical drug-induced cardiomyopathy. This study confirmed that a simultaneous treatment with probucol prevented adriamycin-induced cardiomyopathic changes as well as congestive heart failure, indicated by the improved cardiac function and a reduced mortality. Normalized cardiac structure due to probucol and adriamycin combination therapy has been shown before (Siveski-Iliskovic *et al.*, 1995). However, the next logical step in our research process was to understand the mechanisms by which probucol offered this protection. Data reported in the present study has significantly advanced our understanding of the adriamycin-cardiomyopathy as well as the basis of its prevention by probucol.

Probucol was introduced into clinical practice in the early 1970's (Zimetbaum *et al.*, 1990). For a number of years it was used as a lipid lowering drug. However, its use was discontinued because it was shown to lower not only triglycerides and LDL, but HDL as well (Zimetbaum *et al.*, 1990). An interesting observation made by Wissler and Vesselinovitch (1983) was that probucol and cholestiramin (another lipid lowering drug) both significantly

lowered cholesterol levels in monkeys, while only probucol caused regression of atherosclerotic plaques. That pointed out towards possible other mechanisms of action of probucol, besides lipid lowering. Its chemical structure has two phenolic rings, that way resembling vitamin E, another well known antioxidant which has only one phenolic group. Indeed, probucol was found to be a potent antioxidant (Mao *et al.*, 1991; Pryor et al, 1988). In addition, we reported that probucol acts as a promoter of endogenous antioxidants (Siveski-Iliskovic *et al.*, 1994; 1995).

Adriamycin due to uniqueness of its chemical structure, is very prone to generation of free radicals (Doroshow, 1983; Kalyanaraman *et al.*, 1980). There is impressive evidence in literature in support of the role of free radicals in the pathogenesis of adriamycin-induced cardiomyopathy (Singal *et al.*, 1987; Singal *et al.*, 1995). Hence, antioxidant properties of probucol as well as promotion of endogenous antioxidants might have had a role in the protection against adriamycin-induced cardiomyopathy observed by probucol (Siveski-Iliskovic *et al.*, 1995; Singal *et al.*,1995). However, contribution of lipid lowering properties of probucol in this protection could not be dismissed without solid research evidence.

Probucol has three characteristic properties in that it is: 1) a lipid-lowering drug; 2) an antioxidant and 3) a promoter of endogenous antioxidants. Any one or all of these in some combination may be involved in the protective effects of probucol. We compared lipid-lowering effects of probucol with that of lovastatin, and antioxidant effects with that of trolox to resolve this issue. Data presented in this thesis show that in fact all three properties of probucol are important and are required to make an ideal protective agent against adriamycin-cardiomyopathy.

The occurrence of adriamycin-induced congestive heart failure in patients (Lefrak *et al.*, 1973; Yeung *et al.*, 1991) and its reproduction in different animal models is now a well established phenomenon (Singal *et al.*, 1987). Characteristic hemodynamic as well as myocardial cell structural changes associated with adriamycin-induced cardiomyopathy have been documented in humans (Lefrak *et al.*, 1973; Buja *et al.*, 1973; Bristow *et al.*, 1978), and have also been reproduced in a variety of animal species, including rat (Chalcroft *et al.*, 1973; Singal, 1985). Some of the features noted in common in patients and different animal models include refractory heart failure as well as loss of myofibrills and cytoplasmic vacuolization in the cardiac myocytes (Lefrak *et al.*, 1973; Deally and Singal, 1990; Weinberg and Singal, 1987). As the rat seems to mimic many structural and functional features of adriamycin-cardiomyopathy in humans, its use as an animal model has been frequent as well as reliable (Singal *et al.*,1987; Siveski-Iliskovic *et al.*, 1994; Siveski-Iliskovic *et al.*, 1995; Deally and Singal, 1990; Olson and Capen , 1977; Mettler *et al.*, 1977; Zbinden *et al.*, 1978). The model used in our study is highly reproducible (Siveski-Iliskovic *et al.*, 1994b; Deally and Singal, 1990).

