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Hepatocellular toxicity and pharmacological effect of amiodarone and amiodarone derivatives

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Running title: Toxicity and pharmacology of amiodarone and analogues

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Abbreviations: CHO: chinese hamster ovary, DCFH-DA: 2,7-dichlorofluorescin diacetate, DMSO: dimethylsulfoxide, FACS: fluorescence activated cell scanning, hERG: human ether-a-go-go related gene, HPLC: high performance liquid chromatography, JC-1: 5,5',6,6,'-tetraethylbenzimidazolylcarbocyanidde iodide, LDH: lactate dehydrogenase, NMR: nuclear magnetic resonance, PI: propidiumiodide, RCR: respiratory control ratio, ROS: reactive oxygen species

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Abstract

The aim of this work was to compare hepatocellular toxicity and pharmacological activity of amiodarone (2-n-butyl-3-[3,5 diiodo-4-diethylaminoethoxybenzoyl]-benzofuran = B2-O-Et-N-diethyl) and of eight amiodarone derivatives. Three amiodarone metabolites were studied, namely mono-N-desethylamiodarone (B2-O-Et-NH-ethyl), di-N-desethylamiodarone (B2-O-Et-NH_2) and B2 carrying an ethanol side chain (B2-O-Et-OH). In addition, five amiodarone analogues were investigated, namely N-Dimethylamiodarone (B2-O-Et-N-dimethyl), N-Dipropylamiodarone (B2-O-Et-N-dipropyl), B2-O carrying an acetate side chain (B2-O-Acetate), B2-O-Et- carrying an propionamide side chain (B2-O-Et-Propionamide) and B2-O- carrying an ethyl side chain (B2-O-Et). A concentration-dependent increase in LDH leakage from HepG2 cells and isolated rat hepatocytes was observed in the presence of amiodarone and of most analogues, confirming their hepatocellular toxicity. Using freshly isolated rat liver mitochondria, amiodarone and most analogues showed a dose-dependent toxicity on the respiratory chain and on β-oxidation, significantly reducing the respiratory control ratio and oxidation of palmitate, respectively. The ROS concentration in hepatocytes increased time-dependently and apoptotic/necrotic cell populations were identified using flow cytometry and annexinV/propidiumiodide staining. The effect of the three least toxic amiodarone analogues on the hERG channel was compared to amiodarone. Amiodarone, B2-O-Acetate and B2-O-Et-N-dipropyl (each 10 µmol/L) significantly reduced the hERG tail current amplitude, whereas B2-O-Et (10 µmol/L) displayed no detectable effect on hERG outward potassium currents. In conclusion, three amiodarone analogues (B2-O-Et-N-dipropyl, B2-O-Acetate and B2-O-Et) showed a lower

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hepatocellular toxicity profile than amiodarone and two of these analogues (B2-O-Et-N-dipropyl and B2-O-Acetate) retained hERG channel interaction capacity, suggesting that amiodarone analogues with class III antiarrhythmic activity and lower hepatic toxicity could be developed.

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Introduction

Amiodarone (2-n-butyl-3-[3,5 diiodo-4-diethylaminoethoxybenzoyl]-benzofuran, B2-O-Et-N-diethyl) is a class III antiarrhythmic used in the treatment of a wide spectrum of cardiac arrhythmias (Singh, 1996). Amiodarone has been shown to be at least as efficacious as sotalol in patients with atrial fibrillation (Singh et al., 2005) and to reduce mortality in patients with a high risk for of arrhythmia, e.g. patients with severe congestive heart failure (Doval et al., 1994) or after acute myocardial infarction (1997). Amiodarone is a class III antiarrhythmic drug which blocks hERG channels, leading to prolongation of the refractoriness and resulting in QT prolongation (Singh, 1996). In addition, it has an inhibitory effect on fast sodium as well as on calcium channels (Singh, 1996). Similar to its pharmacological action, amiodarone's adverse reaction profile is complex, ranging from thyroidal (Harjai and Licata, 1997) to pulmonary (Jessurun et al., 1998), ocular (Pollak, 1999) and/or liver toxicity (Morse et al., 1988; Lewis et al., 1989). Amiodarone is a mitochondrial toxicant, uncoupling oxidative phosphorylation and inhibiting the electron transport chain and ß-oxidation of fatty acids (Fromenty et al., 1990a; Fromenty et al., 1990b; Spaniol et al., 2001a; Kaufmann et al., 2005).

Amiodarone is composed of a benzofuran ring carrying a C_4H_9 side chain and a highly lipophilic di-iodobenzene ring (B2) with a diethylaminoethoxy side chain (see Table 1). It is metabolized by desalkylation of the diethylaminoethoxy group to mono-N-desethylamiodarone (B2-O-Et-NH-ethyl) (Flanagan et al., 1982) and to di-N-desethylamiodarone (B2-O-Et-NH_2) (Ha et al., 2005), which may be transaminated and reduced to B2-O-Et-OH (see Table 1) (Ha et al., 2005).

In earlier studies, we have investigated the significance of the 2-butyl-benzofuran group and O-dealkylation of the amiodarone molecule with respect to mitochondrial

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toxicity (Spaniol et al., 2001a). These studies revealed that the benzofuran ring and the presence of iodines were important for mitochondrial toxicity. More recent studies (Kaufmann et al., 2005) showed, however, that not the benzofuran ring alone is responsible for hepatocellular toxicity of amiodarone, but that a side chain in position 2 and/or 3 of the benzofuran ring was necessary.

The principal aim of the current studies was to find out amiodarone derivatives without or with minimal mitochondrial toxicity, but still exhibiting inhibitory activity towards the hERG channel. We therefore synthesized eight amiodarone derivatives (including 3 metabolites) with different lipid solubilities (see Table 1). All of the derivatives synthesized contained a benzofuran ring carrying a butyl group and differed from each other only by their side chain. Amiodarone (B2-O-Et-N-diethyl), B2-O-Et-NH-ethyl, B2-O-Et-NH_2, B2-O-Et-N-dimethyl, B2-O-Et-N-dipropyl and B2-O-Et-Propionamide had side chains differing from each other by the substituents coupled to the nitrogen atom (see Table 1). In comparison, B2-O-Acetate, B2-O-Et-OH and B2-O-Et did not carry a nitrogen atom in the side chain coupled to the diiodophenyl ring (see Table 1). Hepatocellular toxicity was studied using freshly isolated rat liver mitochondria, primary rat hepatocytes and HepG2 cells. The effect on the blockade of the voltage-gated potassium channel hERG was tested for amiodarone and the least toxic analogues (B2-O-Et-N-dipropyl, B2-O-Et and B2-O-Acetate) in order to estimate their class III antiarrhythmic activity.

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Materials and methods

Amiodarone and amiodarone derivatives

Amiodarone hydrochloride was purchased from Sigma-Aldrich (Buchs, Switzerland). All of the amiodarone metabolites or analogues were synthesized starting from B2 as shown in Figure 1.

Chemistry, General Methods. All chemicals used in the synthesis work were purchased from Aldrich (Buchs, Switzerland) and were used without further purification. All melting points given are uncorrected. NMR spectra were obtained for all substances synthesized and will be reported elsewhere.

Synthesis of (2-Butyl-benzofuran-3-yl)-(4-hydroxy-3,5-diiodophenyl)-methanone (B2). This compound was prepared with a yield of 60% as described previously (Ha et al., 2000). Melting point (146.4-146.9°C) and NMR data (not shown) were in agreement with the previous report. As shown in Figure 1, B2 is the origin of the synthesis of all amiodarone metabolites and analogues used in this study.

Synthesis of (2-Butyl-benzofuran-3-yl)-4-[2-(ethylamino-ethoxy)-3,5-diiodophenyl]-methanone • hydrochloride (B2-O-Et-NH-ethyl). To a mixture of B2 (2 g, 3.66 mmol) and K_2CO_3 (1.66 g, 12 mmol) in toluene:water (2:1, v/v, total volume 75 mL) heated to 55-60 °C N-ethyl-2-chloroethylamine hydrochloride (2.66 g, 18.5 mmol) was added portionwise. N-ethyl-2-chloroethylamine hydrochloride had been prepared by Lasselle's method (Lasselle and Sundet, 1941). After the addition, the temperature was raised to reach reflux over 30 minutes, until the yellow color of B2 had disappeared. The reaction was refluxed for 1 additional hours and the phases were separated quickly by a separation funnel at 60°C. The toluene phase was washed three times with 25 mL of

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water at this temperature. The organic phase was evaporated to dryness; the residue suspended in 10 mL of 5% NH_3 and B2-O-Et-NH-ethyl was extracted three times with 15 mL toluene. The organic phases were separated by centrifugation, combined and evaporated to dryness under reduced pressure. Two mL of 10N HCl and 15 mL of toluene were added, and the liquids were removed under reduced pressure at 80°C. A white solid was obtained after three additional treatments with 10 mL toluene. The obtained residue was then crystallized from toluene, yielding 1.55 g (65%) of analytically pure B2-O-Et-NH-ethyl. The melting point was 176.8-177.7°C.

