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Protective effect of amiodarone but not N-desethyl-amiodarone on postischemic hearts through the inhibition of mitochondrial permeability transition

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Abbreviations: $\Delta\psi$: mitochondrial membrane potential; CsA: Cyclosporin A; ROS: reactive oxygen species; FCCP: carbonylcyanide *p*-trifluoro-methoxyphenylhydrazone; MTT^+: (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide).

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Abstract

Amiodarone is a widely used and potent antiarrhytmic agent which is metabolised to desethylamiodarone. Both amiodarone and its metabolite possesses antiarrhytmic effect and both compounds can contribute to toxic side-effects. Here, we compare the effect of amiodarone and desethylamiodarone on mitochondrial energy metabolism, membrane potential, permeability transition and on mitochondria related apoptotic events. Amiodarone but not desethylamiodarone protects the mitochondrial energy metabolism of the perfused heart during ischemia in perfused hearts. At low concentrations, amiodarone stimulated state 4 respiration due to an uncoupling effect, inhibited the Ca^{2+}-induced mitochondrial swelling, while it dissipated the $\Delta\Psi$, and prevented the ischemia-reperfusion induced release of AIF. At higher concentrations, amiodarone inhibited the mitochondrial respiration and simulated a CsA independent mitochondrial swelling. In contrast to these, desethylamiodarone did not stimulate state 4 respiration, did not inhibit the Ca2+-inducedmitochondrial permeability transition, did not induce the collapse of $\Delta\Psi$ in low concentrations and did not prevent the nuclear translocation of AIF in perfused rat hearts, but it induced a CsA independent mitochondrial swelling at higher concentration like amiodarone. That is, desethylamiodarone lack the protective effect of amiodarone seen at low concentrations like the inhibition of calcium induced mitochondrial permeability transition and inhibition of the nuclear translocation of the proapoptotic AIF. On the other hand, both amiodarone and DEA at higher concentration induced a CsA independent mitochondrial swelling resulting in apoptotic death which explain their extracardiac toxic effect.

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Amiodarone (2-butyl-3-benzofuranyl 4-[2-(diethylamino)-ethoxy]-3,5-diiodophenyl-ketone hydrochloride), is one of the most effective antiarrhythmic drugs, and is frequently used in the clinical practice for treating ventricular and supraventricular arrhythmias. It is a class III antiarrhythmic agent, prolonging action potential duration which effect may involve blocking of β-adrenergic receptors, sodium channels and L-type calcium channels (Singh et al., 1970; Nokin et al., 1983; Nattel et al., 1987; Varro et al., 1996). It may also have a role in preventing mortality following myocardial infarction (Singh, 1996; Julian et al., 1997). Despite its effective antiarrhytmical properties, the use of amiodarone is often limited by its toxic side effects, including thyroid dysfunction, liver and pancreas fibrosis (Amico et al., 1994; Martin and Howard, 1985). However the most severe adverse effect of the drug is pulmonary fibrosis, occurring in up to 13 % of the patients receiving the amiodarone in doses higher than 400 mg day^{-1} (Martin and Rosenow, 1988). The etiology of the amiodarone induced pulmonary toxicity is unknown.

Desethylamiodarone, the major metabolite of amiodarone also has antiarrhythmic activity, significantly increasing the action potential duration (Class III antiarrhythmic effect) and decreasing the maximum rate of depolarization (Class I antiarrhythmic effect) at clinically relevant concentrations (Pallandi and Campbell, 1987). This antiarrhythmic effect was shown to be in part dependent on gene expression rather than a direct effect on cell-membrane channels or receptors (Drvota et al., 1998). Desethylamiodarone rapidly accumulates in the lung following amiodarone treatment, sometimes in higher concentrations than amiodarone itself (Daniels et al., 1989). It proved to be more toxic than amiodarone in pulmonary cell types (Ogle and Reasor, 1990), suggesting, that desethylamiodarone may play an important role in the development of the amiodarone-treatment induced pulmonary fibrosis.

Whereas numerous studies support the toxic effect of amiodarone and it's metabolite desethylamiodarone on extracardiac tissues such as lung, thyroid gland, liver and pancreas, reports on the effects of amiodarone on cardiac function have been variable. Some emphasized its beneficial effect on cardiac functions and arrhythmia following ischemia and reperfusion (Nokin et al., 1987; Vander-Elst et al., 1990), however other reports found it worsened the damages to the mitochondrial energy metabolism caused by ischemia-reperfusion (Moreau et al., 1999). Moreover there is only limited data about the effect of desethylamiodarone on ischemic heart. We have previously demonstrated that amiodarone increases the level of the high-energy phosphate metabolites by directly influencing the mitochondria following ischemia and reperfusion in perfused rat hearts (Varbiro et al., 2003). Myocardial ischemia can lead to a severe arrhythmia, that may necessitate amiodarone administration, therefore it is important to assess the effect of amiodarone and it's metabolite desethylamiodarone on postischemic heart. Since ischemia most of all deteriorates the energy metabolism of the mitochondria, it is especially important to evaluate their effect on the mitochondrial functions. In addition to their critical role in energy metabolism, mitochondria are known to regulate cell viability as well as cell death (Kroemer and Reed, 2000) through pathways like mitochondrial permeability transition with the dissipation of the mitochondrial membrane potential ($\Delta\psi$) and release of proapoptotic factors like cytochrome c or the apoptosis inducing factor (AIF), the disruption of ATP production, and the generation of reactive oxygen species (ROS).

In this study we aimed to verify that in low concentrations amiodarone but not desethylamiodarone has cardioprotective features, while at higher concentrations they both exhibit toxic properties especially to extracardiac tissues. Therefore we examined the effect of amiodarone and desethylamiodarone on mitochondrial energy-metabolism during ischemia-reperfusion of Langendorff-perfused rat hearts by *in situ* ^{31}P-NMR spectroscopy. We assessed

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and compared the cytotoxicity of amiodarone and desethylamiodarone on cardiomyocytes as well as on extracardiac cells in culture. In an attempt to reveal the underlying mechanism of amiodarone and desethylamiodarone in these paradigms, we studied their effect on the translocation of the proapoptotic AIF from the mitochondria to the nucleus of the perfused heart cells, their direct mitochondrial effects including the induction of ROS production, the effect on the mitochondrial membrane potential ($\Delta\Psi$) and the opening of mitochondrial permeability transition pore (PTP), and their effect on the mitochondrial respiration.

Materials and Methods

Chemicals. CsA was from Biomol Research Labs. Inc. (Plymouth Meeting, PA); Rhodamine 123 (Rh123), Dihydrorhodamine123 (DRh123) was from Molecular Probes (Eugene, OR), Desethylamiodarone (DEA) was a gift from Professor Varro; all other compounds were from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Animals. Wistar rats were purchased from Charles River Hungary Breeding LTD (Budapest, Hungary). The animals were kept under standardized conditions; tap water and rat chow were provided *ad libitum*. Animals were treated in compliance with approved institutional animal care guidelines.

Cell culture. PANC-1 human pancreatic epithelioid carcinoma cells, BRL 3A rat liver cells, H9C2 mouse cardiomyocytes and WRL 68 human liver cells were from American Type Culture Collection (Manassas, VA). The cell lines were grown in humidified 5 % CO_2 atmosphere at 37 °C. The cells were maintained as monolayer adherent culture in Dulbecco's Modified Eagle's Medium containing 1% antibiotic-antimycotic solution (Sigma) and 10 % fetal calf serum. Cells were passaged at intervals of 3 days.

Heart Perfusion. Pretreatment with amiodarone or desethylamiodarone was done exactly as described previously (Nokin et al., 1987). Briefly, adult male Wistar rats (weighing 300-350 g; $n = 4$ in each group), were anesthetized with 200 mg/kg ketamine i.p., then either 400 μl/kg isotonic saline (sham-operated), 20 mg/kg amiodarone (400 μl/kg Cordarone, Sanofi-Synthelabo Budapest, Hungary) or 20 mg/kg desethylamiodarone was injected into the femoral vein. Thirty minutes after the treatment, the animals were heparinized with sodium heparin (100 IU/rat i.p.), their hearts were excised and merged into ice-cold Krebs-Henseleit buffer. Hearts were perfused via the aorta according to the Langendorff method at a constant pressure of 70 mm Hg, at 37 °C as described before (Szabados et al., 1999). The perfusion medium was a modified phosphate-free Krebs-Henseleit buffer consisting of 118 mM NaCl, 5

mM KCl, 1.25 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM glucose, and 0.6 mM octanoic acid. The perfusate was adjusted to pH 7.4 and bubbled with 95% O_2, 5% CO_2 through a glass oxygenator. After a washout (non-recirculating period of 15 min), hearts were perfused under normoxic conditions for 10 minutes; the flow was subsequently discontinued for 30 minutes by inflating a balloon (ischemia), which was followed by 15 minutes of reperfusion. Levels of high-energy phosphate intermediates were monitored in the magnet of a ^{31}P NMR spectroscope during the entire perfusion.

Determination of drug concentrations. The concentrations of amiodarone and N-desethylamiodarone in the plasma and in the heart mitochondria of rats 30 minutes after the pretreatment by either 20 mg/kg amiodarone or desethylamiodarone (*n* = 3 in each group) were determined by a high-pressure liquid chromatographic procedure as described previously (Kannan et al., 1987).

NMR Spectroscopy. NMR spectra were recorded with a Varian ^{UNITY}INOVA 400 WB instrument. ^{31}P measurements (161.90 MHz) of perfused hearts were run at 37 °C in a Z·SPEC 20-mm broadband probe (Nalorac Co. Martinez , CA), applying WALTZ proton decoupling (γB_2 = 1.6 kHz) during the acquisition only. Field homogeneity was adjusted by following the ^1H signal ($w_{1/2}$ = 10-15 Hz). Spectra were collected with a time resolution of 3 min by accumulating 120 transients in each free induction decay. 45° flip angle pulses were used after a 1.25-s recycle delay, and transients were acquired over a 10-kHz spectral width in 0.25 s, and the acquired data points (5000) were zero-filled to 16384.

Under the above circumstances, relative concentrations of the species are proportional to the corresponding peak areas, since interpulse delays exceeded 4 to 5 times the T_1 values of the metabolites that were analyzed in the ^{31}P experiments. Data were acquired from 5 independent experiments for sham-operated and amiodarone-treated groups each.

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Western blot analysis of AIF. Myocardial specimens (n = 3 in each group) were snap-frozen immediately after surgical removal or at the end of the Lagendorff-perfusion experiment and stored at -80 °C until analyzed. Frozen heart muscle samples were mechanically homogenized in liquid nitrogen and the nuclear fraction was prepared as described (Schmitt et al., 2002). Equal amounts of nuclear extracts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12% gel) and then transferred to a nitrocellulose filter. Membranes were blocked using 5% dry milk. The blot was probed with monoclonal antibodies against AIF (Oncogene; Boston, MA.) overnight at the temperature of 4°C. The antigen–antibody complex was visualized on an X-ray film using secondary antibodies linked to horseradish peroxidase (1:1000; Sigma, St. Louis, MO) and a chemiluminescence kit (ECL; Amersham, Piscataway, NJ.). The experiments were repeated three times for each group and the results are demonstrated by photomicrograph of a representative blot.

Cell viability assay. PANC 1, BRL-3A, WRL 68 and H9C2 cells were seeded into 96-well plates at a starting density of 2.5 x 10^4 cell/well and cultured overnight in humidified 5 % CO_2 atmosphere at 37 °C. The following day, amiodarone or desethylamiodarone at the indicated concentrations were added to the medium. Forty-eight hours later, 0.5 % of the water soluble mitochondrial dye, (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; MTT^+) was added. Incubation was continued for 3 more hours, the medium was removed, and the water insoluble blue formosan dye formed stochiometrically from MTT^+ was solubilized by acidic isopropanol. Optical densities were determined by an Anthos Labtech 2010 ELISA reader (Wien, Austria) at 550 nm wavelength. All experiments were run in at least 4 parallels and repeated 3 times.

Isolation of mitochondria. Liver and heart mitochondria were prepared according to standard protocol (Schneider and Hageboom, 1950). Only difference among the organs were in the primary homogenization protocol; liver was squeezed through a liver press, while

pooled heart tissue from 5 rats was minced with a blender. All isolated mitochondria were purified by Percoll gradient centrifuging (Sims, 1990).

Mitochondrial oxygen consumption was detected by a Clark electrode. Briefly, isolated mitochondria were suspended in 20 mM Tris buffer, pH 7.4 containing 20 mM KCl, 220 mM mannitol, 70 mM sucrose and 1 mM EGTA. The mitochondrial respiration (state 4) was assessed with either 10 mM pyruvate (Complex I respiration) or 10 mM succinate (in the presence of 1 μM rotenone; Complex II respiration) and different concentrations of amiodarone or desethylamiodarone. Before performing experiments, RCR (respiratory control ratio) values of the mitochondria were determined and were found to be in the range of 2.1 ± 0.4 and 3.7± 0.6 for the mitochondria from the heart or liver respectively (Elimadi et al., 1997; Garcia et al., 1997). The results are expressed as mean values ± S.E.M. from three independent experiments.

Mitochondrial permeability transition was monitored by following the accompanying large amplitude swelling via the decrease in absorbance at 540 nm (Cassarino et al., 1999) measured at room temperature by a Perkin-Elmer fluorimeter (London, UK) in reflectance mode. Briefly, mitochondria at the concentration of 1 mg protein/ml were pre-incubated in the assay buffer (70 mM sucrose, 214 mM mannitol, 20 mM N-2-hydroxyethyl piperasine-N'-2-ethanesulfonic acid, 5 mM glutamate, 0.5 mM malate, 0.5 mM phosphate) containing the studied substances for 60 seconds. Mitochondrial permeability transition was induced by the addition of either 60 μM of Ca^{2+}, or amiodarone or desethylamiodarone at the indicated concentration. Decrease of E_{540} was detected for 20 min. The results are demonstrated by representative original registration curves from at least five independent experiments, each repeated three times using mitochondria prepared from the same liver or pool of rat hearts respectively. The results are demonstrated by representative original registration curves from

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five independent experiments, each repeated three times using mitochondria prepared from the same liver or pool of rat hearts respectively.