In the present study, presence of congestive heart failure in the adriamycin group was indicated by a significant decrease in the aortic systolic (ASP) and left ventricular peak systolic (LVPSP) pressures as well as a significant increase in the left ventricular end diastolic pressure (LVEDP). Similar findings in adriamycin-treated rats have been reported before (Weinberg and Singal, 1986; Deally and Singal, 1990). These data, as well as ascites and congested liver, confirmed the existence of the congestive heart failure subsequent to the failure of cardiac and/or extracardiac compensatory mechanisms. Animals entered a

refractory congestive heart failure spiral (Singal *et al.*, 1992). Increase in sympathetic tone in response to ADR stress, reported in early stages of failure, becomes inadequate in the later stages (Tong *et al.*, 1991). Ultrastructural damage is massive and refractoriness of the heart failure to therapeutical procedures is suggested to be due to the depressed contractile function at the cardiac myofiber level. Thus, depressed cardiac function and reduced responsiveness are suggested to be myogenic in origin (Deally and Singal, 1990).

A significant research has been devoted to understand the mechanism of these myocardial defects and thus of adriamycin cardiomyopathy. These efforts have resulted in several postulates including interaction of adriamycin with deoxyribonucleic acid and inhibition of nucleic acid as well as protein synthesis (Arena *et al.*, 1974; Buja *et al.*, 1973), release of vasoactive amines (Bristow *et al.*, 1980), changes in adrenergic mechanisms (Tong *et al.*, 1991), abnormalities in high energy phosphate metabolism (Gosalvez *et al.*, 1979), free radical formation and lipid peroxidation (Doroshow, 1983; Kalyanaraman *et al.*, 1980; Singal *et al.*, 1987; Singal *et al.*, 1985), alterations in sarcolemma and membrane bound enzymes (Singal and Pierce, 1986; Singal and Panagia, 1984), lysosomal alterations and imbalance of myocardial electrolytes (Singal *et al.*, 1985; Olson *et al.*, 1974) cardiac sympathetic denervation (Takano et al., 1995) as well as occurrence of calcium overload (Singal and Panagia, 1984). Pathogenesis of adriamycin cardiomyopathy appears to be multifactorial. However, free radical mediated injury seems to be a common denominator in the majority of the listed postulates (Singal *et al.*, 1987; 1995; Hasinoff *et al.*, 1988; Costa *et al.*, 1988; Doroshow , 1983; Kalyanaraman *et al.*, 1980). Due to the semiquinone ring in its structure, adriamycin increases oxygen radical activity and lipid peroxidation of polyunsaturated fatty

acids. That might explain adriamycin-induced membrane defects. In this regard, probucol's antioxidant properties might have contributed to the observed protective effect (Mao *et al.*, 1991; Pryor *et al.*, 1988).

Probucol is transported predominantly by LDL, VLDL and HDL in the plasma (Zimetbaum *et al.*, 1990). 1 g/day of probucol increases its level in the blood as well as adipose tissue (Taylor *et al.*, 1978). It is hard to draw any parallel between the dosage used by us in rats (12 x 10 mg/kg, i.p.) and therapeutic dosage (2 x 500 mg/day, 3-6 months). However, the protocol treatment used in our study was generally well tolerated by the rats. No absolute correlation between the plasma levels of probucol and the extent of cholesterol lowering effect was found (Polachek *et al.*, 1970).

Although probucol was used because of its LDL-cholesterol-lowering properties, it was soon noted that the drug lowered HDL-cholesterol to a greater extent than it lowered LDL-cholesterol (Zimetbaum *et al.*, 1990). Probucol treatment in patients with heterozygous familial hypercholesterolemia caused regression of xanthomas in the manner which did not correlate with the observed level of cholesterol reduction (Yamamoto *et al.*, 1986). Both probucol and cholestyramine, which is another cholesterol-lowering drug, sharply lowered serum cholesterol levels in the non-human primate experimental model while only probucol led to a regression of atherosclerotic lesions in these animals (Wissler *et al.*, 1983). These observations point to the possibility that the beneficial effects of probucol may be independent of its cholesterol-lowering effect. Probucol has no apparent structural similarity to other agents which actively lower cholesterol levels. It is a bis-phenol which resembles vitamin E, another well-known antioxidant, but with only one phenolic group. As it has two phenolic

groups in its molecular structure, probucol has been reported to be a strong antioxidant (Parthasarathy *et al.*, 1986; Pryor *et al.*, 1988; Mao *et al.*, 1991) and this realization has opened a whole new perspective for the use of this drug. Beneficial effect of probucol against restenosis after percutaneous transluminal coronary angioplasty has also been suggested to be due to its antioxidant property (Schneider *et al.*, 1993; Miyauchi *et al.*, 1993).

