

ASSESSING HEPATOPROTECTIVE EFFECTS OF ANTIOXIDANTS ON AMIODARONE-INDUCED CYTOTOXICITY IN HUMAN HEPATOMA *HEPARG* CELL LINE

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Effective therapy of amiodarone-induced hepatotoxicity requires studying the mechanisms of the toxic effects of amiodarone on hepatocytes and assessing the potential impact of hepatoprotective agents. The study was aimed to assess hepatoprotective effects of antioxidants on the amiodarone-induced hepatotoxicity with the use of immortalized human hepatoma cells of the *HepaRG* cell line. Cell viability was evaluated upon exposure to amiodarone and in the mixture with vitamin E, N-acetylcysteine and S-adenosylmethionine by impedance measurement; the levels of some hepatotoxicity biomarkers were defined using the Luminescence MAP technology. As a result of the research, the dose-dependent toxic effects of amiodarone were established. The IC_{50} value of amiodarone in the *HepaRG* cell line was 3.5 μ M. It is shown that cytotoxic effects decrease and the IC_{50} value increases in the presence of vitamin E, N-acetylcysteine and S-adenosylmethionine. Amiodarone reduces the activity of cell cycle regulators: AKT, JNK kinases, and p53 protein. Exposure to amiodarone results in reduced intracellular ATP levels and the release of intracellular enzymes (malate dehydrogenase 1, glutathione S-transferase, sorbitol dehydrogenase, 5'-nucleotidase) into conditioned medium, indicating the necrotic cell death. Thus, vitamin E, S-adenosylmethionine and N-acetylcysteine reduce amiodarone cytotoxicity in the model of amiodarone-induced damage to hepatocytes and can be considered as hepatoprotective agents in case of the need to protect liver against the hepatotoxic effects of amiodarone.

Keywords: *HepaRG*, amiodarone, drug hepatotoxicity, vitamin E, N-acetylcysteine, S-adenosylmethionine

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ОЦЕНКА ГЕПАТОПРОТЕКТОРНОГО ЭФФЕКТА АНТИОКСИДАНТОВ НА АМИОДАРОН-ИНДУЦИРОВАННУЮ ЦИТОТОКСИЧНОСТЬ В КЛЕТКАХ ГЕПАТОМЫ ЧЕЛОВЕКА ЛИНИИ *HEPARG*

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Для эффективной терапии амиодарон-индуцированной гепатотоксичности необходимы изучение механизмов токсического действия амиодарона на гепатоциты и оценка возможного влияния гепатопротекторов. Целью работы было исследовать гепатопротекторный эффект антиоксидантов на амиодарон-индуцированную цитотоксичность с использованием immortalized гепатомы человека линии *HepaRG*. Жизнеспособность клеток оценивали при действии амиодарона и в смеси с витамином Е, N-ацетилцистеином и S-аденозилметионином методом импедансометрии, а также определяли содержание некоторых биомаркеров гепатотоксичности с использованием технологии Luminescence MAP. В результате исследования установлен дозозависимый эффект токсического действия амиодарона, IC_{50} амиодарона для линии *HepaRG* составил 3,5 мкМ. Показано, что в присутствии витамина Е, N-ацетилцистеина и S-аденозилметионина снижается цитотоксический эффект и увеличивается значение IC_{50} . Амиодарон снижает активность регуляторов клеточного цикла: киназ AKT, JNK и белка p53. В результате действия амиодарона уменьшается содержание АТФ в клетках и наблюдается выход внутриклеточных ферментов (малатдегидрогеназы 1, глутатион-S-трансферазы, сорбитолдегидрогеназы, 5'-нуклеотидазы) в conditioned medium, что свидетельствует о клеточной гибели по типу некроза. Таким образом, витамин Е, S-аденозилметионин и N-ацетилцистеин снижают цитотоксичность амиодарона в модели амиодарон-индуцированного повреждения гепатоцитов и могут быть рассмотрены в качестве гепатопротекторов при необходимости защиты тканей печени от гепатотоксических эффектов амиодарона.

Ключевые слова: *HepaRG*, амиодарон, лекарственная гепатотоксичность, витамин Е, N-ацетилцистеин, S-аденозилметионин

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The symptoms of drug-induced liver injury constitute about 10% of adverse reactions, caused by medications, and are considered the main reason for clinical trial termination or for withdrawal of medications already used in therapy. Drugs of various pharmacological classes possess hepatotoxic effects: antibiotics (amoxicillin/clavulanate), analgesics (acetaminophen), antiepileptic drugs (valproate), etc. [1].

Drug dosage reduction or permanent discontinuation of the drug allows for prevention of irreversible liver damage. However, in some cases it is necessary to continue using hepatotoxic drug in parallel with the use of hepatoprotective drugs. The efficiency of hepatoprotective drugs used in clinical practice results from the presence of components, having various mechanisms of action. There remains a need for developing new pharmaceutical products to protect the liver against the

possible toxic effects, since hepatoprotective drugs are not always able to minimize the adverse effects of xenobiotics, and the use of specific antidotes is limited to the use of N-acetylcysteine in paracetamol overdose [2]. Developing the efficient methods for the drug-induced liver injury treatment requires studying the toxin-induced hepatic cell damage mechanisms, and selecting the potential hepatoprotective agents and specific antidotes.

