Amifostine protection against doxorubicin cardiotoxicity in rats

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Aminothiol amifostine (AMI) protects against toxic effects of both ionizing radiation and numerous anticancer drugs. The aim of this study was to investigate the potential protective effects of AMI against doxorubicin (DOX)-induced cardiotoxicity in rats. Male Wistar rats were treated with AMI (75 mg/kg i.p.) and/or DOX (1.25 mg/kg i.p.), 4 times per week, for 4 weeks. Mortality, general condition and body weight of the animals were observed during the whole treatment, and for a further 4 weeks, until the end of experiment. Evaluation of cardioprotective efficacy of AMI was performed by analyzing the electrocardiographic parameters and response to the pro-arrhythmic agent aconitine, as well as activity registration of the *in situ* rat heart preparations. Necropsy was also performed at the end of the experiment, and heart excision, weight and macroscopic examination were done before histological evaluation. Doxorubicin caused rat heart disturbances manifested by prominent electrocardiographic changes (Sα–T prolongation and T-wave flattening), significantly enhanced response to aconitine, decrease of the heart rate and contractility, as well as histopathologically verified myocardial lesions. The heart changes were accompanied by 40% mortality rate, significant decline in body mass and severe effusion intensity score in 66.6% of the animals. Application of AMI before each dose of DOX significantly reduced or completely prevented its toxic effects. Therefore, since AMI had very good protective effects against a high dose of DOX given as a multiple, low, unitary dose regimen, not only on the heart but on the whole rat as well, it could be recommended for further investigation in this potentially new indication for clinical application. *Anti-Cancer Drugs* 15:169–178 © 2004 Lippincott Williams & Wilkins.

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Introduction

The anthracycline antibiotic doxorubicin (DOX) is one of the most effective antitumor agents used against a variety of human malignancies [1,2]. However, its therapeutic success is limited by the development of irreversible cardiac toxicity manifested as a dilated cardiomyopathy leading to congestive heart failure [3–5]. Each dose of DOX produces an increase of myocardial damage and the normal heart can compensate for this until a lifetime dose of approximately 450 mg/m^2 has been reached. Consequently, the amount of drug that can be used in the therapy is usually restricted to less than 550 mg/m^2.

The exact causal mechanism of DOX-induced cardiomyopathy remains unclear. Many investigators consider its toxic effects just as results of particular subcellular structural alterations and consequently functional alterations which are directly connected with them [6–8]. Its effects on the plasma membrane cause altered sarcolemmal Ca^{2+} transport, changes in adenylate cyclase, Na^+/K^+-ATPase, Ca^{2+}/K^+-ATPase and Ca^{2+}-ATPase, and increased membrane permeability which eventually leads to irreversible cell injury. DOX also has profound effects on sarcoplasmic reticulum (SR) function [6–8], but new data accentuate inhibition of SR Ca^{2+}-ATPase gene transcription, leading to impaired Ca^{2+} handling and reduced cardiac function [9,10]. On the other hand, there is growing evidence demonstrating that mitochondria are the principal targets in the development of DOX-induced cardiotoxicity [6–8,11–13]. Interference with mitochondrial calcium regulation that leads to depletion of cytosolic ATP appears to be the cause, with special serious consequences in the heart as compared with other organs. Structural disintegration of myofibrils is also one of the primary ultrastructural changes observed in endomyocardial biopsy from humans treated with DOX [14]. It appears that suppression of cardiac-specific contractile protein genes, which has been shown, can lead to defective maintenance of the contractile apparatus [15,16]. However, most of the data favor the hypothesis that actually all these effects are the result of the free radical production, which induces lipid peroxidation and oxidative damage of the heart [5–8,13,17]. In the presence of oxygen, redox cycling of

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DOX-derived quinone-semiquinone yields superoxide radicals, hydrogen peroxide and, most dangerously, hydroxyl radicals which react with polyunsaturated fatty acids yielding lipid hydroperoxides. On the other hand, in the presence of transition metal ions, the chain reaction continues, and free iron appears to play a particularly important role in DOX-induced lipid peroxidation and altered cellular membrane integrity.

In a view of all these facts, one of the approaches to minimize DOX-induced cardiotoxicity has been the preventive usage of free radical scavengers and other antioxidants, as well as iron-chelating agents, but without compromising its antineoplastic activity [18–22].

Amifostine (AMI), a thiophosphate derivative of cysteamine, was originally developed by the US Army as a radioprotective agent [23,24]. Soon it became clear that if administered before ionizing radiation or cytotoxic chemotherapy it provides cytoprotection of various normal tissues without attenuating their antitumor response [25,26]. WR-1065, an active metabolite of AMI, is selectively produced in normal tissues through the dephosphorylation of the parent compound by membrane-bound alkaline phosphatase. Its protective effects appear to be mediated by scavenging free radicals, hydrogen donation, binding directly to active species of antineoplastic agents, liberation of endogenous non-protein sulfhydryls from their bonds with cell proteins, the formation of mixed disulfide to protect normal cells, induction of hypoxia, DNA binding and acceleration of damaged DNA repair, etc. [24,27,28]. AMI is a broad-spectrum cytoprotector which preserves the integrity of almost all normal tissue with the exception of the central nervous system [24,27–29]. It protects the bone marrow against the harmful effects of ionizing radiation as well as cyclophosphamide, nitrogen mustard, melphalan, mitomycin C, carmustine, 5-fluorouracil, carboplatin and cisplatin. Protection from cisplatin nephrotoxicity and ototoxicity has been shown, as well as protection of peripheral neural tissue from cisplatin, paclitaxel, vincristine and vinblastine toxicity. However, data concerning AMI efficacy in preventing the toxic effects of DOX is still insufficient. *In vitro* studies conducted in culture of neonatal rat heart cells have suggested that both AMI and WR-1065 significantly reduce DOX-induced cardiomyocytes toxicity [30]. *In vivo* studies have shown that AMI can reduce to a certain degree the general and cardiotoxic effects of DOX in rats and mice [31–34]. In our previous study we evaluated the cardioprotective effects of AMI and/or selenium in rats treated by large single doses of DOX [35].