Synthesis of (2-Butyl-benzofuran-3-yl)-4-[2-(dimethylamino-ethoxy)-3,5-diiodophenyl]-methanone • hydrochloride (B2-O-Et-N-dimethyl) and (2-Butyl-benzofuran-3-yl)-4-[2-(dipropylamino-ethoxy)-3,5-diiodophenyl]-methanone • hydrochloride (B2-O-Et-N-dipropyl). B2-O-Et-N-dimethyl and B2-O-Et-N-dipropyl were prepared in a similar manner as described for B2-O-Et-NH-ethyl, but 2-(dimethylamino)ethyl chloride and 2-(diisopropylamino)ethyl chloride were used instead of N-ethyl-2-chloroethylamine hydrochloride. For B2-O-Et-N-dimethyl, the melting point was 89.5-89.7°C and for B2-O-Et-N-dipropyl 146.8-148.7°C.

Synthesis of [4-(2-Aminoethoxy)-3,5-diiodophenyl]-(2-butylbenzofuran-3-yl)-methanone • hydrochloride (B2-O-Et-NH_2). A mixture of B2 (1.2 g; 2 mmol), 2 mL (20 mmol) of 2-ethyl-2-oxazoline and 3 mL of toluene was heated at reflux for 1 h and cooled to room temperature. The mixture was taken up in 10 mL methylene chloride, washed with 4 N potassium hydroxide (3x20 mL), dried (Na_2CO_3) and concentrated in vacuo to give a brown oil. The oil was solidified by trituration with petroleum ether followed by recrystallization from ethyl acetate:hexane to give a white solid. Thin layer chromatography analysis using Merck precoated silica gel 60-F_{254} plates and

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hexane:isopropyl alcohol:25% NH_3 (84:15:1 v/v) as an eluant revealed only one spot with an Rf value of 0.42 with UV detection at 254 nm. The corresponding Rf value of B2 was 0.05. This intermediate compound corresponded to B2-O-Propionamide (see Figure 1) by NMR analysis (data not shown).

To this compound 10 mL of 6N HCl were added. The mixture was heated to 130°C for 3.5 h and then cooled to room temperature. Impurities were washed out by diethyl ether until the organic phase was colorless (5 x 5 mL). The precipitate was collected by filtration and washed with water. The compound was flash-chromatographed using silica gel Merck 60 (40 - 60 µm, 230 – 400 mesh) with hexane:isopropyl alcohol:25% NH_3 (84:15:1 v/v) as an eluant, yielding 0.6 g (60%) of hydrochloride salt of B2-O-Et-NH_2 as a white solid with a melting point of 200.5-201.4°C. B2-O-Et-NH_2 was found as a minor metabolite of amiodarone in humans, and its spectroscopic data have been reported in a previous study (Ha et al., 2005).

Synthesis of (2-Butyl-benzofuran-3-yl)-(4-ethoxy-3,5-diiodophenyl)-methanone (B2-O-Et). To a mixture of B2 (2.0 g, 3.66 mmol) in dry acetone (50 mL) iodoethyl (2.34 g, 15 mmol) was added over 20 min. The reaction was stirred at 50°C for 16 h. The insoluble salt was removed by filtration and the filtrate concentrated in vacuo to give 1.7 g (yield 75%) of B2-O-Et as a white solid. The melting point was 67.0-69.4°C.

Synthesis of (2-Butyl-benzofuran-3-yl)-[4-(2-hydroxyethoxy)-3,5-diiodophenyl]-methanone (B2-O-Et-OH). This compound was prepared in a similar manner as B2-O-Et, but iodoethyl was replaced by 2-chloroethanol. The final product was obtained as an oil. When stored in a closed vial at room temperature, it solidified after 10 days. The melting point was 69.6-72.9°C. B2-O-Et-OH was found as a minor metabolite of

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amiodarone in humans, and its spectroscopic data have been reported in a previous study (Ha et al., 2005).

Synthesis of [4-(2-butyl-benzofuran-3-carbonyl)-2,6-diiodophenyl]-acetic acid (B2-O-Acetate). The ethyl ester of B2-O-Acetate (not shown in Figure 1) was prepared from B2 and α-bromoethyl acetate as described by Carlsson et al. (Carlsson et al., 2002). The hydrolysis of the ester was performed in the presence of 0.1M NaOH at 22°C for 16 h. The melting point of the product was 186.4-187.8°C.

Amiodarone and the analogues were dissolved in dimethylsulfoxide (DMSO). The end concentration of DMSO in the experiments never exceeded 1% and control incubations contained the same DMSO concentration.

Determination of the octanol/water partition coefficient of amiodarone and derivatives

The octanol/water partition of the compounds synthesized was determined using reversed phase HPLC as described by Braumann (Braumann, 1986). HPLC of the substances was performed at 37°C using different 0.005M phosphate buffer (pH7.4)/methanol mixtures as an eluant. The octanol/water partition of a substance can be calculated based on the retention times obtained in the presence of different concentrations of methanol in the eluant (Braumann, 1986).

Other chemicals

1-[^{14}C]Palmitic acid was purchased from Amersham Pharmacia Biotech (Dübendorf, Switzerland), collagenase type 2 from BioConcept (Allschwil, Switzerland). Propidium iodide was from Molecular Probes (Eugene, OR, USA) and Alexa Fluor 633 labeled annexin V was a generous gift from Dr. Felix Bachmann, Aponetics Ltd.

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(Witterswil, Switzerland). All other chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland) and were of best quality available when not otherwise stated. All cell culture media, all supplements and fetal calf serum were from Gibco (Paisley, UK), except for Williams E, which was purchased from Cambrex Bio Science (Verviers, Belgium). The 96-well-plates and the 12-well-plates were purchased from Beckton Dickinson (Franklin Lakes, NJ, USA).

Animals

Male Sprague-Dawley rats were purchased from Charles River Laboratories (L'Arbresle, France), and kept in the animal facility of the University Hospital Basel, Switzerland. Rats were kept in a temperature-controlled environment with a 12-h light-dark cycle and food and water ad libitum. The study protocol had been accepted by the Animal Ethics Committee of the Canton Basel Stadt.

Isolation of rat hepatocytes

The isolation of rat hepatocytes by a two step collagenase perfusion was based on a method described by Berry (Berry, 1974) and performed as described earlier (Kaufmann et al., 2005). Cell viability was determined by trypan blue exclusion and was always greater than 80%. Cells were seeded into cell culture dishes in Williams E medium supplemented with 10% heat-inactivated fetal calf serum, 10mmol/L HEPES buffer (pH 7.4), 2mmol/L GlutaMax® and 1000 U/mL penicillin/streptomycin. The mean rat weight was 395 ± 115 g.

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Isolation of rat liver mitochondria

Rat liver mitochondria were isolated by differential centrifugation according to the method of (Hoppel et al., 1979). The mitochondrial protein content was determined using the biuret method with bovine serum albumin as a standard (Gornall et al., 1949). The mean rat weight was 343 ± 91 g and the mean rat liver weight was 14.12 ± 2.81 g.

Cell lines and cell culture

HepG2 cells were kindly provided by Professor Dietrich von Schweinitz (University Hospital Basel, Switzerland). The cell line was cultured in Dulbecco's Modified Eagle Medium supplemented with 10% (v/v) inactivated fetal calf serum, 10 mmol/L HEPES buffer (pH 7.4), 2 mmol/L GlutaMAX (Invitrogen, Basel, Switzerland), nonessential amino acids, and penicillin/streptomycin (100 U/mL). The culture conditions were 5% CO_2 and 95% air atmosphere at 37°C.

Cell viability

Lactate dehydrogenase (LDH) leakage in the supernatant was determined as a measure of cell viability as described by Vassault (Vassault, 1983) and calculated as described by Huang et al. (Huang et al., 2000). LDH leakage from the cells treated with the test compounds was compared to the LDH leakage of cells lysed with the detergent NP40 (0.01% w/v). The activity in the supernatant of lysed cells was set at 100%.

Apoptosis and necrosis detection by annexin V binding and propidium iodide uptake

After an 18-h-incubation with the test compounds, hepatocytes were stained with 0.5 µl Alexa Fluor 633 labeled annexin V and 2 µl propidium iodide (final concentration:

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1.5 µg/L). After 15 minutes of incubation at 37ºC, the cells were analyzed by flow cytometry (FACS Calibur flow cytometer: Beckton Dickinson, San José, CA, USA). As positive controls for apoptosis, deoxycholic acid (100 µmol/L) was used. As a positive control for necrosis, cells were treated with the surfactant NP40 (final concentration 0.1%, v/v).