Amiodarone, being the anti-arrhythmic agent, is characterized by the frequent side effects, manifested by the symptoms of drug-induced liver injury. Amiodarone is one of the most frequently prescribed anti-arrhythmic medication due to high efficiency and broad spectrum of action [3].

In 10–15% of cases, administration of amiodarone is compounded by side effects in the form of elevated serum

transaminase levels, phospholipidosis and steatohepatitis. The long-term use of amiodarone may result in acute liver failure, which is sometimes fatal [4].

Liver injury, resulting from administration of amiodarone, is due to cytotoxic effects of the medication. It is known that amiodarone and its metabolites (mostly mono- and di-N-desethylamiodarone) inhibit the electron transport chain function and uncouple oxidative phosphorylation, which should be complemented by the reactive oxygen species accumulation and the development of oxidative stress [5, 6]. Oxidative stress is indicated by the elevated lipid peroxidation marker levels after exposure to amiodarone [7]. Furthermore, amiodarone is able to inhibit phospholipase A and β -oxidation of long-chain fatty acids, which promotes lipid accumulation in hepatic cells [8, 9]. There is also a decline in the intracellular ATP levels and calcium ions; endoplasmic reticulum is damaged [10].

Earlier, the study involving the L929 mouse fibroblast cell line showed that the use of antioxidant agents, vitamin C and N-acetylcysteine, contributed to reduced amiodarone cytotoxicity [11]. We have studied the ability of nonspecific hepatoprotective compounds, possessing antioxidant activity (vitamin E and the sulfur-containing N-acetylcysteine and S-adenosylmethionine), to affect cytotoxic properties of amiodarone.

Vitamin E has long been known as a cytoprotective agent and recommended for therapy of inflammatory and degenerative liver diseases, such as non-alcoholic fatty liver disease and steatohepatitis [12]. N-acetyl-L-cysteine (NAC) is the effective antidote to treat paracetamol overdose. NAC is also proposed to be used in management of non-paracetamol drug-induced

liver injury [13]. S-adenosyl-L-methionine (SAM) is the main donor of methyl groups in the body, it also plays an important role in the xenobiotic metabolism in the liver. Therefore, SAM is also viewed as a hepatoprotective agent for various liver diseases. However, in clinical practice, hepatoprotective effects of SAM has not been supported by randomized placebo-controlled trials [14].

Test systems based on primary human hepatocyte culture or immortalized cell lines are used for *in vitro* investigation of cytotoxic effects on hepatocytes. During the study, we used the human hepatoma *HepaRG* cell line. Cells of this cell line ensure the expression of biotransformation enzymes (in particular, cytochromes P450) and transport proteins at the levels close to those of primary human hepatocytes. This gives us ground to consider this cell line to be the optimal choice for *in vitro* hepatotoxicity modeling [15, 16]. The *HepaRG* cell line has shown maximum sensitivity in assessing the potential hepatotoxicity of medications with the use of multiparametric assay [17, 18].

Thus, the study was aimed to assess cytoprotective effects of antioxidants in the human hepatoma *HepaRG* cell line-based model of amiodarone-induced cytotoxicity.

METHODS

Human hepatoma cells of the *HepaRG* cell line (Gibco; USA) were cultured in the complete growth medium, consisting of William's E medium with 10% fetal bovine serum, 5 $\mu\text{g/mL}$ of insulin, 10 U/mL of penicillin, 100 $\mu\text{g/mL}$ of streptomycin, 50 μM of hydrocortisone hemisuccinate, and 2 mM of L-glutamine, in

