

Hepatocellular Toxicity and Pharmacological Effect of Amiodarone and Amiodarone Derivatives

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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-NH₂), and (2-butyl-benzofuran-3-yl)-(4-hydroxy-3,5-diiodophenyl)-methanone (B2) carrying an ethanol side chain [(2-butyl-benzofuran-3-yl)-[4-(2-hydroxyethoxy)-3,5-diiodophenyl]-methanone; B2-O-Et-OH]. In addition, five amiodarone analogs were investigated, namely, *N*-dimethylamiodarone (B2-O-Et-*N*-dimethyl), *N*-dipropylamiodarone (B2-O-Et-*N*-dipropyl), B2-O carrying an acetate side chain [[4-(2-butyl-benzofuran-3-carbonyl)-2,6-diiodophenyl]-acetic acid; B2-O-acetate], B2-O-Et carrying an propionamide side chain (B2-O-Et-propionamide), and B2-O carrying an ethyl side chain [(2-butyl-benzofuran-3-yl)-(4-ethoxy-3,5-diiodophenyl)-methanone; B2-O-Et]. A concentration-dependent increase in lactate dehydrogenase leakage from HepG2 cells and isolated rat hepatocytes was observed in the presence of amiodarone and

of most analogs, confirming their hepatocellular toxicity. Using freshly isolated rat liver mitochondria, amiodarone and most analogs 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 reactive oxygen species concentration in hepatocytes increased time-dependently, and apoptotic/necrotic cell populations were identified using flow cytometry and annexin V/propidium iodide staining. The effect of the three least toxic amiodarone analogs on the human *ether-a-go-go*-related gene (*hERG*) channel was compared with amiodarone. Amiodarone, B2-O-acetate, and B2-O-Et-*N*-dipropyl (each 10 μ M) significantly reduced the *hERG* tail current amplitude, whereas 10 μ M B2-O-Et displayed no detectable effect on *hERG* outward potassium currents. In conclusion, three amiodarone analogs (B2-O-Et-*N*-dipropyl, B2-O-acetate, and B2-O-Et) showed a lower hepatocellular toxicity profile than amiodarone, and two of these analogs (B2-O-Et-*N*-dipropyl and B2-O-acetate) retained *hERG* channel interaction capacity, suggesting that amiodarone analogs with class III antiarrhythmic activity and lower hepatic toxicity could be developed.

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 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 that 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

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ABBREVIATIONS: B2-O-Et-*N*-diethyl, 2-*n*-butyl-3-[3,5 diiodo-4-diethylaminoethoxybenzoyl]-benzofuran; *hERG*, human *ether-a-go-go*-related gene; B2, 2-butyl-benzofuran-3-yl-(4-hydroxy-3,5-diiodophenyl)-methanone; B2-O-Et-NH-ethyl, (2-butyl-benzofuran-3-yl)-4-[2-(ethylaminoethoxy)-3,5-diiodophenyl]-methanone-hydrochloride; B2-O-Et-*N*-dimethyl, 2-butyl-benzofuran-3-yl)-4-[2-(dimethylaminoethoxy)-3,5-diiodophenyl]-methanone-hydrochloride; B2-O-Et-*N*-dipropyl, 2-butyl-benzofuran-3-yl)-4-[2-(dipropylaminoethoxy)-3,5-diiodophenyl]-methanone-hydrochloride; B2-O-Et-NH₂, [4-(2-aminoethoxy)-3,5-diiodophenyl]-(2-butylbenzofuran-3-yl)-methanone-hydrochloride; B2-O-Et-OH, (2-butyl-benzofuran-3-yl)-[4-(2-hydroxyethoxy)-3,5-diiodophenyl]-methanone; B2-O-Et, 2-butyl-benzofuran-3-yl)-(4-ethoxy-3,5-diiodophenyl)-methanone; B2-O-acetate, [4-(2-butyl-benzofuran-3-carbonyl)-2,6-diiodophenyl]-acetic acid; DMSO, dimethyl sulfoxide; HPLC, high-performance liquid chromatography; NP-40, Nonidet P-40; ROS, reactive oxygen species; DCFH, 2,7-dichlorofluorescein; RCR, respiratory control ratio; CHO, Chinese hamster ovary.

(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 β -oxidation of fatty acids (Fromenty et al., 1990a,b; Spaniol et al., 2001a; Kaufmann et al., 2005).

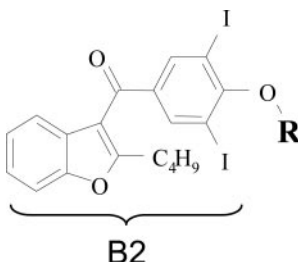
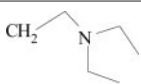
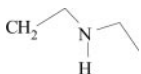
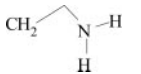
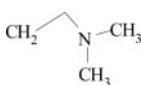
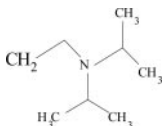
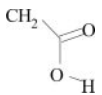
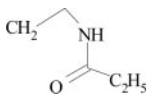

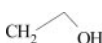
Amiodarone is composed of a benzofuran ring carrying a C_4H_9 side chain and a highly lipophilic diiodobenzene ring (B2) with a diethylaminoethoxy side chain (Table 1). It is metabolized by dealkylation of the diethylaminoethoxy group to mono-*N*-desethylamiodarone (B2-*O*-Et-NH-ethyl) (Flanagan et al., 1982) and to di-*N*-desethylamiodarone (B2-*O*-Et-NH₂) (Ha et al., 2005), which may be transaminated and reduced to B2-*O*-Et-OH (Table 1) (Ha et al., 2005).

