Original Article

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The Hepatoprotective Role of Thiol Reductants against Mitoxantrone-Induced Liver Injury

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Abstract

Mitoxantrone is an anthracycline antibiotic highly effective against various human cancers. Hepatotoxicity is associated with mitoxantrone administration. On the other hand, there is no effective therapeutic option against chemotherapy-induced liver injury. The current investigation was designed to evaluate the effect of thiol reductants on mitoxantrone-induced liver injury in two experimental models. As an *ex vivo* model, the isolated rat liver was exposed to increasing concentrations of mitoxantrone (100, 250, 750, and 1000 µM) alone or in combination with thiol-reductants (Dithiothreitol; DTT, and N-acetyl cysteine; NAC). In addition, rats (*in vivo*) received mitoxantrone (2.5 mg/kg, i.p, at days 1, 10, and 20), NAC (100 and 300 mg/kg/day, i.p, for 20 consecutive days), and DTT (15 and 30 mg/kg/day, i.p, for 20 consecutive days), then liver and serum pathological changes were monitored. Mitoxantrone-induced liver injury was evident in both *ex vivo* and *in vivo* experiments as assessed by pathological changes in biomarkers of liver injury, along with tissue histopathological changes. Furthermore, an increase in liver tissue markers of oxidative stress was detected in the mitoxantrone-treated group. It was found that thiol reductants significantly mitigated mitoxantrone hepatotoxicity. The data indicate that thiol reductants might serve as hepatoprotective agents against chemotherapy-induced liver injury.

Keywords: Antineoplastic agents, Chemotherapy, Drug-Induced Liver Injury (DILI), Glutathione, Hepatotoxicity

1. Introduction

Many xenobiotics, including several pharmaceuticals, might affect liver function due to the central role of this organ in metabolizing foreign compounds (1). Antineoplastic agents are among the most cytotoxic drugs and chemotherapy-induced hepatotoxicity is a major clinical challenge (2). Hence, finding new therapeutic strategies against this complication might have clinical value.

Mitoxantrone is an anthracycline antineoplastic agent used against different types of tumor cells in human (Figure 1). Mild to moderate transient elevations of serum transaminases without clinical toxicity has been often occurred in cancer chemotherapy regimens (2-4). On the

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other hand, severe and potentially fatal hepatotoxicity might occur (5-8). Several cases of mitoxantrone-induced liver injury have been reported (3, 4, 9, 10).

Mitoxantrone structurally resembles doxorubicin (Figure 1). Doxorubicin is a well-known oxidative stress provoking agent and a mitochondrial toxin, which induces cardiotoxicity, hepatotoxicity, and lung injury (11-14). It has been well established that oxidative stress plays a pivotal role in chemotherapy-induced toxicity (12, 15). Oxidative stress plays a major role in the pathogenesis of drug-induced liver injury (16, 17). Drugs and toxins that induce oxidative stress are able to affect a vast range of cellular targets and finally causing organ injury (16-18). Oxidative stress also seems to play a central role in the pathogenesis of mitoxantrone-induced cytotoxicity (19). Hence, antioxidants and thiol reducing agents might provide protection against this complication.

The current study aimed to evaluate the effects of thiol reducing agents (N-acetyl cysteine and Dithiothreitol) on mitoxantrone-induced liver injury in two different experimental models.

2. Materials and methods

2.1. Chemicals

N-acetyl cysteine (NAC), and Dithiothreitol were obtained from Acros (New Jersey, USA). Bovine serum albumin fraction IV, Trichloroacetic acid (TCA), and Ethylenediaminetetraacetic acid (EDTA) were obtained from Merck (Darmstadt, Germany). Kits for evaluating biomarkers of liver injury were purchased from Pars Azmun® (Tehran, Iran). Thiobarbituric acid (TBA) was obtained from SERVA (Heidelberg, New York). All salts used for preparing buffer solutions were of analytical grade and obtained from Merck (Darmstadt, Germany).

2.2. Animals

Male Sprague-Dawley rats (200-300 g) were purchased from Laboratory Animal Breeding Center, Shiraz University of Medical Sciences and allowed a free access to food and tap water. The animals were handled and used according to the animal handling protocol approved by a local ethics committee of Faculty of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran (#95-01-36-13606).