Mitochondrial membrane potential was monitored by fluorescence of Rh123, released from the mitochondria following the induction of permeability transition at room temperature by using a Perkin-Elmer fluorimeter (London, UK) at an excitation wavelength of 495 and an emission wavelength of 535 nm. Briefly, mitochondria at the concentration of 1 mg protein/ml were pre-incubated in the assay buffer (70 mM sucrose, 214 mM mannitol, 20 mM N-2-hydroxyethyl piperasine-N'-2-ethanesulfonic acid, 5 mM glutamate, 0.5 mM malate, 0.5 mM phosphate) containing 1 μM Rh123 and the studied substances for 60 seconds. Alteration of the mitochondrial membrane potential ($\Delta\psi$) was induced by the addition of either 60 μM of Ca^{2+}, or amiodarone or desethylamiodarone at the indicated concentration. Changes of fluorescence intensity were detected for 4 min. The results are demonstrated by representative original registration curves from five independent experiments, each repeated three times using mitochondria prepared from the same liver or pool of rat hearts respectively.

The determination of ROS-formation. ROS formation was detected as described (Varbiro et al., 2001) by the fluorescence of Rh123 formed by ROS-induced oxidation of the non-fluorescent DRh123 in situ at an excitation wavelength of 495 nm and an emission wavelength of 535 nm by a Perkin-Elmer fluorimeter (London, UK). The ROS-induced oxidation of N-acetyl-8-dodecyl-3,7-dihydroxyphenoxazine forms N-acetyl-8-dodecyl-resorufin (resorufin) which exhibits strong red fluorescence. This product is well retained in living cells and organelles by virtue of its lipophilic tail, making it possible to detect ROS production in the lipid phase. The method is the same as described above except for changing the excitation wavelength to 578 nm and the emission wavelength to 597 nm. ROS-formation was calculated from the slope of the registration curves

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Statistical analysis. Data were presented as means ± S.E.M. For multiple comparisons of groups ANOVA was used. Statistical difference between groups was established by paired or unpaired Student's *t* test, with Bonferroni correction.

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Results

Effect of amiodarone and desethylamiodarone on the energy metabolism during ischemia-reperfusion in perfused hearts. Concentrations of high-energy phosphate intermediates were monitored during ischemia-reperfusion in Langendorff-perfused hearts by using ^{31}P NMR spectroscopy. In order to study the effect of amiodarone or desethylamiodarone on the energy-metabolism of the perfused hearts, a single i.v. injection of 20 mg/kg amiodarone or 20 mg/kg desethylamiodarone was administered to a group of rats, 30 min before the start of the heart-perfusion, a protocol that was previously reported to be optimal for cardioprotection by amiodarone. This resulted in a mitochondrial amiodarone and desethylamiodarone concentrations of 2.42 ± 0.29 and 2.69 ± 0.28 μg/mg mitochondrial protein respectively (Table 2). Thirty minutes of global ischemia induced the disappearance of creatine phosphate (Fig. 1A) and ATP (Fig. 1B), and a gradual increase of inorganic phosphate (Fig. 1C) signal. During reperfusion, all hearts restarted working, while creatine phosphate concentrations in hearts of sham-operated animals recovered to 35% ± 4 of their normoxic level. Amiodarone pretreatment resulted in a significantly higher recovery ($p < 0.001$) of creatine phosphate concentrations during the ischemia-reperfusion cycle (74% ± 6, Fig. 1A) when compared to control (35% ± 4, Fig. 1A), while the pretreatment with desethylamiodarone had no significant effect on the recovery of creatine phosphate concentration following ischemia (32% ± 4, Fig. 1A). In addition, amiodarone pretreatment slightly but significantly ($p < 0.05$) delayed the decrease of ATP concentrations during ischemia, while significantly ($p < 0.001$) improved the recovery of ATP level (45% ± 6 vs. 28% ± 5, Fig. 1B). The pretreatment with desethylamiodarone had no significant effect on the recovery of ATP level (28% ± 5, Fig. 1B) when compared to control. Amiodarone pretreatment facilitated the significantly ($p < 0.001$) faster and more complete utilization of inorganic phosphate (31% ± 6 vs. 62% ± 6, Fig. 1C) during reperfusion. The pretreatment

with desethylamiodarone did not prove to be effective in the utilization of inorganic phosphate (63% ± 4, Fig. 1C).

Effect of amiodarone and desethylamiodarone on apoptosis inducing factor (AIF) translocation in perfused hearts following ischemia-reperfusion. The release and nuclear translocation of AIF was detected from the nucleus of Langendorff-perfused rat heart tissue following ischemia and reperfusion by Western blot. The results, demonstrated by a photomicrograph of a representative blot, are presented in Fig. 2. The removal and normoxic perfusion of the heart increases the level of AIF in the nucleus of the heart cell compared to the control (Fig. 2, lane 1 and 2), however this is further increased by 30 minutes of ischemia and 60 minutes of reperfusion (Fig. 2, lane 3). Pretreatment with a single i.v. injection of 20 mg/kg amiodarone prevented the increase in the level of AIF stimulated by ischemia-reperfusion (Fig. 2, lane 4). The pretreatment with a single i.v. injection of 20 mg/kg desethylamiodarone however does not have any attenuative effect on the increase of AIF level following ischemia-reperfusion (Fig. 2, lane 5).

Effect of amiodarone and desethylamiodarone on cultured cell lines. Viability of H9C2, BRL-3A, WRL-68 and PANC-1 cells exposed to different concentrations of amiodarone or desethylamiodarone for 48h were assessed by the MTT^{+} method. In each of the cell lines desethylamiodarone proved to be more toxic than amiodarone in a specific concentration range (Fig. 3). This concentration range seemed to be variable in the different cell lines. The significant difference ($p < 0.001$) between the toxicity of amiodarone and desethylamiodarone observed in H9C2 cardiomyocytes was in a concentration range between 10 to 60 μM (Fig. 3A). In the normal rat (BRL-3A) and human (WRL-68) liver cell lines the significant difference ($p < 0.001$ and $p < 0.01$ respectively) between the toxicity of the drugs appeared in a lower concentration range between 3 to 20 μM (Fig. 3A and 3B). In PANC-1 human pancreatic epithelioid carcinoma cells, significant difference ($p < 0.01$) between the

toxicity of amiodarone and desethylamiodarone was observed in a concentration range of 20 to 80 μM as revealed by the viability data (Fig. 3D). The various cell lines presented different sensitivity towards the toxicity of amiodarone or desethylamiodarone (Table 1). The drugs were shown to be the least toxic in H9C2 cardiomyocyte cells. BRL-3A and WRL-68 hepatocytes were much more sensitive to amiodarone or desethylamiodarone toxicity than the PANC-1 human pancreatic epithelioid carcinoma cells, however, BRL-3A and WRL-68 were normal, while PANC-1 was a cancer cell line.

When H9C2 cardiomyocytes were exposed to different concentrations of amiodarone, desethylamiodarone or FCCP for only 1h, the results detected by the formation of water insoluble blue formasan dye from the yellow mitochondrial dye, MTT^+ by the functionally active mitochondria reflect the respiratory state, rather than the number of viable cells. Amiodarone, up to the concentration of 30 μM, exerted an uncoupling effect as indicated by the increase of optical densities, similarly to that of the equimolar concentrations of FCCP, a widely used uncoupling agent (Fig. 4A). However, at the concentration of 100 μM, the stimulatory effect of amiodarone declined significantly ($p < 0.001$), when compared to equimolar concentrations of FCCP (114% ± 7 vs 141% ± 6, Fig. 4A). Desethylamiodarone did not exhibit any stimulatory effect on the respiration, when present in low concentrations, whereas it significantly ($p < 0.001$) decreased the MTT^+ formation, when compared to equimolar amount of amiodarone above the concentration of 10 μM (Fig. 4A). In WRL-68 liver cells, amiodarone, did not show any stimulatory effect up to the concentration of 30 μM, while it decreased the respiration, when present in higher concentration (Fig. 4B). The effect of both desethylamiodarone and FCCP on the respiration of WRL-68 were similar like in the case of H9C2, with desethylamiodarone showing inhibition, while FCCP manifesting an uncoupling effect above the concentration of 10 μM (Fig. 4B). The values of the optical densities in this concentration range for both desethylamiodarone and FCCP were

significantly different ($P < 0.001$) from those of the equimolar concentrations of amiodarone (Fig. 4B).

The effect of amiodarone and desethylamiodarone on the mitochondrial oxygen consumption. The oxygen consumption of isolated mitochondria (state 4 respiration) was measured by a Clark electrode, with 10 mM pyruvate (Complex I supported respiration) or 10 mM succinate in the presence of 1 μM rotenone (Complex II supported respiration) exposed to different concentrations of amiodarone or desethylamiodarone. In isolated rat heart mitochondria, at low concentrations of up to 6 μM in the case of Complex I and Complex II supported respiration, amiodarone did not have any significant effect, as compared to the control. In an intermediate concentration range of 6 to 30 μM amiodarone increased both the Complex I and Complex II supported oxygen consumption indicating an uncoupling effect, while, the drug gradually inhibited respiration at higher concentrations (Fig. 5A and 5B).

Desethylamiodarone did not have any significant effect on the mitochondrial oxygen consumption at low concentrations of up to 10 μM. Above this concentration it gradually inhibited the respiration supported by succinate and in the concentration above 30 μM the respiration supported by pyruvate, without presenting an uncoupling effect, as indicated by the absence of the stimulation of both Complex I and Complex II supported respiration in the concentration range of 6 to 30 μM. In case of the Complex I supported respiration, above the concentration of 30 μM, desethylamiodarone presented a similar rate of inhibition that was observed with equimolar concentrations of amiodarone (Fig. 5A). However in the case of Complex II supported respiration, above the concentration of 30 μM, desethylamiodarone presented a significantly higher rate of inhibition ($p< 0.01$) than the one observed by equimolar concentrations of amiodarone (Fig. 5B)

The effect of amiodarone or desethylamiodarone on isolated rat liver mitochondria was basically the same (data not shown).

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Effect of amiodarone and desethylamiodarone on permeability transition in isolated mitochondria. In order to demonstrate the direct effect of amiodarone and desethylamiodarone on the mitochondrial permeability transition, we monitored mitochondrial swelling from isolated, Percoll gradient purified rat liver mitochondria. High-amplitude swelling of the mitochondria due to permeability transition was monitored by the decrease of reflectance of 540 nm light.

In isolated liver mitochondria, the swelling induced by 60 μM of Ca^{2+} (Fig. 6A, line 2; Fig. 6B, line 2) was completely inhibited by 2.5 μM of CsA (Fig. 6A, line 3; Fig. 6B, line 3) or by 1 μM of FCCP (data not shown). Depending on its concentration, amiodarone had a biphasic effect on mitochondrial swelling. Up to the concentration of 10 μM, amiodarone inhibited the rapid swelling induced by Ca^{2+} in a concentration dependent manner (Fig. 6A, lines 4-7) with the IC_{50} of 3.9 ± 0.8 μM. The most pronounced inhibitory effect of amiodarone on the swelling induced by 60 μM of Ca^{2+} was at the concentration of 10 μM (Fig. 6A, line 7). At higher concentrations, amiodarone proved to be less effective in delaying the Ca^{2+}-induced swelling (Fig. 6A, line 8). In contrast to amiodarone, desethylamiodarone did not show any inhibitory effect on the mitochondrial permeability transition induced by 60 μM of Ca^{2+} up to the concentration of 10 μM (Fig. 6B, lines 4-8).

At concentrations above 10 μM, amiodarone induced mitochondrial swelling by its own (Fig. 7A, lines 5 and 7), that was not inhibited by 2.5 μM of CsA (Fig. 7A, lines 6 and 8). In contrast to 30 μM of amiodarone which developed swelling with a rate significantly slower than that of the Ca^{2+}-induced swelling, desethylamiodarone, at the concentration of 30 μM induced swelling with a rate similar to that of the Ca^{2+}-induced one (Fig. 7B, line 7). The swelling induced by desethylamiodarone was not inhibited by 2.5 μM of CsA (Fig. 7B, lines 6 and 8).

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The effect of amiodarone or desethylamiodarone on isolated rat heart mitochondria was basically the same (data not shown).

Effect of amiodarone and desethylamiodarone on membrane potential in isolated mitochondria Sixty μM Ca^{2+} caused the dissipation of $\Delta\Psi$, as detected by the release of the membrane potential sensitive dye, Rh123 from isolated liver mitochondria (Fig. 8.A, line 2). When the mitochondrial membrane was depolarized by Ca^{2+} in the presence of 2.5 μM CsA, after a transient depolarization lasting for about a minute, $\Delta\Psi$ returned to the value identical to the one before the addition of Ca^{2+} (Fig. 8.A, line 4). Ten μM of amiodarone depolarized the mitochondrial membrane in a similar extent as did the 60 μM of Ca^{2+}, (Fig. 8A, line 3), however it's depolarizing effect was not influenced at all by 2.5 μM of CsA (Fig. 8.A, line 5). Amiodarone caused a concentration dependent release of Rh123 from liver mitochondria with a calculated EC_{50} value of 4.2 ± 0.7 μM (Fig. 8.B, lines 2-6). In contrast to this, desethylamiodarone, up to the concentration of 10 μM did not induce the dissipation of $\Delta\Psi$ (Fig. 8.C, lines 2-5). However desethylamiodarone, at the concentration of 20 μM (Fig. 8.C, line 6) caused Rh123 release from the isolated mitochondria as did 20 μM of amiodarone (Fig. 8.B, line 6), 60 μM of Ca^{2+} (Fig. 8A, line 2), or as did 1 μM of FCCP (data not shown). The calculated EC_{50} value for desethylamiodarone was 16.3 ± 2.3 μM. The depolarizing effect of 20 μM of desethylamiodarone was not influenced at all by 2.5 μM of CsA (data not shown).

The effect of amiodarone and desethylamiodarone on mitochondrial ROS production. Since ROS formation can induce mitochondrial permeability transition, we studied the effect of amiodarone and desethylamiodarone on ROS production in isolated, Percoll gradient purified rat heart and liver mitochondria. ROS formation was measured by monitoring the green or red fluorescence of Rh123 or resorufin oxidized by the ROS from non-fluorescent DRh123 or N-acetyl-8-dodecyl-3,7-dihydroxyphenoxazine *in situ*. By virtue of its dodecyl

group, resorufin is localized in membranous regions and detect ROS formation in lipid phase while Rh123 fluorescence reflects to ROS levels in aqueous phase. Amiodarone and desethylamiodarone did not induce ROS production in either case in the concentration range of 1 μM to 100 μM (data not shown).