In previous studies, we investigated the significance of the 2-butyl-benzofuran group and *O*-dealkylation of the amiodarone molecule with respect to mitochondrial 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 study was to find amiodarone derivatives with minimal mitochondrial toxicity or without mitochondrial toxicity that still exhibit inhibitory activity toward the *hERG* channel. We therefore synthesized eight amiodarone derivatives (including three metabolites) with different lipid solubilities (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. B2-*O*-Et-*N*-diethyl, B2-*O*-Et-NH-ethyl, B2-*O*-Et-NH₂, 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 (Table 1). In comparison, B2-*O*-acetate, B2-*O*-Et-OH, and B2-*O*-Et did not

TABLE 1

Compound	 B2		
	R	ClogP	logP
B2- <i>O</i> -Et- <i>N</i> -diethyl (amiodarone)		7.3	4.92 ± 0.25
B2- <i>O</i> -Et-NH-ethyl		6.6	4.47 ± 0.12
B2- <i>O</i> -Et-NH ₂		5.9	3.83 ± 0.10
B2- <i>O</i> -Et- <i>N</i> -dimethyl		6.7	4.40 ± 0.07
B2- <i>O</i> -Et- <i>N</i> -dipropyl		8.2	5.51 ± 0.12
B2- <i>O</i> -Acetate		6.4	4.69 ± 0.08
B2- <i>O</i> -Et-propionamide		6.3	4.31 ± 0.16
B2- <i>O</i> -Et		7.0	4.83 ± 0.06
B2- <i>O</i> -Et-OH		6.2	4.18 ± 0.15

carry a nitrogen atom in the side chain coupled to the diiodophenyl ring (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 analogs (B2-*O*-Et-*N*-dipropyl, B2-*O*-Et, and B2-*O*-acetate) to estimate their class III antiarrhythmic activity.

Materials and Methods

Amiodarone and Amiodarone Derivatives

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

Chemistry and 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 (data not shown).

Synthesis of 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 Fig. 1, B2 is the origin of the synthesis of all amiodarone metabolites and analogs used in this study.

Synthesis of B2-*O*-Et-NH-ethyl. To a mixture of B2 (2 g; 3.66 mmol) and K₂CO₃ (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 was prepared by Lasselle's method (Lasselle and Sundet, 1941). After the addition, the temperature was raised to reach reflux over 30 min, until the yellow color of B2 disappeared. The reaction was refluxed for 1 additional hour, and the phases were separated quickly by a separation funnel at 60°C. The toluene phase was washed three times with 25 ml of water at this temperature. The organic phase was evaporated to dryness; the residue suspended in 10 ml of 5% NH₃ and B2-*O*-Et-NH-ethyl was extracted three times with 15 ml of toluene. The organic phases were

separated by centrifugation, combined, and evaporated to dryness under reduced pressure. Two milliliters of 10 N 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 B2-*O*-Et-*N*-dimethyl and 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, the melting point was 146.8–148.7°C.

Synthesis of B2-*O*-Et-NH₂. 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 of methylene chloride, washed with 4 N potassium hydroxide (3 × 20 ml), dried (Na₂CO₃), 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-F₂₅₄ plates and hexane/isopropyl alcohol/25% NH₃ [84:15:1 (v/v)] as an eluant revealed only one spot with a retention factor value of 0.42 with UV detection at 254 nm. The corresponding retention factor value of B2 was 0.05. This intermediate compound corresponded to B2-*O*-propionamide (Fig. 1) by NMR analysis (data not shown).

To this compound, 10 ml of 6 N HCl was 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 (five washes with 5 ml each). 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% NH₃ [84:15:1 (v/v)] as an eluant, yielding 0.6 g (60%) of hydrochloride salt of B2-*O*-Et-NH₂ as a white solid with a melting point of 200.5–201.4°C. B2-*O*-Et-NH₂ 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).