ATP content of the cells

Freshly isolated hepatocytes were seeded on a 12-well-plate (200,000 cells/well). Subsequently, they were treated for 18 hours with the test compounds. After the incubation, cells were trypsinized, the pellet was resuspended in 600 µL reagent grade water and snap-frozen in liquid nitrogen (Yang et al., 2002). Intracellular ATP concentration of the cells was determined using a luciferin-luciferase assay (Sigma, Deissenhofen, Germany).

Measurement of reactive oxygen species (ROS)

A fluorescence-based microplate assay (Wang and Joseph, 1999) was used for the evaluation of oxidative stress in primary hepatocytes treated with the test compounds. DCFH-DA is a membrane permeable, non-polar and non-ionic molecule. In the cytoplasm, it is hydrolyzed by intracellular esterases to non-fluorescent DCFH, which is oxidized to fluorescent dichlorofluorescein in the presence of reactive oxygen species (H_2O_2 and $O_2^{\cdot -}$). Hepatocytes were simultaneously exposed to test compounds and to DCFH-DA dissolved in ethanol (final concentration 5 µmol/L) and incubated for 2, 4, 6 and 18 hours. The fluorescence was measured using a microtiter plate reader (HTS 700

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Plus Bio Assay Reader, Perkin Elmer, Buckinhamshire, UK) in incubations containing cells and exposure medium at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Oxygen consumption and β-oxidation of intact mitochondria

Oxygen consumption by intact mitochondria was measured in a chamber equipped with Clark-type oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH) at 30ºC (Hoppel et al., 1979). The final concentration of L-glutamate was 20 mmol/L. The mitochondrial respiratory control ratio (RCR) as well as state 3 and state 4 of respiration were determined according to Estabrook (Estabrook, 1967) and as described by Krähenbühl et al. (Krahenbuhl et al., 1991). Uncoupling of the oxidative phosphorylation was investigated by monitoring oxygen consumption in the presence of test substances and oligomycin, an inhibitor of F_1F_0-ATPase (final concentration of 5 µg/mL) (Kaufmann et al., 2005).

Mitochondrial β-oxidation

Mitochondrial β-oxidation of [1-^{14}C] palmitic acid in the presence of test compounds was determined usingg freshly isolated liver mitochondria according to the method of Freneaux et al. (Freneaux et al., 1988) with some modifications as described by Spaniol et al. (Spaniol et al., 2001a).

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Effect of amiodarone, DIP-AMI, B2-O-AcOH and B2-O-Et on the inhibition of hERG currents

CHO hERG cells

The 3 least toxic compounds (B2-O-Et-N-dipropyl, B2-O-Acetate and B2-O-Et) and amiodarone were chosen for examination concerning their antiarrhythmic effect. The interaction of the test substances (final concentration 10 µmol/L) with the hERG channel was examined using CHO cells stably expressing this potassium channel. Briefly, two separate human cardiac plasmid cDNA libraries were prepared from freshly isolated tissue and the hERG alpha subunit PCR product released from the pCR2.1-TOPO vector (Invitrogen, Basel, Switzerland) for ligation into a modified pcDNA5/FRT/TO vector (Invitrogen, Basel, Switzerland) with excluded BGH site. Restriction analysis and complete sequencing confirmed the correct composition and expression of the hERG alpha subunit in the plasmid. CHO cells were transfected with the calciumphosphate precipitation method (Invitrogen, Basel, Switzerland). Clones were selected with 700µg/mL Hygromycin B (Invitrogen, Basel, Switzerland) and checked back electrophysiologically for the presence of sufficient potassium current, longterm recording stability and sealing properties. Using limited dilution, clone CHO hERG K2, which displayed an average tail current amplitude of approximately 700-1500pA, was isolated. This clone was successfully cultivated over 40 passages without detectable loss of current density and was used in the current studies. The cells were generally maintained and passaged in HAM/F12 with GlutaMax I (GIBCO, Paisley, UK) supplemented with 9% fetal bovine serum (GIBCO, Paisley, UK), 0.9% Penicillin/Streptomycin solution (GIBCO, Paisley, UK) and 500µg/mL Hygromycin B

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(Invitrogen, Basel, Switzerland). For electrophysiological measurements, the cells were seeded onto 35 mm sterile culture dishes containing 2 mL culture medium (without antibiotics or antimycotics). Confluent clusters of CHO cells are electrically coupled. Because responses in distant cells are not adequately voltage clamped and because of uncertainties about the extent of coupling, cells were cultured at a density enabling single cells (without visible connections to neighbouring cells) to be used for the experiments.

Electrophysiology

hERG currents were measured by means of the patch-clamp technique in the whole-cell configuration. The incubation buffer contained (in mmol/L) NaCl 137, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, D-glucose 10, HEPES 10, pH (NaOH) 7.40. The pipette solution consisted of (in mmol/L) KCl 130, $MgCl_2$ 1, MgATP 5, HEPES 10, EGTA 5, pH (KOH) 7.20. After formation of a Gigaohm seal between the patch electrodes and individual hERG stably transfected CHO cells, the cell membrane across the pipette tip was ruptured to assure electrical access to the cell interior. All solutions applied to cells were continuously perfused (1mL/min) and maintained at room temperature. As soon as a stable seal could be established, hERG outward tail currents were measured upon depolarization of the cell membrane to +20mV for 3 s (activation of channels) from a holding potential of -80mV and subsequent partial repolarization to -40 mV for 4s. This voltage protocol was run a minimum of 10 times at intervals of 10 s before application of the test compound. After equilibration (typically taking approximately 3 min), the voltage protocol was run continuously until the steady state level of current block was reached. At least three individual measurements were run per test compound. The steady state

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level currents were compared to those from control conditions (0.01% DMSO) and the residual current calculated as percentage of control.

Statistical methods

Data are presented as mean ± standard error of the mean (SEM) of at least three individual experiments. Differences between groups (control and test compound incubations) were analyzed by analysis of variance (ANOVA) and Dunnett´s post hoc test was performed if ANOVA showed significant differences. A p value ≤ 0.05 was considered to be significant.

Results

Oxygen consumption

Oxygen consumption by isolated mitochondria can roughly be divided into two states. In state 3, a substrate donates electrons to the electron transport chain and ADP is being phosphorylated to ATP. In state 4, ADP is depleted and therefore no ATP can be produced. Since the RCR is the ratio between these two states, a decrease in the RCR can be due to reduced state 3 of respiration (impairment of the function of electron transport chain) or an increase in state 4 of respiration (in most cases uncoupling of the activity of electron transport chain from production of ATP).

In order to get an overview about the effect on mitochondria, the influence of 1, 10 and 100µmol/L amiodarone and analogues on oxidative metabolism on isolated rat liver mitochondria was tested in the presence of L-glutamate as a substrate (see table 2). For

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all test compounds, the RCR decreased dose-dependently, and for all compounds, except for B2-O-Et, the decrease was statistically significant in comparison to control incubations. B2-O-Et did not have a significant effect on mitochondrial respiration at any concentration.

Most of the analogues and amiodarone were associated with an increase in state 4 respiration, suggesting uncoupling properties. In order to test this possibility, oxygen consumption was studied in the presence of test compounds and oligomycin. Oligomycin is an inhibitor of the F_1F_0-ATPase, and induces a so called state 4u when incubated with mitochondria. Under these conditions, an increase in oxygen consumption can only reflect uncoupling and not production of ADP by other mechanisms. After an incubation in the presence of oligomycin, amiodarone, B2-O-Et-N-dimethyl, B2-O-Et-N-dipropyl and B2-O-Acetate significantly increased oxygen consumption at 10μmol/L, and B2-O-Et-N-dipropyl and B2-O-Et-Propionamide at 100μmol/L (data not shown). In contrast, B2-O-Et-OH did not change the state 4u significantly up to 100μmol/L. B2-O-Et was not investigated, since it did not change the RCR (see Table 2). These data match well with the observed effect on state 4 respiration (compare with data in Table 2).

Mitochondrial β-oxidation

Since amiodarone is known to impair mitochondrial β-oxidation (Fromenty et al., 1990b; Kaufmann et al., 2005), the effect on the metabolism of palmitate was investigated. In comparison to the control incubations, amiodarone and most of the derivatives significantly decreased palmitate metabolism in a dose-dependent fashion

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(Figure 2). The exceptions were B2-O-Et-N-dipropyl and B2-O-Et, which did not affect mitochondrial β-oxidation up to 100µmol/L.

Production of ROS

Since increased ROS formation can be one of the consequences of the inhibition of the electron transport chain (Kaufmann et al., 2005), this was determined using isolated rat hepatocytes. ROS formation was measured after incubation for 2, 4, 6 or 18 hours with 100µmol/L amiodarone or its analogues. The emitted fluorescence increased time-dependently for all derivatives and for amiodarone as shown in Figure 3. After 18 hours of incubation, ROS production was significantly increased in comparison to control incubations for all compounds except for B2-O-Et-N-dipropyl and B2-O-Et.