The aim of the present study was to establish and further elucidate the potential cardioprotective effect afforded by AMI against toxicity that accompanies treatment of rats with low, unitary doses of DOX which, as previously shown, when cumulatively administered eventually lead to progressive cardiomyopathy [36].

Methods

Animals and treatment

Male Wistar rats, weighing 200–250 g, were used. The animals were housed in plastic cages, under standard laboratory conditions (21°C, 12/12 h light/dark cycle, commercial food and tap water *ad libitum*) before being randomized into four experimental groups of animals each treated 4 times per week for 4 weeks. Group 1 was the control (saline); group 2 was treated with 1.25 mg/kg i.p. of DOX; group 3 was treated with 75 mg/kg i.p. of AMI 20 min before DOX; group 4 was treated with 75 mg/kg i.p. of AMI 20 min before saline.

The study protocol was based on the Guidelines for Animal Study no. 282-12/2002 (Ethics Committee of the Military Medical Academy, Belgrade, Serbia and Montenegro).

AMI (Chemical Department, Military Technical Institute, Belgrade, Serbia and Montenegro) and DOX (Adriblastina; kind gift from Farmitalia, Carlo Erba, Milan, Italy) were dissolved in saline immediately prior to injection.

The total cumulative dose of DOX administered to rats (20 mg/kg i.p.) was estimated as being sufficient to induce a progressive cardiomyopathy according to Jensen *et al.* [36].

Survival study and general toxicity

Mortality and general condition of the animals were observed daily throughout the whole experiment lasting 8 weeks. Body weights were recorded 4 times per week for 4 weeks, during the treatment, and once a week for further 4 weeks, until the end of experiment. Postmortem examination, heart excision and weighing were also done at that time. Fluid accumulation in the abdominal, pleural and pericardial cavities was also determined, and quantitated according to a graded scale of 0 to 3+: 0, none; 1+, mild; 2+, moderate; 3+, severe. Each experimental group consisted of 20 animals.

Myocardial alterations

DOX cardiotoxicity as well as protective efficacy of AMI were evaluated by following electrocardiographic (ECG) parameters, response to the pro-arrhythmic agent aconitine, activity of the *in situ* rat heart preparation, as well as by histopathologic evaluation at the end of experiment.

ECG recordings

ECG was recorded before the beginning of the treatment and 4 weeks after the last dosing. For ECG recording

(Model T-120; EI Nis, Serbia) rats were under light ether anesthesia. Needle electrodes were inserted under the skin for the limb lead at position II and paper speed was 50 mm/s. For each ECG tracing heart rate (beats/min), QRS complex and Sα–T segment (expressed in ms) as well as T-wave (expressed in μV) were measured because it has been found that they are the most reliable ECG parameters for the assessment of DOX-induced cardiotoxicity in rats [37]. Each experimental group consisted of six to 10 animals.

Aconitine test

The procedure for this test has been described in detail elsewhere [35]. Briefly, after described standard ECG records, the Aconitin (acetylbenzoylaconina, aconitine) solution (Fluka, Buchs, Switzerland) (50 μg/ml) was infused via the jugular vein (8 μg/ml/min). The appearance of the first ventricular extrasystoles (VES) in the ECG was taken as an indicator of the arrhythmogenic action of aconitine. The dose of aconitine needed to evoke VES was calculated in order to evaluate the influence of described treatment protocols on the rat heart susceptibility to aconitine-induced arrhythmias.

Registration of the *in situ* rat heart preparation activity

The amplitude of contraction and the heart rate were studied on the *in situ* heart preparation according to the procedure as described elsewhere [38]. Briefly, 4 weeks after the last dose of DOX and/or AMI rats were anaesthetized by 25% urethane (Sigma, St Louis, MO) (7 ml/kg i.p.). After a few minutes a tracheotomy was performed and a glass cannula connected with an artificial breathing pump with positive pressure (Hugo Sachs Elektronik, Breisgan, Germany) was placed in position. After careful surgical approach to the heart and removing the pericardium, the apex of the heart was connected through an isometric transducer (Unirecord 7050; Ugo Basile, Italy) to a printer recording system whose sensitivity was the same throughout the whole series of experiments. After a stabilizing period of 15 min, heart activity was recorded for 30 min. Each experimental group consisted of six to 10 animals.