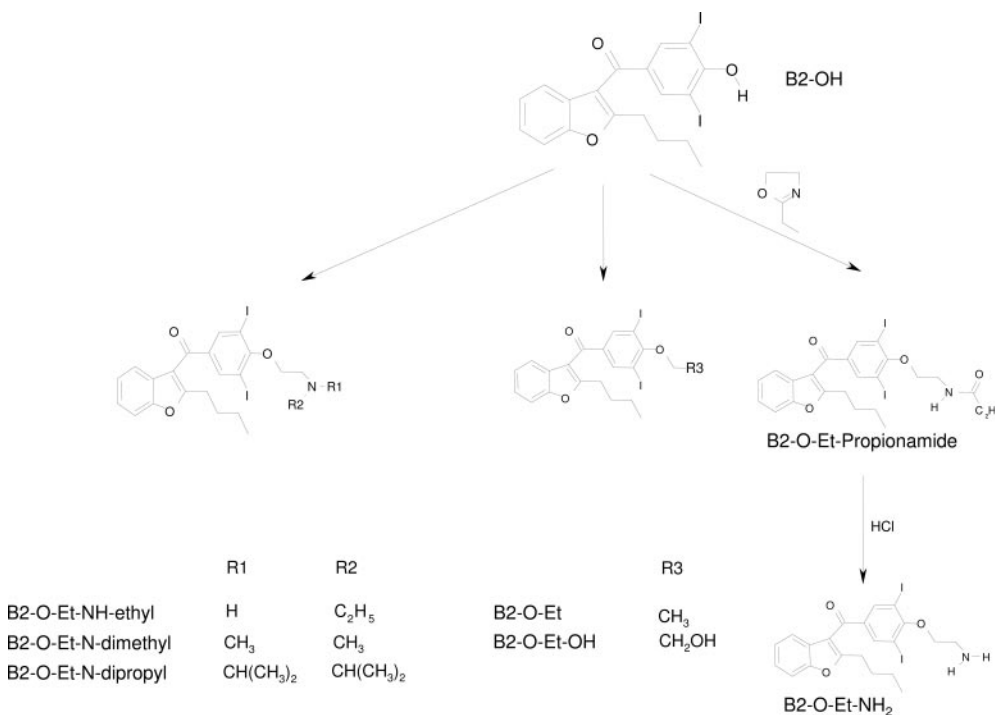


Fig. 1. General scheme of the synthesis of amiodarone derivatives. All the amiodarone metabolites or derivatives used were synthesized from B2. B2 was synthesized as described previously (Ha et al., 2000). The derivatives B2-*O*-Et-NH-ethyl, B2-*O*-Et-NH₂, B2-*O*-Et-*N*-dimethyl, and B2-*O*-Et-*N*-dipropyl were prepared by condensing B2 with the respective 2-chloroethyl amine hydrochloride salts in the presence of K₂CO₃. Using the same procedure, B2-*O*-Et-OH, B2-*O*-acetate and B2-*O*-Et were obtained from the reaction of B2 with iodoethanol, α -bromo-ethyl acetate (subsequently hydrolyzed in 0.1 M 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 $\geq 97\%$ as assayed by HPLC. See *Materials and Methods* for more details on synthesis and characterization of the compounds.

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

Synthesis of B2-O-Acetate. The ethyl ester of B2-O-acetate (not shown in Fig. 1) was prepared from B2 and α -bromoethyl acetate as described by Carlsson et al. (2002). The hydrolysis of the ester was performed in the presence of 0.1 M NaOH at 22°C for 16 h. The melting point of the product was 186.4–187.8°C.

Amiodarone and the analogs were dissolved in dimethyl sulfoxide (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 (1986). HPLC of the substances was performed at 37°C using different 0.005 M phosphate buffer, pH 7.4/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-[¹⁴C]Palmitic acid was purchased from Amersham Pharmacia Biotech (Dübendorf, Switzerland), and collagenase type 2 was from BioConcept (Allschwil, Switzerland). Propidium iodide was from Molecular Probes (Eugene, OR), and Alexa Fluor 633-labeled annexin V was a generous gift from Dr. Felix Bachmann (Aponetics Ltd., 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 S.p.r.l. (Verviers, Belgium). The 96-well plates and the 12-well plates were purchased from BD Biosciences (Franklin Lakes, NJ).

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 (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 (1974) and performed as described previously (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, 10 mM HEPES buffer, pH 7.4, 2 mM GlutaMAX (Invitrogen, Basel, Switzerland), and 1000 U/ml penicillin/streptomycin. The mean rat weight was 395 \pm 115 g.

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 \pm 91 g and the mean rat liver weight was 14.12 \pm 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's medium supplemented with 10% (v/v) inactivated fetal calf serum, 10 mM HEPES buffer, pH 7.4, 2 mM GlutaMAX (Invitrogen), nonessential amino acids, and 100 U/ml penicillin/streptomycin. The culture conditions were 5% CO₂ 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 (1983) and calculated as described by Huang et al. (2000). LDH leakage from the cells treated with the test compounds was compared with the LDH leakage of cells lysed with the detergent Nonidet P-40 [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 μ l of Alexa Fluor 633-labeled annexin V and 2 μ l of propidium iodide (final concentration 1.5 μ g/l). After 15 min of incubation at 37°C, the cells were analyzed by flow cytometry (FACSCalibur flow cytometer; BD Biosciences). As positive controls for apoptosis, 100 μ M deoxycholic acid was used. As a positive control for necrosis, cells were treated with the surfactant Nonidet P-40 [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 h with the test compounds. After the incubation, cells were trypsinized, the pellet was resuspended in 600 μ l of 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 Chemie, Deissenhofen, Germany).

Measurement of Reactive Oxygen Species

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-diacetate is a membrane-permeable, nonpolar, and nonionic molecule. In the cytoplasm, it is hydrolyzed by intracellular esterases to nonfluorescent DCFH, which is oxidized to fluorescent dichlorofluorescein in the presence of reactive oxygen species (H₂O₂ and O₂⁻). Hepatocytes were simultaneously exposed to test compounds and to DCFH-diacetate dissolved in ethanol (final concentration 5 μ M) and incubated for 2, 4, 6, and 18 h. The fluorescence was measured using a microtiter plate reader (HTS 700 Plus Bio Assay Reader; PerkinElmer, Beaconsfield, Buckinghamshire, 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 (YSI, Yellow

Springs, OH) at 30°C (Hoppel et al., 1979). The final concentration of L-glutamate was 20 mM. The mitochondrial respiratory control ratio (RCR) as well as state 3 and state 4 of respiration were determined according to Estabrook (1967) and as described by Krähenbühl 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 μ g/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 using freshly isolated liver mitochondria according to the method of Freneaux et al. (1988) with some modifications as described by Spaniol et al. (2001a).