Cell viability

Impaired mitochondrial ß-oxidation and/or impaired function of oxidative phosphorylation with ROS production can be associated with necrosis and apoptosis of the affected cells (Kaufmann et al., 2005). In order to investigate these possibilities, HepG2 cells or primary rat hepatocytes were treated for 18 hours with amiodarone or its analogues in rising concentrations (1, 10 and 100 µmol/L) and the extracellular LDH activity was measured in the supernatant as a marker of cell death (see Figure 4 for HepG2 cells). Amiodarone, its metabolites B2-O-Et-NH-ethyl, B2-O-Et-NH_2 and B2-O-Et-OH, as well as B2-O-Et-N-dimethyl, B2-O-Acetate and B2-O-Et-Propionamide showed a dose-dependent toxicity, which, at a concentration of 10 and/or 100µmol/L,

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was significant in comparison to control incubations. A similar pattern was obtained, when this experiment was repeated with primary rat hepatocytes (results not shown).

Mechanism of cell death

The mechanism of cell death was investigated using staining with annexin V/PI, which can differentiate between early apoptosis and late apoptosis/necrosis (Kaufmann et al., 2005). After 18 hours of incubation, hepatocytes showed a significant increase in late apoptosis/necrosis for all substances investigated, except for B2-O-Et-N-dimethyl, B2-O-Et-N-dipropyl, B2-O-Acetate and B2-O-Et (Figure 5). In agreement with these findings, the ATP content of the hepatocytes was reduced at this time point in the incubations containing amiodarone, B2-O-Et-NH-ethyl, B2-O-Et-NH_2, B2-O-Et-Propionamide and B2-O-Et-OH (data not shown). With the exception of B2-O-Et-N-dimethyl (significant LDH release and non-significant increase in late apoptosis/necrosis), these findings agree well with the results of the LDH leakage assay. The discrepancy between LDH release and annexin V/PI staining may be associated with extent of cell damage needed to obtain a positive result.

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Effects on the cardiac rapid delayed rectifier K^+ current (I_{Kr})

Since one of the important antiarrhythmic mechanisms of amiodarone is the inhibition of hERG channels, the effect of amiodarone and of the three least toxic amiodarone derivatives (B2-O-Et-N-dipropyl, B2-O-Acetate and B2-O-Et) on the K^+ current was investigated in CHO cells overexpressing hERG channels. As shown in Figure 6, amiodarone (10µmol/L) rapidly and robustly blocked hERG tail currents (6.28 ±

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4.05% tail current relative to control, mean ± SEM of n=3 experiments). The derivatives B2-O-Acetate (10μmol/L) and B2-O-Et-N-dipropyl (10μmol/L) also significantly inhibited the tail current, but less than amiodarone (remaining tail current 81.9 ± 2.6% and 76.2 ± 6.5%, respectively, relative to control values, mean ± SEM of n=3 experiments). No significant effect was observed for 10μmol/L B2-O-Et (94.6 ± 3.5% tail current relative to control, mean ± SEM of n=3 experiments). As expected, at higher concentrations (≥30μmol/L) the inhibitory effects of B2-O-Acetate and B2-O-Et-N-dipropyl was more pronounced with the exception of B2-O-Et, which again displayed no significant hERG blockage (data not shown). Also the vehicle (0.01% DMSO) did not interfere with hERG channel activity (96.70 ± 0.49%, relative tail current, mean ± SEM of n=3 experiments).

Discussion

In our current studies, amiodarone and most of its analogues demonstrated a similar toxicity pattern towards hepatic mitochondria as described earlier in similar investigations (Fromenty et al., 1990a; Fromenty et al., 1990b; Spaniol et al., 2001b; Kaufmann et al., 2005). As shown in Table 3, amiodarone and its analogues inhibited the function of the respiratory chain, impaired mitochondrial β-oxidation and/or uncoupled oxidative phosphorylation. Most substances were cytotoxic, exceptions were B2-O-Et and B2-O-Et-N-dipropyl.

Regarding B2-O-Et and the B2-O-Et-NR_2 derivatives, the pattern of cytotoxicity (strong cytotoxicity of the amiodarone metabolites B2-O-Et-NH-ethyl and B2-O-Et-NH_2, lower cytotoxicity for amiodarone and very low or lacking cytotoxicity for B2-O-Et) was very similar to the toxicity found on alveolar macrophages, as reported in a recent investigation (Quaglino et al., 2004). As shown for B2-O-Et-N-dipropyl, uncoupling of the

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respiratory chain was not sufficient to induce cytotoxicity. Incomplete uncoupling (mitochondria are still able to produce some ATP) and extramitochondrial production of ATP (e.g. by glycolysis) could serve as explanations for this finding. As evidenced e.g. by amiodarone and by the amiodarone metabolites B2-O-Et-NH-ethyl and B2-O-Et-NH_2, cytotoxicity is associated primarily with substances that affect the function of the respiratory chain and/or mitochondrial β-oxidation. Inhibition of the respiratory chain can be associated with ROS production (Kaufmann et al., 2005), which can trigger opening of the mitochondrial permeability transition pore, leading to the release of cytochrome c and other substances into the cytoplasm and triggering apoptosis and/or necrosis, depending on the ATP content of the cell (Eguchi et al., 1997; Leist et al., 1997). In combination with the concomitant drop in the cellular ATP content, the results in Figure 4 therefore indicate that after 18 hours of incubation most hepatocytes had undergone necrosis in the presence of 100µmol/L amiodarone, B2-O-Et-NH-ethyl, B2-O-Et-NH_2, B2-O-Et-Propionamide or B2-O-Et-OH.

Amiodarone and the amiodarone metabolites B2-O-Et-NH-ethyl and B2-O-NH_2 were shown to be strong inhibitors of mitochondrial β-oxidation and of the respiratory chain (B2-O-Et-NH-ethyl and B2-O-NH_2) or uncouplers of oxidative phosphorylation (amiodarone). Regarding amiodarone, mitochondrial toxicity explains the histological findings in liver biopsies from patients (Lewis et al., 1990) and mice (Fromenty et al., 1990b) with amiodarone-associated hepatotoxicity, revealing micro- and macrovesicular accumulation of fat in hepatocytes as a hallmark of their toxicity. Accumulation of small lipid droplets in hepatocytes (microvesicular steatosis) is considered to be a consequence of the inhibition of β-oxidation in hepatocellular mitochondria (Fromenty and Pessayre, 1995). It can therefore be predicted that beside amiodarone, also its

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metabolites B2-O-Et-NH-ethyl, B2-O-Et-NH_2 and B2-O-Et-OH as well as the other amiodarone analogues synthesized and tested (all of them except B2-O-Et and B2-O-Et-N-dipropyl) will most probably be associated with microvesicular steatosis. For amiodarone, it has been shown that inhibition of carnitine palmitoyltransferase I (CPT I) is a mechanism for the inhibition of β-oxidation (Kennedy et al., 1996). This may also be the case for the amiodarone metabolites and analogues, but formal proof is so far lacking.

For amiodarone, the toxicity found in the current investigations (strong uncoupling activities and inhibition of mitochondrial β-oxidation) is in agreement with previous investigations (Fromenty et al., 1990a; Spaniol et al., 2001a; Kaufmann et al., 2005). Since the two metabolites B2-O-Et-NH-ethyl and B2-O-NH_2 are strong inhibitors of the respiratory chain and are both associated with ROS production (which may be a consequence of the inhibition of the respiratory chain (Kaufmann et al., 2005)) and with a remarkable cytotoxicity, it appears to be possible that they are at least partially responsible for the hepatic toxicity in patients treated with amiodarone. If this were the case, induction of CYP3A4, the main CYP isozyme responsible for amiodarone deethylation (Fabre et al., 1993), may be a risk factor for hepatotoxicity associated with amiodarone. While a high dosage and/or high plasma levels of amiodarone are considered to represent risk factors for hepatotoxicity associated with this drug (Pollak et al., 1990; Bravo et al., 2005), induction of CYP3A4 has so far not been reported to be a risk factor for amiodarone-associated liver injury (Rigas et al., 1986; Flaharty et al., 1989; Lewis et al., 1989). Since CYP3A4 inducers (e.g. antiepileptics such as phenytoin, phenobarbital and carbamazepine as well as rifampicin) are used quite frequently and

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since hepatotoxicity associated with amiodarone is potentially fatal (Lewis et al., 1989), this question is clinically important and should therefore be investigated and answered.

As shown in Table 3, the toxicity of the investigated substances showed tendencies but no clear correlation with their lipophilicity profile. Inhibition of the respiratory chain (state 3 respiration), of mitochondrial β-oxidation, ROS production and cytotoxicity were preferentially associated with less lipophilic substances. On the other hand, uncoupling of oxidative phosphorylation was associated preferentially with substances showing a higher lipophilicity. Since all substances investigated showed some mitochondrial toxicity (at least uncoupling of oxidative phosphorylation or inhibition of β-oxidation), all of the compounds studied had to be able to penetrate the inner mitochondrial membrane. Lack of penetration of the mitochondrial membranes is therefore no probable explanation for a low toxicity.