Histopathologic analysis

Four weeks after the last DOX and/or AMI administration, the animals were euthanized in light ether anesthesia, and the hearts were excised, weighted and fixed in 10% formalin. Transmural tissue samples from the left and right ventricular free walls were embedded in paraffin blocks. Tissue samples 5-μm thick were stained with hematoxylin & eosin and heart sections were analyzed (× 40; Olympus-2B microscope). Cardiomyocytes exhibiting cytoplasmic vacuolation and/or myofibrillar loss, according to modified histopathological evidence for DOX cardiomyopathy previously described [14], were considered as damaged. The grading system was as follows: 0 = no damage, 1 = < 5%, 2 = 16–25%, 3 = > 35%. Six hearts from each group were available for evaluation and five sections from each heart were analyzed. The sections were evaluated without prior knowledge of the treatments given to the animals.

Statistical analysis

Statistical evaluation was performed using the χ^2-test (frequency distribution comparison) and one-way ANOVA + Tukey test (multi-group comparison). A Mann–Whitney test was utilized to determine the significance of the difference in the severity of the cardiac damage scores among the various treatment groups. Student's *t*-test was used to assess differences in ECG parameters as well as dose of aconitine in the aconitine test between the experimental groups. For all tests, differences with values of $p < 0.05$ were considered significant.

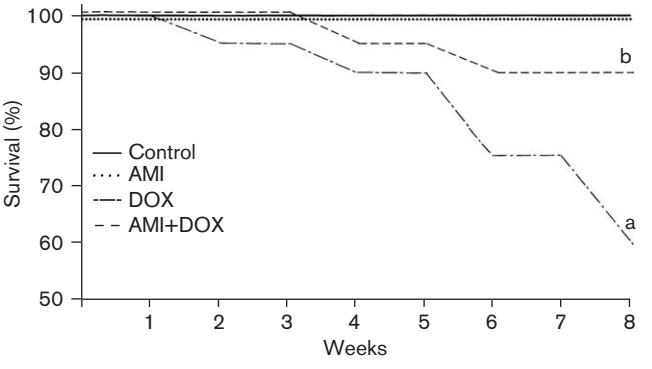
Results

Evaluation of general toxicity and/or corresponding protection

Mortality data during the course of the study are summarized in Fig. 1. Forty percent of the animals died before termination of the experiment in the DOX-only-treated group. First death occurred after the fifth dose of the drug and most of them died starting from 1 week after cessation of DOX treatment. Pretreatment with 75 mg/kg i.p. of AMI significantly reduced mortality at the end of the experiment. The first death occurred after the 14th dose of drugs and another one took place in the second week after stopping application of DOX.

Rats treated with AMI-only and saline controls did not experience body weight loss, and showed a steady weight gain from the second week of treatment to the end of the

Fig. 1

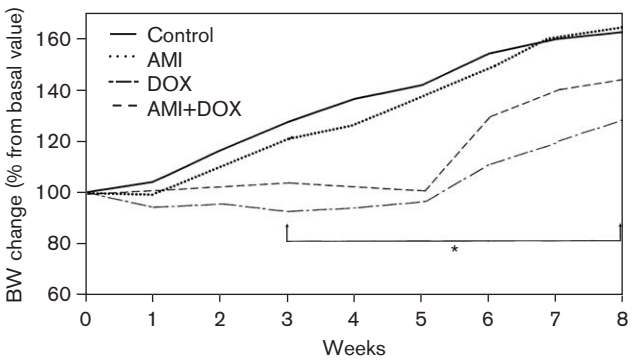


Survival study of Wistar rats given DOX (1.25 mg/kg i.p.) and/or AMI (75 mg/kg i.p.) 4 times a week, 4 weeks (in a group treated with the combination, AMI was given 20 min before DOX). Total follow-up time was 8 weeks. Each curve represents a treatment group of 20 animals. Statistical evaluation was performed using the χ^2-test. [a]Statistically significant compared to control, saline-treated group ($p<0.05$); [b]statistically significant compared to DOX group ($p<0.05$).

experiment (Fig. 2). Animals given DOX lost body weight during the first 3 weeks of treatment and then showed slow weight gain, but it was significantly ($p < 0.05$) below that of the control throughout the rest of the 8 weeks of the study. The weight of the rats in the AMI-pretreated group showed slow, but steady, weight gain from the beginning of the experiments. It was above that of the DOX-only-treated group, although not significantly, especially during the last 3 weeks of the experiment.

At necropsy, the most prominent gross pathologic change in rats treated with DOX was excessive amounts of pericardial, pleural and peritoneal fluid. Effusion intensity score was severe in 66.6% of DOX-treated animals, compared with 5.5% in AMI-pretreated rats (Table 1). Moreover, even 50% of the surviving animals in the AMI-protected group had no presence of fluid accumulations, while there were no such animals in the DOX-treated group.

Fig. 2



Percentage change in body weight of rats treated with DOX (1.25 mg/kg i.p.) and/or AMI (75 mg/kg i.p.) 4 times a week, 4 weeks (same as in Fig. 1). Total follow-up time was 8 weeks. Each curve represents a treatment group of 20 animals. Statistical evaluation was performed using the one-way ANOVA + Tukey test. *$p < 0.05$ for both the DOX and AMI + DOX group compared to the control, saline-treated group.