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Discussion

Both amiodarone and desethylamiodarone have damaging effect on extracardiac tissues as lung, thyroid, liver and pancreas (Amico et al., 1984; Martin and Howard, 1985; Card et al., 1998). Whereas desethylamiodarone has similar antiarrhythmic activity as amiodarone (Pallandi and Campbell, 1987), their effect on postischemic heart has not been compared. To assess this issue, we monitored real time *in situ* concentrations of ATP, creatine phosphate and inorganic phosphate during ischemia-reperfusion of Langendorff-perfused rat hearts by ^{31}P-NMR spectroscopy. A single i.v. injection of 20 mg/kg amiodarone or desethylamiodarone administered 30 min before the removal of the heart resulted in a rather low blood concentration of the drugs, whereas their concentrations in the heart mitochondria were 3.5 and 4.1 µM for amiodarone and desethylamiodarone respectively. Under these conditions, amiodarone had a significant protecting effect on the energy metabolism of perfused hearts during ischemia-reperfusion indicated by the faster and more complete recovery of the high-energy phosphate concentrations. However in the case of desethylamiodarone a similar dose and timing of pretreatment had no effect on the concentrations of creatine phosphate, ATP and inorganic phosphate during ischemia-reperfusion, when compared to the control (Fig. 1A-C). These indicate that desethylamiodarone does not contribute to amiodarone's cardioprotective effect.

To assess cytotoxicity of both amiodarone and desethylamiodarone on cardiomyocytes and extracardiac cells, we selected cell lines with comparable doubling time, originating from extracardiac tissues exhibiting an increased vulnerability toward amiodarone administration, namely normal liver and pancreas cancer cells. The effect of the drugs on thyroid cells has been reported previously (Di Matola et al., 2000), while normal pancreatic as well as non-fibroblast lung cell lines are not available. We performed viability studies utilizing the mitochondrial dye, MTT^{+}. The water insoluble blue formosan dye reflects not only the

number of viable cells, but also the number and respiratory function of mitochondria. This can explain the moderate increase of the optical density detected in the presence of low concentrations (10 to 20 μM) of amiodarone when compared to the control in H9C2 cardiomyocytes (Fig. 3A), which is due to the uncoupling effect of the drug, rather than the increase in the number of cells. This is more pronounced in the case when H9C2 or WRL-68 cell lines were exposed to amiodarone, desethylamiodarone and FCCP for only one hour (Fig. 4A-B). Amiodarone exhibited an uncoupling effect similar to FCCP up to the concentration of 30 μM, in H9C2 but not in WRL-68 cells, whereas desethylamiodarone lacked the uncoupling effect, presenting inhibition in both cell lines above the concentration of 10 μM. In each of the cell lines examined, desethylamiodarone proved to be significantly more toxic than the equimolar concentrations of amiodarone in a specific concentration range (Fig. 3A-D). This is in accord with the results of other reports (Bolt et al., 2001; Di Matola et al., 2000; Daniels et al., 1989; Ogle and Reasor, 1990). Extracardiac cell lines exhibited a marked sensitivity toward amiodarone toxicity in the whole concentration range tested when compared to the toxicity in H9C2 cell line, as demonstrated by their lower LD_{50} values. Desethylamiodarone also was more toxic in case of the BRL 3A and WRL 68 cells, than in H9C2 cardiomyocytes, but not in the case of the pancreas cell line, which presented a similar LD_{50} value for desethylamiodarone as did the cardiomyocytes (Table 1). PANC-1 cell line is however a carcinoma line, representing a more immature state of differentiation than the liver cells, which could explain its decreased susceptibility towards the drug, indicated by a higher LD_{50} value. The examined concentration range of 1-100 μM is in accord with the levels of either amiodarone or desethylamiodarone observed in different tissues following long-term amiodarone treatment. These results suggest that desethylamiodarone is unlikely to contribute to the cardioprotective effect of its parent drug.

While the molecular mechanism of the antiarrhythmic effect of amiodarone is well established (Singh et al., 1970; Nattel et al., 1987), the mechanism of cardioprotectivity and cytotoxicity is obscure. The observation, that amiodarone protected the energy metabolism in perfused hearts suggested, that the cardioprotectivity of the drug could be consequence of it's direct mitochondrial effect, by influencing the $\Delta\Psi$, the ROS production, the respiration or the permeability transition. While some studies addressed the effect of amiodarone and desethylamiodarone on mitochondrial respiration and ROS production (Fromenty et al., 1990; Di Matola et al., 2000), the effect of the drugs on permeability transition has not been fully disclosed (Varbiro et al., 2003), although the involvement of permeability transition in the collapse of oxidative phosphorylation and ion homeostasis, as well as in mediation of both necrotic and apoptotic cell death is well established (Kroemer and Reed, 2000). Therefore we studied the direct effect of the drugs on isolated, Percoll-gradient purified mitochondria from rat liver and heart, as well as the effect of amiodarone on the release of AIF from mitochondria and it’s translocation to the nucleus in prefused rat hearts.

We revealed that amiodarone has a mixed effect on mitochondrial functions. In low concentration range, where it exhibited cardioprotective effect and inhibited the release of AIF from the mitochondria in perfused hearts, it stimulated state 4 respiration due to an uncoupling effect, inhibited the Ca^{2+}-induced mitochondrial swelling, while it caused a moderate dissipation of the $\Delta\Psi$. However at higher concentrations it exerted an inhibitory effect on the mitochondrial respiration, and simultaneously induced a mitochondrial swelling, that was not inhibited by CsA. In contrast to this, desethylamiodarone did not stimulate state 4 respiration, did not inhibit the Ca^{2+}-induced mitochondrial permeability transition and did not induce the collapse of $\Delta\Psi$ in low concentrations, and did not prevent the release and nuclear translocation of AIF in perfused rat hearts. At higher concentrations, similar to amiodarone, it induced a mitochondrial swelling that was not inhibited by CsA and inhibited the respiration.

This inhibitory effect was more prominent on Complex II supported respiration than that of the amiodarone (Bolt et al., 2001).

The mitochondrial permeability transition following the opening of the permeability transition pore causes swelling of the matrix, leading to membrane disruption and finally cell death, while the release of mitochondrial proteins like cytochrome-c or AIF lead to the activation of the apoptotic pathway (Green and Reed, 1998; Kroemer and Reed, 2000). While the dissipation of $\Delta\Psi$ was previously suspected to be a phenomenon tightly associated with permeability transition, recent reports have revealed, the collapse of the mitochondrial membrane potential does not induce, but rather prevents the mitochondrial swelling (Aronis et al., 2002; Kahlert and Rieser, 2002). We also found, that FCCP, a widely used uncoupling agent, inhibits the Ca^{2+}-induced mitochondrial swelling, while it dissipated the $\Delta\Psi$. Amiodarone similarly to FCCP exhibits uncoupling effect, inhibits swelling and dissipates the $\Delta\Psi$. A theory suggests, that during uncoupling, the mitochondrial respiratory chain works more efficiently, leading to less leakage of electrons and thus to lower levels of ROS generation (Budd et al., 1997), obscuring the mechanism by which FCCP or lower membrane potential promotes pore opening. Previous reports (Ribeiro et al., 1997; Di Matola et al., 2000), and our findings that amiodarone does not induce ROS production in isolated mitochondria is also in accord with this theory. In the case of desethylamiodarone however, none of the above described effects of amiodarone were found, more over it was reported to increase the intracellular cytosolic free Ca^{2+} concentration (Himmel et al., 2000), which could also contribute to it's enhanced toxicity.

In conclusion, we present clear evidence that amiodarone but not desethylamiodarone protects the mitochondrial energy metabolism of the perfused heart during ischemia and reperfusion as detected by real time *in situ* ^{31}P NMR measurement. We demonstrate for the first time, that amiodarone prevents the mitochondrial AIF release induced by ischemia-

reperfusion in perfused hearts. We also demonstrated, that cardiac and extracardiac cells are more susceptible to desethylamiodarone than amiodarone. We also report, that amiodarone exerts a biphasic effect on the mitochondria, with protective effects in lower concentration and toxic properties manifesting, when present at higher concentrations, whereas desethylamiodarone does not have this dual feature. Amiodarone when present in low concentrations protects the energy mechanism of postischemic heart by inhibiting the mitochondrial permeability transition, and attenuating the ROS generation. These properties of amiodarone are due to the presence of its ethyl group, since it's major metabolite, desethylamiodarone, does not exhibit the cardioprotective and beneficial mitochondrial features of the parent drug. In higher concentrations, amiodarone as well as desethylamiodarone, besides inhibiting the mitochondrial respiration can induce a CsA independent mitochondrial swelling, thus contributing to the toxic property of the drug. While both amiodarone and desethylamiodarone have similar antiarrhythmic properties, only amiodarone possesses with cardioprotective effect, and the frequently manifesting side effects during long-term amiodarone therapy could be related, at least in part, to the accumulation of desethylamiodarone.

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Footnotes

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Legends for figures

Fig. 1. The effect of amiodarone and desethylamiodarone on the high-energy phosphate metabolism in Langendorff-perfused rat hearts. Groups of 5 rats were treated by a single shot of physiological salt solution (sham-operated), 20 mg/kg amiodarone or 20 mg/kg desethylamiodarone iv. 30 min before sacrifice. Their hearts were removed, applied to a Langendorff perfusion apparatus, which was inserted into the magnet of an NMR spectrometer. Concentrations of creatine phosphate (A), ATP (B) and inorganic phosphate (C) were measured *in situ* by ^{31}P NMR spectroscopy in the perfused hearts subjected to 30 min of ischemia followed by 15 min of reperfusion. Data represents average ± S.E.M. Note that the time axis is not proportional. *, significant difference ($p < 0.05$; mean ± S.E.M., repeated measures ANOVA); ***, significant difference ($p < 0.001$; mean ± S.E.M., repeated measures ANOVA) of amiodarone from equimolar concentrations of desethylamiodarone.

Fig. 2. The effect of amiodarone and desethylamiodarone on the nuclear AIF level in Langendorff-perfused rat hearts. Western blot analysis of the apoptosis inducing factor (AIF) level in the nuclear fraction of rat heart tissue following ischemia-reperfusion in Lagendorff-perfused rat hearts. *Lane 1*: control (no perfusion).; *lane 2*: 30 min of normoxic perfusion.; *lane 3*: 30 minutes of ischemia followed by 60 minutes of reperfusion.; *lane 4*: pretreatment with 20 mg/kg of amiodarone for 30 minutes followed by excision of the heart and 30 minutes of ischemia and 60 minutes of reperfusion.; *lane 5*: pretreatment with 20 mg/kg of desethylamiodarone for 30 minutes followed by excision of the heart and 30 minutes of ischemia and 60 minutes of reperfusion.

Fig. 3. The effect of amiodarone and desethylamiodarone on viability of cell lines. The effect of amiodarone (open bars) and desethylamiodarone (filled bars) on viability of H9C2

(A), BRL-3A (B), WRL-68 (C) and HeLa cells (D) were detected by the formation of water insoluble blue formasan dye from the yellow mitochondrial dye, MTT^+ by the functionally active mitochondria of the cells. The cells were exposed to different concentrations of amiodarone or desethylamiodarone for 48 hours before the addition of the MTT^+ dye. Data represents average ± S.E.M. of three independent experiments running in 4 parallels. **, significant difference ($p < 0.01$; mean ± S.E.M., paired *t* test); ***, significant difference ($p < 0.001$; mean ± S.E.M., paired *t* test) of amiodarone from equimolar concentrations of desethylamiodarone.

Fig. 4. The effect of amiodarone, desethylamiodarone and FCCP on the cellular respiration. The effect of amiodarone, desethylamiodarone and FCCP on respiration of H9C2 (A) and WRL-68 (B) cells were detected by the formation of water insoluble blue formasan dye from the yellow mitochondrial dye, MTT^+ by the functionally active mitochondria of the cells. The cells were exposed to different concentrations of amiodarone, desethylamiodarone or FCCP for 1 hours before the addition of the MTT^+ dye. Data represents average ± S.E.M. of three independent experiments running in 4 parallels. +++, significant difference ($p < 0.001$; mean ± S.E.M., ANOVA) of FCCP from equimolar concentrations of amiodarone; ***, significant difference ($p < 0.001$; mean ± S.E.M., ANOVA) of desethylamiodarone from equimolar concentrations of amiodarone.

Fig. 5. The effect of amiodarone and desethylamiodarone on mitochondrial oxygen consumption. Mitochondrial respiration was measured by a Clark electrode and was supported with either 10 mM pyruvate (Complex I respiration) or 10 mM succinate (Complex II respiration) in the presence of different concentrations of amiodarone or desethylamiodarone. Data represents average ± S.E.M. of three independent experiments

repeated twice using mitochondria prepared from the same heart samples. Note that the concentration axis is logarithmic. *, significant difference ($p < 0.05$; mean ± S.E.M., ANOVA); **, significant difference ($p < 0.01$; mean ± S.E.M., ANOVA); ***, significant difference ($p < 0.001$; mean ± S.E.M., ANOVA) of amiodarone or desethylamiodarone from control.

Fig. 6. The effect of amiodarone or desethylamiodarone on Ca^{2+}-induced mitochondrial swelling. Mitochondrial swelling was demonstrated by monitoring E_{540} in isolated rat liver mitochondria. Amiodarone (Ad) at the indicated concentration or 2.5 μM cyclosporin A (CsA) was present throughout the experiment(A). Alternatively desethylamiodarone (Dea) at the indicated concentration or 2.5 μM cyclosporin A (CsA) was present throughout the experiment (B). The mitochondrial permeability transition (swelling) was induced by adding 60 μM Ca^{2+} at arrow

(A) Line 1: baseline swelling (no agent); line 2: 60 μM Ca^{2+}-induced swelling (no Ad or CsA); line 3: CsA; line 4: 1 μM Ad; line 5: 2.5 μM Ad; line 6: 5 μM Ad; line 7: 10 μM Ad; line 8: 20 μM Ad.

(B) Line 1: baseline swelling (no agent): line 2: 60 μM Ca^{2+}-induced swelling (no Dea or CsA); line 3: CsA; line 4: 1 μM Dea; line 5: 2.5 μM Dea; line 6: 5 μM Dea; line 7: 10 μM Dea; line 8: 20 μM Dea.

Fig. 7. The mitochondrial swelling induced by amiodarone or desethylamiodarone. Mitochondrial swelling was demonstrated by monitoring E_{540} in isolated rat liver mitochondria. Cyclosporin A (CsA), at the concentration of 2.5 μM, where indicated, was present throughout the experiment. Swelling was induced by adding amiodarone (Ad) at the indicated concentration (A) or desethylamiodarone (Dea) at the indicated concentration (B), or 60 μM Ca^{2+} at the arrow.