The lack of a clear relationship between lipophilicity of the substances and their cytotoxicity may be explained at least partially by the rather small differences in their lipophilicity. The log P values of the substances tested were between 3.83 and 5.51, indicating that all compounds investigated are lipophilic, and that the most lipophilic substance (B2-O-Et-N-dipropyl) has an approximately 50 times lower solubility in water than the compound with the lowest lipophilicity (B2-O-Et-NH_2). In addition to their lipophilicity profile, the observed differences in hepatic mitochondrial toxicity between the compounds tested may therefore reflect also the composition of the side chain attached to B2. This is for instance substantiated by B2-O-Et, which has a quite high lipophilicity (log P 4.83), but almost no cytotoxicity. The cytotoxicity increases, however, when functional groups are attached to B2-O-Et, e.g. a hydroxyl group or an amino group with or without substituents. Regarding the amino groups, alkylation gradually

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decreases its toxicity (as shown by the comparison of the -NH_2, -N-dimethyl, -N-diethyl and -N-dipropyl derivatives), but increases the uncoupling activity. The increase in the uncoupling activity associated with substituents at the amino group may be explained by the positive inductive effect of the alkyl groups, rendering the amino group more basic and therefore better suitable as a proton carrier. Due to their better lipid solubility, derivatives with large alkyl substituents at the amino group may diffuse better out of the mitochondrial matrix after having been deprotonated in the basic environment of the mitochondrial matrix, thereby explaining as well the observed tendency for a lower toxicity on the electron transport chain and on β-oxidation.

Similar to mitochondrial toxicity, also the effect on hERG channels did not show a clear relation to the lipophilicity of the compounds tested. Although the four substances investigated had a similar lipophilicity profile (log P values between 4.69 and 5.51), their effect on hERG channels was quite different. B2-O-Et had no inhibitory effect, whereas B2-O-Acetate and B2-O-Et-N-dipropyl had a median and B2-O-Et-N-diethyl a strong inhibitory activity on the hERG channels. The functional groups may therefore not only be important for the toxicity of these substances, but also for their inhibitory activity on the hERG channels. Inhibition of hERG channels is associated with prolongation of the refractoriness of cardiac tissue and QT prolongation, resulting in an antiarrhythmic (class III) activity (Singh, 1996). However, in the case of overdosage and/or presence of certain risk factors such as electrolyte dysbalances, QT prolongation may become excessive and turn into so called torsade de pointes, a specific form of ventricular fibrillation which may be fatal (Hohnloser et al., 1994).

In conclusion, despite similar lipophilicity profiles, amiodarone and the investigated amiodarone metabolites and analogues show large differences in mitochondrial toxicity

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and inhibition of hERG channels, accentuating the importance of the functional groups attached to the side chain of B2. Our studies reveal the possibility to detect amiodarone analogues with activity against hERG channels but with a lower mitochondrial toxicity than amiodarone, potentially offering the possibility to find safer antiarrhythmic drugs.

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Footnotes

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Legends for Figures

Figure 1.

General scheme of the synthesis of amiodarone derivatives. All the amiodorane metabolites or derivatives used were synthesized from B2. B2 was synthesized as described previously (Ha et al., 2000). The derivatives B2-O-Et-NH-ethyl, B2-O-Et-NH_2, B2-O-Et-N-dimethyl, and B2-O-Et-N-dipropyl were prepared by condensing B2 with the respective 2-chloroethyl amine hydrochloride salts in the presence of K_2CO_3. Using the same procedure, B2-O-Et-OH, B2-O-Acetate and B2-O-Et were obtained from the reaction of B2 with iodoethanol, alpha-bromo-ethylacetate (subsequently hydrolyzed in 0.1M NaOH) and iodoethyl, respectively. The compound B2-O-Et-NH-Propionamide was synthesized from B2 and 2-ethyl-2-oxazoline. The yields of the reactions were between 70 and 85% and the purity of the final substances was ≥ 97% as assayed by HPLC. See Method section for more details on synthesis and characterization of the compounds.

Figure 2.

Effect of amiodarone and its analogues on ß-oxidation by isolated rat liver mitochondria. With the exception of B2-O-Et-N-dipropyl and B2-O-Et, amiodarone (B2-O-Et-N-diethyl) and all amiodarone derivatives impaired mitochondrial β-oxidation at a concentration of 10 to 100µmol/L. Incubations were performed as described in the Method section. Results are expressed as acid soluble products produced from [^{14}C]-palmitic acid and represent mean ± SEM of at least 4 experiments. All incubations contained 1% DMSO; *p<0.05 and **p<0.01 versus control incubations.

Figure 3.

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ROS formation by primary hepatocytes. ROS formation by primary hepatocytes treated with 100 µmol/L amiodarone and analogues increased time-dependently for all compounds tested, reaching statistical significance for amiodarone, B2-O-NH-ethyl, B2-O-Et-NH_2, B2-O-Et-N-dimethyl, B2-O-Acetate, B2-O-Et-Propionamide and B2-O-EtOH. Incubations were performed as described in Methods. Results are shown as mean ± SEM of at least three individual experiments in quadruplicate. All incubations contained 1% DMSO; *$p<0.05$ and **$p<0.01$ versus control incubations.

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Figure 4.

Cytotoxicity of amiodarone and amiodarone derivatives. Cytotoxicity was studied using HepG2 cells under the conditions described in the Method section. Amiodarone (B2-O-Et-N-diethyl), B2-O-Et-NH-ethyl, B2-O-Et-NH_2, B2-O-Et-N-dimethyl, B2-O-Acetate, B2-O-Et-Propionamide and B2-O-Et-OH increased the LDH leakage into the cell culture media dose-dependently after an incubation for 18 hours. In contrast, for B2-O-Et-N-dipropyl and B2-O-Et, the cell membrane remained tight under each concentration used for the incubations. Similar data were obtained using primary rat hepatocytes. Data are given as mean±SEM of at least three individual experiments in triplicates. All incubations (except “no treatment”) contained 1% DMSO; *p<0,05 and **p<0.01 versus control incubations.

Figure 5.

Mechanism of cell death in primary hepatocytes. Cell death was assessed using staining with Annexin V/propidiumiodide followed by flow cytometry. The assays were carried out as described in the Method section and quantified as described previously (Kaufmann et

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al., 2005). Deoxycholate (100 µmol/L) was used as a positive control for early apoptosis and the detergent NFP40 (0.01%, v/v) as a positive control for late apoptosis/necrosis. During apoptosis, phosphatidylserine is externalized and can be bound by Annexin V, which can be detected by flow cytometry. During late apoptosis or necrosis, propidiumiodide is able to enter the cells across disintegrated membranes and to stain DNA, which can be differentiated by flow cytometry from early apoptosis (Kaufmann et al., 2005). After 18 hours of incubation with the compounds shown in figure, the positive control (deoxycholate 100 µmol/L) was associated with early apoptosis, but none of the test compounds investigated. In comparison, late apoptosis/necrosis was detected in the presence of the positive control (NP40 0.01%, v/v), and also in the presence of amiodarone (B2-O-Et-N-diethyl), B2-O-Et-NH-ethyl, B2-O-Et-NH_2, B2-O-Et-Propionamide and B2-O-Et-OH (all at a concentration of 100µmol/L). The results are presented as mean±SEM of three individual experiments. With the exception of the incubation labelled "no treatment", all incubations contained 1% DMSO; *p<0,05 and **p<0.01 versus control incubations.

Figure 6.

Inhibition of potassium current. Representative current traces of potassium currents through hERG channels stably expressed in CHO cells are shown. Measurements were accomplished in the whole-cell patch-clamp configuration at room temperature. Outward currents were activated upon depolarization of the cell membrane from -80mV to +20mV for 3s. Partial repolarization to -40mV for 4s evoked large tail currents. At least three cells were recorded per test compound. The vehicle (0.1% DMSO) as well as 10µmol/L B2-O-Et had no significant effect on hERG channel activity (traces at the top). In

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contrast, amiodarone, B2-O-Acetate and B2-O-Et-N-dipropyl had a clear inhibitory effect on the hERG channels. The upper line in the figures depicts the control incubations (0.1% DMSO) and the lower line the incubations containing the test compounds.

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Table 1.

Chemical structures of amiodarone and of the amiodarone metabolites and analogues used in the current investigations. The chemical formulas of the substances given in Table 1 are spelled out in the Introduction. ClogP is the calculated octanol/water partition coefficient using Marvin software (www.chemaxon.com). LogP was determined using reversed phase HPLC at 37°C as described by Braumann (Braumann, 1986). The mobile phase consisted of different mixtures of 0.005M phosphate buffer pH7.4 and methanol (n=3 determination per compound, mean±SEM).