Effect of Amiodarone, B2-O-Et-N-dipropyl, B2-O-Acetate, and B2-O-Et on the Inhibition of hERG Currents

Chinese Hamster Ovary hERG Cells. The three 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 μ M) with the hERG channel was examined using CHO cells stably expressing this potassium channel. In brief, two separate human cardiac plasmid cDNA libraries were prepared from freshly isolated tissue and the hERG α subunit PCR product released from the pCR2.1-TOPO vector (Invitrogen) for ligation into a modified pcDNA5/FRT/TO vector (Invitrogen) with excluded BGH site. Restriction analysis and complete sequencing confirmed the correct composition and expression of the hERG α subunit in the plasmid. CHO cells were transfected with the calcium phosphate precipitation method (Invitrogen). Clones were selected with 700 μ g/ml hygromycin B (Invitrogen) and checked back electrophysiologically for the presence of sufficient potassium current, long-term recording stability, and sealing properties. Using limited dilution, clone CHO hERG K2, which displayed an average tail current amplitude of approximately 700 to 1500 pA, 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's F-12 with GlutaMAX I (GIBCO) supplemented with 9% fetal bovine serum (GIBCO), 0.9% penicillin/streptomycin solution (GIBCO), and 500 μ g/ml hygromycin B (Invitrogen). For electrophysiological measurements, the cells were seeded onto 35-mm sterile culture dishes containing 2 ml of 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 neighboring 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 137 mM NaCl, 4 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 10 mM D-glucose, and 10 mM HEPES, pH (with NaOH) 7.40. The pipette solution consisted of 130 mM KCl, 1 mM MgCl_2 , 5 mM MgATP, 10 mM HEPES, and 5 mM EGTA, pH (with 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 ensure electrical access to the cell interior. All solutions applied to cells were continuously perfused (1 ml/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 +20 mV for 3 s (activation of channels) from a holding potential of -80 mV and subsequent partial repolarization to -40 mV for 4 s. 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 level currents were compared with those from control conditions (0.01% DMSO), and the residual current was calculated as percentage of control.

Statistical Methods

Data are presented as mean \pm S.E.M. of at least three individual experiments. Differences between groups (control and test compound incubations) were analyzed by analysis of variance and Dunnett's post hoc test was performed if analysis of variance 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; 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 to an increase in state 4 of respiration (in most cases, uncoupling of the activity of electron transport chain from production of ATP).

To get an overview about the effect on mitochondria, the influence of 1, 10, and 100 μ M amiodarone and analogs on oxidative metabolism on isolated rat liver mitochondria was tested in the presence of L-glutamate as a substrate (Table 2). For all test compounds, the RCR decreased dose dependently, and for all compounds, except for B2-O-Et, the decrease was statistically significant in comparison with control incubations. B2-O-Et did not have a significant effect on mitochondrial respiration at any concentration.

Most of the analogs and amiodarone were associated with an increase in state 4 respiration, suggesting uncoupling properties. 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 10 μ M, and B2-O-Et-N-dipropyl and B2-O-Et-propionamide at 100 μ M (data not shown). In contrast, B2-O-Et-OH did not change the state 4u significantly up to 100 μ M. B2-O-Et was not investigated, since it did not change the RCR (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 with the control incubations, amiodarone and most of the derivatives significantly decreased palmitate metabolism in a dose-dependent manner (Fig. 2). The exceptions were B2-O-Et-N-dipropyl and B2-O-Et, which did not affect mitochondrial β -oxidation up to 100 μ M.

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

TABLE 2

Effects of amiodarone and amiodarone metabolites and analogs on oxidative metabolism in isolated rat liver mitochondria

The values represent mean \pm S.E.M. of at least three experiments. Units for states 3 and 4 oxidation are nanoatoms/milligram of mitochondrial protein/minute. All incubations contained 1% DMSO; *, $p < 0.05$ and **, $p < 0.01$ versus control incubations.

	State 3	State 4	RCR
Control (1% DMSO)	78.9 \pm 15.0	2.30 \pm 0.37	7.91 \pm 1.28
B2-O-Et-N-diethyl (amiodarone) (μ M)			
1	72.9 \pm 9.3	2.5 \pm 0.5	6.60 \pm 0.53
10	72.2 \pm 7.7	4.17 \pm 1.53*	4.21 \pm 1.38**
100	64.8 \pm 8.1	9.83 \pm 2.25**	1.52 \pm 0.31**
B2-O-Et-NH-ethyl (μ M)			
1	68.5 \pm 16.9	3.67 \pm 2.25	5.06 \pm 2.17*
10	69.7 \pm 8.0	3.33 \pm 1.44	5.10 \pm 1.42*
100	3.7 \pm 1.3**	3.7 \pm 1.3	1.0 \pm 0.0**
B2-O-Et-NH ₂ (μ M)			
1	76.7 \pm 3.2	2.50 \pm 0.5	7.07 \pm 1.5
10	75.2 \pm 8.8	2.50 \pm 0.5	7.01 \pm 2.09
100	3.4 \pm 1.6**	3.4 \pm 1.6	1.0 \pm 0.0**
B2-O-Et-N-dimethyl (μ M)			
1	75.1 \pm 12.3	2.67 \pm 0.58	6.40 \pm 0.66
10	66.8 \pm 6.0	3.67 \pm 0.58*	4.15 \pm 0.67**
100	5.2 \pm 1.3**	5.2 \pm 1.3**	1.0 \pm 0.0**
B2-O-Et-N-dipropyl (μ M)			
1	77.3 \pm 11.4	3.17 \pm 0.29	5.55 \pm 1.24*
10	72.1 \pm 10.1	3.67 \pm 1.26	4.70 \pm 1.31**
100	68.4 \pm 3.4	5.17 \pm 2.08**	3.32 \pm 1.27**
B2-O-Acetate (μ M)			
1	69.1 \pm 8.9	2.50 \pm 0.87	6.50 \pm 1.39
10	49.1 \pm 10.2*	3.67 \pm 0.58*	3.00 \pm 0.38**
100	9.7 \pm 5.2**	5.13 \pm 1.15**	1.25 \pm 0.43**
B2-O-Et-propionamide (μ M)			
1	63.2 \pm 26.1	2.50 \pm 2.71	5.67 \pm 2.34*
10	63.9 \pm 9.3	3.83 \pm 0.29*	3.77 \pm 0.79**
100	47.6 \pm 6.8*	47.6 \pm 6.8**	1.0 \pm 0.0**
B2-O-Et (μ M)			
1	84.7 \pm 21.3	2.33 \pm 0.29	8.40 \pm 3.22
10	81.0 \pm 15.2	2.50 \pm 0.25	7.27 \pm 1.36
100	73.6 \pm 19.8	2.67 \pm 0.29	6.12 \pm 0.99
B2-O-Et-OH (μ M)			
1	72.9 \pm 13.4	2.50 \pm 0.50	6.55 \pm 0.18
10	75.8 \pm 7.7	2.67 \pm 0.29	6.37 \pm 0.40
100	20.0 \pm 3.2**	20.0 \pm 3.2**	1.0 \pm 0.0**