(A) Line 1: baseline swelling (no agent); line 2: 60 μM Ca^{2+}-induced swelling (no Ad or CsA); line 3: 10 μM Ad; line 4: 10 μM Ad + CsA; line 5: 20 μM Ad; line 6: 20 μM Ad+ CsA; line 7: 30 μM Ad; line 8: 30 μM Ad + CsA.

(B) Line 1: baseline swelling (no agent); line 2: 60 μM Ca^{2+}-induced swelling (no Dea or CsA); line 3: 10 μM Dea; line 4: 10 μM Dea + CsA; line 5: 20 μM Dea; line 6: 20 μM Dea+ CsA; line 7: 30 μM Dea; line 8: 30 μM Dea + CsA.

Fig. 8. Effect of amiodarone or desethylamiodarone on the mitochondrial membrane potential in isolated mitochondria. Membrane potential was monitored by measuring the fluorescence intensity of the cationic fluorescent dye rhodamine 123. Isolated rat liver mitochondria, added at the first arrow, takes up the dye in a voltage dependent manner and quenches its fluorescence. Amiodarone (Ad), desethylamiodarone (Dea) at the concentrations indicated or 60 μM Ca^{2+} (either added at second arrow) induces depolarization resulting in release of the dye and increase of the fluorescence intensity.

(A) Line 1: no agent; line 2: Ca^{2+}; line 3: 10 μM Ad; line 4: Ca^{2+} + 2.5 μM CsA ; line 5: 10 μM Ad + 2.5 μM CsA; line 6: 10 μM Dea.

(B) Line 1: no agent; line 2: 1 μM Ad; line 3: 2.5 μM Ad; line 4: 5 μM Ad; line 5: 10 μM Ad; line 6: 20 μM Ad

(C) Line 1: no agent; line 2: 1 μM Dea; line 3: 2.5 μM Dea; line 4: 5 μM Dea; line 5: 10 μM Dea; line 6: 20 μM Dea.

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Tables

TABLE 1

LD_{50} values for amiodarone and desethylamiodarone in different cell lines following 48 hours of incubation

Cell lines	Amiodarone	Desethylamiodarone
	μM ± S.E.M.	*μM ± S.E.M.*
H9C2	49.1 ± 5.8	17.2 ± 3.9[a]
BRL-3A	13.3 ± 4.2	4.2 ± 1.1[a]
WRL-68	2.4 ± 0.4	2.5 ± 0.3
PANC-1	41.4 ± 3.7	23.5 ± 1.4[a]

[a] Significant differences between the LD_{50} of amiodarone and desethylamiodarone ($p < 0.01$, paired *t* test, $n = 8$).

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TABLE 2

Concentration values for amiodarone and desethylamiodarone in the plasma and the heart mitochondria of rats measured by HPLC 30 min after the iv administration of either 20mg/kg amiodarone or desethylamiodarone.

	Plasma	Heart mitochondria
	μg/ml ± S.E.M.	*μg/mg ± S.E.M.*
Amiodarone	4.1 ± 0.8	2.42 ± 0.29
Desethylamiodarone	4.3 ± 4.2	2.69 ± 0.28

Results are expressed as mean ± S.E.M.(*n* = 3)

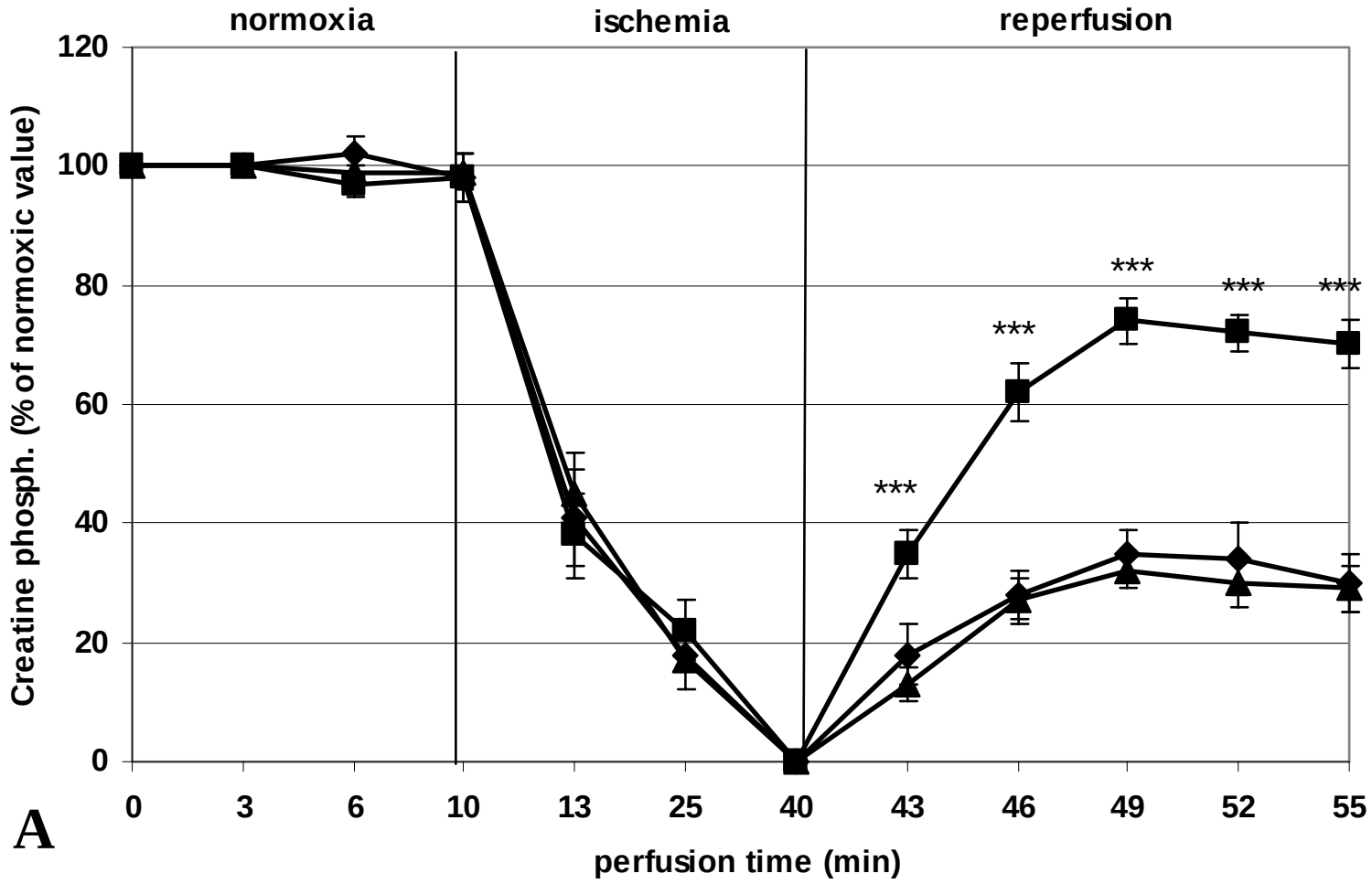
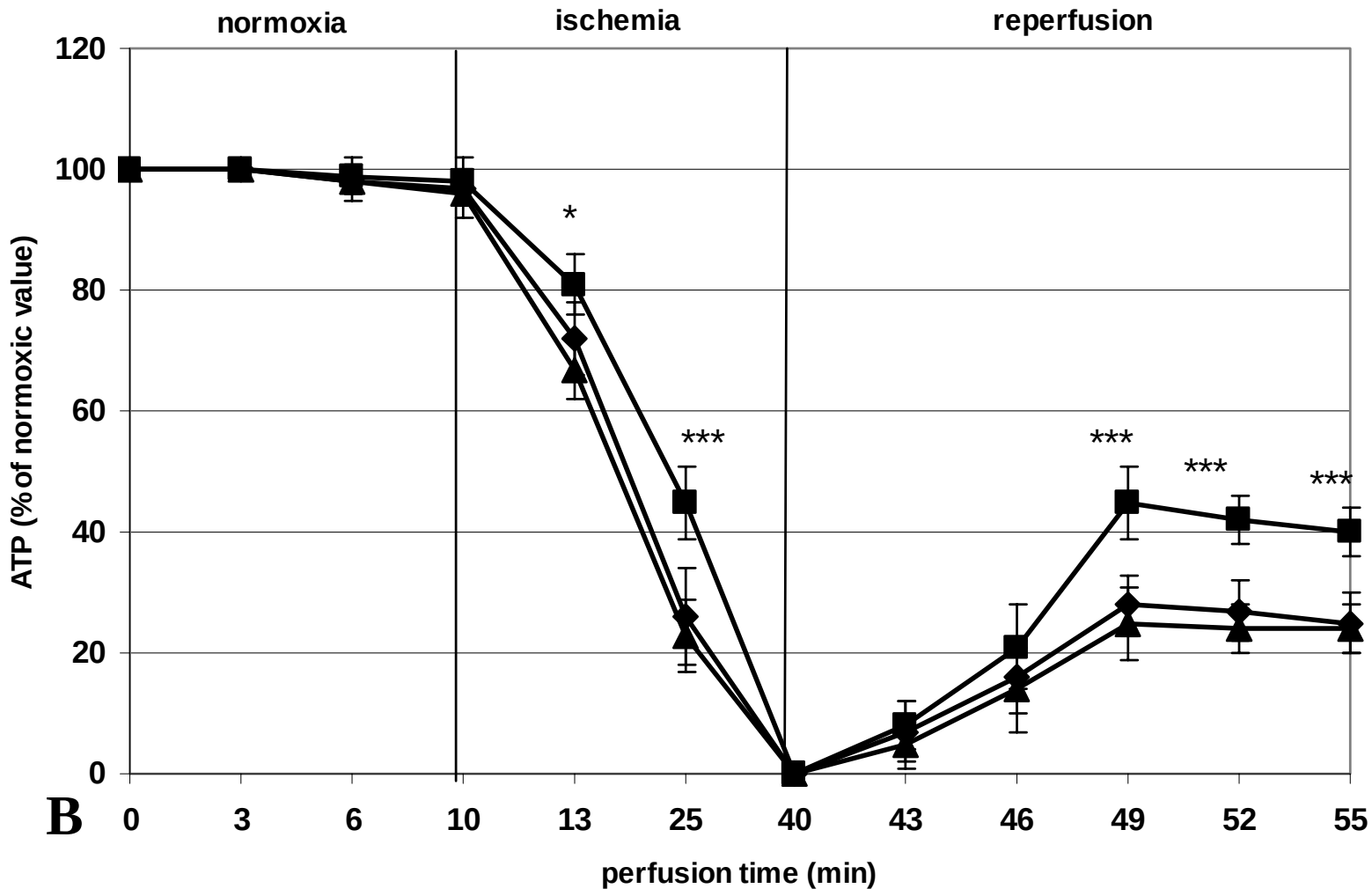


Fig. 1



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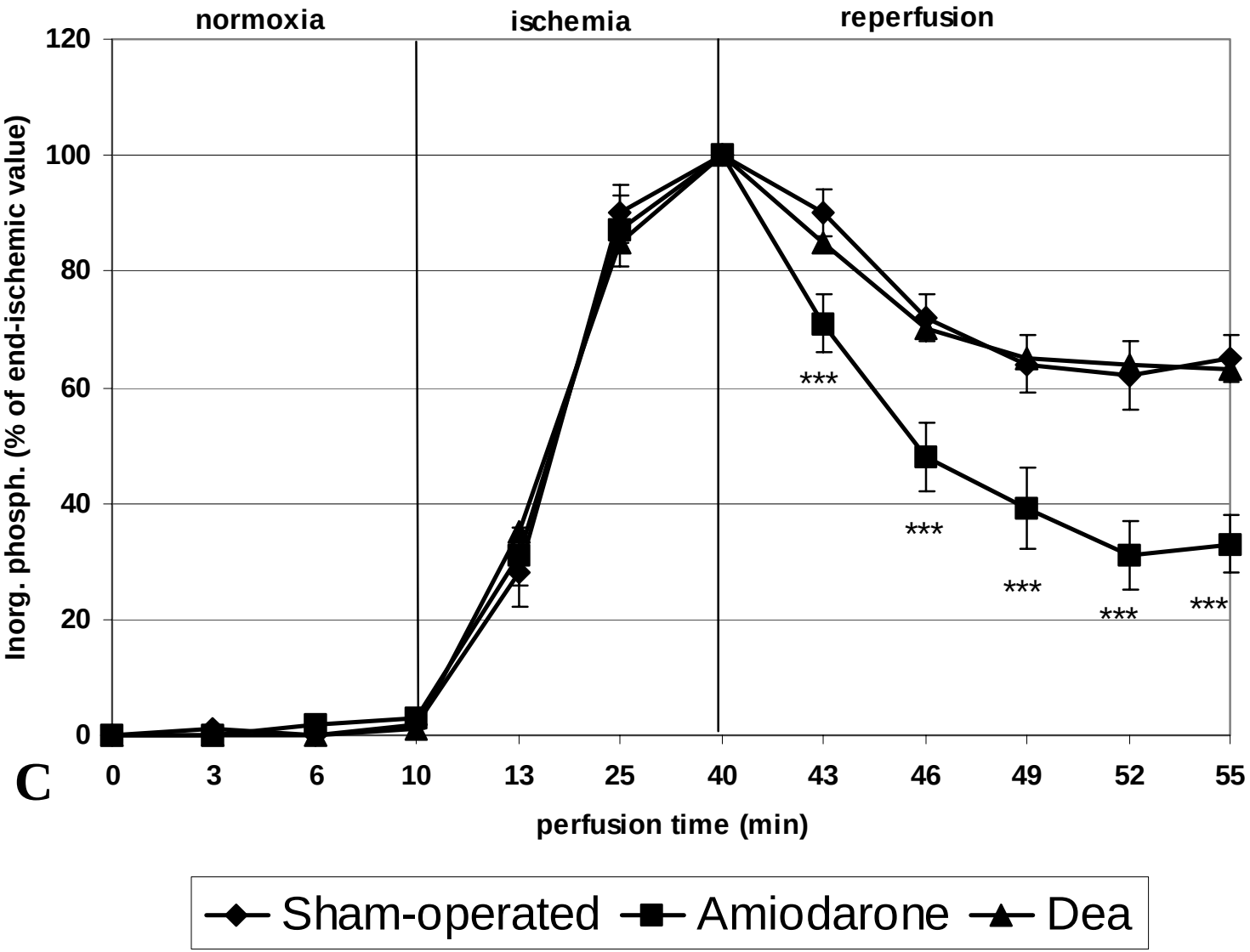