Evaluation of myocardial alterations

ECG alterations and aconitine test findings

The influence of treatment with AMI and/or DOX on rat ECG parameters is shown in Table 2. Sα–T segment prolongation as well as T-wave flattening in DOX-treated rats was statistically significant compared to the control ($p < 0.01$). In AMI-pretreated animals, the Sα–T segment duration significantly decreased and T-wave voltage significantly increased compared to DOX-treated rats. Also, 50% of the animals treated with DOX had depression of the ST segment that was not present in the rats pretreated with AMI. On the other hand, no changes were observed in heart rate and QRS complex duration in all experimental groups.

However, differences in heart rate became manifest during the aconitine infusion (Fig. 3), i.e. increasing doses of aconitine led to the heart rate decreasing in both experimental groups (DOX-only- and AMI-pretreated rats) compared to control animals. Moreover, the VES-inducing dose of aconitine was significantly reduced in DOX-treated rats compared to control (30.09 versus 49.30 μg/kg, respectively; $p < 0.001$) (Fig. 4). Pretreatment with AMI reversed the arrhythmogenic dose of aconitine to values not significantly different from the control one.

AMI given before saline injection had no effects on the monitored parameters (Table 2 and Figs 3 and 4).

Assessment of the activity of the in situ rat heart preparation

Evaluation of the contractile properties and heart rate of the *in situ* rat heart preparation was also done 4 weeks after the last AMI and/or DOX administration. It was found that the cumulative dose of 20 mg/kg of DOX significantly depressed the heart rate (not shown) and the amplitude of contraction (Fig. 5) not earlier than 25 min after the period of adaptation of the *in situ* heart preparation. Pretreatment with AMI did not influence DOX-induced effects on the heart rate (not shown), but it antagonized its depressive effects on the contractility

Table 1 The influence of AMI on DOX-induced abdominal, pleural and pericardial effusion intensity score in surviving rats

Treatment	Total no. of animals	Effusion intensity score							
		0		+		+ +		+ + +	
		N	%	*N*	%	*N*	%	*N*	%
Control	20	20	100.0	0	0.0	0	0.0	0	0.0
AMI	20	20	100.0	0	0.0	0	0.0	0	0.0
DOX	12	0	0.0	2	16.7	2	16.7	8	66.6[a]
AMI + DOX	18	9	50.0	5	27.8	3	16.7	1	5.5[b]

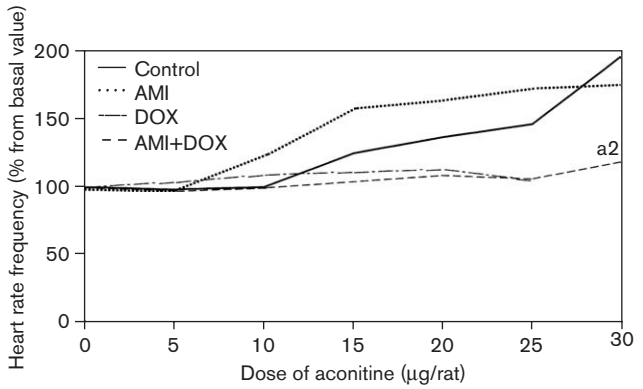
AMI (75 mg/kg i.p.) was given 20 min before DOX (1.25 mg/kg i.p., 4 times per week, 4 weeks) or saline (the control group, 1 ml/kg i.p., 4 times per week, 4 weeks). Rats were sacrificed 4 weeks after the last injection. Score: (0) none; (+) mild; (+ +) moderate; (+ + +) severe. Statistical evaluation was performed using the χ^2-test.
[a]$p < 0.01$ versus control.
[b]$p < 0.05$ versus DOX.

Table 2 **The influence of AMI on ECG parameters of the rats treated with DOX (mean ± SD)**

Treatment	QRS (ms)	Sα–T (ms)	T-wave (μV)	Frequency (beats/min)
Control	14 ± 4.2	12.22 ± 2.64	255 ± 30	361.33 ± 98.33
AMI	14.5 ± 2	11.66 ± 2.58	245 ± 70	352.3 ± 70.53
DOX	16.2 ± 5	37.5 ± 4.86[a3]	183 ± 50[a1]	408.33 ± 86.53
AMI + DOX	13.2 ± 4.7	25 ± 5.48[a3,b3]	267 ± 60[b1]	382 ± 41.58

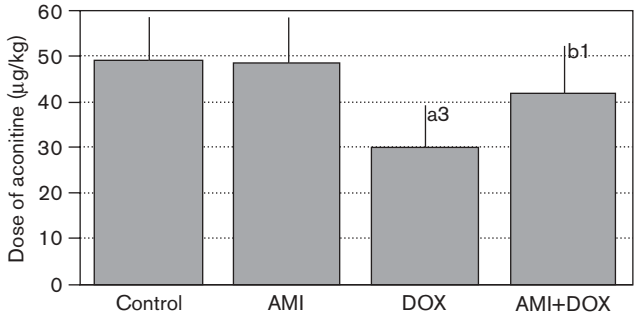
Treatment as shown in Table 1. Each experimental group consisted of six to 10 animals. Statistical evaluation was performed using Student's *t*-test.
[a1,a3] $p < 0.05$; 0.001 versus control group.
[b1,b3] $p < 0.05$; 0.001 versus DOX group.

