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ORIGINAL ARTICLE

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New aspects in probucol cardioprotection against doxorubicin-induced cardiotoxicity

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Abstract *Purpose*: Doxorubicin (DOX) is a broad-spectrum anticancer drug with dose-dependent cardiotoxicity. Probucol has been reported to completely prevent DOX-induced cardiomyopathy. The aim of the present study was to determine the possible effect of probucol pretreatment on the pharmacokinetics of DOX and its role in cardioprotection as well as the possible contribution of the lipid-lowering effect of probucol on the disposition of DOX in cardiac tissue. *Methods*: Two groups of male albino rats were given either probucol (10 mg/kg, i.p.) or corn oil daily for 12 days followed by a single dose of DOX (15 mg/kg, i.p.). The concentration-time profile of DOX in plasma and its concentration in different tissues, and plasma and myocardial lipids were determined. *Results*: A rapid and significant increase in plasma DOX clearance was observed in rats pretreated with probucol. Probucol induced a significant increase in DOX concentration in both liver and kidney tissues and a significant decrease in DOX concentration in the spleen. However, heart and lung DOX concentrations were not affected. Also, probucol pretreatment resulted in a significant reduction in cardiotoxicity indices including peak serum creatine kinase (CK) concentration and the area under the CK concentration-time curve. Moreover, probucol pretreatment not only counteracted significantly the decrease in the ATP/ADP ratio induced by DOX, but also induced a significant increase as compared with the control group. In addition, probucol significantly reduced plasma total cholesterol and low-density lipoprotein, but it did not induce any significant changes in myocardial lipids. *Conclusions*: The present study demonstrated, for the first time, that probucol pretreatment alters the pharmacokinetics of DOX. Besides its antioxidant properties, the cardioprotective effect of probucol may be related to its enhancing action on the ATP/ADP ratio.

Keywords Probucol · Doxorubicin · Pharmacokinetics · Cardiotoxicity

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Introduction

Doxorubicin (DOX) is an anthracycline antibiotic with a wide spectrum of antitumor activity. Unfortunately, DOX therapy is associated with acute as well as cumulative dose-related cardiotoxicity [19]. Considerable research has focused on elucidating the mechanisms of DOX-induced cardiomyopathy as well as on finding ways to prevent the development of cardiotoxicity. A large body of evidence supports the role of oxygen free radicals in DOX-induced toxicity [11]. It has been proposed that DOX is converted to a semiquinone free radical by NADPH-dependent cytochrome P450 which could then lead to generation of superoxide and hydroxyl radicals, causing membrane lipid peroxidation, DNA strand breaks and enzyme inactivation [14, 17]. The role of the oxyradical hypothesis in DOX-induced cardiotoxicity has been confirmed by the reduction of cardiomyopathy using certain antioxidants [7, 33].

On the other hand, there is ongoing experimental and clinical research to improve the therapeutic index of DOX. Some trials have been directed towards reducing cardiotoxicity by using dose fractionation or long-term intravenous infusion [23]. Other attempts have been made to control the pharmacokinetics and disposition of DOX by using a variety of specific drug delivery systems [24] or alternatively by coadministration of other drugs

that modify DOX pharmacokinetics, such as tamoxifen [31] and phenobarbitone [1].

Probucol, an antioxidant as well as a lipid-lowering drug used clinically, has been reported to completely prevent DOX-induced cardiomyopathy [8, 28]. As DOX depresses myocardial antioxidants and probucol counters this effect, the observed protection has been suggested to be due to the enhancement of endogenous antioxidants, particularly glutathione peroxidase and superoxide dismutase [27, 28]. However, until now there are no data concerning the possible effect of probucol on the pharmacokinetic distribution of DOX and its role in cardioprotection. Accordingly, the purpose of this study was to examine the effect of probucol pretreatment on the pharmacokinetics and tissue disposition of DOX and its role in cardioprotection.

In addition, cholesterol is a known modulator of membrane function. Mason [10] reported that changes in myocardial membrane lipid composition (mainly cholesterol) substantially modulate the partitioning of lipophilic drugs such as calcium channel antagonists. Doxorubicin is a well-known lipophilic drug [35]. In this regard, Lliskovic and Singal [9] found that probucol treatment at a total cumulative dose of 120 mg/kg induces a significant decrease in cardiac total cholesterol. Accordingly, we investigated the possible contribution of the lipid-lowering effect of probucol on the disposition of DOX in cardiac tissue.