2.3. Ex vivo experimental setup

Animals were anesthetized (thiopental, 70 mg/kg, i.p.), and the liver was cannulated and perfused via portal vein with a hemoglobin- and albumin-free Krebs Henseleit buffer (pH=7.4, 37 °C) gassed with carbogen (95% O_2, 5% CO_2) (20-22). The perfusate was pumped through the liver with a peristaltic pump (Heidolph, Germany) at a constant flow rate of 3 mL/min/g liver weight, in a re-circulating mode. The perfusate buffer volume was 200 mL in all experiments (23, 24).

The isolated rat liver was exposed to increasing concentrations of mitoxantrone alone or in combination with the investigated hepatoprotective agents for 180 min (22). Hepatic injury was determined at scheduled time intervals during the experiment (every 1 hr) to evaluate the effects of the various concentration of mitoxantrone on the liver. The injurious concentration of the drug is reported as a concentration value, which leads to a significant rise in all assessed biomarkers of liver injury 120 min after organ perfusion (Table 1) (23-25). Samples were taken from liver perfusate at different times and assessed for biomarkers of liver injury. At the end of each experiment (180 min), liver samples were applied for lipid peroxidation, glutathione content, and

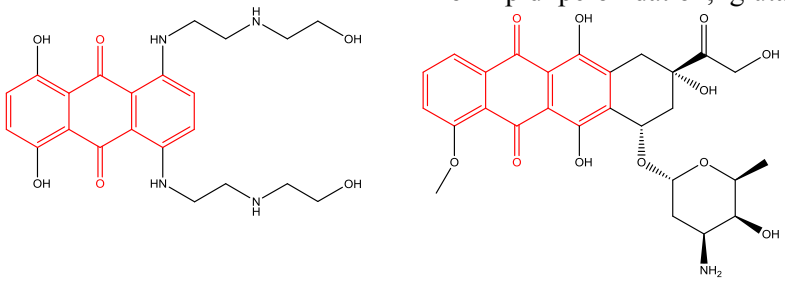

Figure 1. Resemblance between the chemical structure of mitoxantrone and doxorubicin.

Table 1. Concentration-response of mitoxantrone-induced liver injury in the isolated perfused rat liver.

Treatment	Perfusate LDH (U/l)	Perfusate ALT (U/l)	Perfusate AST (U/l)
Control (Only KBH buffer)	14±3	4±1	80±26
+Mitoxantrone 100 µM	290±14*	6±2	69±14
+Mitoxantrone 250 µM	446±23*	13±4	170±25*
+Mitoxantrone 500 µM	520±39*	16±4	130±32*
+Mitoxantrone 750 µM	752±109*	30±7	125±42*
+Mitoxantrone 1000 µM	3934±221*	190±14*	570±45*

Data are given as Mean±SD (n=5), which were assessed 120 min after liver perfusion. KBH: Krebs Henseleit. *Indicates significantly higher as compared with control (Only KBH buffer) group (P<0.05).

histopathological tests.

2.4. In vivo experiments

Mitoxantrone (2.5 mg/kg, i.p) was administered at days 0, 10, and 20. The animals were sacrificed on day 22 to evaluate drug-induced liver injury (26). The treatments were as follow:

1) Control (Vehicle-treated);

2) Mitoxantrone;

3) Mitoxantrone+DTT (15 mg/kg/day, i.p, for 20 consecutive days);

4) Mitoxantrone+DTT (30 mg/kg/day, i.p, for 20 consecutive days);

5) Mitoxantrone+NAC (150 mg/kg/day, i.p, for 20 consecutive days);

6) Mitoxantrone+NAC (300 mg/kg/day, i.p, , for 20 consecutive days).

2.5. Serum and liver perfusate biochemistry

Mindray Random Access BS-200® auto analyzer (Mindray chemistry analyzers for low-volume laboratories, Guangzhou, China), and Pars Azmun® standard kits (Tehran, Iran) were used to measure alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) activities in liver perfusate at different time points (22, 27).

