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Synthesis and evaluation of the permeability transition inhibitory characteristics of paramagnetic and diamagnetic amiodarone derivatives

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Abstract—Several amiodarone analogues were synthesized varying the 2-substituent on the benzofuran ring and diethylaminoethyl side chain of phenolether by introducing 2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole and 1,2,5,6-tetrahydropyridine nitroxides or their amino or hydroxylamino precursors. The new compounds were screened on isolated mitochondria and perfused heart and their toxicity was evaluated on WRL-68 liver cells and H9C2 cardiomyocytes. Most of the newly synthesized derivatives exerted uncoupling effect on the mitochondrial oxidative phosphorilation at higher concentrations, compared to amiodarone and one of the modified amiodarone analogues showed an effect similar to that of amiodarone on the mitochondrial permeability transition and on restoring of mitochondrial high-energy phosphate metabolites in perfused hearts. This amiodarone analogue can be new leading compound among the experimental amiodarone analogues with the same or enhanced efficiency of amiodarone, but with less side effects.

1. Introduction

Amiodarone (2-butyl-3-benzofuranyl-4-[2-(diethylamino)-ethoxy]-3,5-diiodophenyl-ketone hydrochloride) is a benzfuran derivate (Fig. 1) with dominantly class III antiarrhythmic activity.[1] It is frequently used in controlling intractable cardiac arrhythmias.[2] There is also data suggesting, that an important component of amiodarone's antiarrhythmic action might be mediated via inhibition of thyroid hormone action in the heart.[3] Amiodarone, however, has several toxic adverse effects, the most important of which are thyroid, pulmonary and hepatic toxicity. Pulmonary toxicity is reversible if it is recognized in time, but still fatal in 10% of the cases. Liver injury is also common, but mild, although several fatal cases were also reported.[4] The most serious long-term toxicity of amiodarone derives from its kinetics of distribution and elimination.[5]

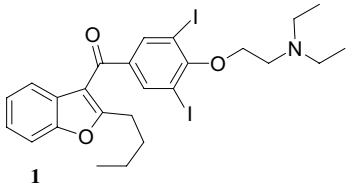

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Figure 1. Chemical structure of amiodarone.

Apart from its antiarrhythmic properties, it also possesses beneficial effect on ischemia-reperfusion injuries.[6,7] This effect of amiodarone is due—at least in part—to the inhibition of mitochondrial permeability transition (MPT) in lower concentrations.[8] However, when administrated in higher concentrations, it induced mitochondrial swelling as well as the collapse of the

Keywords: Amiodarone; Nitroxides; Mitochondria; Permeability transition; Langendorff-perfused heart.
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mitochondrial membrane potential ($\Delta\psi$).[8] *N*-Desethylamiodarone, the major metabolite of amiodarone—reported by some authors to be the major cause of the amiodarone administration induced toxicity[9]—does not present these biphasic characteristics, with no inhibitory effect on the MPT in lower concentrations and the induction of swelling and the collapse of the membrane potential in higher concentrations.[10] The difference between the effect of amiodarone and *N*-desethylamiodarone is due to the absence of an ethyl side chain from the amino group of *N*-desethylamiodarone.[10] This led us to the conclusion that the structural modification of amiodarone can improve its inhibitory effect on MPT as well as its beneficial effect in ischemia-reperfusion injuries. In concert with this, recently, numerous agents were shown to have an effect on the MPT, like heterocyclic antidepressants and antipsychotics with structural similarity presenting inhibitory effect,[11] or curcumin and its derivates showing the induction of mitochondrial swelling.[12]

Considering the advantages of amiodarone, a lot of effort has been made in the past decade to improve pharmacokinetic properties of amiodarone, mainly by chemical alterations of the original molecule such as synthesis of monoiodo derivatives,[3] introduction of carboxymethoxy side chain instead of tertiary amine[13] or substitute the original *n*-butyl group for an isobutyl ester.[14] During the past decade, it has turned out that drugs modified with nitroxides or their precursors have an additional, more advantageous role beyond their original purpose. Namely, they have some antioxidant properties capable of scavenging most of the toxic ROS and RNS highly damaging to biomolecules (e.g., proteins, lipids and nucleic acids).[15–17] This prompted us to connect the trend of amiodarone modifications with the idea of incorporating 2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrole, 1,2,5,6-tetrahydropyridine nitroxides or their amine precursors to evaluate their influence on pharmacological properties of amiodarone in the hope that these new derivatives may reduce the undesirable side effects of the original drug.