Development of adriamycin cardiomyopathy was accompanied by an increase in the free radical production (measured indirectly by increased TBARS levels) and decrease in endogenous antioxidants activity, mainly glutathione peroxidase (GSHPx). Cardiac GSPHx was also reported to be inhibited (Doroshow *et al.*, 1980) and depleted by adriamycin (Olson *et al.*, 1980). In our study, probucol prevented adriamycin-induced increase in TBARS and a decrease in GSHPx activity. Probucol by itself caused an increase in SOD and somewhat in GSHPx activities. Increase was even higher (88%) in PROB + ADR treated group. Hence, probucol improved "endogenous antioxidant reserve", which has been suggested to have beneficial effect on myocardial structure and function (Singal and Kirshenbaum, 1990). Another important observation was a small, statistically not significant, increase in the SOD activity in the ADR group. Adriamycin was reported to cause induction of SOD activity in lymphocytes as well as neutrophils (Niwa *et al.*, 1993). Higher increase in SOD activity in PROB + ADR group might be due to some synergistic effect between adriamycin and probucol. Catalase activity did not show any change in any group and its activity is known to be generally low in the heart tissue (Doroshow *et al.*, 1980).

Mechanisms for adriamycin-induced decrease in GSHPx and probucol-induced increase in antioxidants (GSHPx and SOD) are not clear. These results demonstrate that

probucol might be providing protection by acting as an antioxidant as well as promoter of endogenous antioxidants. In this regard, the LDL isolated from the plasma of patients treated with probucol was found to be resistant to oxidative modification (Parthasarathy *et al.*, 1986), supporting the increased antioxidant ability in our experimental animals. Probucol-induced oxidative inhibition of isolated LDL was found to be maximal after 2 weeks of the beginning of treatment and returned to baseline values 4-6 weeks after treatment was finished (Dujovne *et al.*, 1994). Commencement of adriamycin administration in our study might have coincided with optimal antioxidant protection achieved after two weeks of probucol pretreatment.

Different pathological situations are characterized by peroxidation of membrane lipids by different drugs (Plaa and Witsch, 1976). Consequences are changes in membrane microarchitecture and permeability as well as alterations in different membrane-bound enzyme activities (Kaul *et al.*, 1993). Adriamycin has a high affinity for cardiolipin (Nicolay *et al.*, 1985), hence mitochondria are particularly susceptible to adriamycin-induced peroxidation. We have shown previously that structural integrity of the myocytes, including that of mitochondria, was maintained by probucol (Siveski-Iliskovic *et al.*,1995).

In our study, adriamycin treatment decreased levels of reduced glutathione (GSH) and increased oxidized (GSSG) glutathione, resulting in decreased GSH/GSSG ratio. Different studies have described different changes in glutathione levels due to adriamycin administration (Boor, 1979; Julicher *et al.*,1986; Jackson *et al.*,1984). While acute studies described both increase (Jackson *et al.*, 1984) and decrease (Boor, 1979) in GSH levels, chronic administration of adriamycin mainly caused increase in cardiac glutathione levels (Jackson *et al.*,1 984). When discussing and comparing our glutathione data with that described in the

literature, one has to take into account the experimental model and stage of the cardiac dysfunction. It appears that glutathione changes due to adriamycin administration depend on the animal species used, dose of the drug used, length of the treatment and the post-treatment duration. Probucol treatment improved adriamycin decreased GSH/GSG ratio mainly through normalization of GSSG levels. This observation is in accord with previously described beneficial effect of probucol on glutathione levels in the renal cortex of rats with bilateral urethral obstruction (Modi *et al.*,1990).