2.6. Lipid peroxidation in the liver tissue

The level of lipid peroxidation was assessed by measuring thiobarbituric acid reactive substances (TBARS) in the liver tissue (24, 28). Briefly, the reaction mixture was consisted of trichloroacetic acid (15%, w/v), thiobarbituric acid (0.375%, w/v), HCl (0.25 N), sodium dodecyl sulfate (SDS; 0.1 % w:v), and 0.2 ml of tissue homogenate (10 % w:v). Samples were mixed well and heated in boiling water (100 °C) for 45 min (29). When the samples were cooled (4 ºC), 2 ml of n-butanol was added and vigorously mixed. Samples were centrifuged (10,000 g, 5 min) and the absorbance of the n butanol phase was read at 532 nm using an Ultrospec 2000® UV spectrophotometer (30, 31).

2.7. Hepatic glutathione content

The liver glutathione (GSH) level was assessed by determining non-protein sulphydryl contents with the Ellman reagent (32, 33). Briefly, samples of the liver (200 mg) were homogenized in ice-cooled EDTA (20 mM; 4 ºC). Five ml of liver homogenate was mixed with 4 ml of distilled water and 1 ml of trichloroacetic acid (50% w/v). The mixture was centrifuged (10,000 g, 4 °C, for 20 min). Two ml of the supernatant was mixed with 4 ml of Tris-HCl buffer (pH=8.9). Then, 100 µl of DTNB (0.01 M in methanol) was added (24, 28, 34). The absorbance of the developed color was measured at 412 nm using an Ultrospec 2000® UV spectrophotometer (Pharmacia Biotech, Uppsala, Sweden) (35).

2.8. Liver histopathology

For histopathological assessments, samples of liver were fixed in the buffered formalin solution (0.4% sodium phosphate monobasic, NaH_2PO_4, 0.64% sodium phosphate dibasic, Na_2HPO_4, and 10% formaldehyde in double distilled water). Paraffin-embedded sections of tissue (5 µm) were prepared and stained with hematoxylin and eosin (H&E) before light microscope view-

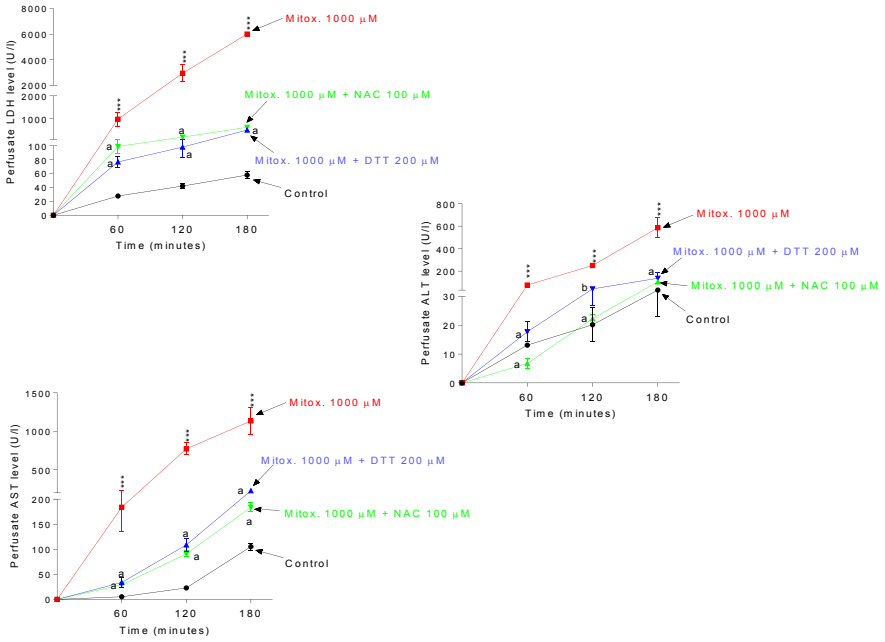

Figure 2. Liver perfusate biochemistry in mitoxantrone-treated isolated rat liver. Mitox.: Mitoxantrone; DTT: Dithiothreitol; NAC: N-acetylcysteine. Data are given as Mean±SD (n=5). *Indicates significantly different as compared with control group (P<0.001). a Indicates significantly different as compared with the mitoxantrone-treated group (P<0.001). b Indicates significantly different as compared with the mitoxantrone-treated group (P<0.05).

ing (28, 36). Samples were analyzed by a pathologist in a blind fashion.