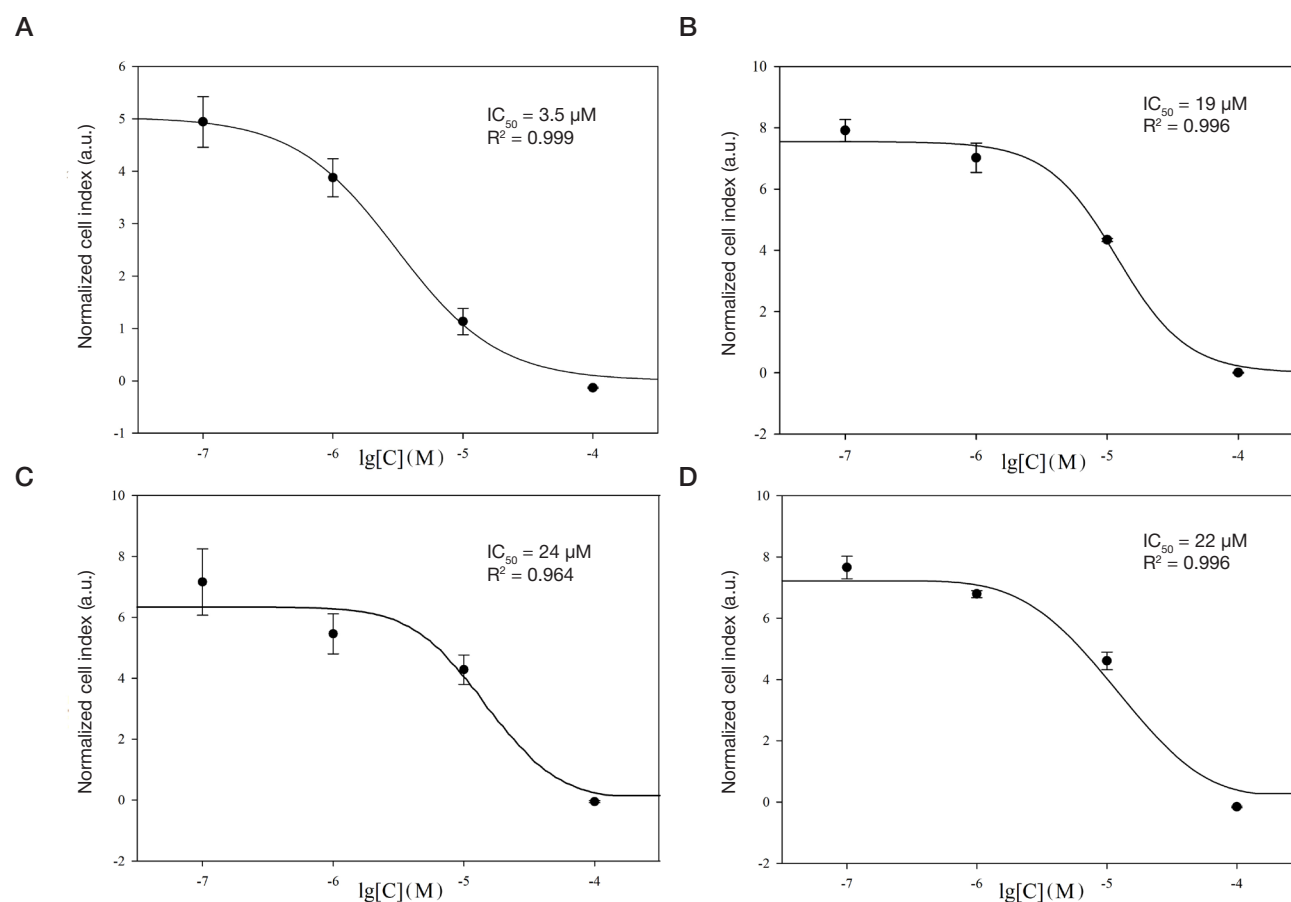


Fig. 1. Relationship between cell index of *HepaRG* cells and log concentration of amiodarone (cell index has been normalized in terms of the time when the medium containing the studied substance has been added). **A.** Changes in cell index upon exposure to amiodarone at a concentration of 0.1, 1, 10 and 100 μM . **B.** Changes in cell index upon exposure to amiodarone dilution series in the presence of 100 μM of vitamin E. **C.** Changes in cell index upon exposure to amiodarone dilution series in the presence of 100 μM of N-acetylcysteine. **D.** Changes in cell index upon exposure to amiodarone dilution series in the presence of 100 μM of S-adenosylmethionine