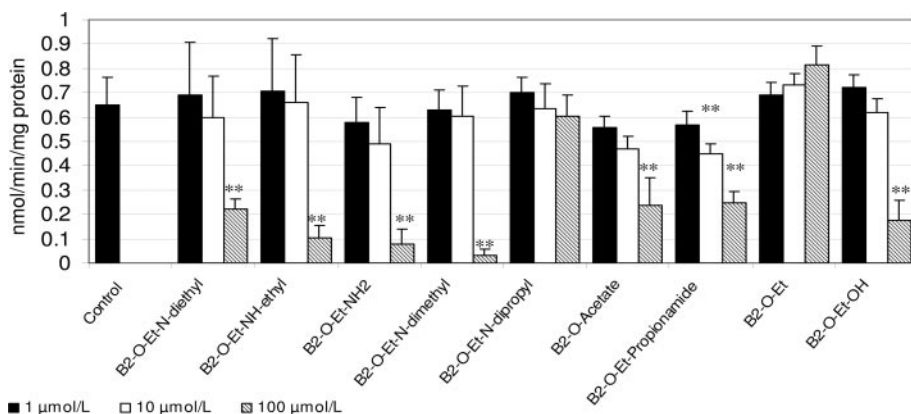


Fig. 2. Effect of amiodarone and its analogs on β -oxidation by isolated rat liver mitochondria. With the exception of B2-O-Et-N-dipropyl and B2-O-Et, amiodarone (B2-O-Et-N-diethyl), and all amiodarone derivatives impaired mitochondrial β -oxidation at a concentration of 10 to 100 μ M. Incubations were performed as described under *Materials and Methods*. Results are expressed as acid-soluble products produced from [14 C]palmitic acid and represent mean \pm S.E.M. of at least four experiments. All incubations contained 1% DMSO; *, $p < 0.05$ and **, $p < 0.01$ versus control incubations.

determined using isolated rat hepatocytes. ROS formation was measured after incubation for 2, 4, 6, or 18 h with 100 μ M amiodarone or its analogs. The emitted fluorescence increased time-dependently for all derivatives and for amiodarone as shown in Fig. 3. After 18 h of incubation, ROS production was significantly increased in comparison with control incubations for all compounds except for B2-O-Et-N-dipropyl and B2-O-Et.

Cell Viability. Impaired mitochondrial β -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). To investigate

these possibilities, HepG2 cells or primary rat hepatocytes were treated for 18 h with amiodarone or its analogs in rising concentrations (1, 10, and 100 μ M) and the extracellular LDH activity was measured in the supernatant as a marker of cell death (see Fig. 4 for HepG2 cells). Amiodarone, its metabolites B2-O-Et-NH-ethyl, B2-O-Et-NH₂, 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 100 μ M, was significant in comparison with control incubations. A similar pattern was obtained when this experiment was repeated with primary rat hepatocytes (data not shown).