The present study shows for the first time that lovastatin, a lipid-lowering drug without any known antioxidant property, also has a significant beneficial effect against adriamycin cardiomyopathy. However, this protection with lovastatin with respect to the hemodynamics, ascites and mortality was only partial and this may have to do with the lack of any coexisting antioxidant effect. In this regard, lovastatin unlike probucol had no effect on any of the endogenous antioxidant enzyme activities. Although three different doses (4, 8 and 12 mg/kg) of lovastatin were used by us in a pilot study, a maximum lipid-lowering effect was seen at 4 mg/kg of lovastatin, thus ruling out the possibility that a lack of complete protection with lovastatin was not due to an inadequacy of the dose used.

The adriamycin-induced hyperlipidemia seen in this study has also been reported by others (Kunitomo *et al.*, 1985; Joles *et al.*,1993; Washio *et al.*, 1994). In this regard, rats injected twice with a relatively low dose of adriamycin (2 mg/kg each injection) at a 20-day interval developed marked hyperlipidemia indicated by about five fold increase in total cholesterol and up to 9 time increase in plasma triglycerides (Washio *et al.*, 1994). An increase in total serum cholesterol, triglycerides and phospholipid levels was also seen in rats injected with a cumulative dose of

24 mg/kg of adriamycin and this increase was accentuated if rats were fed high-cholesterol diet (Kunitomo *et al.*, 1985). We also observed a 3 fold increase in plasma triglycerides, and about fold increase in total cholesterol and LDL in the ADR group. Adriamycin is reported to cause the development of chronic glomerulonephritis leading to progressive glomerulosclerosis associated with the nephrotic syndrome (Washio *et al.*, 1994). Typically, nephrotic syndrome is characterized by the presence of persistent proteinuria, hypoalbuminemia, hyperlipidemia and lipiduria (Joles *et al.*,1993; Washio *et al.*,1994). Thus it is likely that hyperlipidemia observed due to adriamycin treatment is basically the result of adriamycin-induced nephrotic syndrome (Kunitomo *et al.*, 1985; Washio *et al.*, 1994).

Probucol and lovastatin, lipid-lowering drugs used in this study, had overall beneficial effects on adriamycin-induced lipid changes in the plasma as well as the heart. Trolox treatment did not have any influence on adriamycin-induced plasma and heart lipid changes. Probucol decreased triglycerides to a much greater extent than lovastatin. Effects of the two drugs on total cholesterol and LDL levels were similar while HDL were decreased due to probucol and increased due to lovastatin treatment. Comparable effects of probucol and lovastatin on total cholesterol have been described in rats with bilateral urethral obstruction, while both drugs had very limited effect on triglyceride levels (Modi *et al.*, 1990). Probucol improved renal function in rats with bilateral urethral obstruction and lovastatin did not (Modi *et al.*, 1990). Patients with hyperlipidemia due to nephrotic syndrome, caused by a variety of renal pathologies, have benefited from probucol treatment through the significant lowering of serum total cholesterol, triglycerides, HDL and LDL levels (Iida *et al.*, 1987). However, probucol had no effect on urine and serum protein, serum albumin levels and renal function (Iida *et al.*, 1987). Since lipid-lowering with probucol as well as lovastatin had a modulatory effect on adriamycin-induced

cardiomyopathic changes, it is suggested that hyperlipidemia is deleterious for heart function and appears to contribute in the adriamycin-induced heart failure.

Concerns that discouraged the use of probucol as a lipid-lowering agent, i.e. its HDL lowering property (which was confirmed in our study as well) should not matter in its safe use in cancer patients. Decrease in HDL could be an issue only in a chronic use of probucol, while in the proposed protocol the usage would be for a limited time. Furthermore, the benefit of preventing the cardiomyopathy and congestive heart failure definitely outweighs consequences of temporary decrease in plasma HDL levels of cancer patients.

Adverse effects of high concentration of free fatty acids on cardiac contractility have been described before (Willebrands *et al.*, 1973). Perfusion of isolated rat hearts with solutions containing different free fatty acids/albumin ratios showed that not only high concentrations of free fatty acids have deleterious effects on cardiac contractility but the study also suggested that an increase in free fatty acids/albumin ratio was an important negative factor (Willebrands *et al.*,1973).