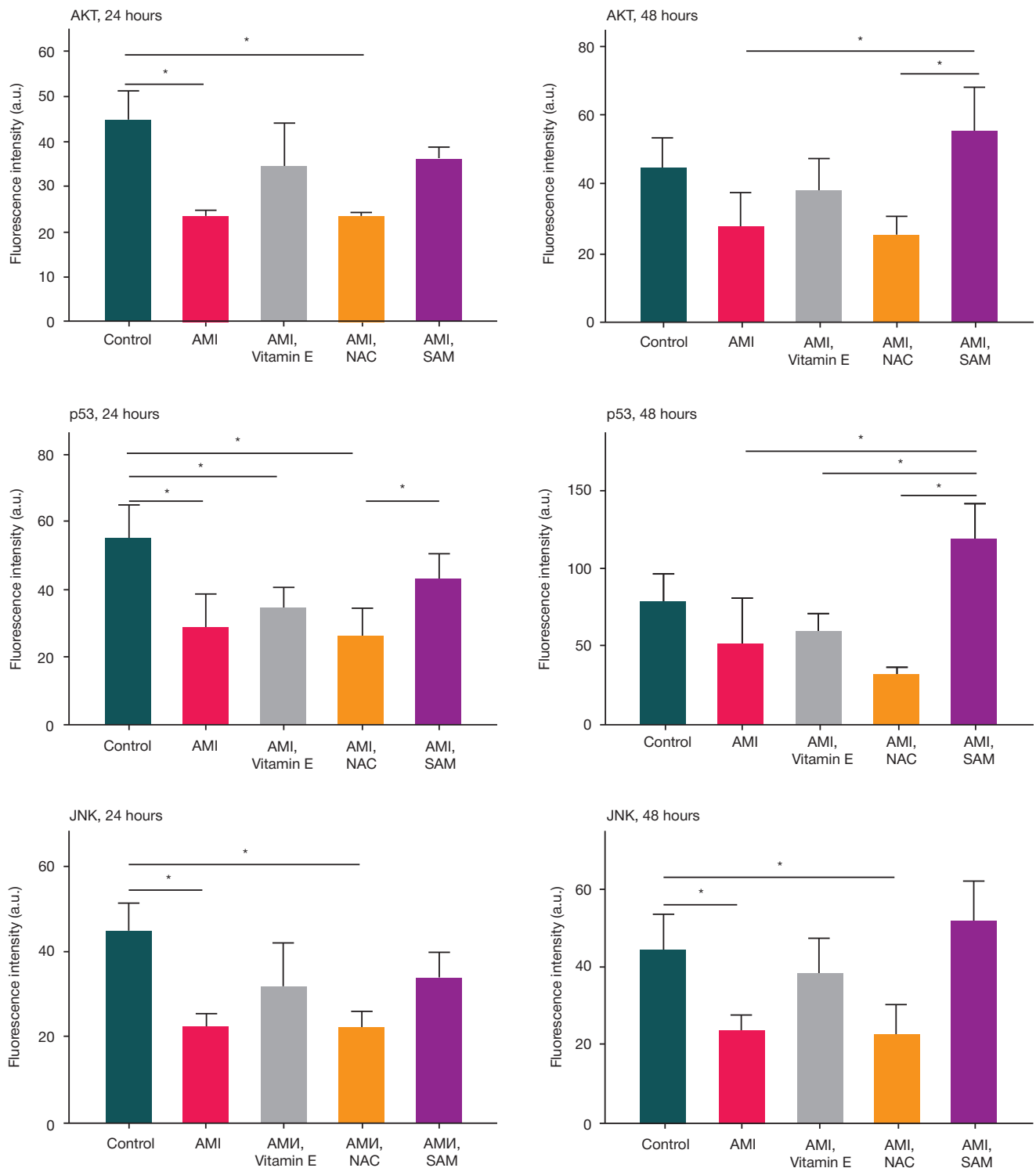


Fig. 2. Phosphorylated AKT kinase (Ser473), p53 protein (Ser46), JNK kinase (Thr183/Tyr185) fluorescence intensity in the *HepaRG* cell lysates after exposure to amiodarone at a concentration of 10 μ M (AMI), and against the effects of 100 μ M of vitamin E, 100 μ M of N-acetylcysteine (NAC), 100 μ M of S-adenosylmethionine (SAM) during the 24- and 48-hour incubation. * — $p < 0.05$

the atmosphere of CO₂ incubator (5% CO₂) at a temperature of 37 °C and saturation humidity.

The xCELLigence RTCA system (ACEA; USA) was used to define amiodarone cytotoxicity. Cell index, being the indicator of cell viability, was calculated with the RTCA Software 2.0 (ACEA; USA) based on the analysis of changes in impedance over time. Prior to the experiment, the impedance of the growth medium in the absence of cells was measured. Next, a total of 10,000 *HepaRG* cells were added to each well of the 96-well plate and cultured for 24 hours. After that amiodarone (at a

concentration of 0.1–100 μ M) and studied antioxidants (vitamin E, NAC and SAM at a concentration of 100 μ M) were added to the medium. Cells cultured in complete growth medium were used as a control.

Molecular markers of cell damage were assessed with the Bio-Plex 200 suspension array system (Bio-Rad; USA). Markers of apoptosis were assessed in cell lysates, and markers of hepatocyte damage were assessed in conditioned media. In order to obtain conditioned media and lysates, human hepatoma cells of the *HepaRG* cell line were added to the 24-well plate

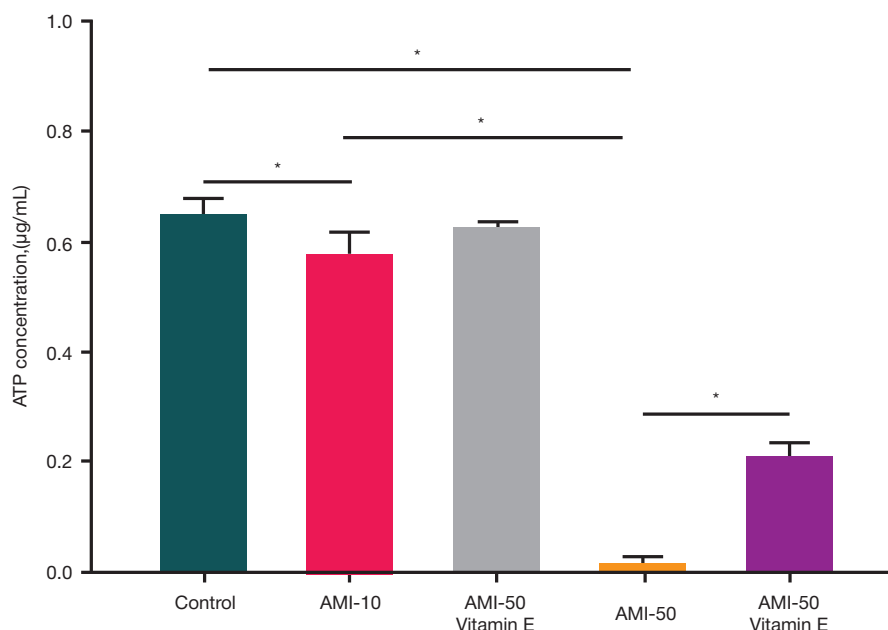


Fig. 3. ATP levels in HepaRG cell lysates after the 24-hour exposure to amiodarone at a concentration of 10 µM (AMI-10) and 50 µM (AMI-50) against the background of exposure to vitamin E. * — $p < 0.05$

(400,000 cells per well) and cultured in the complete growth medium. The next day after passage, the medium was replaced with the medium, containing amiodarone at a concentration of 10 µM together with the studied antioxidants at a concentration of 100 µM. The samples were incubated for 24 and 48 hours. Each experimental concentration of the studied substances was added in at least three iterations. The following kits were used for the multiplex analysis: MILLIPLEX MAP Human Liver Injury Magnetic Bead Panel (Cat. HLINJMAG-75K; Merck, USA), MILLIPLEX MAP Human Early Apoptosis Magnetic Bead 6-Plex Kit (Cat. 48-669MAG; Merck, USA), and Bio-PlexPro™ Human Cytokine 27-plex Screening Panel (Cat. M500KCAFOY; Bio-Rad, USA).