Materials and methods

Animals and drugs

The investigation was performed on 44 male albino rats weighing 160–200 g from our institute's own outbreed stock. The animals were housed in a conditioned atmosphere and kept on a standard diet and water ad libitum.

Doxorubicin (Adriablastine; Pharmacia & Upjohn, Milan, Italy) was dissolved in saline and injected i.p. as a single dose of 15 mg/kg. Probucol (Sigma, Deisenhofen, Germany) was dissolved in corn oil and administered i.p. at a dose of 10 mg/kg daily for 12 days (total cumulative dose of 120 mg/kg as in the study by Siveski-Iliskovic et al. [28]). The animal treatment protocol was approved by the Ethical and Animal Care Committee of the National Cancer Institute, Cairo University, before starting the experiments.

Experimental design

Pharmacokinetic study

For the pharmacokinetic study, 20 male albino rats were divided into two equal groups. One of the two groups was given probucol (10 mg/kg, i.p.) daily for 12 days while the other group was considered the control group and given corn oil (0.5 ml/150 g body weight, i.p.) daily. On the 13th day, the animals were anesthetized with chloralhydrate (300 mg/kg) [16] and each animal in both groups was given a single dose of DOX (15 mg/kg, i.p.). Blood samples were collected from the retro-orbital sinus of the eye into Eppendorf tubes containing EDTA, at 0, 10 and 30 min, and 1, 2, 4, 6 and 24 h after DOX injection. Plasma samples were immediately obtained by centrifugation at 3000 rpm for 10 min and stored at −45°C.

After withdrawal of the last blood sample, the animals were killed by cervical dislocation and selected tissues (heart, liver, kidney, spleen and lung) were dissected out immediately and washed with ice-cold saline. The tissues were homogenized (10%) in water using a Branson Sonifier (model 250; VWR Scientific, Danbury, Ct.). DOX concentrations in plasma and tissue homogenate were analyzed by the method of Formelli et al. [6] as modified by Rhaman et al. [20]. In brief, 0.2 ml silver nitrate (33% w/v) was added to 1 ml tissue homogenate. Plasma (0.25 ml) was diluted to 1 ml with distilled water, and added to 0.2 ml silver nitrate. The tubes were vortexed vigorously and 3 ml *n*-butyl alcohol saturated with water was added. Each tube was vortexed for 1 min and then centrifuged at 5000 rpm for 10 min. The organic layer was removed followed by further extraction of the residue with 2 ml *n*-butyl alcohol. The tubes were vortexed for 30 s and then centrifuged for 10 min again at 5000 rpm. The second organic layer was removed and pooled with the first. The butanol extract was read in a spectrophotofluorometer at 470 nm excitation and 585 nm emission. Fresh DOX samples were prepared in *n*-butyl alcohol each day for calculation of the concentration of DOX in the samples.

In addition, plasma triglycerides (TG), total cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) as well as myocardial TG and total cholesterol were determined enzymatically according to the methods of Richmond [21] (for total cholesterol, LDL and HDL) and Bucolo and David [3] (for TG).

Toxicodynamic studies

For the toxicodynamic studies, 24 male albino rats were divided into four equal groups. The rats of group 1 and 2 were given probucol (10 mg/kg, i.p.) daily for 12 days while the other two groups (3 and 4) were given corn oil (0.5 ml/150 g body weight, i.p.) daily. On the 13th day, the animals were anesthetized with chloralhydrate (300 mg/kg) and each rat in groups 1 and 3 was given a single dose of DOX (15 mg/kg, i.p.) while rats in groups 2 and 4 were given a single dose of saline (0.5 ml/150 g body weight, i.p.). Blood samples were collected from the retro-orbital sinus of the eye into Eppendorf tubes, before DOX injection (t = 0) and 1, 2, 4, 6 and 24 h after DOX injection. Blood samples were allowed to clot, and the serum was separated by centrifugation at 3000 rpm for 10 min and stored at −45°C until analysis (usually within 1 week of collection). Serum creatine kinase (CK) was analyzed according to the method of Dawson et al. [4].