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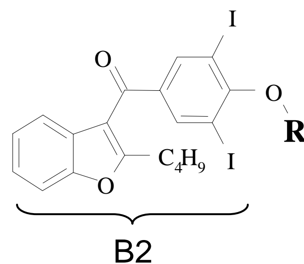

Compound	R	ClogP	logP
B2-O-Et-N-diethyl (amiodarone)	CH_2 N	7.3	4.92±0.25
B2-O-Et-NH-ethyl	CH_2 N H	6.6	4.47±0.12
B2-O-Et-NH_2	CH_2 N H H	5.9	3.83±0.10
B2-O-Et-N-dimethyl	CH_2 N CH_3 CH_3	6.7	4.40±0.07
B2-O-Et-N-dipropyl	CH_2 N CH_3 CH_3 H_3C CH_3	8.2	5.51±0.12
B2-O-Acetate	CH_2 O O H	6.4	4.69±0.08
B2-O-Et-Propionamide	CH_2 NH O C_2H_5	6.3	4.31±0.16
B2-O-Et	CH_2 CH_3	7.0	4.83±0.06
B2-O-Et-OH	CH_2 OH	6.2	4.18±0.15

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Table 2.

Effects of amiodarone, and amiodarone metabolites and analogues on oxidative metabolism in isolated rat liver mitochondria. The values represent mean±SEM of at least three experiments. All incubations contained 1%DMSO; *p<0.05 and **p<0.01 versus control incubations.

	State 3	State 4	RCR
	natoms O x min^{-1} x mg^{-1}		
Control (1%DMSO)	78.9 ± 15.0	2.30 ± 0.37	7.91 ± 1.28
B2-O-Et-N-diethyl (amiodarone) (µmol/L)			
1	72.9 ± 9.3	2.5 ± 0.5	6.60 ± 0.53
10	72.2 ± 7.7	4.17 ± 1.53*	4.21 ± 1.38**
100	64.5 ± 8.1	9.83 ± 2.25**	1.52 ± 0.31**
B2-O-Et-NH-ethyl (µmol/L)			
1	68.5 ± 16.9	3.67 ± 2.25	5.06 ± 2.17*
10	69.7 ± 8.0	3.33 ± 1.44	5.10 ± 1.42*
100	3.7 ± 1.3**	3.72 ± 1.29	1.00± 0.00**

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B2-O-Et-NH_2 (µmol/L)			
1	76.7 ± 3.2	2.50 ± 0.5	7.07 ± 1.5
10	75.2 ± 8.8	2.50 ± 0.5	7.01 ± 2.09
100	3.4 ± 1.6**	3.37 ± 1.61	1.00± 0.00**
B2-O-Et-N-dimethyl (µmol/L)			
1	75.1 ± 12.3	2.67 ± 0.58	6.40 ± 0.66
10	66.8 ± 6.0	3.67 ± 0.58**	4.15 ± 0.67**
100	5.2 ± 1.3**	5.20 ± 1.29**	1.00 ± 0.00**
B2-O-Et-N-dipropyl (µmol/L)			
1	77.3 ± 11.4	3.17 ± 0.29	5.55 ± 1.24*
10	72.1 ± 10.1	3.67 ± 1.26	4.70 ± 1.31**
100	68.4 ± 3.4	5.17 ± 2.08**	3.32 ± 1.27**

	State 3	State 4	RCR
	natoms O x min^{-1} x mg^{-1}		
B2-O-Acetate (µmol/L)			
1	69.1 ± 8.9	2.50 ± 0.87	6.50 ± 1.39
10	49.1 ± 10.2**	3.67 ± 0.58**	3.00 ± 0.38**
100	9.7 ± 5.2**	5.13 ± 1.15**	1.25 ± 0.43**
B2-O-Et-Propionamide (µmol/L)			

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1	63.2 ± 26.1	2.5 ± 2.71	5.67 ± 2.34*
10	63.9 ± 9.3	3.83 ± 0.29	3.77 ± 0.79**
100	47.6 ± 6.8*	47.57 ± 6.81**	1.00 ± 0.00**
B2-O-Et (μmol/L)			
1	84.7 ± 21.3	2.33 ± 0.29	8.40 ± 3.22
10	81.0 ± 15.2	2.50 ± 0.00	7.27 ± 1.36
100	73.6 ± 19.8	2.67 ± 0.29	6.12 ± 0.99
B2-O-Et-OH (μmol/L)			
1	72.9 ± 13.4	2.50 ± 0.50	6.55 ± 0.18
10	75.8 ± 7.7	2.67 ± 0.29	6.37 ± 0.04
100	20.0 ± 3.2**	20.07± 3.15**	1.00 ± 0.00**

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Table 3

Overview of the toxicological properties of amiodarone and analogues and of their effect on hERG channels. The compounds are listed with increasing lipophilicity. The graduation was as follows. For state3, state4, state4u, β-oxidation and cytotoxicity: + = $p<0.05$ at 100μmol/L, ++ = $p<0.01$ at 100μmol/L and +++ = $p<0.05$ at 10μmol/L. For ROS production: + = $p<0.05$ at 18h, ++ = $p<0.05$ at 6h and +++ = $p<0.05$ at 4h. For the effect on hERG channels: + = $p<0.05$ at 10μmol/L, ++ = $p<0.01$ at 10μmol/L and +++ = $p<0.001$ at 10μmol/L.

	logP	State3	State4	State4u	β-oxida-tion	ROS	Cytotoxi-city[1]	Effect on hERG channels
B2-O-Et-NH_2	3.83	++	0	ND	++	+++	++	ND
B2-O-Et-OH	4.18	0	0	0	++	+	++	ND
B2-O-Et- Pro-pionamide	4.31	+++	+++	++	+++	+	+	ND
B2-O-Et-N-dimethyl	4.40	++	+++	+++	++	+	++	ND
B2-O-Et-NH-ethyl	4.47	++	0	ND	++	+	+++	ND
B2-O-Acetate	4.69	+	++	+++	++	+++	+	+
B2-O-Et	4.83	++	++		0	0	0	0
B2-O-Et-N-diethyl	4.92	0	+++	+++	++	+	++	+++

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(Amiodarone)								
B2-O-Et-N-dipropyl	5.51	0	++	+++	0	0	0	+

[1]Cytotoxicity was determined using the data of Figure 4

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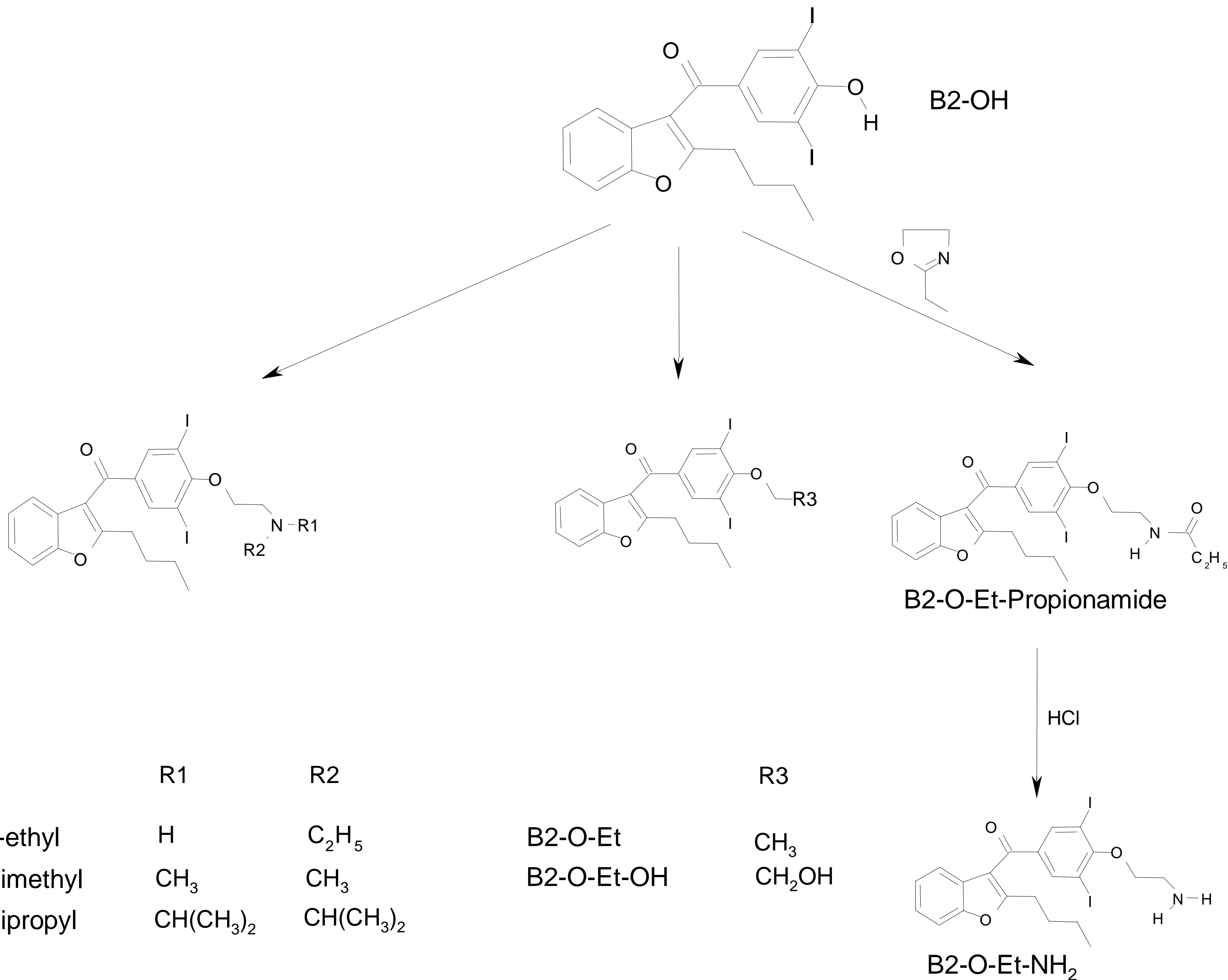
Figure 1.