ATP was assessed in the HepaRG cell lysates using the ATP Bioluminescent Assay Kit (Cat. FLASC; Sigma-Aldrich, USA). The intensity of luminescent emission was measured with the FLx800 Microplate Fluorescence Reader (BioTek; USA). Data preprocessing was performed with the Gen5 1.10 software (BioTek; USA).

Statistical analysis and charting were carried out using the SigmaPlot 12.5 software (SystatSoftware Inc; USA). The sample was tested for normality with the Shapiro–Wilk test. The significance of differences between means was assessed by univariate analysis of variance for normal distributions and Kruskal–Wallis test for distributions other than normal. The differences were considered significant when $p < 0.05$. The results were presented as mean \pm standard deviation.

RESULTS

The IC_{50} value, calculated based on the monitoring of amiodarone cytotoxicity for the human hepatoma cells of the HepaRG cell line, was 3.5 µM (Fig. 1). The use of hepatoprotective agents, possessing antioxidant activity, resulted in significantly increased cell index compared to amiodarone. Thus, the IC_{50} value obtained for amiodarone in the presence of vitamin E was 19 µM; the value for N-acetylcysteine was 24 µM, and the value for S-adenosylmethionine was 22 µM.

The levels of some kinases involved in the cell cycle regulation in cell lysates, and the levels of intracellular enzymes in the conditioned media were analyzed in order to

assess the mechanisms of cytotoxicity. The exposure of cells to amiodarone (10 µM) during 24 and 48 hours resulted in reduced levels of phosphorylated AKT and JNK kinases together with the p53 protein (Fig. 2). In other words, amiodarone contributes the decreased activity of enzymes, involved in the cell cycle regulation, and triggers cell death. This can explain high cytotoxicity of amiodarone. The levels of AKT and JNK kinase active forms in the cell lysates after treating with amiodarone (10 µM) in the presence of vitamin E or S-adenosylmethionine were the same as in control samples.

The study found that amiodarone concentrations of 10 and 50 µM caused a significant decline in the ATP levels in the HepaRG cell lysates; the decline in the ATP levels was dose dependent (Fig. 3). Vitamin E provided a significant increase in the intracellular ATP levels upon amiodarone cell treatment.

Assessment of extracellular enzyme levels showed that there was no significant increase in the levels of arginase 1 after treating cells with amiodarone (Fig. 4). After the 24 hour incubation with amiodarone, elevated levels of malate dehydrogenase 1, glutathione S-transferase and sorbitol dehydrogenase in the conditioned medium were observed (Fig. 4, 5). The levels of 5'-nucleotidase significantly increased in 48 hours. In the presence of vitamin E and S-adenosylmethionine, decline in the levels of MDH1, GSTα and SDH intracellular enzymes in the conditioned medium was detected.

Analysis of cytokine levels after the HepaRG line cells' incubation in the medium with amiodarone (10 µM) showed the significant and the most prominent increase in the levels of IL1β, IL6, IL8, IFNγ, TNFα pro-inflammatory factors, resulting from exposure to amiodarone (Fig. 6). At the same time, elevated levels of anti-inflammatory cytokine IL10 were noted. The effects of antioxidants were reflected in the reduced levels of the assessed cytokines in the conditioned media.

DISCUSSION

Side effects of amiodarone were reported in the late 20th century, after the beginning of its widespread use as an antiarrhythmic agent [19]. Based on its structure, amiodarone belongs to the class of cationic amphiphilic substances. Moreover, amiodarone has a long half-life in the terminal elimination phase, which can