Our current studies were focused on synthesis of several new amiodarone analogues with varying 2-substituent on benzofuran ring and diethylaminoethyl side chain of phenolether, and investigation of their effect on MPT in isolated mitochondria and Langendorff-perfused heart and monitoring their toxicity on WRL-68 liver cells and H9C2 cardiomyocytes.

2. Results and discussion

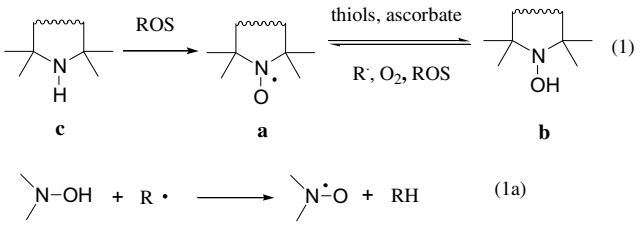
2.1. Chemistry

The paramagnetic amiodarone derivatives were achieved by alkylating **2**[13] and **12**[3] diiodophenoles in the presence of K_2CO_3 and 18-crown-6 in dry acetone with the corresponding paramagnetic bromides **3a**[18] and **4a**[19] or with *N*-chloroethylpiperidine **5**[20] or mesylate such as **6a**. The primary starting compound was **2**, which is readily available from the commercially available 2-methylbenzofuran. Compound **12** was synthesized only for coupling with the most promising side chain (**6a**, see biological data). We got mesylate **6a** by treatment of **11** paramagnetic alcohol with methanesulfonyl chloride in the presence of Et_3N. Compound **11** was synthesized by alkylation of *N*-ethylethanolamine with **3a** allylic bromide. To achieve better water-soluble derivatives for biological study from **7a**, **8a**, **10a** and **13a** *N*-oxyl derivatives we reduced the diamagnetic *N*-hydroxylamine hydrochloride salts **7b**, **8b**, **10b** and **13b** by refluxing them in ethanol saturated with HCl gas.[15] These derivatives in buffer solution at basic or at neutral pH are readily oxidized back to nitroxide by O_2 and by reactive oxygen species (ROS). On the other hand, nitroxides are reduced to *N*-hydroxylamines by any putative reducing agents of a living organism such as ascorbate or thiols; ultimately we can assume an equilibrium between nitroxide '**a**' form and *N*-hydroxylamine '**b**' form (Scheme 1, Eq. 1), albeit *N*-hydroxylamines may act as H donor antioxidants immediately upon administration, eliminating harmful radical species giving a non-reactive, resonance stabilized nitroxide free radical (Eq. 1a).

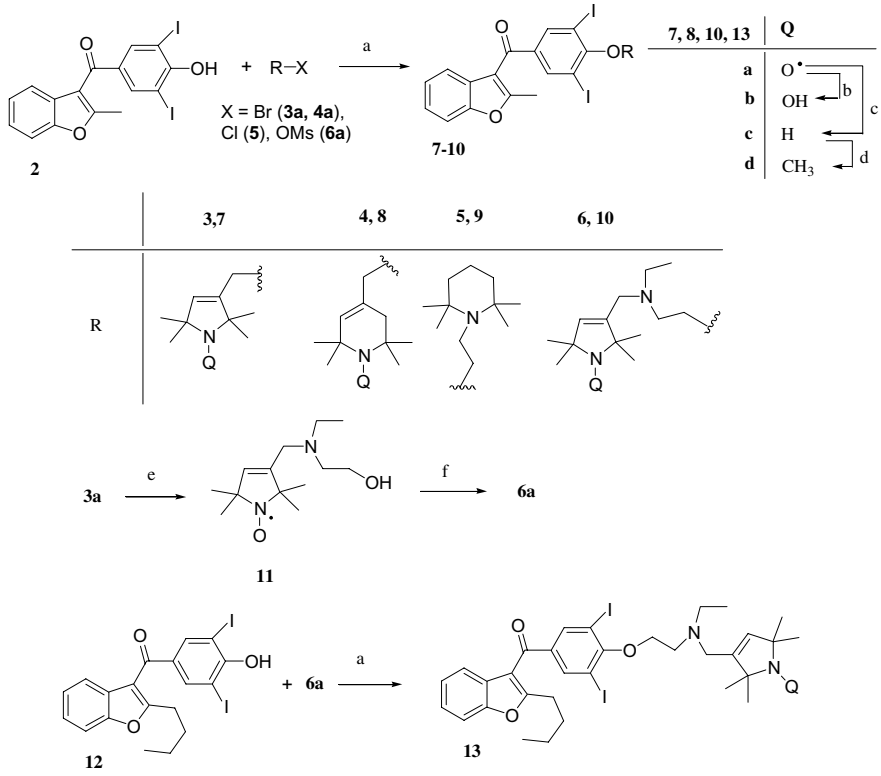
The other method for improving water solubility and antioxidant properties of the new derivatives is reducing nitroxide to the secondary amine **7c**, **8c**, **10c** and **13c**. It could be executed with Fe powder in acetic acid[21] without any alteration on the diiodobenzene ring. These secondary amine derivatives may also act as ROS scavengers by oxidizing to nitroxide as proved experimentally in the case of a cardioprotective drug[22] and in singlet oxygen scavenging in plant (Eq. 1).[23] From compound **7c** we made the *N*-methyl derivative **7d** by refluxing this molecule with excess MeI in the presence of K_2CO_3 (Scheme 2).