Fig. 1

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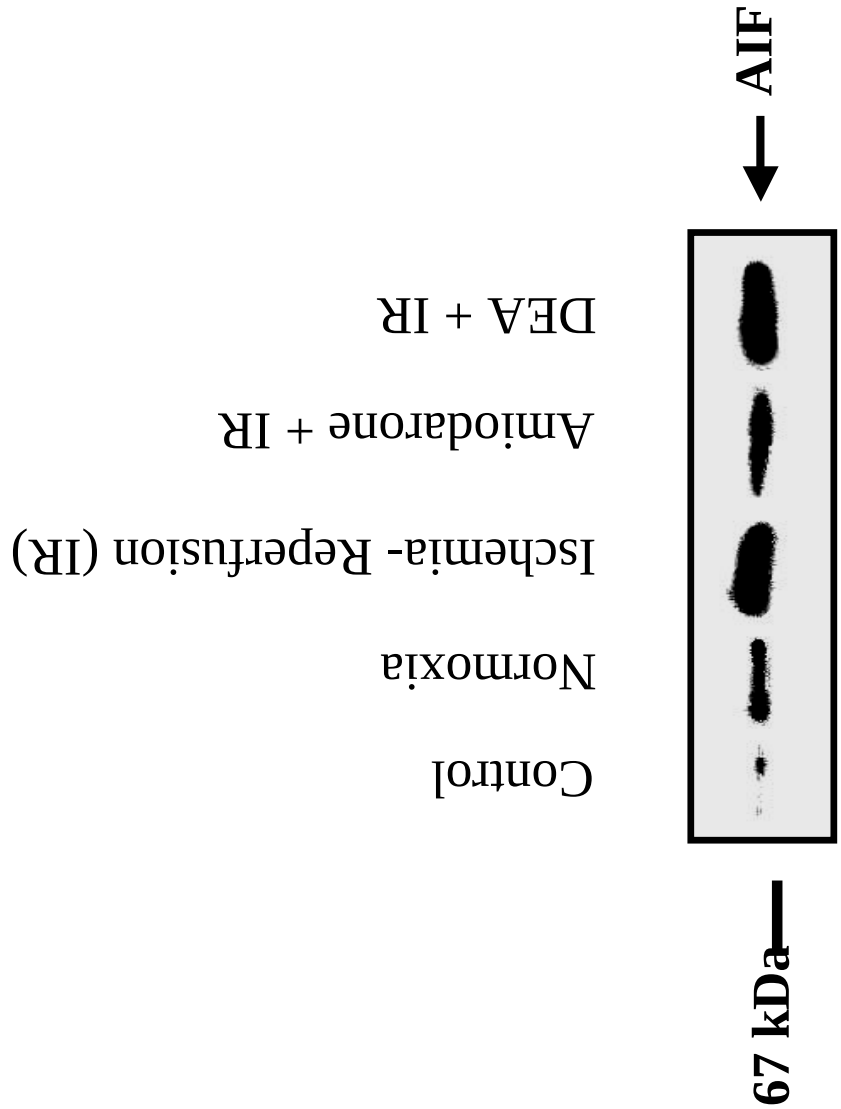
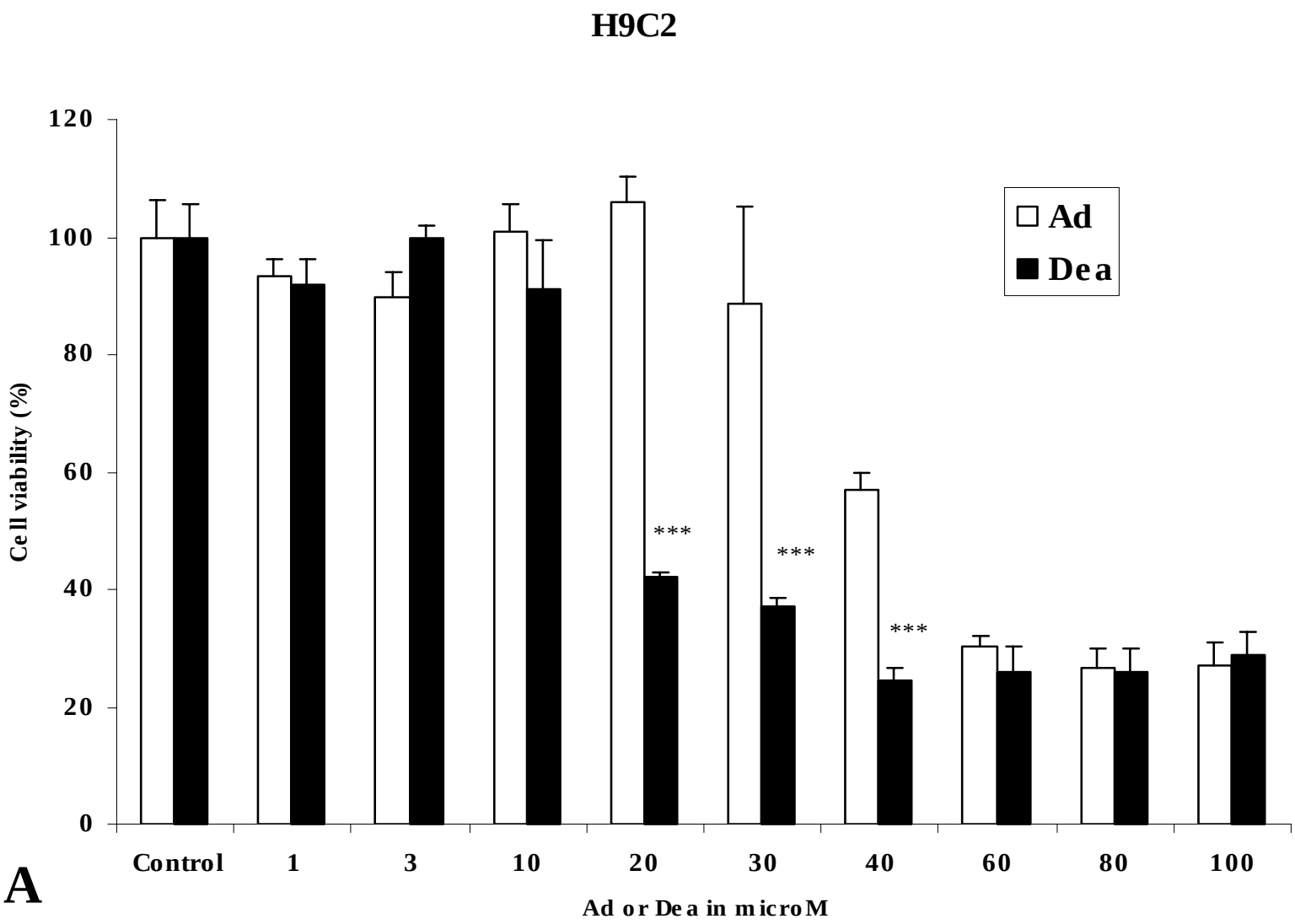
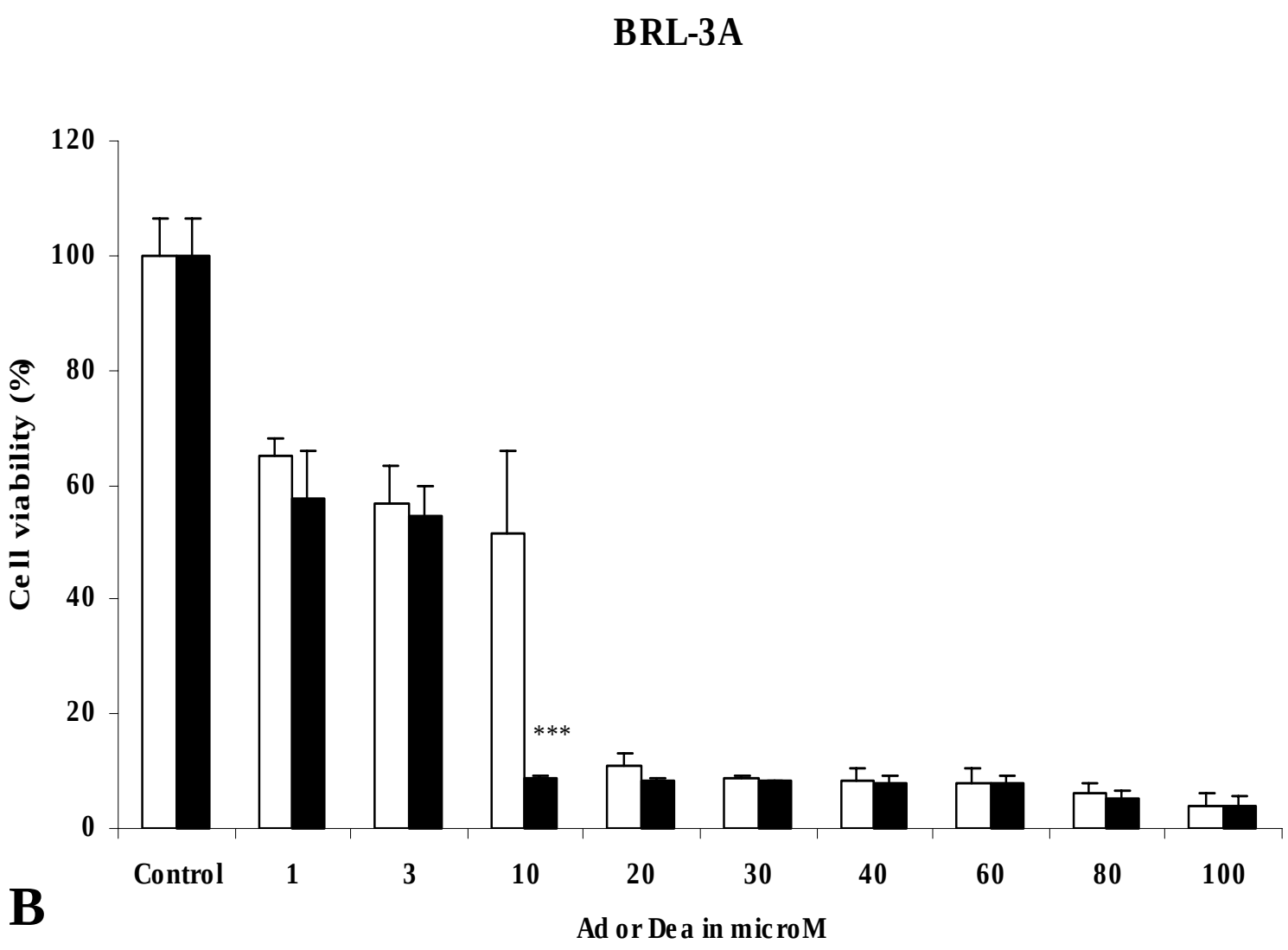


Fig. 2.