2.9. Statistical analysis

Data are presented as Mean±SEM. A comparison of data sets was performed by employing the one-way analysis of variance (ANOVA) with Tukey's as the *post hoc* test. Differences were considered significant when P<0.05.

3. Results

Mitoxantrone caused an elevation in biomarkers of liver injury in the liver perfusate (*ex vivo*) dose dependently (Table 1). It was found that 1000 µM of mitoxantrone caused a significant elevation in all the assessed biomarkers including LDH, ALT, and AST (Table 1). The concentration of 1000 µM of mitoxantrone was considered as the hepatotoxic dose of the drug in the *ex vivo* system and used for further investigations.

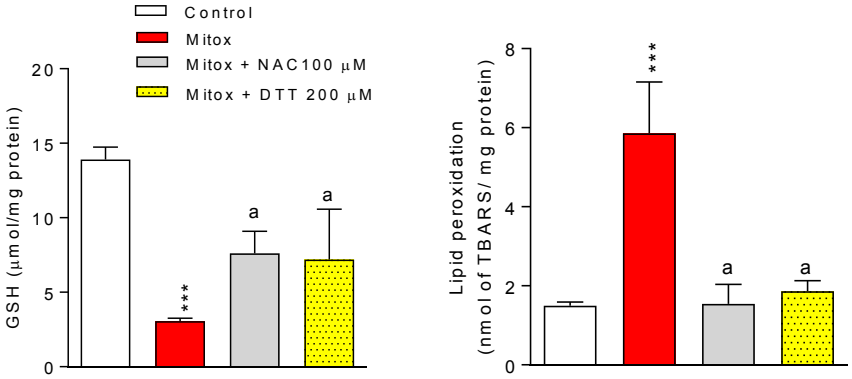

Figure 3. Hepatic glutathione content and tissue lipid peroxidation in mitoxantrone-treated isolated perfused rat liver. Mitox: Mitoxantrone; NAC: N-acetyl cysteine; DTT: Dithiothreitol. Data are shown as Mean±SD (n=5). *Significant difference as compared with control group (P<0.05). a Significant as compared with the mitoxantrone-treated group (P<0.05).

Table 2. Liver histopathological changes in mitoxantrone-treated rats.

	Congestion	Inflammation	Hydropic Changes	Apoptosis	Vaculization	Total grade
Control (vehicle-treated)	-	-	-	-	-	0 (Normal liver)
Mitox.	+++	+	+	+	+	7
Mitox.+NAC 300 mg/kg	++	-	++	-	-	4
Mitox.+NAC 600 mg/kg	++	-	-	-	-	2
Mitox.+DTT 15 mg/kg	++	+	-	+	-	4
Mitox.+DTT 30 mg/kg	++	-	-	+	-	3

Mitox.: Mitoxantrone; NAC: N-acetyl cysteine; DTT: Dithiothreitol.

Liver injury biomarkers were assessed in different experimental groups after administrating thiol reducing agents (NAC and DTT) (Figure 2). It was found that NAC (100 µM) significantly decreased elevation of ALT, AST, and LDH in mitoxantrone-treated perfused livers (P<0.05) (Figure 2). Administration of DTT as another thiol-reducing agent also mitigated elevation in the perfusate biomarkers of liver injury at the concentration of 200 µM (Figure 2) (P<0.05).

Lipid peroxidation and hepatic glutathione content were assessed at the end of each *ex vivo* experiment. It was found that a significant amount of TBARS were formed when mitoxantrone was perfused to the rat liver (P<0.05) (Figure 3). Moreover, a significant decrease in hepatic glutathione stores was detected in the mitoxantrone-treated isolated perfused liver (P<0.05) (Figure 3). It was found that administration of thiol-reducing agents significantly decreased the level of lipid peroxidation (P<0.05) (Figure 3). Furthermore, hepatic glutathione reservoirs were maintained at a sig-