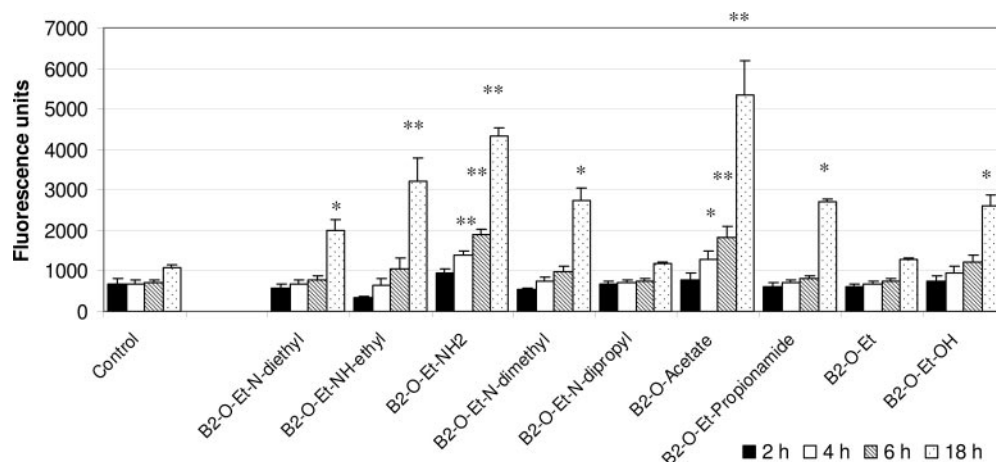


Fig. 3. ROS formation by primary hepatocytes. ROS formation by primary hepatocytes treated with 100 μM amiodarone and analogs increased time-dependently for all compounds tested, reaching statistical significance for amiodarone, B2-O-Et-NH-ethyl, B2-O-Et-NH₂, B2-O-Et-N-dimethyl, B2-O-acetate, B2-O-Et-propionamide, and B2-O-Et-OH. Incubations were performed as described under *Materials and Methods*. Results are shown as mean ± S.E.M. of at least three individual experiments in quadruplicate. All incubations contained 1% DMSO; *, $p < 0.05$ and **, $p < 0.01$ versus control incubations.

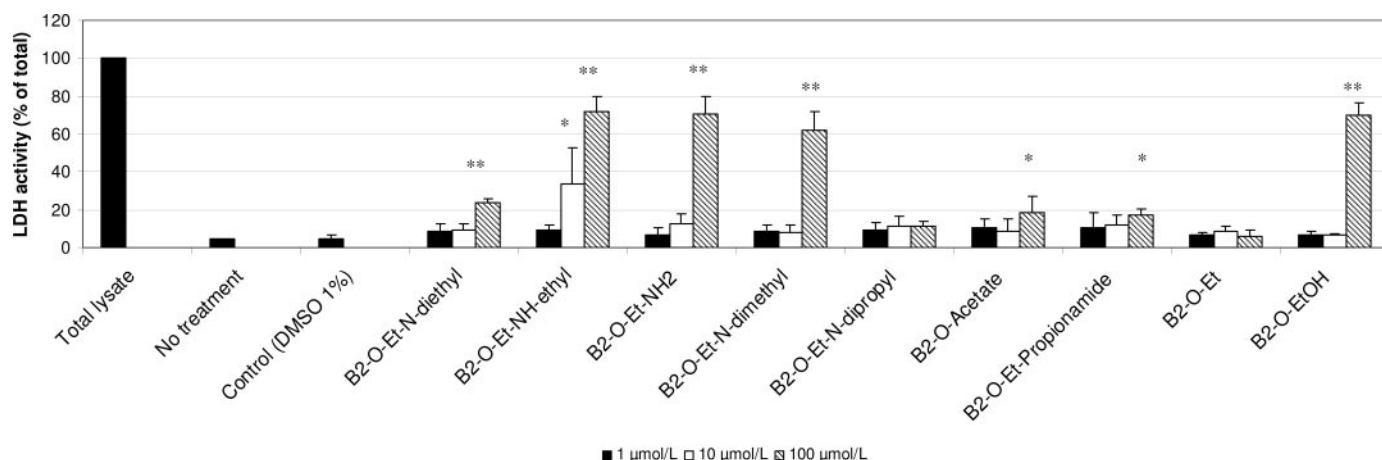


Fig. 4. Cytotoxicity of amiodarone and amiodarone derivatives. Cytotoxicity was studied using HepG2 cells under the conditions described under *Materials and Methods*. Amiodarone (B2-O-Et-N-diethyl), B2-O-Et-NH-ethyl, B2-O-Et-NH₂, B2-O-Et-N-dimethyl, B2-O-acetate, B2-O-Et-propionamide, and B2-O-Et-OH increased the LDH leakage into the cell culture media dose-dependently after an incubation for 18 h. 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 ± S.E.M. 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.

Mechanism of Cell Death. The mechanism of cell death was investigated using staining with annexin V/propidium iodide, which can differentiate between early apoptosis and late apoptosis/necrosis (Kaufmann et al., 2005). After 18 h 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 (Fig. 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-NH₂, 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 nonsignificant 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/propidium iodide staining may be associated with extent of cell damage needed to obtain a positive result.

Effects on the Cardiac Rapid Delayed Rectifier K⁺ Current. 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 overex-

pressing hERG channels. As shown in Fig. 6, 10 μM amiodarone rapidly and robustly blocked hERG tail currents ($6.28 \pm 4.05\%$ tail current relative to control, mean ± S.E.M. of $n = 3$ experiments). The derivatives B2-O-acetate (10 μM) and B2-O-Et-N-dipropyl (10 μM) also significantly inhibited the tail current but less so than amiodarone (remaining tail current 81.9 ± 2.6 and $76.2 \pm 6.5\%$, respectively, relative to control values, mean ± S.E.M. of $n = 3$ experiments). No significant effect was observed for 10 μM B2-O-Et ($94.6 \pm 3.5\%$ tail current relative to control, mean ± S.E.M. of $n = 3$ experiments). As expected, at higher concentrations (≥ 30 μM) 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). In addition, the vehicle (0.01% DMSO) did not interfere with hERG channel activity ($96.70 \pm 0.49\%$, relative tail current, mean ± S.E.M. of $n = 3$ experiments).

Discussion

In our current studies, amiodarone and most of its analogs demonstrated a similar toxicity pattern toward hepatic mitochondria as described in similar investigations (Fromenty et al., 1990a,b; Spaniol et al., 2001b; Kaufmann et al., 2005).