	R1	R2
B2-O-Et-NH-ethyl	H	C_2H_5
B2-O-Et-N-dimethyl	CH_3	CH_3
B2-O-Et-N-dipropyl	$CH(CH_3)_2$	$CH(CH_3)_2$

	R3
B2-O-Et	CH_3
B2-O-Et-OH	CH_2OH

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Figure 2.

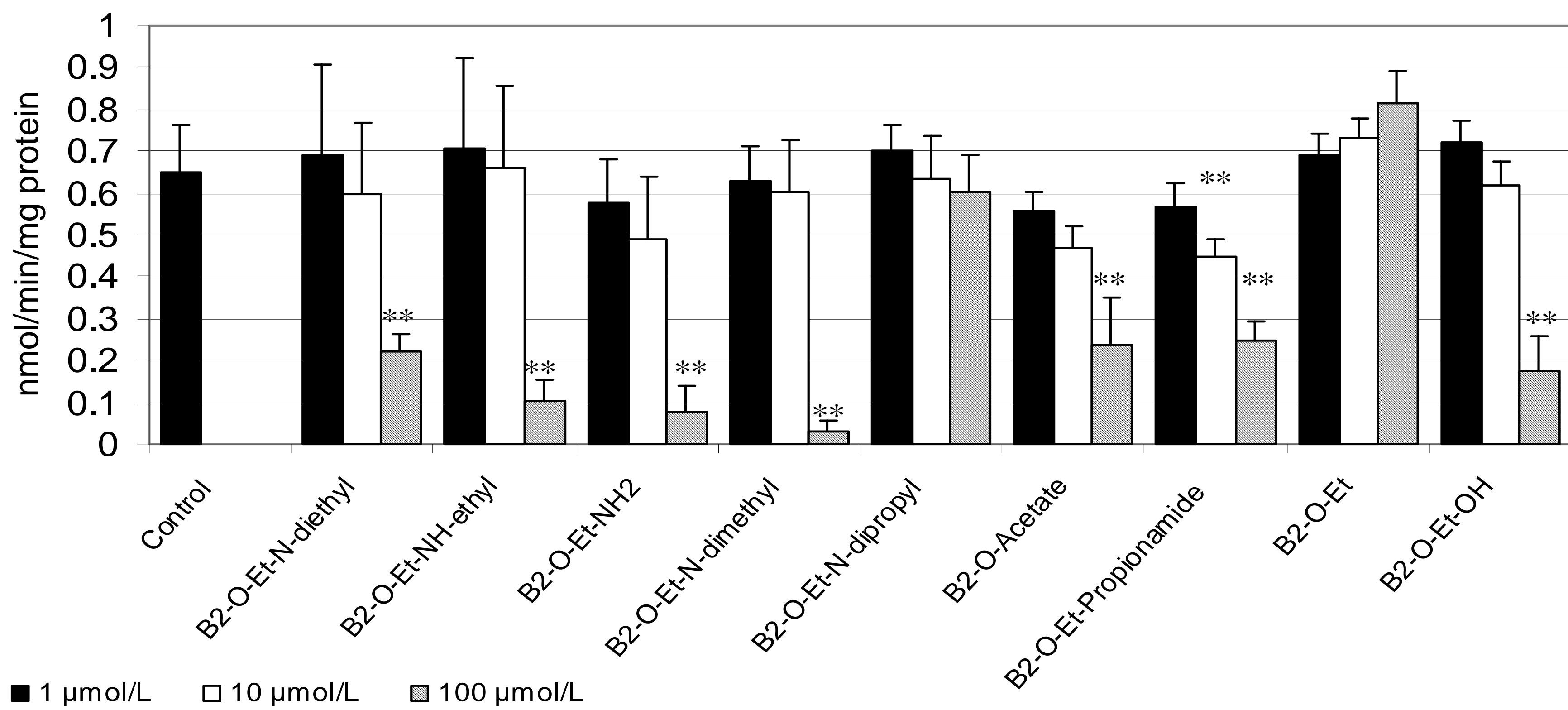

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Figure 3.

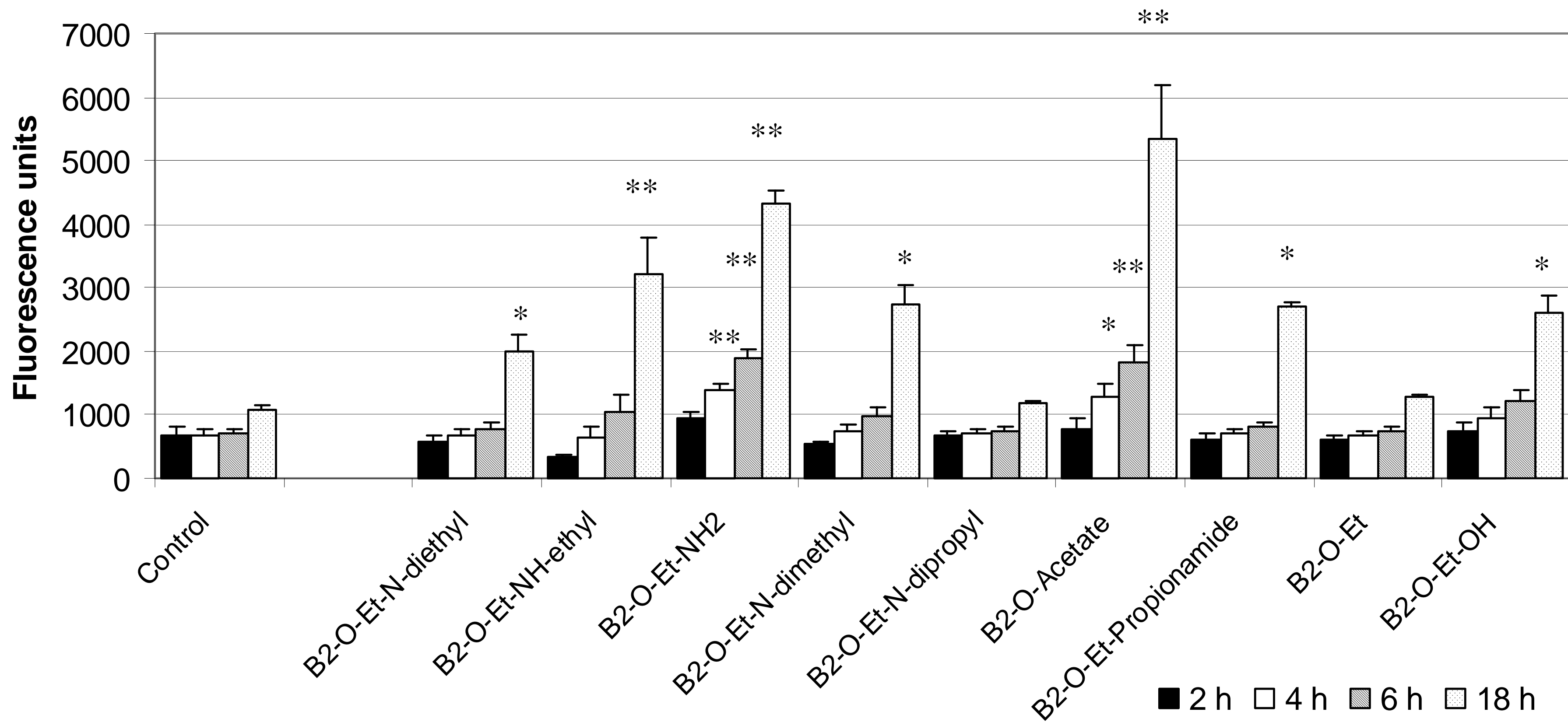

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Figure 4.