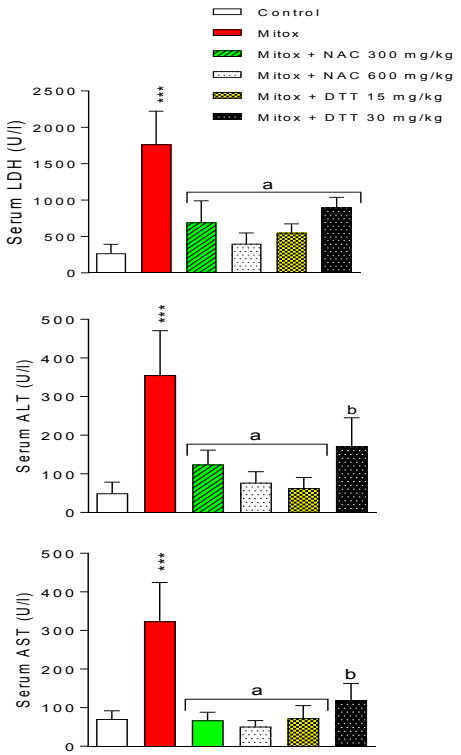

Figure 4. Serum biochemistry of mitoxantrone-treated animals.
Mitox: Mitoxantrone; NAC: N-acetyl cysteine; DTT: Dithiothreitol. Data are expressed as Mean±SD (n=5). ***Indicates significantly different as compared with control (P<0.001). a Indicates significantly different as compared with mitoxantrone-treated group (P<0.001). b Indicates significant difference as compared with the mitoxantrone-treated group (P<0.05).

nificantly higher level when thiol reducing agents were administered (P<0.05) (Figure 3).

Mitoxantrone hepatotoxicity was biochemically evident by the elevated serum biomarkers of liver injury in drug-treated rats (Figure 4). It was found that mitoxantrone administration (2.5 mg/kg, i.p) significantly increased serum ALT, LDH, and AST levels (Figure 4). NAC (300 and 600 mg/kg) and DTT (15 and 30 mg/kg) supplementation significantly decreased mitoxantrone-induced elevation in serum liver injury biomarkers (Figure 4).

Mitoxantrone also caused significant glutathione depletion (Figure 5), and increased tissue lipid peroxidation in the rat liver (Figure 5). Moreover, histopathological evaluation of the liver revealed necrosis, inflammation, sinusoidal dilation, and hydropic changes in the mitoxantrone-treated group (Figure 6 and Table 2). It was found that NAC (300 and 600 mg/kg) and DTT (15 and 30 mg/kg) preserved liver glutathione stores (Figure 6), decreased lipid peroxidation (Figure 6), and mitigated mitoxantrone-induced liver histopathological changes (Figure 6 and Table 2).

4. Discussion

Chemotherapy-induced hepatotoxicity is a serious clinical complication (2, 7, 37). On the other hand, there is no safe and promising therapeutic option against this complication. Hence, finding new therapeutic strategies could have clinical value. Mitoxantrone is among the hepatotoxic anticancer agents. Several cases of liver injury have been reported in association with mitoxantrone therapy (4, 9, 10). The objective of the present study was to investigate the role of thiol reducing agents against mitoxantrone-induced hepatic injury in two different experimental models.

Previous investigations proposed oxidative stress as a mechanism for mitoxantrone-induced liver injury (9, 38, 39). In the current study, we found that mitoxantrone caused a significant amount of lipid peroxidation as an index of oxidative biomembranes disruption (Figures 3 and 5). Moreover, hepatic glutathione stores were decreased (Figures 3 and 5). Lipid peroxidation and hepatic glutathione depletion are consequences of oxidative stress in the liver. Mitoxantrone is converted to reactive metabolites that are capable of inducing oxidative stress and interacting with different intracellular components (40). Biomembrane lipids and cell proteins are among the most available intracellular targets for drug reactive metabolites (41). Glutathione conjugation might also serve as a mechanism for mitoxantrone detoxification. Hence, administration of thiol-reducing agents could contract mitoxantrone-induced oxidative stress and its deleterious consequences.

Thiol reductants such as NAC and DTT protect hepatocytes through preserving a high level of intracellular glutathione concentration. Glutathione is the most abundant low molecular weight intracellular thiol. Glutathione serves as a vital molecule to maintain a reduced cellular environment (42). The reduced form of glutathione and the enzymes that use this molecule to detoxify xenobiotics significantly prevent oxidative stress (42). In the current investigation, it was found that thiol-reducing agents effectively mitigated mitoxantrone-induced liver injury. Previous in-