Adriamycin treatment increased serum FFA and decreased serum albumin levels, as well as increased FFA/albumin ratio. Both probucol and lovastatin treatment returned FFA/albumin ratio to the control levels, as a result of a decrease in serum FFA. Trolox had no effect on serum FFA levels, or FFA/albumin ratio. Reported decrease in carnitine levels in the heart due to adriamycin (Senekowitsch *et al.*,1985) may also promote the production of free fatty acids. In this regard, carnitine plays a central role in the transfer of long-chain fatty acids into the mitochondrial matrix where β-oxidation takes place (Bremer, 1983). Adriamycin-induced hyperlipidemia described in our study as well as by others (Kunitomo *et al.*, 1985; Washio *et al.*,

1994) not only increases free fatty acids concentration, but it is also accompanied by hypoalbuminemia (Joles *et al.*,1993; Skutelsky *et al.*,1995). Thus, adriamycin treatment increases free fatty acids/albumin ratio by affecting both components adversely. Modulation of the hyperlipidemia both by probucol and lovastatin improves free fatty acids/albumin ratio which might have a favourable effect on the cardiac function. Since lovastatin treatment in the present study did not have any effect on adriamycin-induced lipid peroxidation, as well as endogenous antioxidants and glutathione changes, it is proposed that lipid-lowering effect itself may offer partial protection against development of adriamycin-induced congestive heart failure. The latter was indicated by reduced mortality and improved hemodynamic function with lovastatin treatment (Iliskovic and Singal, 1997).

Contribution of antioxidant properties and promotion of endogenous antioxidants in the probucol protection was compared with that of trolox – an antioxidant with no known lipid-lowering properties. Trolox is a water soluble form of vitamin E and its chemical structure has a phenolic ring that way resembling probucol. However, one has to take into consideration that finding an antioxidant that would completely duplicate the antioxidant effects of probucol is almost impossible. Probucol is unique in combining lipid modulating properties with antioxidant properties as well as promotion of endogenous antioxidants, that have not been observed with any other antioxidants (Iliskovic and Singal, 1997).

Vitamin E has been used before in the prevention of adriamycin cardiotoxicity, and was found effective only against acute toxic effects (Myers *et al.*, 1977). In that study, a bolus injection of vitamin E was administered to mice 24 h before adriamycin treatment (Myers *et al.*, 1977). However, vitamin E was shown effective in preventing adriamycin

cardiotoxicity only up to 15 days after adriamycin administration, while no protection was observed after a longer post-treatment period (Mimnaugh *et al.*, 1979). We decided to try trolox, because its solubility is different than the solubility of vitamin E, as well as our chronic treatment over 4 weeks offered continuous presence of trolox in the system, in contrast to bolus administration of vitamin E, previously used (Myers *et al.*, 1977). Indeed, trolox offered significant protection against development of adriamycin cardiomyopathy, as manifested by decreased ascites amount, improved hemodynamic function and normalization of cardiac lipid peroxidation. However, LVEDP remained significantly above control as well as PROB + ADR levels, yet significantly lower than ADR group levels, indicating that heart was still affected to a certain level by adriamycin.

Trolox has been shown before to prevent cumene hydroperoxide-induced lipid peroxidation in cultured neonatal rat heart cells (Le *et al.*, 1992). We also found that trolox normalized lipid peroxidation as assessed by TBARS. Trolox reduced the area of infarction in a canine model of coronary artery occlusion, as well as it was more effective than superoxide dismutase or catalase in the protection of myocyte cell cultures from the free radical damage (Mickle *et al.*, 1988). Though trolox normalized adriamycin-induced increase in lipid peroxidation, it did not have any effect on adriamycin-induced decrease in GSHPx activity. Also, trolox treatment did not have any effect on adriamycin-induced decrease in GSH, increase in GSSG and decrease in GSH/GSSG ratio. It might be explained by the finding that trolox actually accelerates GSHPx reaction and its utilization, leading to lowering of reduced glutathione levels as well as reduced/oxidized glutathione ratio in myocytes exposed to free radicals (Le *et al.*, 1995). At the same time, the same group of authors have

reported a decrease in MDA formation in those same trolox exposed myocytes (Le *et al.*, 1995).