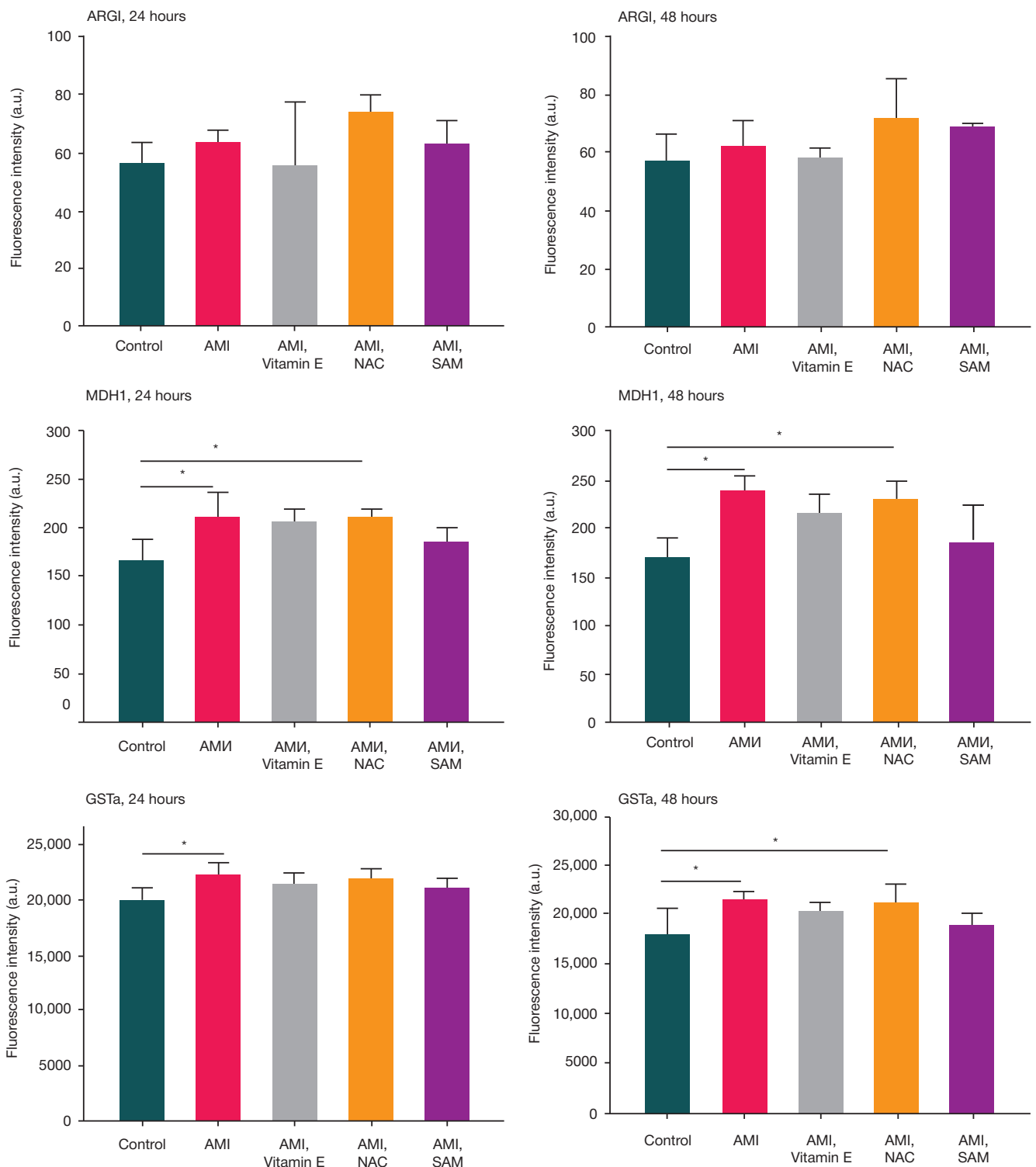


Fig. 4. Arginase 1 (ARG1), malate dehydrogenase 1 (MDH1), glutathione S-transferase (GSTα) fluorescence intensity in the conditioned medium of the *HepaRG* cells after exposure to amiodarone at a concentration of 10 μM (AMI), and against the effects of 100 μM of vitamin E, 100 μM of N-acetylcysteine (NAC), 100 μM of S-adenosylmethionine (SAM) during the 24- and 48-hour incubation. * — $p < 0.05$

be up to 150 days [8]. Amiodarone and its metabolites can accumulate in lungs, skeletal muscles, adipose tissue, liver, and exhibit toxic effects. Meanwhile, the degree of liver injury varies significantly from slightly increased serum transaminase levels to acute liver failure [4].

The long-term oral (per os) administration of amiodarone results in accumulation of fatty acids and polar lipids in hepatocytes due to inhibition of phospholipase A and enzymes for β -oxidation of fatty acids. Accumulation of amiodarone in lysosomal lipid bilayers has been reported, which interferes with

the normal intracellular degradation of membrane phospholipids and results in phospholipidosis [8, 20]. Accumulation of triglycerides and lipid droplets after the 14-day incubation of the human hepatoma *HepaRG* cell line with amiodarone at a concentration of 20 μM has been reported [21].

However, the mechanisms of amiodarone-induced hepatotoxicity are still poorly understood.

We defined the dose-dependent cytotoxic effects of amiodarone on the *HepaRG* cell line for three days and calculated the IC_{50} value, which was 3.5 μM . Earlier, amiodarone