In addition, myocardial ATP and ADP were assessed according to the method of Neri et al. [15]. In brief, after withdrawal of the last blood sample, the animals were killed by cervical dislocation and the hearts were dissected out immediately, washed with ice-cold saline, frozen in liquid nitrogen and homogenized in 6% perchloric acid using a Branson Sonifier (model 250, VWR Scientific). The clear supernatant was then neutralized with potassium hydroxide and used for determination of ATP and ADP by HPLC (model 322; Kontron, Milan, Italy) using a C18 hypersil column and UV detector at 254 nm.

Statistical analysis

Pharmacokinetic parameters were calculated according to the general equation for a two-compartment open model [22]:

$$C_t = Ae^{-\alpha t} + Be^{-\beta t}$$

where C_t is the plasma concentration at time t, A and B are the coefficients of the exponential terms of the concentration in plasma; and α and β are the hybrid rate constants of disposition. Both hybrid rate constants (α and β) and the initial and terminal half-life were determined by least squares linear regression analysis of the log-transformed data (method of residuals).

Clearance was calculated as dose/AUC. Area under plasma DOX concentration-time curve was calculated using the trapezoidal rule from time 0 to ∞ ($AUC_{0-\infty}$). Similarly, area under the

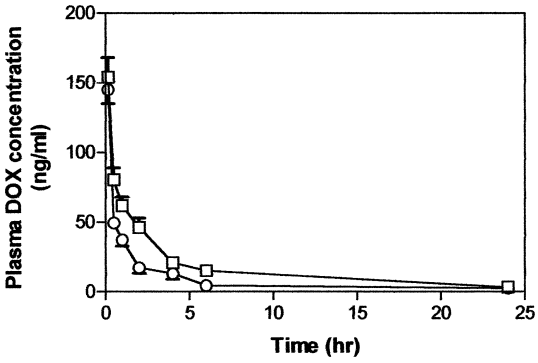

Fig. 1 Comparison of the plasma concentration-time profile of a single dose of DOX (15 mg/kg, i.p.) in rats pretreated with corn oil (□) or probucol (○)

serum CK concentration-time profile was calculated using the trapezoidal rule from time 0 to 24 h (AUC_{CK0-24}) as described by Vora and Boroujerdi [32].

Data are presented as means ± SEM. Individual groups were compared using the two-tailed Student's *t*-test as appropriate. Multiple group comparisons were carried out using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test for post-hoc analysis. Statistical significance was accepted at a level of $P < 0.05$. The data were analyzed using the program SPSS (version 8).

Results

Pharmacokinetic studies

Plasma data

The plasma concentrations of DOX after corn oil or probucol pretreatment were analyzed for each rat (Fig. 1). There was a rapid and significant decline in plasma DOX concentrations (particularly at 0.5, 1, 2 and 6 h after DOX injection) in animals pretreated with probucol as compared to those pretreated with corn oil. Probucol pretreatment also induced a significant alteration in DOX pharmacokinetics, including a significant increase in the second hybrid rate constant (β), the overall elimination rate constant (K_{13}) and the total body clearance (CL), concomitantly with a significant decrease in terminal plasma half-life ($t_{1/2\beta}$) and area under the curve (AUC). The pharmacokinetic parameters for the two groups are presented in Table 1.

Tissue data

DOX disposition in selected tissues of rats pretreated with corn oil or probucol is presented in Table 2. Probucol induced a significant increase in DOX concentration in both liver (345%) and kidney (129%) tissues as compared to those pretreated with corn oil. In contrast, probucol induced a significant decrease in DOX concentration in the spleen (about 3.5-fold less than the control values). However, heart and lung DOX concentrations showed no significant changes.

Plasma and cardiac lipids

Plasma TG, total cholesterol, HDL and LDL were analyzed in blood samples withdrawn before DOX injection. In animals treated with probucol, although there was a trend toward a decrease in all plasma lipids, only the changes in plasma total cholesterol and LDL were significant as compared to the values in animals pretreated with corn oil (Table 3). On the other hand, probucol treatment did not induce any significant change in cardiac TG or total cholesterol (Table 3).