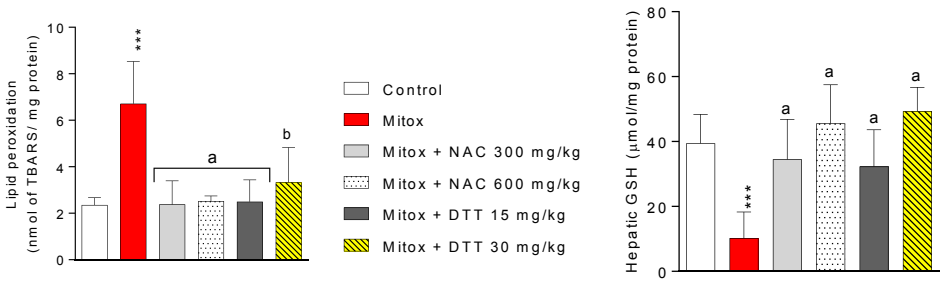

Figure 5. Liver glutathione content and lipid peroxidation in mitoxantrone-treated rats. Mitox: Mitoxantrone; NAC: N-acetyl cysteine; DTT: Dithiothreitol. Data are expressed as Mean±SD (n=5). ***Indicates significantly different as compared with control (P<0.001). a Indicates significantly different as compared with mitoxantrone-treated group (P<0.001).

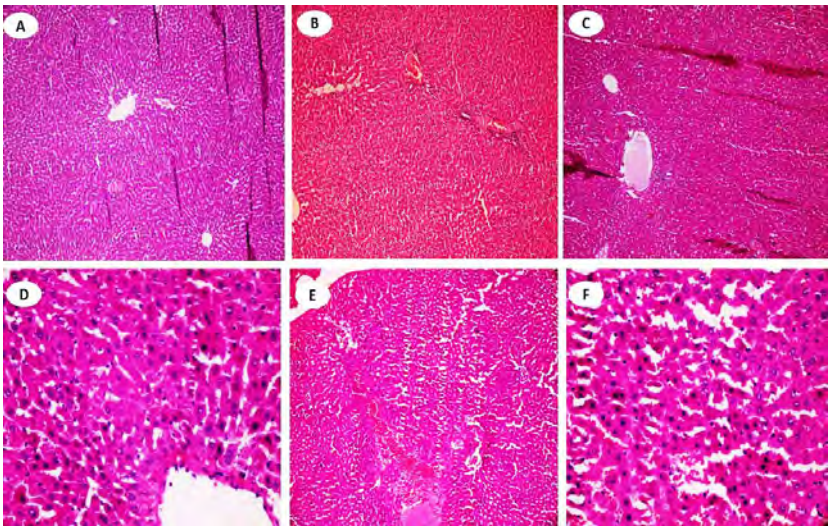

Figure 6. Tissue histopathological changes in mitoxantrone-treated rats. Control rats showed normal liver histopathology (A) (Table 2). The histopathological changes in the liver tissue from mitoxantrone-treated animals included necrosis, inflammation, and hydropic changes (B) (Table 2). Liver histopathological lesions were mitigated when animals received NAC (300 and 600 mg/kg; C and D respectively) or DTT (15 and 30 mg/kg, E and F respectively). Refer to Table 2 for the grade of histopathological changes induced by mitoxantrone.

vestigations also revealed that thiol reductants, such as NAC and DTT, are effective against xenobiotics-induced organ injury and oxidative stress (23). This might implicate the important role of thiol groups and oxidative stress in mitoxantrone-induced hepatotoxicity. This report might mention the potential therapeutic capability of thiol reductants against adverse events accompanied with mitoxantrone administration.

The mechanism of drug-induced liver injury sometimes relies on mitochondrial dysfunction (43, 44). It has been reported that mitoxantrone affected cellular mitochondria in the heart (26). Hence, drug-induced mitochondrial dysfunction might also play a role in mitoxantrone hepatotoxicity. Evaluating the role of mitochondrial dysfunction could help to clarify the precise mechanism of mitoxantrone hepatotoxicity.

Collectively, the data obtained from the current investigation indicate the role of oxidative stress in mitoxantrone-induced liver injury and the potential therapeutic properties of thiol reducing agents and antioxidants against this complication. Clearly, further investigation, especially in tumor-bearing animal models, are essential to exclude that thiol reducing agents such as NAC and DTT may not affect the antitumor efficacy of mitoxantrone.

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Conflict of Interest

None declared.

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