Another study compared effects of five different antioxidants, among them trolox and probucol, on copper-induced oxidation of human LDL (Kogl *et al.*, 1994). Probucol inhibited copper-induced LDL oxidation for over 24 hours while trolox offered protection for only 3 hours (Kogl *et al.*, 1994). As shown by us, probucol improved both plasma and cardiac lipid profile of adriamycin treated rats (Iliskovic and Singal, 1997) but trolox did not have any influence on these adriamycin-induced changes. Hence, one can exclude lipid lowering factor from the protection observed by trolox in this study, and assume that observed improvement in different adriamycin-induced changes is due to trolox antioxidant properties. Normalized myocardial lipid peroxidation is a strong indicator of attenuated free radical-induced injury. However, lack of lipid normalization as well as improvement in endogenous antioxidants might be responsible for signs of cardiomyopathy and heart failure in the TRO + ADR group.

For any practical application of probucol combination with adriamycin, however, it was important to examine whether probucol modified the antitumor properties of adriamycin. In the regard, another important finding in the present study is that in an established tumor model in syngeneic DBA/s mice, probucol had no effect on the antitumor activity of adriamycin. A comparable tumor regression seen in the ADR and the PROB + ADR groups further supports the potential usefulness of combination therapy. These findings also suggest that redox cycling of adriamycin may be unrelated to its antitumor property of the drug.

Transgenic mice, overexpressing different quantities of catalase, were subjected to adriamycin treatment with interesting findings (Kang *et al.*, 1996). One hundred-fold increase

in catalase offered protection against adriamycin cardiotoxicity, two hundred-fold increase offered no protection and five hundred-fold increase actually enhanced adriamycin toxicity (Kang *et al.*, 1996). This study, in which a cytosolic enzyme was increased, emphasized the significance of the appropriate dose, as well as the location of the antioxidant which is aimed at prevention of adriamycin cardiotoxicity. Probucol, with its unique combination of beneficial lipid lowering properties (Iliskovic and Singal, 1997), innate antioxidant activity (in a lipid phase), as well as promotion of endogenous antioxidants, i.e. improvement of cytosolic antioxidant defences (Siveski-Iliskovic *et al.*, 1994a, 1995), appears to have that balanced combination of different characteristics needed for complete neutralization of adriamycin-induced cardiotoxicity.

In conclusion, this study provides a strong evidence that probucol can prevent adriamycin-induced cardiomyopathy. It is proposed that a clinical trial to test the efficacy of this adjunct therapy be undertaken to realize the gains from our laboratory findings.

CONCLUSIONS

- ADR cardiomyopathy and congestive heart failure are associated with ascites formation, high mortality, depressed hemodynamic function, decrease in endogenous antioxidants, decrease in GSH/GSSG ratio, increase in lipid peroxidation, increase in myocardial and plasma lipids as well as serum FFA, decrease in serum albumin and decrease in FFA/albumin ratio.

- Probucol (lipid lowering drug and an antioxidant) normalized ADR-induced hemodynamic changes, prevented mortality, increased endogenous antioxidants, improved GSH/GSSG ratio, normalized lipid peroxidation, decreased myocardial and plasma lipids, decreased FFA and normalized FFA/albumin ratio. Thus offered complete protection against adriamycin-induced cardiomyopathic changes.

- Lovastatin (lipid lowering drug) decreased ascites formation and mortality, partially improved hemodynamic function and decreased myocardial and plasma lipids as well as serum FFA and normalized FFA/albumin ratio. It had no effect on antioxidants changes or lipid peroxidation. Thus offered only partial protection against adriamycin-induced cardiomyopathic changes.

- Trolox (an antioxidant) decreased amount of ascites and mortality rate and improved (although not normalized) hemodynamic function. Trolox decreased lipid

peroxidation to control levels without having any effect on ADR-induced changes in antioxidant reserve. It had no effect on lipid levels or FFA/albumin ratio. Thus offered only partial protection against adriamycin-induced cardiomyopathic changes.

- Administration of probucol in combination with adriamycin did not influence adriamycin antitumor action.

- Protection against ADR-induced cardiomyopathy achieved by probucol appears to be due to unique combination of its lipid-lowering properties, direct antioxidant activity and promotion of endogenous antioxidants.

- Clinical trials are needed to test the applicability of this combination therapy in patients.

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