Fig. 3



Aconitine-induced changes in the heart rate of Wistar rats given DOX (1.25 mg/kg i.p.) and/or AMI (75 mg/kg i.p.) 4 times a week, 4 weeks (same as in Fig. 1). Total follow-up time was 8 weeks after which the experiment was performed. Each curve represents a treatment group of 6 to 10 animals. Statistical evaluation was performed using the one-way ANOVA + Tukey test. [a2] $p < 0.05$ for both the DOX and AMI + DOX group compared to the control, saline-treated group.

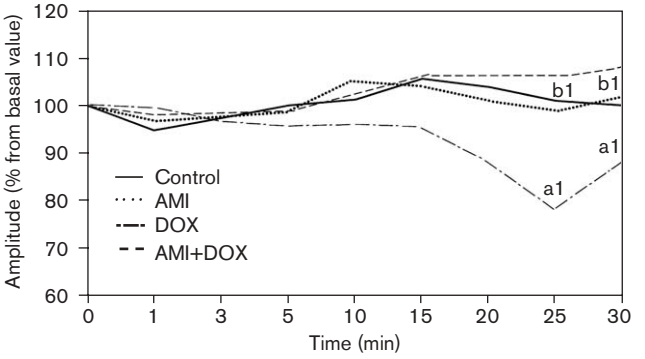
Fig. 4



VES-inducing dose of aconitine in Wistar rats given DOX (1.25 mg/kg i.p.) and/or AMI (75 mg/kg i.p.) 4 times a week, 4 weeks. Total follow-up time was 8 weeks after which the experiment was performed. Each curve represents a treatment group of six to 10 animals. Statistical evaluation was performed using Student's *t*-test. [a3]Statistically significant compared to control ($p < 0.001$); [b1]statistically significant compared to DOX ($p < 0.05$)

(Fig. 5). AMI itself, after its multiple applications before saline, did not significantly change both examined parameters of *in situ* rat heart activity (Fig. 5).

Fig. 5



Changes in the amplitude of contraction of the *in situ* heart activity in rats treated with DOX (1.25 mg/kg i.p.) and/or AMI (75 mg/kg i.p.) 4 times a week, 4 weeks. Total follow-up time was 8 weeks after which the experiment was performed. Each curve represents a treatment group of six to 10 animals. Statistical evaluation was performed using the one-way ANOVA + Tukey test. [a1]Statistically significant compared to control ($p < 0.05$); [b1]statistically significant compared to DOX ($p < 0.05$)

Evaluation of the macroscopic and microscopic findings

There were no significant treatment-related effects on absolute and relative heart weight (results not shown) or on their macroscopic appearance. However, DOX-induced myocardial lesions observed during light microscopic examination were very prominent compared to the control. Large number of myocytes had a fine granular cytoplasm without clearly noticeable nuclei. Also, groups of cardiomyocytes, occupying large areas of cardiac tissue, had numerous small vacuoles and/or pale appearance of the cytoplasm. Grading of the cardiac samples according to the 0–3 scale established by Billingham *et al.* [14], taking into account only myocytes showing cytoplasmic vacuolation and/or myofibrillar loss, revealed a cardiac damage score of 2.50 ± 0.50 in the DOX-treated group (Table 3 and Fig. 6). The score of the saline-treated (control rats) was not significantly different from 0. The difference between the control and DOX-treated group was statistically significant ($p < 0.001$) (Table 3). Moreover, small groups of necrotic cells with pyknotic nuclei were also observed in the DOX-treated group. These irreversible cardiomyocyte injuries were accompanied by

increased numbers of fibroblasts and infiltration with mononuclear cells. AMI appeared to have significant protective effects on rat cardiac myocytes treated with DOX, i.e. myocardial damage was focal and damaged cells with granular cytoplasm were surrounded by those that appeared normal. Furthermore, only limited numbers of isolated cells exhibited cytoplasmic vacuolation and/or early myofibrillar loss (Fig. 7). A mean cardiac damage score of 1.00 ± 0.58 was established in this group, which was significantly reduced compared with the DOX-only-treated group (Table 3). Necrotic cardiomyocytes were very rare, and the presence of mononuclear cells and fibroblasts was decreased in AMI-protected rats compared with the DOX-only-treated group.

Table 3 The influence of AMI on cardiac damage scores in rats treated with DOX

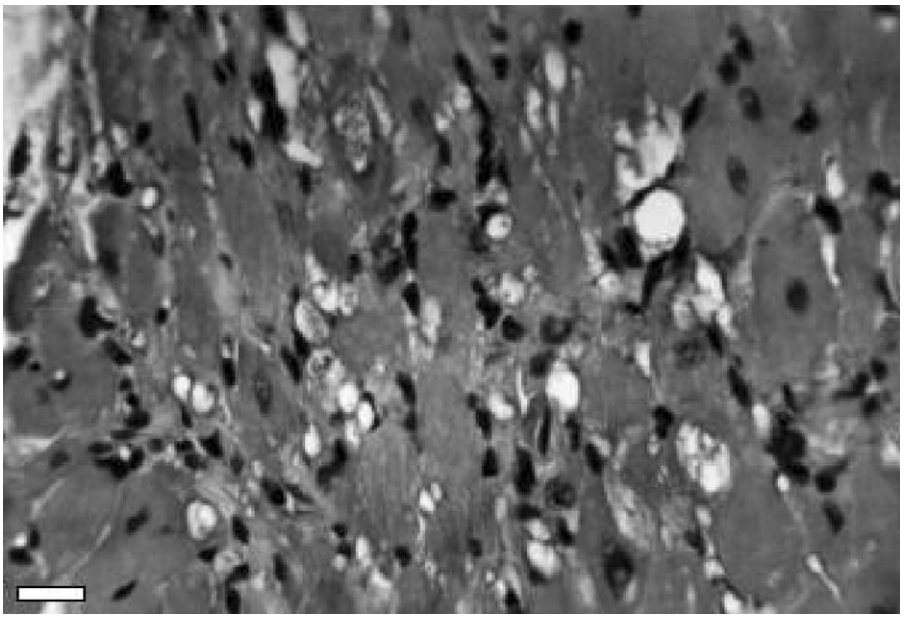
Treatment[a]	Cardiac damage score (6 hearts × 5 sections)				Mean score ± SD
	0	1	2	3	
Control	20	10	0	0	0.33 ± 0.48
AMI	15	15	0	0	0.50 ± 0.51
DOX	0	0	15	15	2.50 ± 0.50[a]
AMI + DOX	5	20	5	0	1.00 ± 0.58[b]