Toxicodynamic studies

Serum creatine kinase

Preliminary studies indicated that chloralhydrate anesthesia did not alter the basal CK levels. Treatment of rats with either probucol or corn oil did not alter the basal levels of CK. On the other hand, a single injection of DOX (15 mg/kg) induced a continuous increase in CK levels, which reached a maximum level 6 h after DOX injection. In addition, probucol pretreatment (10 mg/kg, for 12 days) lowered both the maximum serum concentration of CK by 35% and its area under the curve (AUC_{CK0-24}) by 29% as compared with the corn oil pretreatment (Table 4; Fig. 2).

Table 1 Pharmacokinetic parameters and constants of DOX following a single dose of 15 mg/kg i.p. in rats pretreated with corn oil (control group) or probucol. The values presented are means ± SEM

Parameter/constant	Control group	Probucol group
Coefficient of first exponential, A (μg/l)	190.00 ± 37.28	147.20 ± 15.28
Coefficient of second exponential, B (μg/l)	26.29 ± 2.45	11.72 ± 1.68*
First hybrid rate constant, α (h^{-1})	2.61 ± 0.40	2.09 ± 0.24
Second hybrid rate constant, β (h^{-1})	0.19 ± 0.01	0.44 ± .04*
Initial half-life, $t_{1/2\alpha}$ (h)	0.43 ± 0.12	0.35 ± 0.04
Terminal half-life, $t_{1/2\beta}$ (h)	3.73 ± 0.16	1.61 ± 0.12*
Overall elimination rate constant, K_{13} (h^{-1})	0.97 ± 0.19	1.60 ± 0.16*
Total body clearance, CL (l/h/kg)	36.22 ± 1.70	68.97 ± 3.10*
Area under plasma concentration-time curve, AUC ($\mu g \cdot h \cdot l^{-1}$)	417.90 ± 20.47	219.15 ± 9.29*

*$P \leq 0.05$ vs control group, independent *t*-test

Table 2 Disposition of DOX following a single dose of 15 mg/kg i.p. in selected tissues of rats pretreated with corn oil (control group) or probucol (ten animals per group, the animals were killed 24 h after DOX injection). The values presented are means ± SEM

Tissue	DOX concentration (μg/g wet tissue)	
	Control group	Probucol group
Heart	14.24 ± 1.40	21.10 ± 2.78
Liver	14.76 ± 0.81	51.12 ± 4.75*
Spleen	23.83 ± 1.47	6.45 ± 0.54*
Kidney	14.67 ± 1.03	19.14 ± 1.16*
Lung	10.26 ± 1.45	12.61 ± 2.03

*$P \leq 0.05$ vs control group, independent *t*-test

Cardiac nucleotides

DOX-induced cardiac metabolic damage was evaluated in terms of changes in the myocardial ATP/ADP ratio (Fig. 3). A significant decrease in ATP/ADP ratio was seen 24 h after a single injection of DOX (15 mg/kg). On the other hand, treatment of animals with probucol (10 mg/kg) daily for 12 days induced a highly significant increase in ATP/ADP ratio. The increase reached more than seven-fold the values in corn oil-pretreated animals. Moreover, probucol pretreatment not only counteracted significantly the decrease in ATP/ADP ratio induced by DOX, but also induced a significant increase as compared with corn oil-pretreated animals.

Discussion

DOX is one of the most commonly used antineoplastic agents in cancer therapy. However, its clinical usefulness is seriously limited by the development of cardiomyopathy and congestive heart failure [9]. In previous studies, administration of probucol has been shown to result in complete protection against DOX-induced cardiotoxicity. It has been proposed that probucol cardioprotection may be related to its antioxidant properties and enhancement of endogenous antioxidant enzyme activity [8, 28] as well as its lipid-lowering effect [9].

In the present study, we investigated the possible effect of probucol pretreatment (at a total cumulative dose of 120 mg/kg) on the pharmacokinetics and tissue disposition of a single dose DOX (15 mg/kg) and its role in cardioprotection. In addition, we investigated the possible role of the lipid-lowering effect of probucol on the disposition of DOX in cardiac tissue.