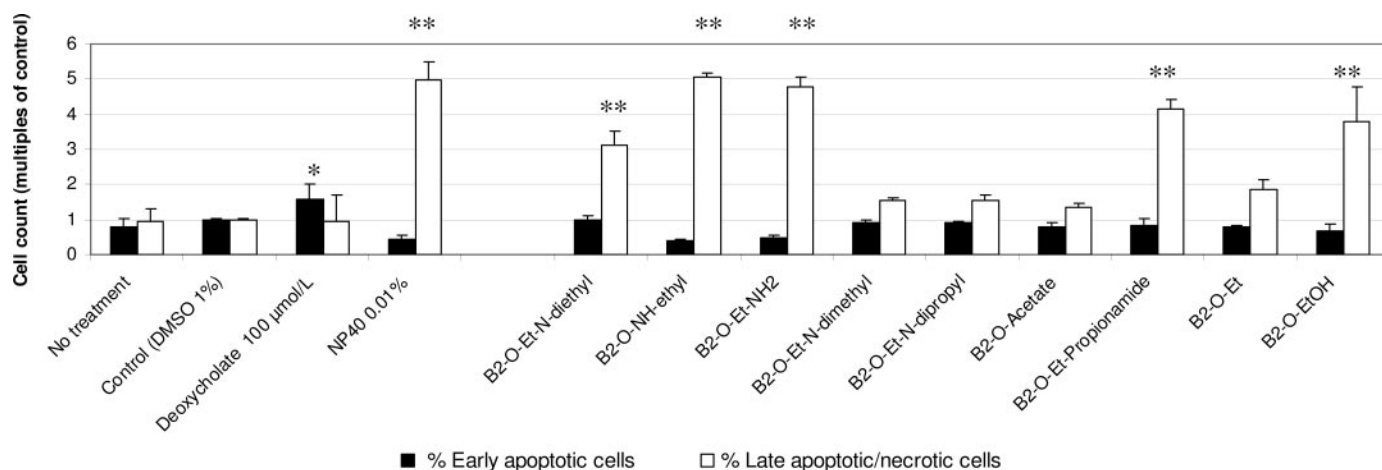


Fig. 5. Mechanism of cell death in primary hepatocytes. Cell death was assessed using staining with annexin V/propidium iodide followed by flow cytometry. The assays were carried out as described under *Materials and Methods* and quantified as described previously (Kaufmann et al., 2005). Deoxycholate (100 µM) was used as a positive control for early apoptosis and the detergent NP-40 [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, propidium iodide 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 h of incubation with the compounds shown in figure, the positive control (100 µM deoxycholate) 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 [NP-40 0.01% (v/v)] and also in the presence of amiodarone (B2-O-Et-N-diethyl), B2-O-Et-NH-ethyl, B2-O-Et-NH₂, B2-O-Et-propionamide, and B2-O-Et-OH (all at a concentration of 100 µM). The results are presented as mean ± S.E.M. of three individual experiments. With the exception of the incubation labeled “no treatment”, all incubations contained 1% DMSO; *, $p < 0.05$ and **, $p < 0.01$ versus control incubations.