Treatment as shown in Table 1. Statistical evaluation was performed using the Mann–Whitney test.
[a] $p < 0.001$ versus control.
[b] $p < 0.001$ versus DOX.

Discussion

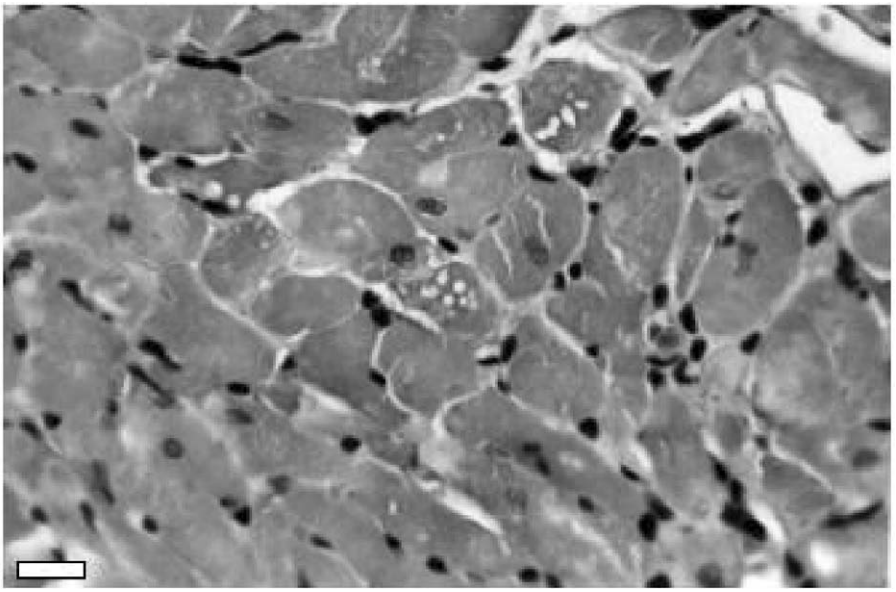
The experimental model adopted for this study was that described by Rossi *et al.* [39] and, similarly, our results have shown that 20 mg/kg cumulative dose of i.p. DOX produced signs of systemic and cardiac toxicity in rats, i.e. 40% of the animals died before termination of the experiment and their body weight was significantly below that of the control during most of the study. Reduction of body weight, debilitation and death associated with multiple, long administration of DOX in experimental animals are considered multifactorial. These are the results of direct toxic effects on intestinal mucosa appearing as mucositis, as well as additional indirect action on the gastrointestinal tract arising from reduced food intake [8,19,32], causing a decrease in secretion of enteral hormones and resulting in decreased trophic effects to the mucosa. However, the majority of authors dealing with this problem consider that cardiomyopathy and nephropathy make the most important contribution to the mortality of experimental animals after long-term treatment of DOX [32,39,40]. In our experiment, excessive fluid accumulation was found in pleural, pericardial and peritoneal cavities in 66.6% of the rats treated by DOX. This finding, together with ventral s.c. edema and enlargement of liver and kidneys, is a manifestation of congestive myocardial failure in experimental animals [41–43]. We studied ECG alterations because it was demonstrated that the severity of changes

Fig. 6



Histological section of the heart from a rat treated with DOX (1.25 mg/kg i.p., 4 times a week, 4 weeks) and sacrificed 4 weeks after the last injection shows widespread and marked vacuolization, as well as some necrotic cardiomyocytes and infiltration of mononuclear cells: 5-μm thick paraffin section, H & E, original magnification × 40. Scale bar = 10 μm.