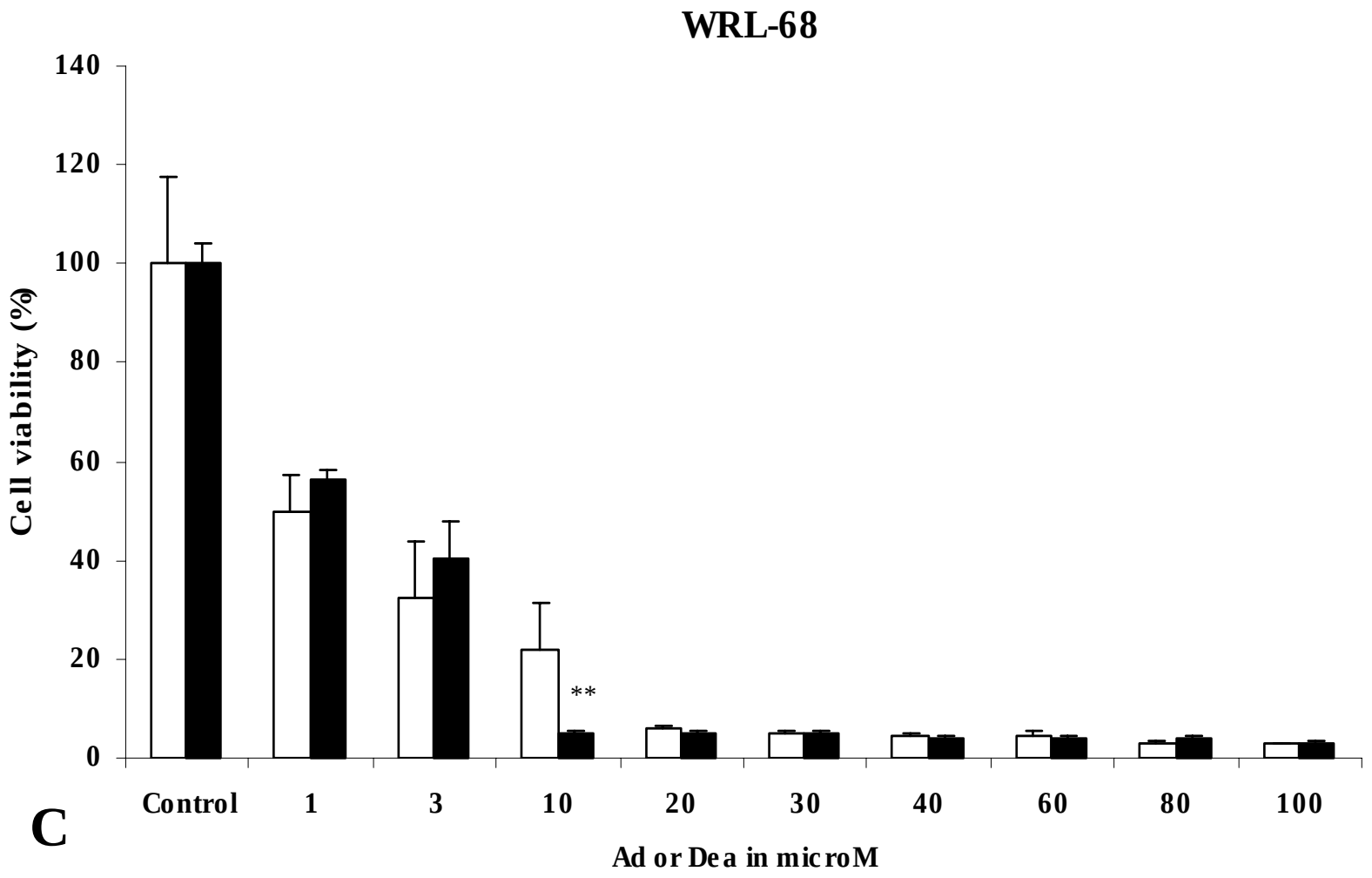
A

Fig. 3



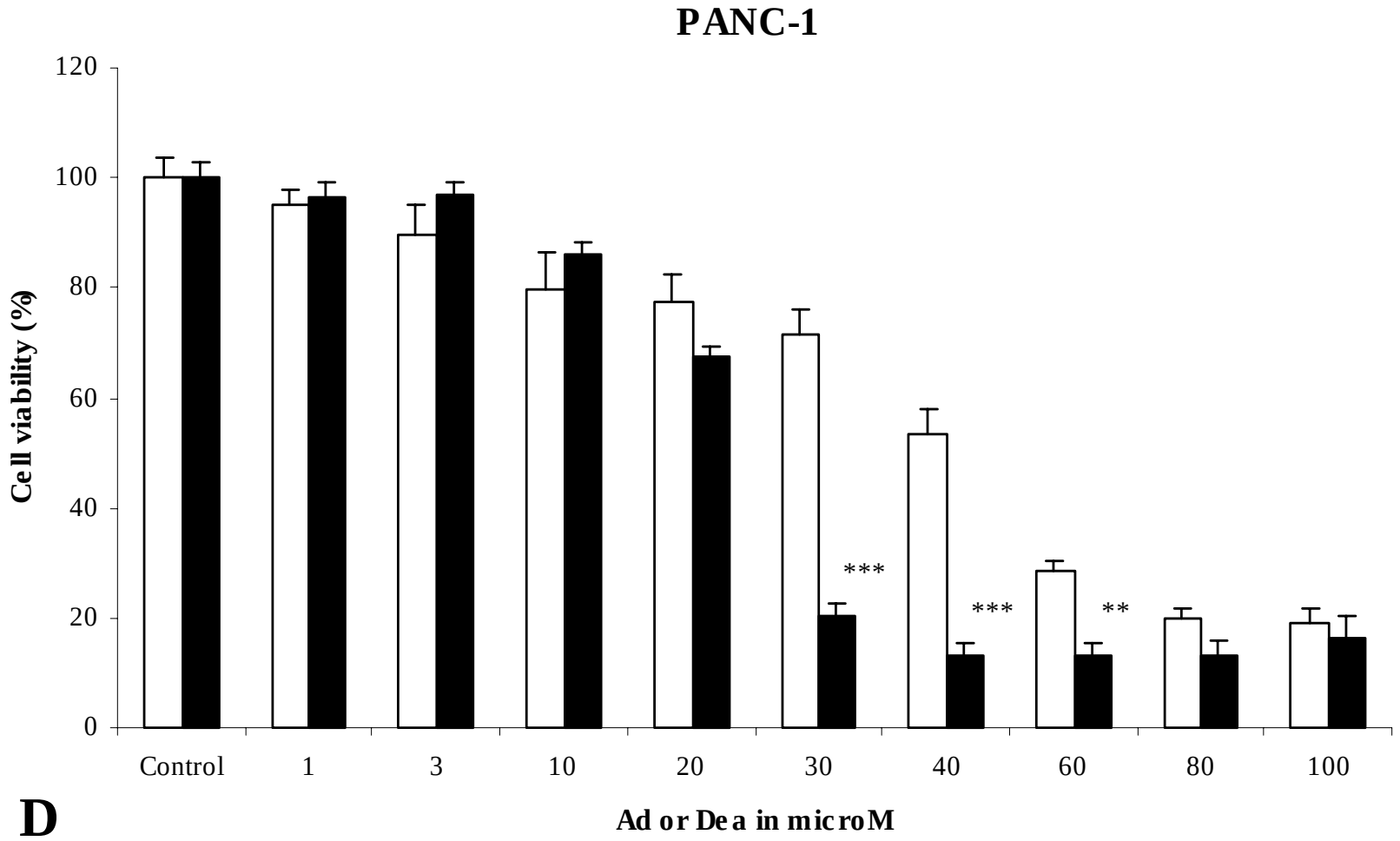
B

Fig. 3



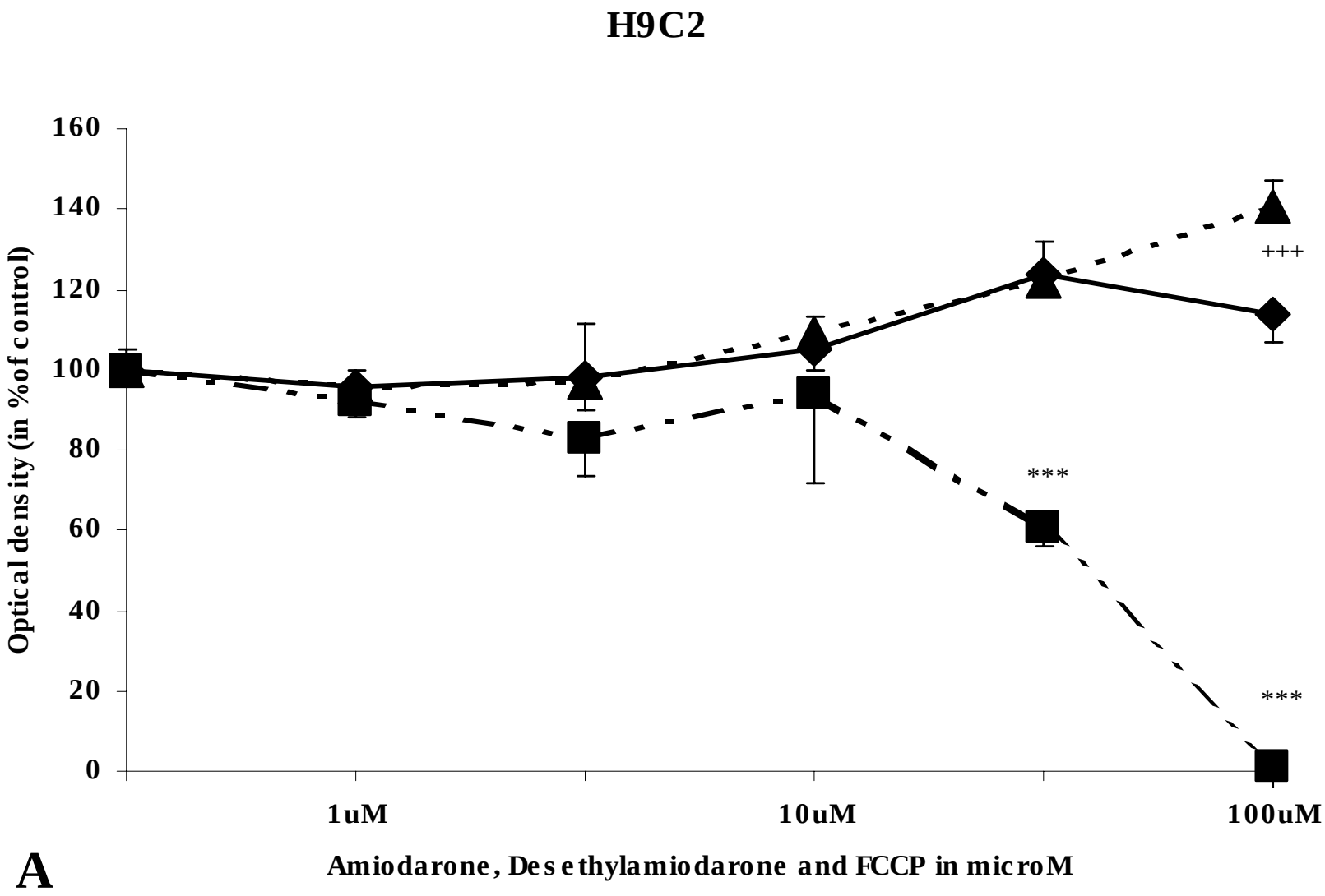
C

Fig. 3



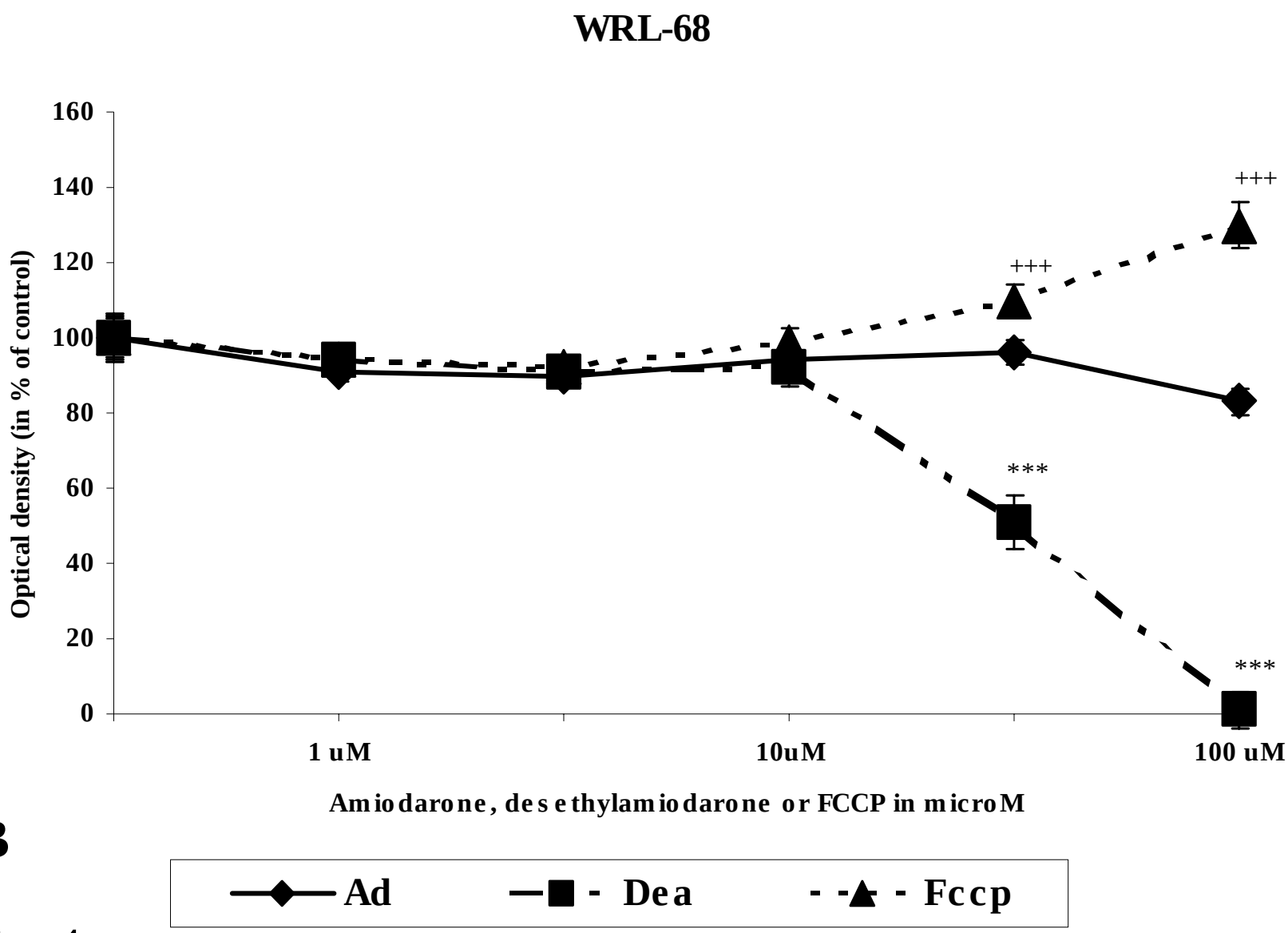
D

Fig. 3



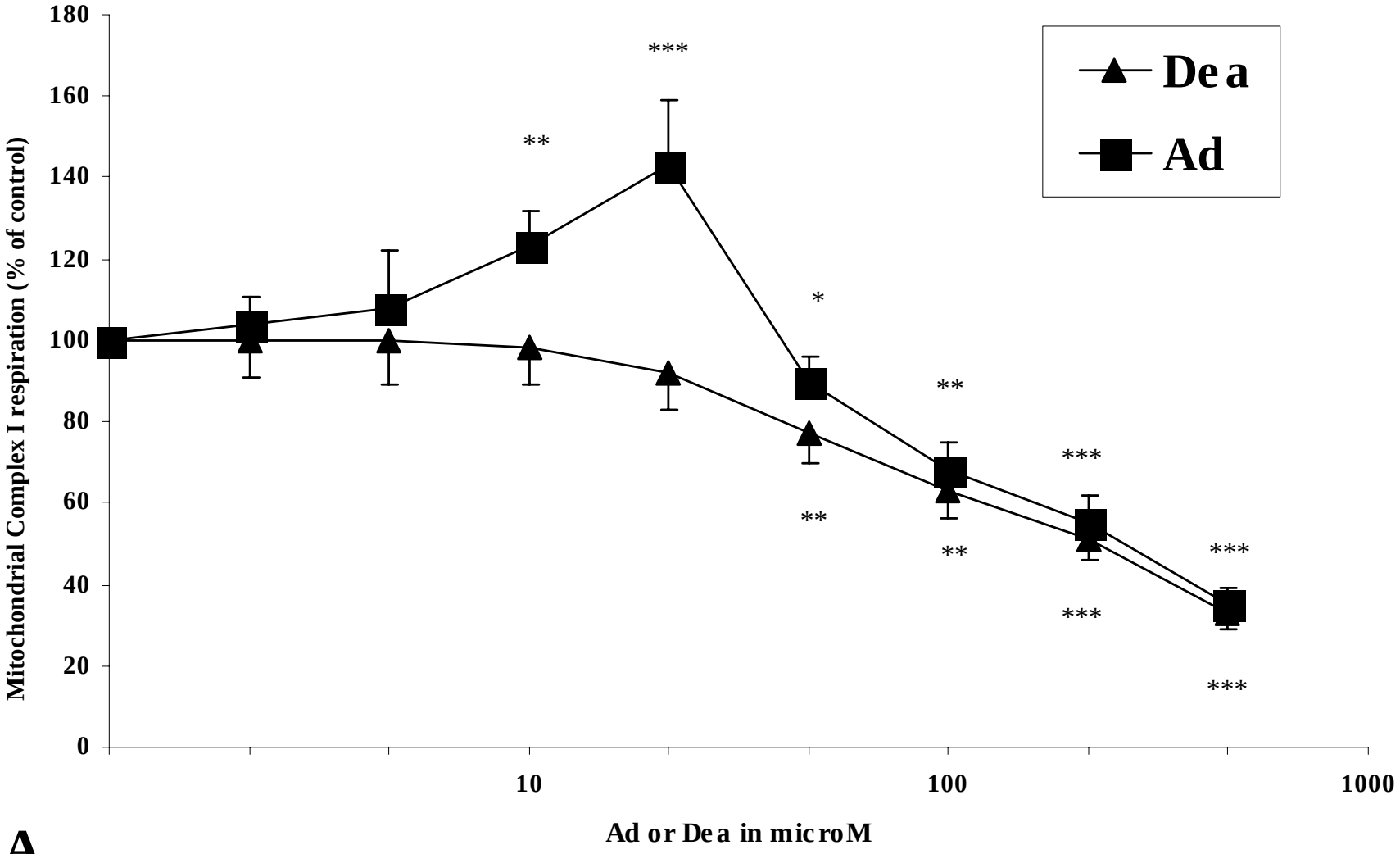