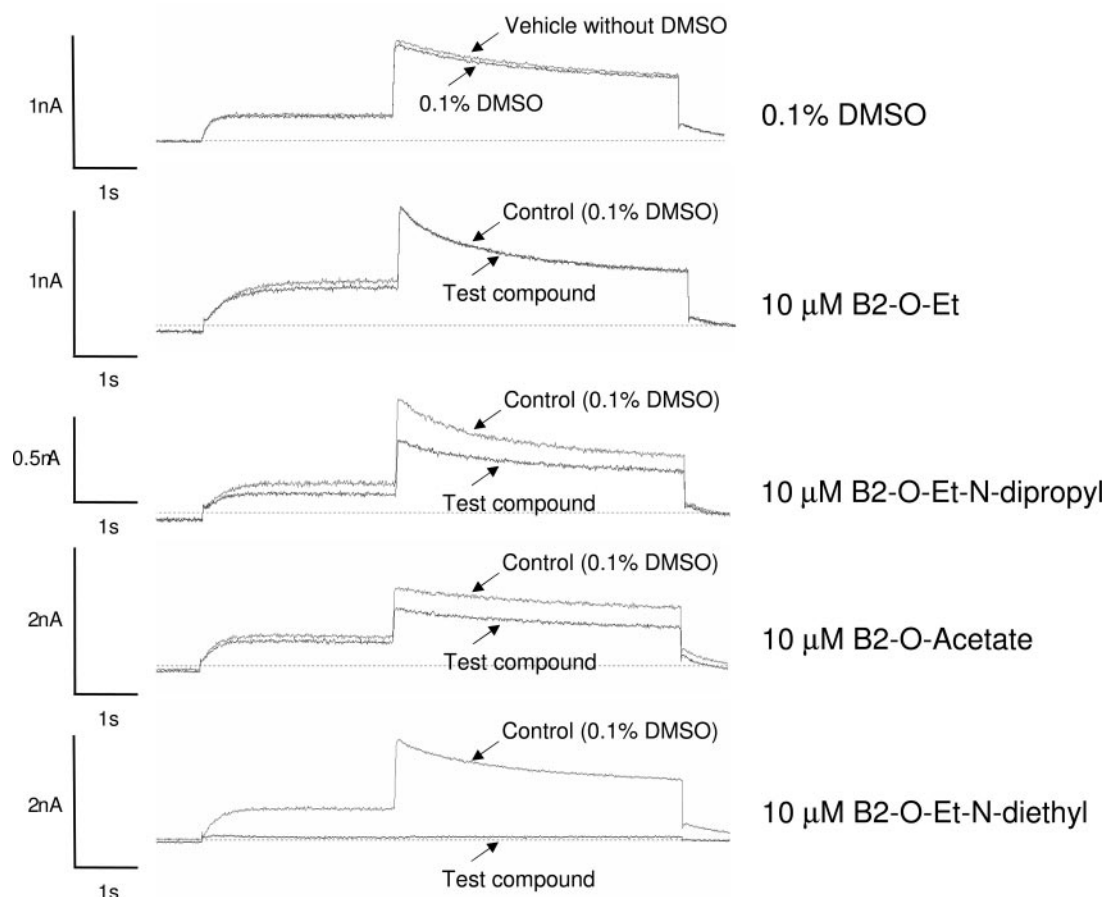


Fig. 6. Inhibition of potassium current. Representative current traces of potassium currents through *hERG* channels stably expressed in CHO cells are shown. Measurements were accomplished in the whole-cell patch-clamp configuration at room temperature. Outward currents were activated upon depolarization of the cell membrane from -80 to $+20$ mV for 3 s. Partial repolarization to -40 mV for 4 s evoked large tail currents. At least three cells were recorded per test compound. The vehicle (0.1% DMSO) as well as 10 µM B2-O-Et had no significant effect on *hERG* channel activity (traces at the top). In contrast, amiodarone, B2-O-acetate, and B2-O-Et-N-dipropyl had a clear inhibitory effect on the *hERG* channels. The top line in the figures depicts the control incubations (0.1% DMSO), and the bottom line depicts the incubations containing the test compounds.

As shown in Table 3, amiodarone and its analogs 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-NR₂ derivatives, the pattern of cytotoxicity (strong cytotoxicity of the amiodarone metabolites B2-O-Et-NH-ethyl and B2-O-Et-NH₂, 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 (Quagliano et al., 2004). As shown for B2-O-Et-*N*-dipropyl, uncoupling of the 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-NH₂, 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 Fig. 4 therefore indicate that after 18 h of incubation most hepatocytes had undergone necrosis in the presence of 100 μ M amiodarone, B2-O-Et-NH-ethyl, B2-O-Et-NH₂, B2-O-Et-propionamide, or B2-O-Et-OH.

Amiodarone and the amiodarone metabolites B2-O-Et-NH-ethyl and B2-O-NH₂ were shown to be strong inhibitors of mitochondrial β -oxidation and of the respiratory chain (B2-O-Et-NH-ethyl and B2-O-NH₂) 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 metabolites B2-O-Et-NH-ethyl, B2-O-Et-NH₂, and B2-O-Et-OH as well as the other amiodarone analogs synthesized and tested (all of them except B2-O-Et and B2-O-Et-*N*-dipropyl) will probably be associated with microvesicular steatosis. For amiodarone, it has been shown that inhibition of carnitine palmitoyltransferase 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 analogs, but so far formal proof is 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-NH₂ 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 seems 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 cytochrome P450 isozyme responsible for amiodarone deethylation (Fabre et al., 1993), may be a risk factor for hepatotoxicity associated with amiodarone. Although 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 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. In contrast, uncoupling of oxidative phosphorylation was associated preferentially with substances showing a higher lipophilicity. Since all substances

TABLE 3

Overview of the toxicological properties of amiodarone and analogs and of their effect on hERG channels. The compounds are listed with increasing lipophilicity

The graduation was as follows. For states 3, 4, and 4u, β -oxidation and cytotoxicity: + = $p < 0.05$ at 100 μ M; ++ = $p < 0.01$ at 100 μ M; and +++ = $p < 0.05$ at 10 μ M. For ROS production: + = $p < 0.05$ at 18 h; ++ = $p < 0.05$ at 6 h; and +++ = $p < 0.05$ at 4 h. For the effect on hERG channels: + = $p < 0.05$ at 10 μ M; ++ = $p < 0.01$ at 10 μ M; and +++ = $p < 0.001$ at 10 μ M.

	logP	State 3	State 4	State 4u	β -Oxi-dation	ROS	Citotoxicity ^a	Effect on hERG Channels
B2-O-Et-NH ₂	3.83	++	0	N.D.	++	+++	++	N.D.
B2-O-Et-OH	4.18	0	0	0	++	+	++	N.D.
B2-O-Et-propionamide	4.31	+++	+++	++	+++	+	+	N.D.
B2-O-Et- <i>N</i> -dimethyl	4.40	++	+++	+++	++	+	++	N.D.
B2-O-Et-NH-ethyl	4.47	++	0	N.D.	++	+	+++	N.D.
B2-O-Acetate	4.69	+	++	+++	++	+++	+	+
B2-O-Et	4.83	++	++	N.D.	0	0	0	0
B2-O-Et- <i>N</i> -diethyl (amiodarone)	4.92	0	+++	+++	++	+	++	+++
B2-O-Et- <i>N</i> -dipropyl	5.51	0	++	+++	0	0	0	+

N.D., not determined.

^a Cytotoxicity was determined using the data of Fig. 4.

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. Therefore, lack of penetration of the mitochondrial membranes is 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-NH₂). In addition to their lipophilicity profile, the observed differences in hepatic mitochondrial toxicity between the compounds tested may therefore also reflect the composition of the side chain attached to B2. This is, for example, substantiated by B2-O-Et, which has a quite high lipophilicity (log P of 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 decreases its toxicity (as shown by the comparison of the -NH₂, -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 relationship to the lipophilicity of the compounds tested. Although the four substances investigated had a similar lipophilicity profile (logP 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 analogs show large differences in mitochondrial toxicity 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 analogs 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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