Fig. 7



Histological section of the heart from a rat treated with AMI (75 mg/kg i.p.) 20 min before DOX (1.25 mg/kg i.p.) 4 times a week, for 4 weeks, and sacrificed 4 weeks after the last injection shows only moderate, focal vacuolization (cf. Fig. 6): 5-μm thick paraffin section, H & E, original magnification × 40. Scale bar = 10 μm.

in ECG parallels the known DOX cardiotoxicity in patients [44,45]. Also, rat and mouse experimental models like ours were defined as suitable tools not only for characterization of the DOX cardiac toxic effects, but also for the estimation of efficacy of various cardioprotectors [39,40,46]. In the present study, no changes in the QRS complex were found, but Sα–T segment prolongation as well as T-wave flattening in DOX-treated rats were statistically significant in comparison to control animals. According to Rossi *et al.* [39], the most reliable and consistent ECG changes noted with high, cumulative dose of DOX were QRS widening, Qα–T prolongation and T-wave flattening. Other authors [37,40] insisted on the significance of the ST interval in evaluating the ECG signs of toxicity induced in rodents by chronic administration of DOX, which is in accordance with our results. All these ECG changes are related to the prolongation of action potential duration, but it is considered that the recovery phase of the transmembrane action potential is most prominently affected with DOX, influencing preferentially Ca^{2+} movements across the cellular membrane [37,40,46]. As mentioned, cardiotoxic effects of DOX preferentially stem from the formation of reactive oxygen free radical species which interact with and damage cellular membranes including the sarcolemma and its Ca^{2+} transport [5–8,17]. Flattening of the T-wave also reflects alterations in the repolarization process, so measurement and observation of the Sα–T segment and T-wave are most reliable methods for the assessment of DOX-induced cardiotoxicity in rats.

Regarding the heart rate frequency, our results suggest that the myocardium damaged by DOX may maintain its functional capabilities as long as it is exposed to noxious stimuli, such as aconitine and *in situ* heart preparation which we used in our experiments. While the hearts of healthy animals responded to increasing doses of aconitine with increasing heart frequencies, in DOX-treated rats this response was absent. Similarly, in the *in situ* rat heart preparation, the heart rate as well as the heart contractility maintained the control level until 15 min after the period of adaptation, and after that they started to decrease, becoming significantly reduced from 25 min to the end of the observation. The negative inotropic effect of repeated DOX administration has been reported both *in vivo* as well as on *in vitro* preparations [40,47–50]. In addition, in DOX-treated rats, as previously demonstrated [35], increased susceptibility of the myocardium to the aconitine-inducing arrhythmias was noticed. In these animals, the VES-inducing dose of aconitine was significantly reduced in comparison with that in healthy ones.

Functional alterations in our experiments were verified by morphological changes found in these animals, i.e. groups of cardiomyocytes which occupied large areas of cardiac tissue had numerous small vacuoles and/or pale appearance of the cytoplasm (mean pathology score of 2.50 ± 0.50), and small groups of necrotic cells were also observed. It has been known for a long time that this vacuolation seen after DOX application is due to

dilatation of the SR [14,41,42,51]. Results from both experimental and clinical studies suggested that the SR, one of the three intracellular membrane systems responsible for myoplasmic calcium regulation in the adult mammalian heart, is the main target of DOX [9,10,50–52]. New studies have shown that decreased cardiac output in experimental animals caused by cumulative application of DOX is accompanied by a significant decrease of mRNA levels for all SR Ca^{2+} transport proteins, including Ca^{2+}-ATPase 2 (SERCA 2), as well as relative amounts of SERCA protein and Ca^{2+} uptake capacity [9]. It was established, using cultured rat neonatal myocytes, that H_2O_2 and probably other reactive oxygen intermediates mediated the effects of DOX on SERCA 2 gene expression, while it was prevented by using the antioxidant *N*-acetylcysteine [10]. Other *in vitro* experiments also indicated that free radicals play a causal role in the progressive impairment of contractile processes as functional manifestations of acute DOX cardiotoxicity [53,54], i.e. adding superoxide dismutase, catalase and some spin-trapping agents partly reduced the decrease of the heart contractility.

The pale appearance of the cytoplasm in our experiment, as it is well known, reflects myofibrillar loss in cardiomyocytes after treatment with DOX [14,40–43]. *In vitro* and *in vivo* investigations in rats have shown that DOX treatment resulted in an acute, rapid decrease of the level of mRNA for the sarcomeric genes, α-actin, troponin I, myosin light chain 2 and M isoform of creatine kinase in cardiac muscle, and these changes precede classical ultrastructural changes [15]. Also, administration of DOX reduces cell respiration, inhibits oxidative phosphorylation and decreases mitochondrial ATPase activity leading to significant reduction of intracellular ATP [16,55,56]. It is the result not only of the fact that the mitochondrial inner membrane is the primary site of free radical generation and damage of mitochondria and other structures, but also of the suppression of transcripts encoding proteins of energy production pathways after application of DOX. Depletion of cytosolic ATP together with the mentioned down-regulation of contractile proteins as well as all SR Ca^{2+} transport protein gene expression probably leads to depressed activity of the *in situ* rat heart preparation and changes in ECG as well as morphological changes of rat myocardium in our experiments. Therefore, generation of free radicals and lipid peroxidation of plasmalemma probably cause perturbation of intracellular second messenger signaling pathways, and together with direct mitochondria membrane free radical injury lead to DOX-induced cardiomyopathy [6,11,13, 16,22,51].