A

Fig. 4



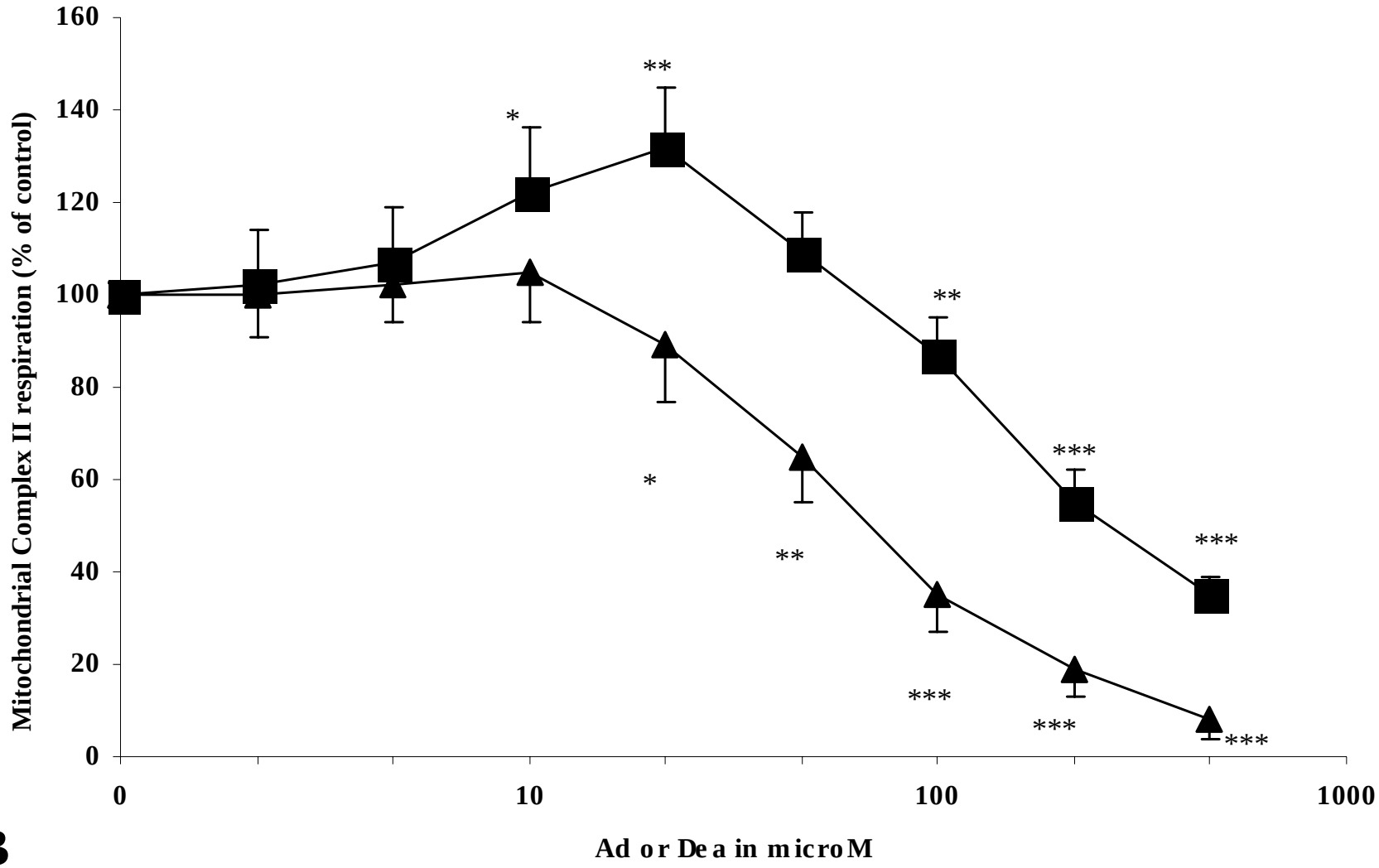
B

Fig. 4



A

Fig. 5



B

Fig. 5

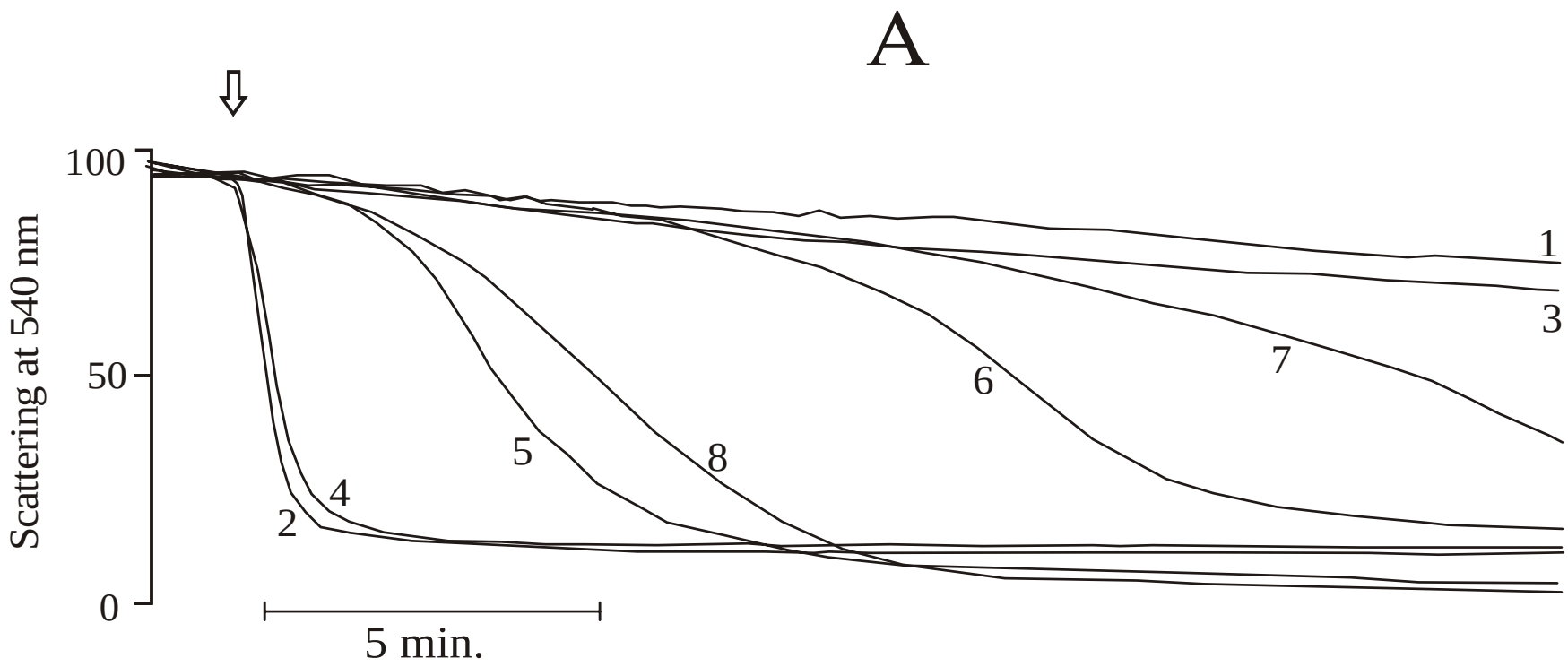


Fig. 6

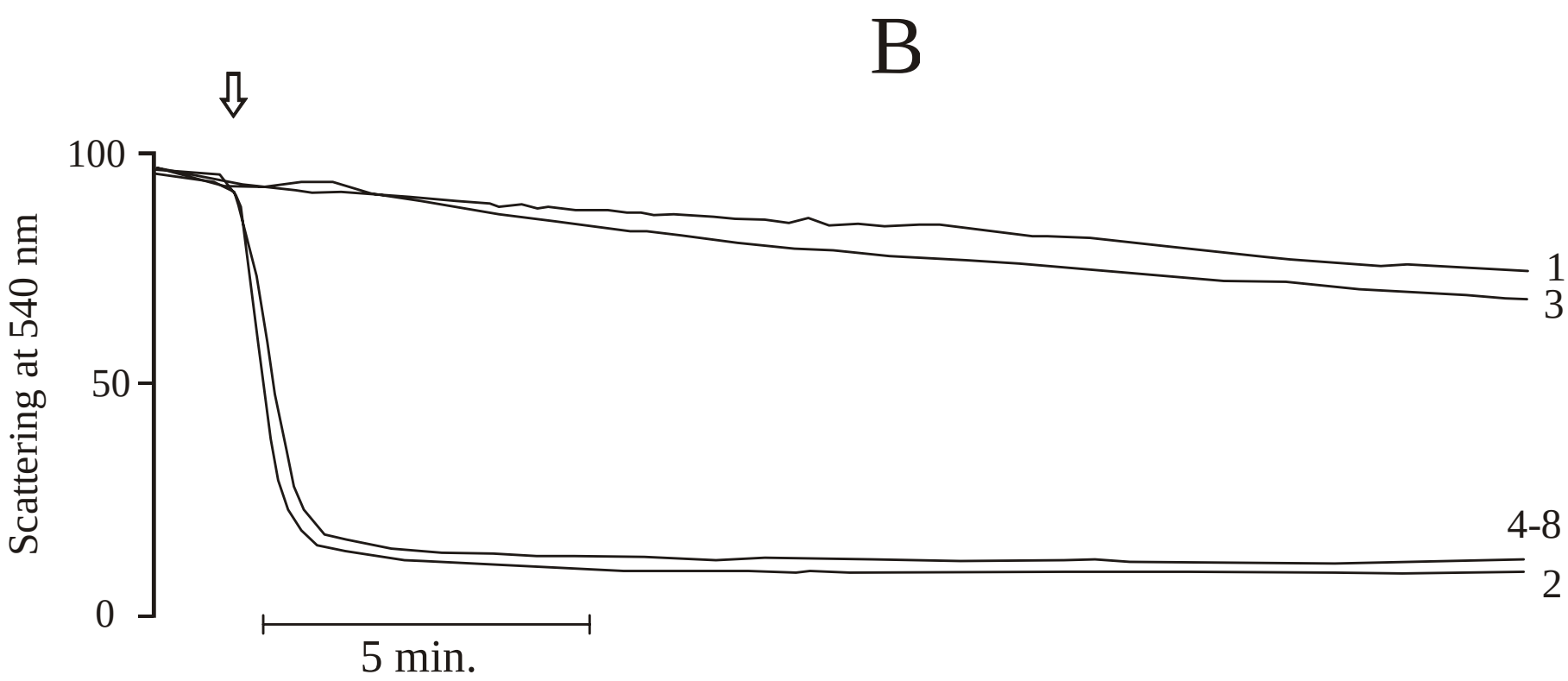


Fig. 6

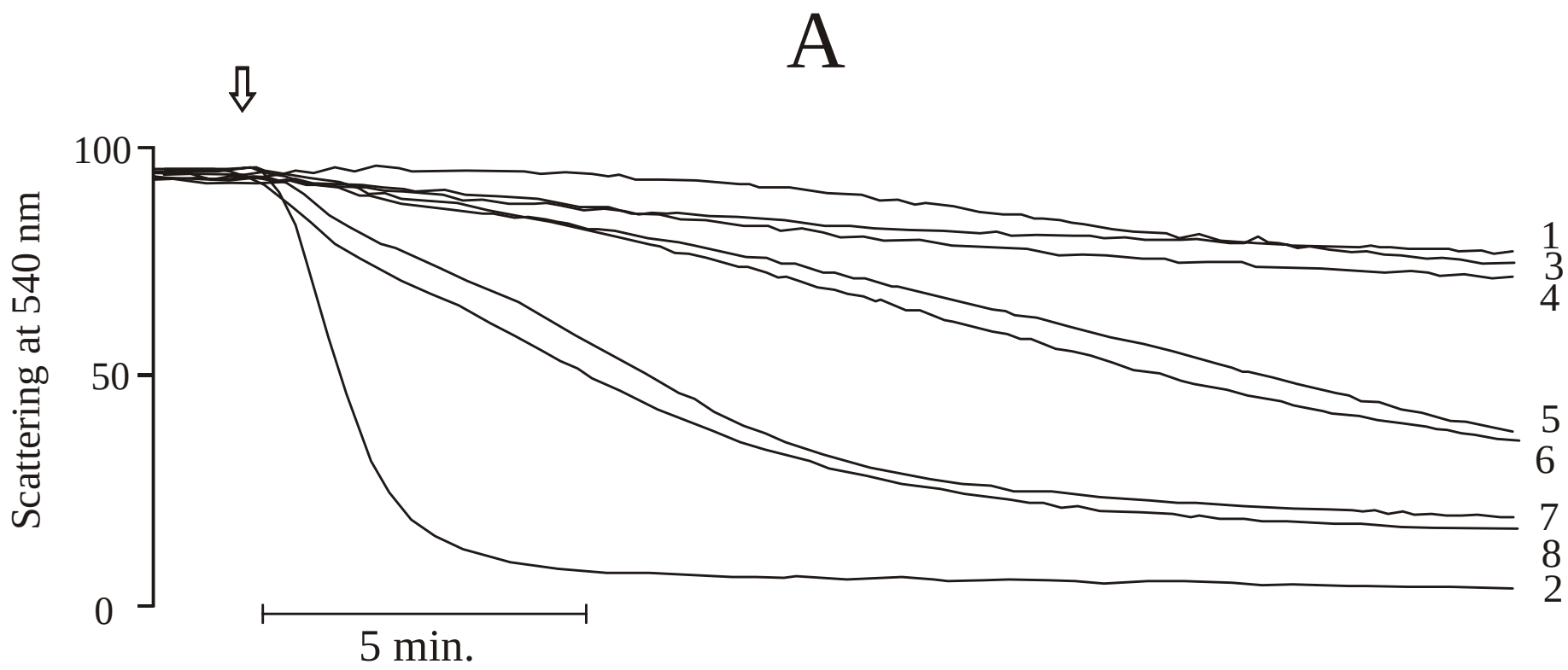


Fig. 7