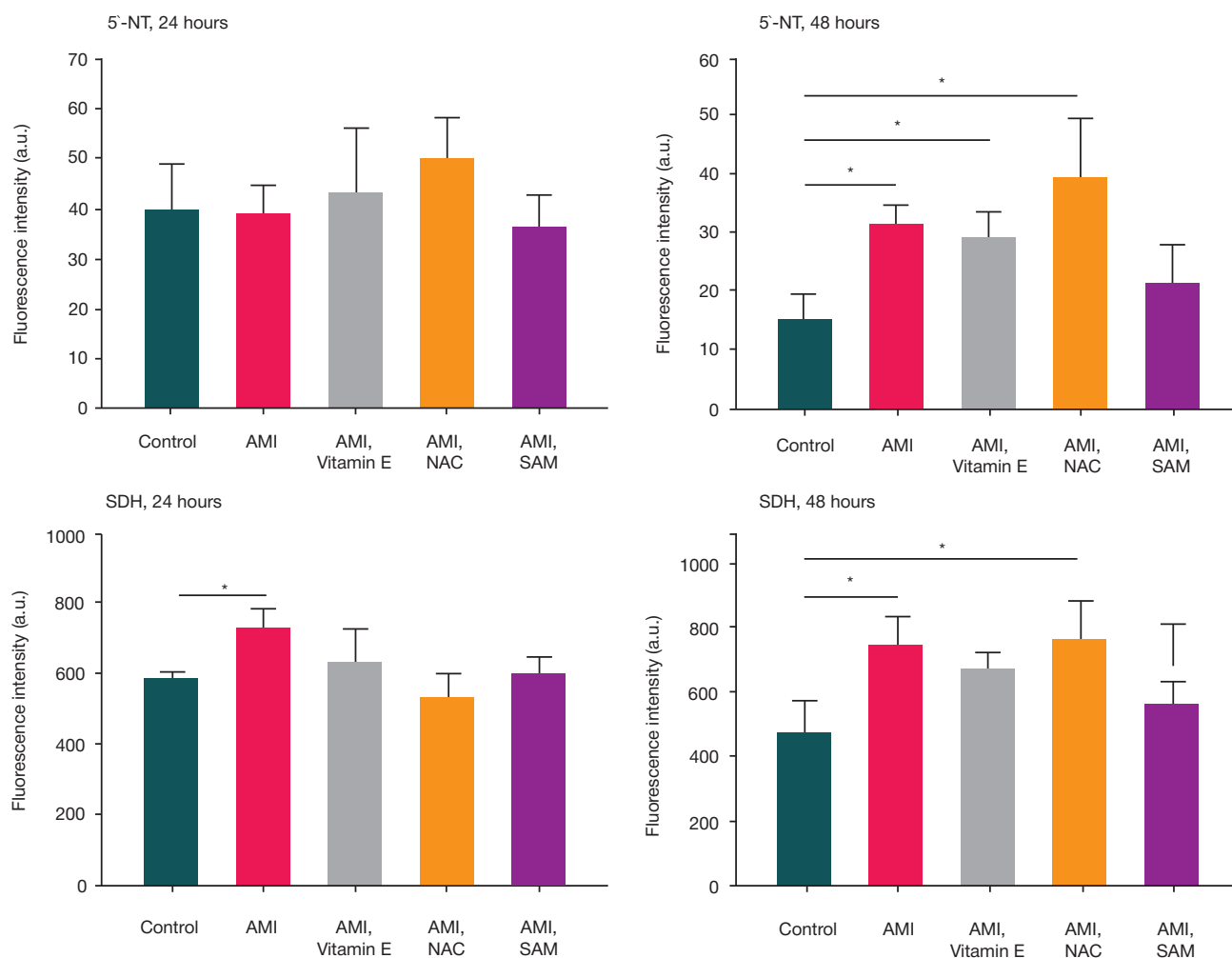


Fig. 5. Nucleotidase (5'-NT), sorbitol dehydrogenase (SDH) fluorescence intensity in the conditioned medium of the *HepaRG* cells after exposure to amiodarone at a concentration of 10 μ M (AMI), and against the effects of 100 μ M of vitamin E, 100 μ M of N-acetylcysteine (NAC), 100 μ M of S-adenosylmethionine (SAM) during the 24- and 48-hour incubation. * — $p < 0.05$

cytotoxicity was demonstrated during the study, involving the *HepG2* cell line, where the IC_{50} value was 105 μ M [7]. However, *HepaRG* cells show higher expression of the cytochrome P450 system enzymes compared to the *HepG2* cell line. Amiodarone metabolites (mono- and di-N-desethylamiodarone), formed after transformation by cytochromes, can exhibit higher hepatotoxicity compared to amiodarone [18].

The increased intracellular levels of active (phosphorylated) forms of kinases, involved in the cell cycle regulation, indicate the type of cell death and the pathways of cell death process activation [22]. During our study, exposure to amiodarone resulted in reduced levels of active forms of AKT and JNK kinases and p53 protein. This indicates that mitochondrial damage is the main mechanism, triggering cell death when exposed to amiodarone. The levels of active initiator caspase-8 are also reduced after threatening cell culture with amiodarone, which makes it possible to speak of necrotic cell death instead of apoptosis.

It is known that intracellular levels of ATP are the measure of cell viability [23]. Depletion of intracellular ATP reserves is considered one of the characteristic features of necrosis [24]. The detected decline in intracellular ATP levels, and the release of intracellular enzymes into conditioned medium also demonstrate the necrotic cell death.

It has been also found that exposure to amiodarone results in elevated levels of pro-inflammatory cytokines IL1 β , IL6, IL8, IFN γ , TNF α and anti-inflammatory cytokine IL10 in the conditioned medium.

Intracellular enzymes, cytokines and danger associated molecular patterns (DAMPs), released when the cells are damaged, activate the innate immunity cells [25]. Activated Kupffer cells and neutrophils together with other resident liver cells secrete various cytokines, which can result in triggering apoptosis through death receptors and the development of inflammatory response [22]. Thus, the exposure to amiodarone may cause both necrosis and apoptosis, which is consistent with the previously published data on cytostatic effects of amiodarone [26, 27].

Thus, the human hepatoma *HepaRG* cell line model has shown that cell death, occurring after the 48-hour exposure to amiodarone, is the result of necrosis.

It is anticipated that oxidative stress is one of the root causes of amiodarone cytotoxicity [7]. Earlier, with the use of various cell models (primary rat hepatocytes, *HepG2*, *L929* cells), it has been shown that antioxidants protect cells against cytotoxic effects of amiodarone [7, 11, 28]. We have assessed the impact of compounds with antioxidant properties on the amiodarone-induced cytotoxicity in the human hepatoma cells of the *HepaRG* cell line. Vitamin E, S-adenosylmethionine and N-acetylcysteine reduce cytotoxicity of amiodarone and increase the IC_{50} value. Vitamin E and S-adenosylmethionine ensure amiodarone-induced reduction of the key pro-inflammatory IL1 β and IL6 cytokine secretion.