In the light of this implication that oxidative stress plays an important role in the injury of the heart in DOX-treated rats we wanted to evaluate the protective efficacy of aminothiol AMI, which has strong oxygen radical scavenging activity [24,27–30]. A direct interaction with reactive oxygen radicals, together with oxygen depletion, has been recognized for a long time as the mechanism responsible for the radioprotective activity of the aminothiol compound [24]. An *in vitro* study using a pure chemical system demonstrated that WR-1065, the active metabolite of AMI with a free thiol group, was able to scavenge hydroxyl radicals and superoxide anions including DOX-derived superoxide anions generated by NADH respiration of heart mitochondria particles [57]. Marzatico *et al.* [58] showed that AMI scavenging activity is exerted mainly against highly reactive $OH^{\cdot}$, the most dangerous reactive oxygen species from a biological point of view. We can suppose that AMI protected cardiomyocytes plasmalemma, thanks to its scavenging activity, and influenced the action potential duration, especially the recovery phase and Ca^{2+} movements across the cellular membrane. There is also some evidence that this protector can actually prevent calcium cellular entry and rise in $[Ca^{2+}]_i$ [59,60]. In our experiment in AMI-pretreated animals, Sα–T segment duration was significantly decreased and T-wave voltage significantly increased compared to DOX-only-treated rats. Also, depression of the ST segment was not present in rats protected with AMI compared with 50% of DOX-treated animals. In the aconitine test, pretreatment with this cytoprotector reversed the arrhythmogenic dose of aconitine to that of controls. This was in accordance with our previous results, when we evaluated the cardioprotective effects of AMI in rats treated by large single doses of DOX [35]. However, this AMI protection of plasmalemma might contribute to reduction of subsequent signaling pathways caused by DOX, which eventually led to myofibrillar degeneration. This was actually substantiated by our microscopic examination. In accordance with this, the protector antagonized the DOX depressive effect on the contractility of the *in situ* rat heart preparation, although it failed to influence its effect on the heart rate. Nazzeyrollas *et al.* [61] have found that direct addition of AMI on perfused isolated rat heart exposed to DOX prevented the heart from any acute decrease in inotropism. Moreover, the same authors [34] recently showed that AMI protected rats from toxicity of DOX at the cumulative dose of 18 mg/kg during 12-day treatment with regard to weight loss and heart contraction. Our own results, as well as others [47,62], support the statement that acute and chronic cardiac toxicity of DOX share the same mechanisms, and, accordingly, chronic toxicity arises from repeated episodes of acute exposure, inducing cumulative damages. Thus, not only formation of highly reactive oxygen species, especially $OH^{\cdot}$, should be blamed for acute, as well as chronic, DOX cardiotoxicity, but also its scavenging could be a main mechanism of AMI protection against both toxicities. According to Luo *et al.* [63], reactive oxygen species after application of DOX by inducing lipid peroxidation

produce cytotoxic aldehydes which result in inflammatory reactions. This eventually leads to increased synthesis of cytokines, infiltration of mononuclear cells and death of heart cells. In accordance with this, in our experiment the presence of mononuclear cells and fibroblasts was decreased in AMI-protected rats compared with the DOX-only-treated group and necrotic cardiomyocytes were very rare. Still, since other antioxidants have not modified this toxicity successfully [18], it is reasonable to suppose that AMI exerts its protection by some other mechanisms in addition to scavenging free radicals. Augmentation of glutathione-based detoxification systems as well as transient supplementation of sulfhydryl levels in cardiac myocytes that can favor detoxification of highly reactive DOX metabolites by multidrug resistance protein/glutathione-S conjugate pump have been suggested [18,24,28,30].

In this paper we presented evidence that AMI (75 mg/kg i.p.) 20 min before each dose of DOX (cumulatively 20 mg/kg) protected rats from signs of systemic and cardiac toxicity 1 month after stopping its application. Body weight reduction of the rats was the only one not prevented or attenuated by AMI. We cannot find adequate explanation for this at the moment, since AMI protected rats against other signs of DOX toxicity in our experimental conditions. Nazeyrollas *et al.* [34] have shown that treatment with 7 and 20 mg/kg i.p., but not with 50 mg/kg, of AMI 30 min before DOX, given at a cumulative dose of 18 mg/kg i.p. during a 12-day treatment of Wistar rats, protected them from significant weight loss observed in DOX-only-treated animals. Herman *et al.* [32] demonstrated that AMI given to spontaneously hypertensive rats at a dose of 200 mg/kg i.p. before 1 mg/kg of DOX i.v. once a week for 12 weeks did not prevent loss of body weight produced by this antineoplastic agent. However, it is difficult to compare these studies, including ours, since the method of application (i.v., i.p.), drug dose and duration of administration were different, and the blood level of the drugs was also unknown.

As mentioned, the protector delayed the onset of animals dying and significantly reduced their mortality at the end of the experiment. Since, as previously mentioned, cardiomyopathy and nephropathy make the most important contribution to the mortality of experimental animals after long-term treatment of DOX [32,39,40], it is reasonable to assume that protection of these organs makes the most contribution to reduced mortality and signs of systemic toxicity in AMI-pretreated rats. In our experiment effusion intensity score was severe in only 5.5% of animals protected with AMI compared to 66.6% in DOX-only-treated rats. This, besides our other presented results, suggests that in AMI protection against DOX-induced toxicity, cardioprotection make the most important contribution. However, since DOX, as a very potent cytotoxic agent damages other normal tissues as well, it would be of interest to assess AMI protective efficacy against other DOX-induced toxicities, as has already been shown for kidneys [32].

In conclusion, our study showed that the radio- and chemoprotector AMI exerted very good protective effects against a high dose of DOX given by a multiple, low, unitary dose regimen, not only on the heart, but on the whole rat as well. Therefore, it can be recommended for further investigation in this potentially new indication for clinical application.

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