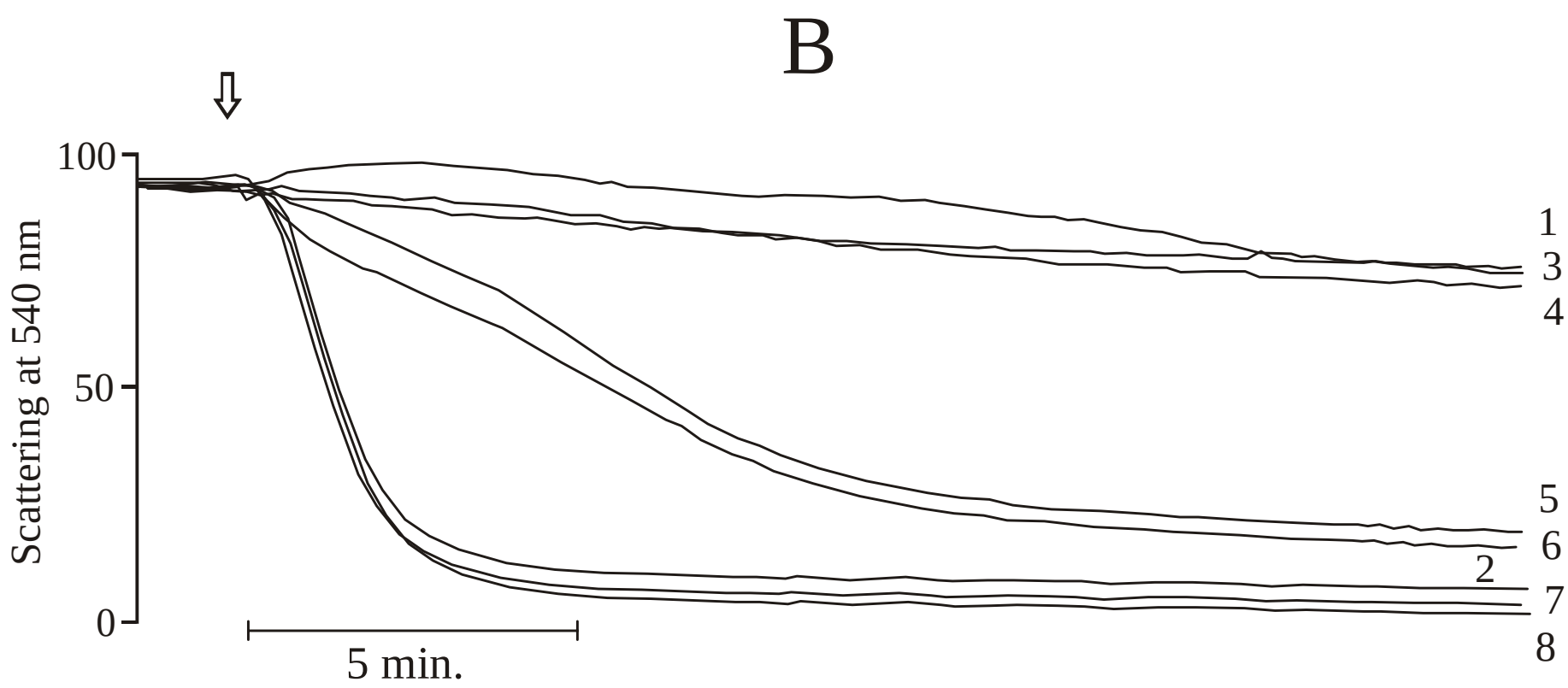


Fig. 7

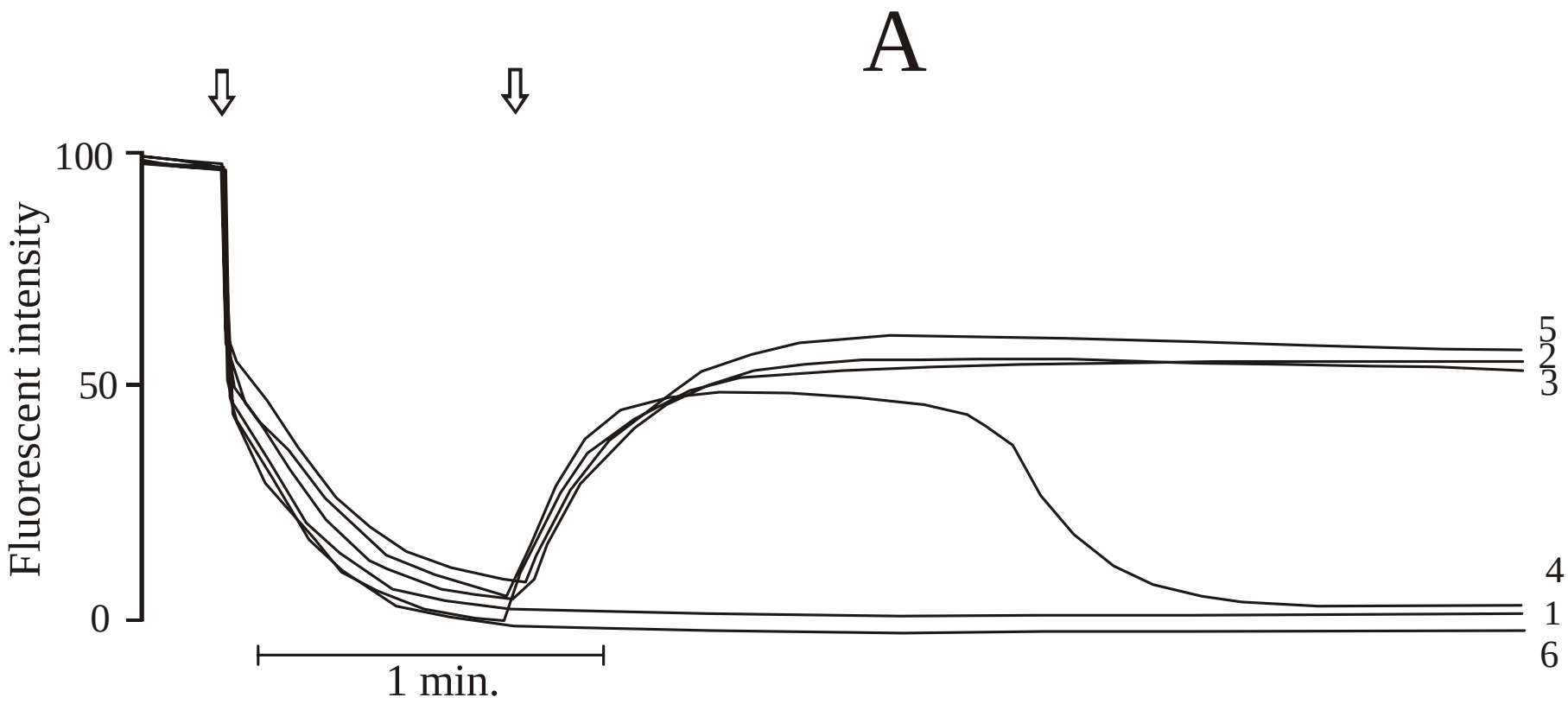


Fig. 8

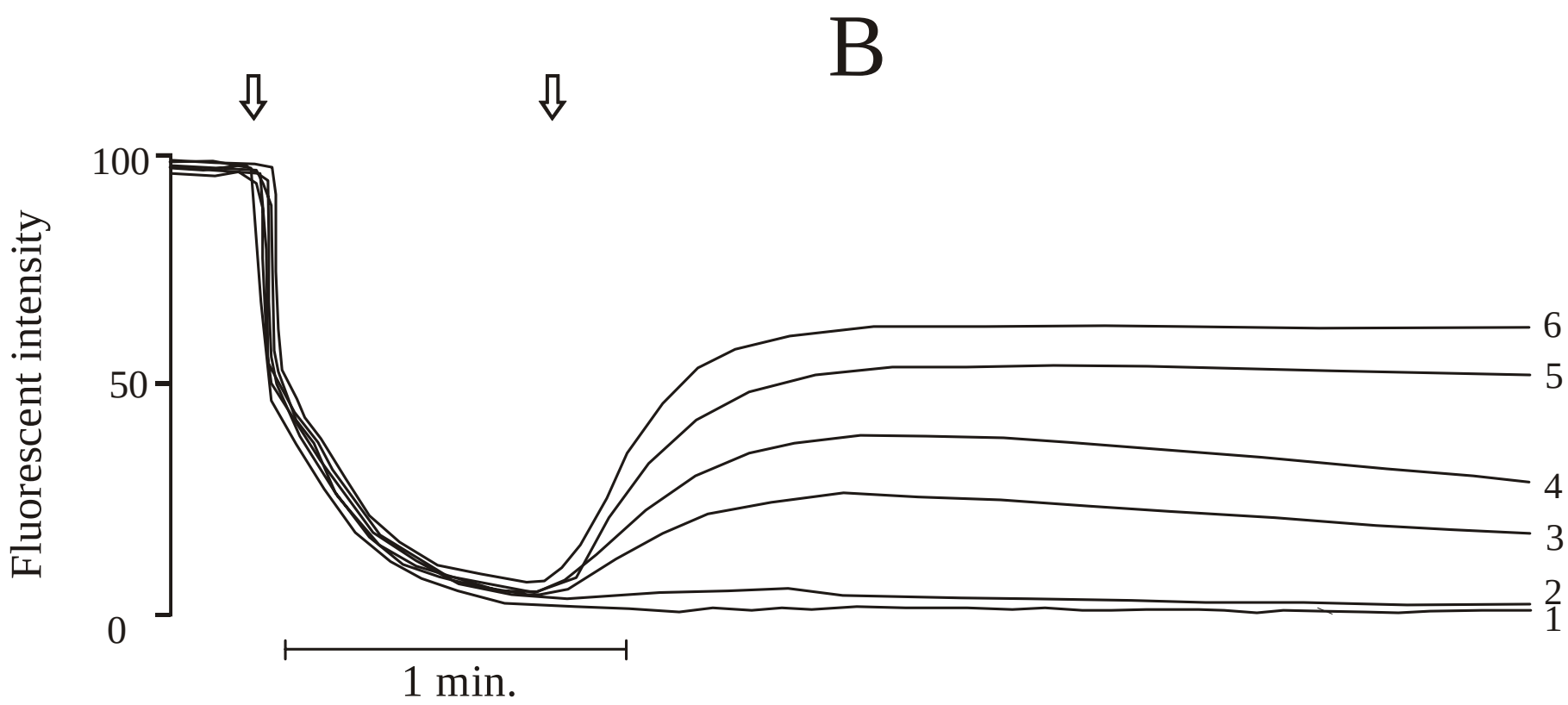


Fig. 8

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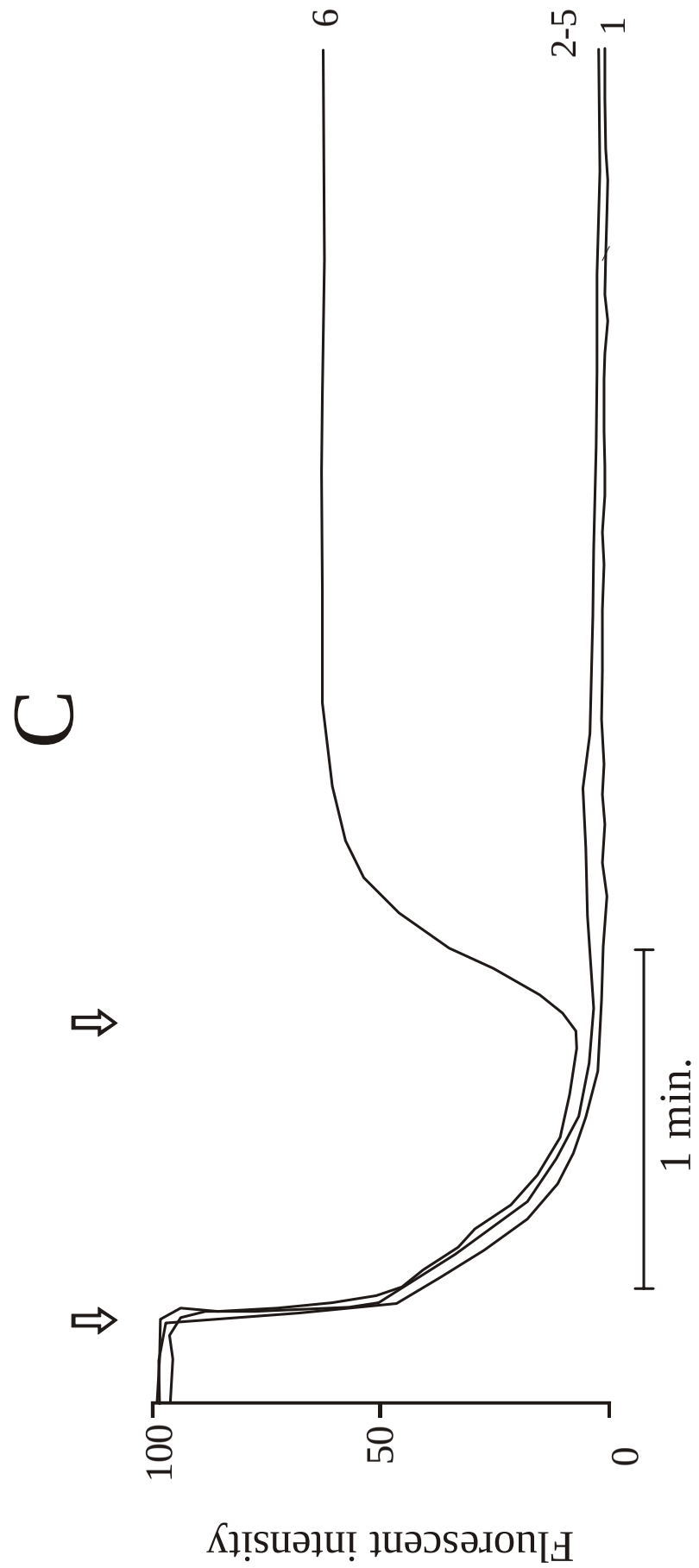

Fig. 8