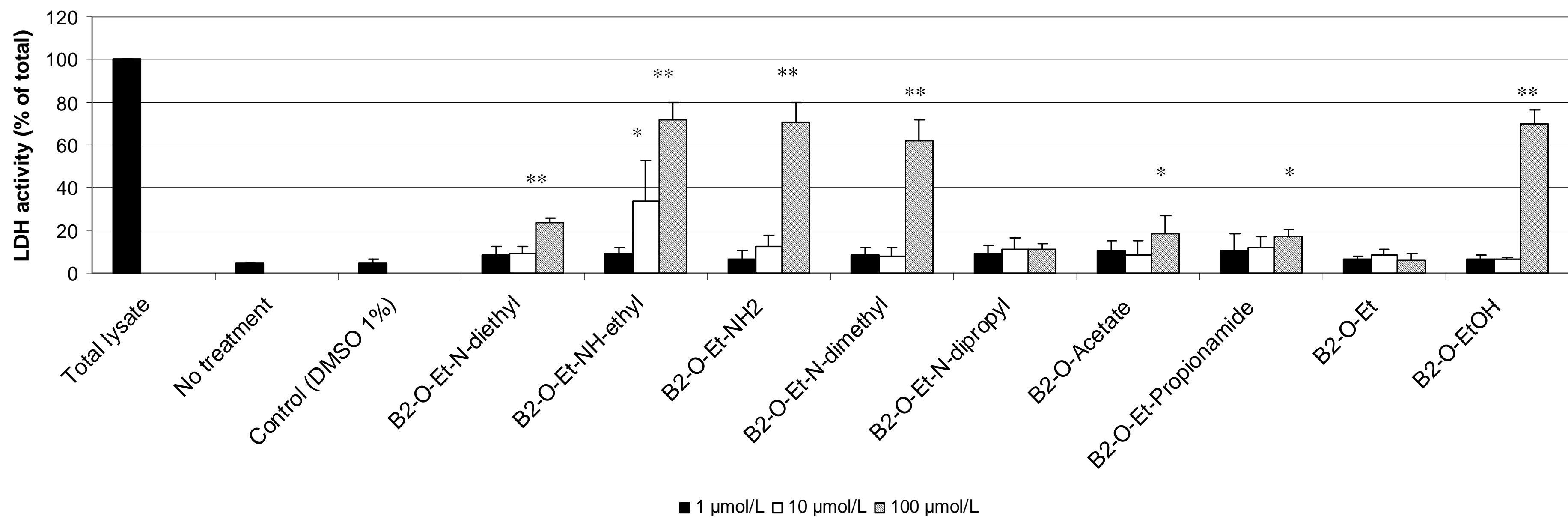

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Figure 5.

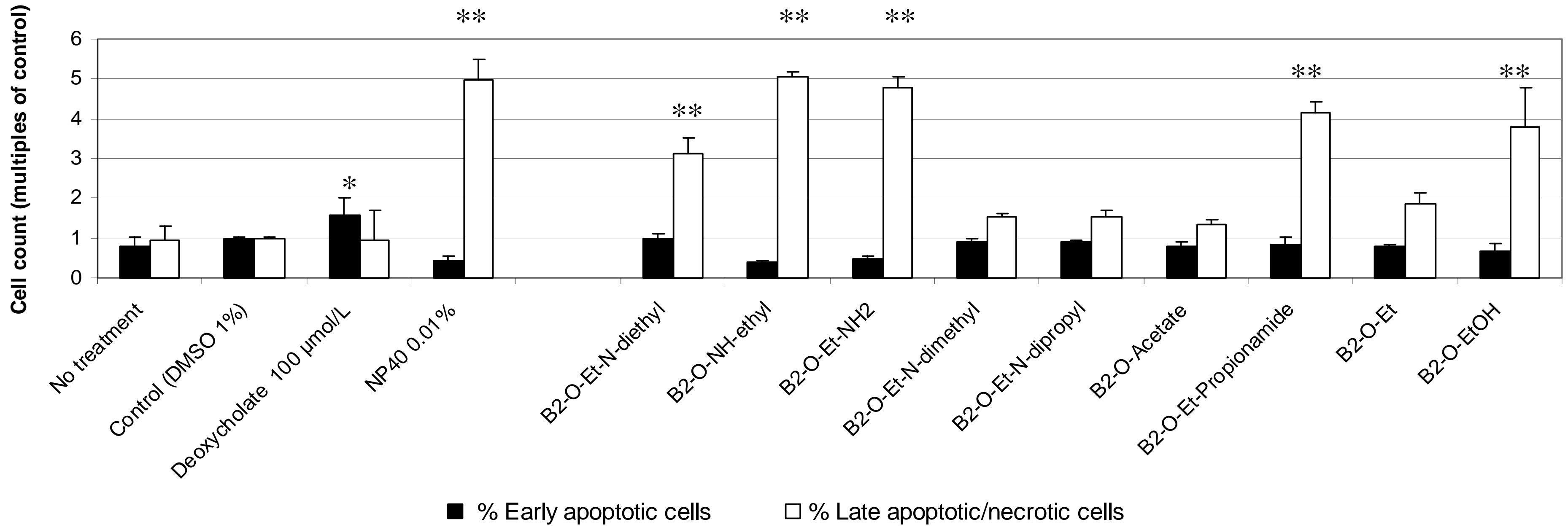

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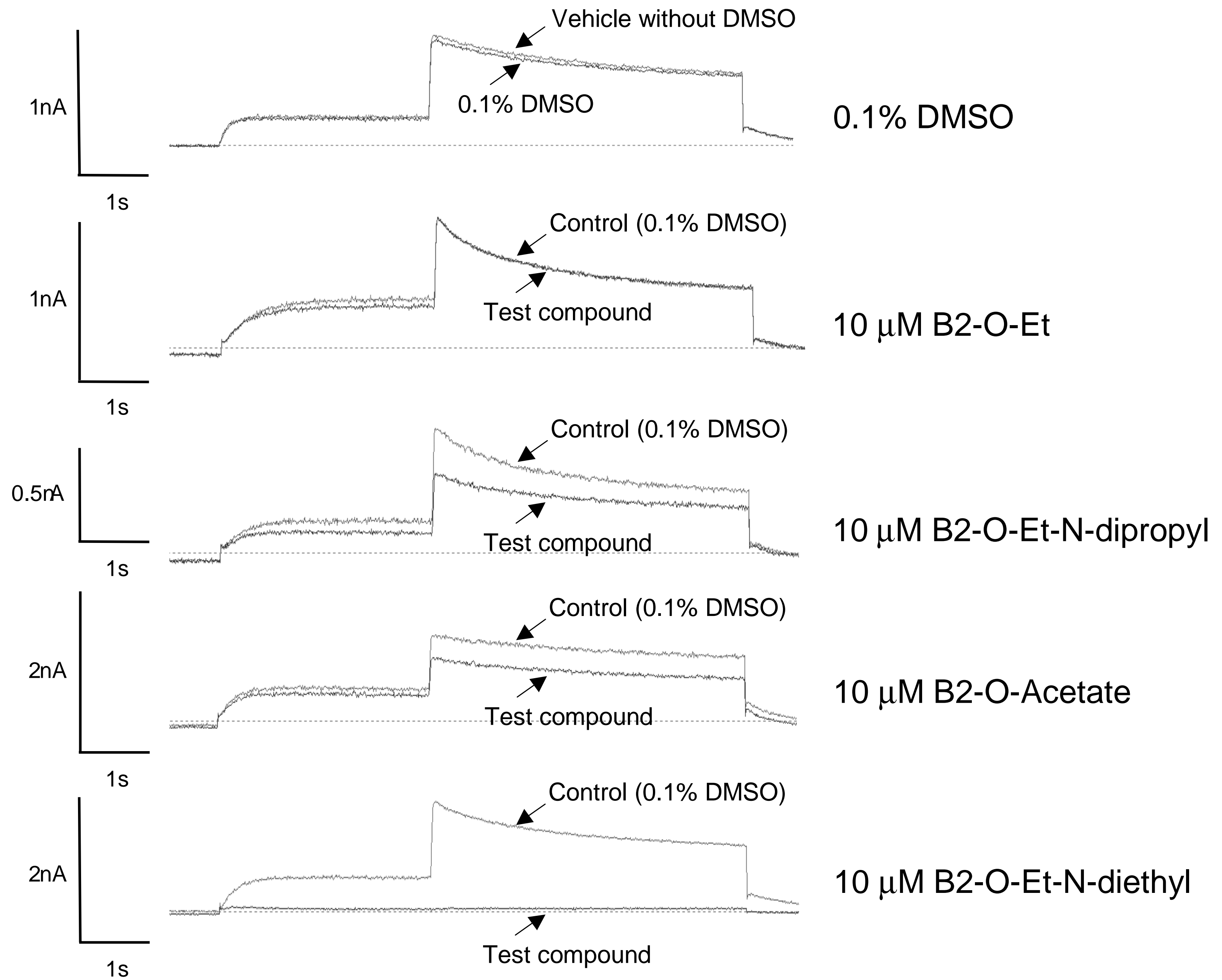
Figure 6.



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