The myocardial damage resulting from DOX administration was investigated by measuring serum CK and cardiac nucleotide levels. It is well known that the magnitude of CK elevation in blood after myocardial injury reflects the extent of damage in the musculature [18]. In the present study, serum CK levels were determined at different time intervals and the area under the CK serum level-time curve and its maximum peak concentration were chosen as toxicodynamic parameters in accordance with the study by Vora and Boroujerdi [32]. It was found that a single injection of DOX induced a continuous increase in CK level, which reached a maximum level 6 h after DOX injection. On the other hand, probucol pretreatment significantly protected the rats from the cardiotoxic effect of DOX as indicated by a significant decrease in peak CK levels (35%) and AUC_{CK} (29%).

Regarding myocardial nucleotide levels, DOX decreased significantly the intracellular ATP/ADP ratio and probucol pretreatment not only counteracted the decrease in ATP/ADP ratio, but also induced a significant increase as compared with the control group. One theory that may explain the cardiotoxic mechanism of DOX depends on mitochondrial dysfunction and ATP depletion induced by DOX [13]. Thus, the enhancing action of probucol on myocardial ATP/ADP ratio may be considered a new mechanism that explains the cardioprotective effect of probucol against DOX-induced cardiotoxicity. It is important to note that Sia and coworkers in two recent studies [25, 26] found that, in chronic congestive heart failure, probucol exerts multiple beneficial pharmacological effects. It attenuates the progression of left ventricular dysfunction and remodeling (dilatation) and improves post-myocardial infarction survival. The enhancing effect of probucol on myocardial nucleotides (as shown in our study) may explain its beneficial effect in congestive heart failure in the previous study.

In an attempt to determine the possible effects of probucol pretreatment on DOX pharmacokinetics, plasma DOX concentration-time profiles and its tissue disposition were analyzed. It was found that plasma pharmacokinetic parameters and constants were greatly altered in animals pretreated with probucol, in particular β, $t_{1/2\beta}$, K_{13} and AUC. Also, plasma clearance of DOX in animals pretreated with probucol showed a

Table 3 Effect of probucol treatment (10 mg/kg, i.p., daily for 12 days) on plasma and cardiac lipids. Plasma lipids were analyzed in blood samples withdrawn before DOX injection, and cardiac lipids were analyzed 24 h after DOX injection. The values presented are means ± SEM

Group ($n = 10$)	Plasma lipids (mg/dl)				Cardiac lipids (mg/g wet tissue)	
	Triglycerides	Total cholesterol	High-density lipoprotein	Low-density lipoprotein	Triglycerides	Total cholesterol
Control	193.44 ± 11.79	120.41 ± 10.36	31.57 ± 2.10	41.15 ± 3.74	22.70 ± 2.22	14.60 ± 1.38
Probucol	155.92 ± 14.86	85.36 ± 3.48*	29.36 ± 2.82	26.21 ± 2.45*	22.02 ± 2.01	11.22 ± 1.10

*$P \leq 0.05$ vs control group, independent *t*-test

Table 4 Cardiotoxicity indices following a single dose of DOX (15 mg/kg, i.p.) in rats pretreated with either corn oil (control group) or probucol. The values presented are means ± SEM (*CK* creatine kinase, AUC_{CK} area under serum CK concentration-time curve)

Cardiotoxicity index	Control group	Probucol group
Peak CK concentration (U/l)	648.60 ± 77.57	418.8 ± 20.64*
AUC_{CK} ($U \cdot h \cdot l^{-1}$)	11.69 ± 0.94	8.30 ± 0.65*

*$P \leq 0.05$ vs control group, independent *t*-test

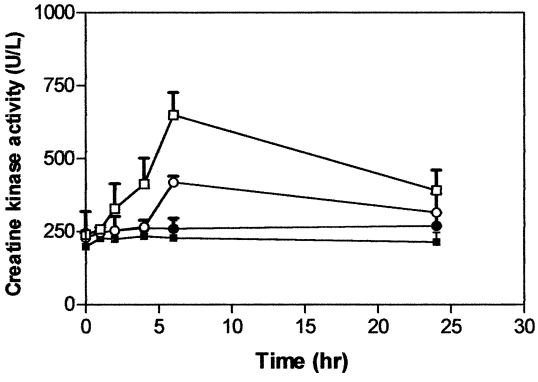

Fig. 2 Profile of serum CK (□ DOX treatment only, ○ probucol pretreatment + DOX, ● probucol pretreatment only, ■ control treatment)