The listed above results confirm that amiodarone is able to induce oxidative stress in cells. The studied vitamin E,

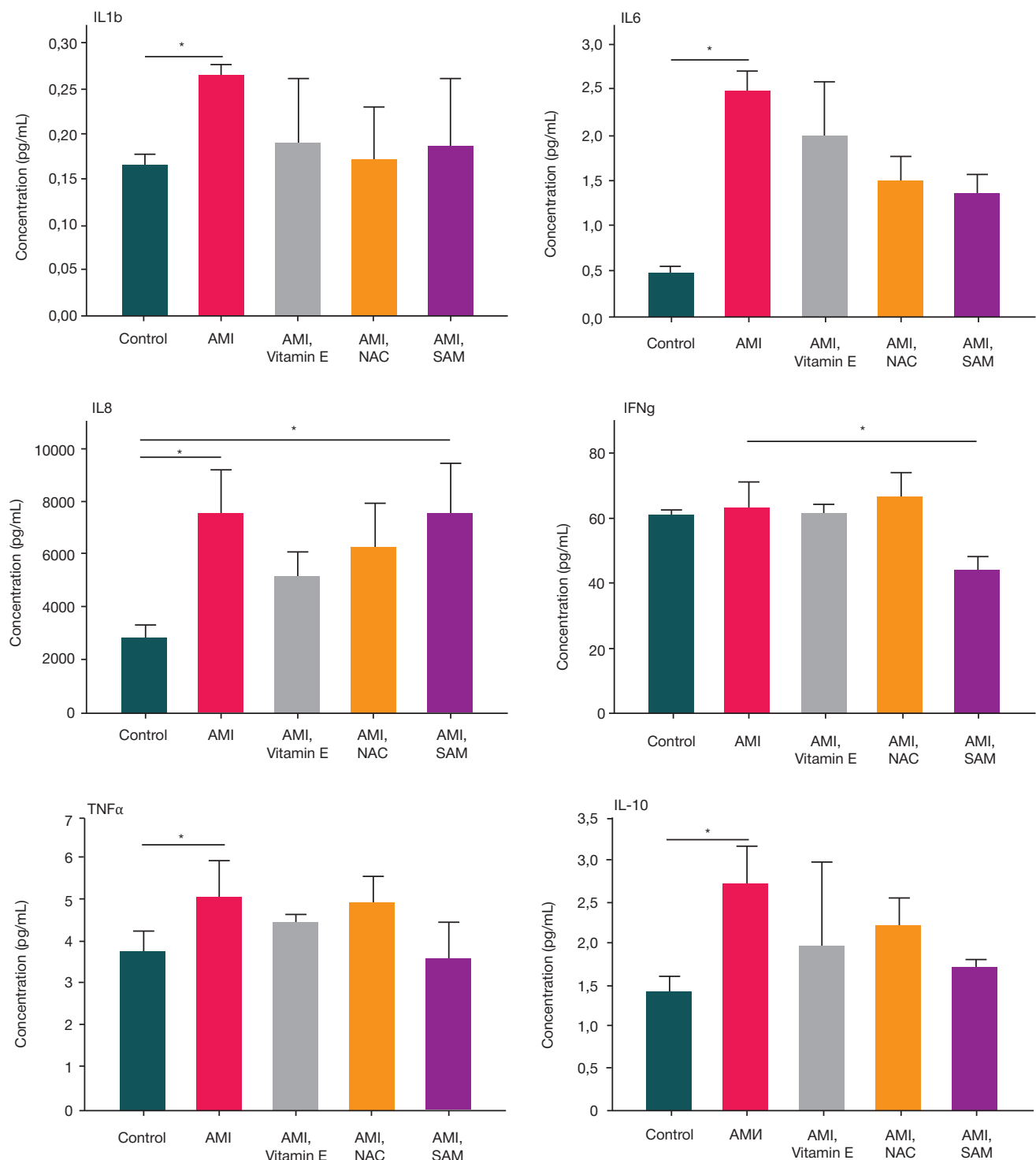


Fig. 6. Concentration of cytokines IL1 β , IL6, IL8, IFN γ , TNF α and IL10 in the conditioned medium of HepaRG cells, incubated during 48 hours and exposed to amiodarone at a concentration of 10 μ M (AMI), and against the effects of 100 μ M of vitamin E, 100 μ M of N-acetylcysteine (NAC), 100 μ M of S-adenosylmethionine (SAM). * — $p < 0.05$

S-adenosylmethionine and N-acetylcysteine are the registered medicinal products and could be recommended as hepatoprotective agents during amiodarone therapy.

CONCLUSIONS

Amiodarone has a cytotoxic effect on the human hepatoma cells of the HepaRG cell line (IC_{50} 3.5 μ M). Cell death occurs with underlying reduction in the levels of active forms of factors,

involved in cell cycle regulation, such as AKT, JNK kinases, and p53 protein. The effects of amiodarone result in cytolysis accompanied by the increase in the levels of intracellular enzymes (MDH1, GST α , SDH and 5'-NT) in the conditioned medium. The studied compounds possessing antioxidant properties, Vitamin E, N-acetylcysteine and S-adenosylmethionine, reduce amiodarone-induced cytotoxicity in the human hepatoma cells of the HepaRG cell line and can be considered as potential hepatoprotective agents during treatment with amiodarone.

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