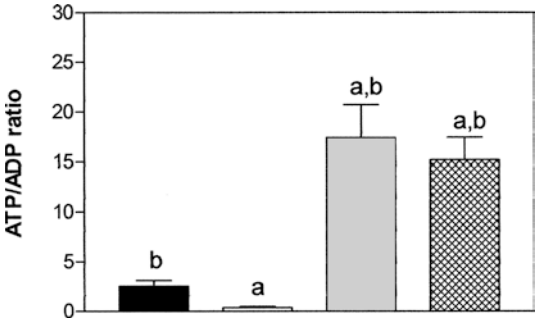

Fig. 3 ATP/ADP ratios following treatment of rats with corn oil (control group, *black bar*), DOX (*unshaded bar*), probucol (*gray bar*), or probucol + DOX (*hatched bar*). [a]Significant difference vs control group; [b]Significant difference vs DOX group

significant increase over that in control animals. In addition, the levels of DOX in tissues, particularly the liver and kidney, were significantly higher, while in the spleen the levels were significantly decreased. These findings need further study to explore the possible effect of probucol on the metabolism and excretion of DOX.

At least two possibilities can be suggested. First, DOX is metabolized mainly into doxorubicinol (the cardiotoxic metabolite) by cytoplasmic NADPH-dependent aldoketoreductase and eliminated predominantly in the bile and moderately in the urine [5]. So, if probucol can inhibit the metabolic transformation of DOX into doxorubicinol this will lead to an increase in DOX levels in liver and heart tissues (the sites of aldoketoreductase activity) and a decrease in the cardiotoxicity. It is important to note that in the probucol-treated animals, there was a small, but statistically non-significant, increase in cardiac DOX levels. In this regard, Behnia and Boroujerdi [1] found that the inhibition of aldoketoreductase may provide a useful approach to improving the safety of DOX by reducing its alcohol metabolite. A further study is therefore necessary to explore the effect of probucol on the metabolism of DOX into doxorubicinol.

Second, because of the two phenolic groups in its molecular structure, probucol is a strong antioxidant [2]. Thus, probucol may increase the excretion rate of DOX (a nephrotoxic drug [30]) by improving kidney function through its antioxidant properties. In this regard it has been reported that probucol improves renal function in rats with bilateral ureteral obstruction. In that study, the role of the antioxidant properties of probucol in the protection of kidney function was emphasized [12]. Therefore, the rapid and significant increase in plasma DOX clearance may be related to an enhancement in the excretion rate of DOX.

In general, there is some evidence that cardiotoxicity is primarily dependent on the peak plasma concentration resulting in high "oxidative stress". In contrast, the antitumor efficacy may be a function of the total exposure, for which the area under the time-concentration curve in plasma (AUC) is the pharmacokinetic correlate [29]. In this regard, in the present study probucol pretreatment induced a significant decrease in area under plasma DOX concentration-time curve (about 50% reduction), that may reflect an interference with DOX antitumor activity. However, in an established tumor model in syngeneic DBA/2 mice, probucol has been proved to have no effect on the antitumor activity of DOX [28]. Nevertheless, further investigation of the effects of probucol on the antitumor properties of DOX is necessary.

Regarding the effect of probucol on plasma and myocardial lipids, probucol treatment induced a significant decrease only in plasma total cholesterol and LDL. On the other hand, probucol did not induce any significant change in cardiac TG or total cholesterol. Lliskovic and Singal [9] have reported the hypolipidemic effect of probucol as seen in our study. However, they found that probucol (at total cumulative dose 120 mg/kg) induces a significant decrease in plasma total cholesterol and HDL (not LDL) as well as myocardial total cholesterol. This difference in the plasma lipid profile is not great because probucol has both LDL- and HDL-lowering effects [34]. Since, in the present study, probucol did not induce a significant change in myocardial total cholesterol nor DOX levels, the hypolipidemic effect of probucol does not appear to play a role in the disposition of DOX in myocardial tissue.

In conclusion, the present study demonstrated that probucol pretreatment alters DOX pharmacokinetics.

Besides its antioxidant properties, the cardioprotective effect of probucol may be related to its enhancing action on ATP/ADP ratio. However, further studies are needed to elucidate the mechanisms by which probucol influences DOX pharmacokinetics and the possible interfering effect of probucol on the antitumor activity of DOX.

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