2.2. Pharmacology

2.2.1. Effect of amiodarone analogues on the mitochondrial permeability transition and the membrane potential ($\Delta\psi$). Amiodarone **1** inhibits the mitochondrial permeability transition (MPT) with an IC_{50} value of 3.9 ± 0.8 μM, however, in concentrations above 10 μM, it induces a swelling of its own.[8] In the case of the modified amiodarone analogues only compounds **8c** and **10c** exerted a biphasic effect as they induced mitochondrial swelling. The other compounds did not induce mitochondrial swelling below the concentration of 100 μM. Furthermore, while amiodarone exerts a temporary inhibitory effect on the Ca^{2+}-induced mitochondrial swelling,[8] compounds **7a**, **8a**, **8b** and **10b** presented an inhibitory effect similar to the one caused by



Scheme 1. Possible transformations of nitroxides or their precursors.



Scheme 2. Synthesis of paramagnetic and diamagnetic amiodarone derivatives. Reagents and conditions: (a) acetone, K_2CO_3 (1.1 or 2.1 equiv in case of **5**), 18-crown-6 (0.05 equiv), stirring at rt for 30 min then addition of **3a**–**6a** (1.1 equiv) reflux for 24 h, 35–62%; (b) EtOH/HCl, reflux for 30 min, 55–71%; (c) Fe (10 equiv)/AcOH, 70 °C, 1 h, then H_2O, K_2CO_3, extraction with $CHCl_3$ 36–51%; (d) **8c**, MeI (2 equiv), K_2CO_3 (2 equiv), $CHCl_3$, reflux, 24 h, 44%; (e) **3a** (1.1 equiv), *N*-ethylethanolamine, K_2CO_3 (1.1 equiv), $CHCl_3$, reflux, 4 h, 75%; (f) MsCl (1.1 equiv), Et_3N (1.1 equiv), CH_2Cl_2, (0 °C → rt), 1 h, 66%.

the classical MPT inhibitor, cyclosporine A (CsA) when the permeability transition was monitored for more than 30 min (data not shown). Amiodarone **1**, due to its uncoupling effect, also induces the collapse of the membrane potential with an ED_{50} value of 4.2 ± 0.7 μM.[10] While a moderate uncoupling proved to be beneficial in decreasing the ROS production,[24] total uncoupling leads to energetic failure of the mitochondria due to the cessation of ATP production. The effect of the different amiodarone analogues on the permeability transition and membrane potential in isolated mitochondria with IC_{50} (Fig. 2) and ED_{50} (Fig. 3) values, respectively, are presented in Table 1. Based on the results obtained from these experiments, three agents had IC_{50} values below 10 μM, **8c**, **10b** and **10c**, showing similarity with amiodarone, however, each of these drugs exerted uncoupling effect on the mitochondrial oxidative phosphorylation in higher concentrations, compared to amiodarone, as it is characterized by elevated ED_{50} values.

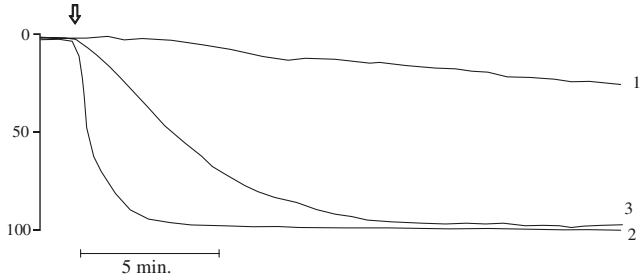


Figure 2. Mitochondrial permeability transition was induced by the administration of 60 μM of Ca^{2+} (indicated with the arrow) in the absence (line 2) and presence of inhibitors. Line 3 represents the swelling in the presence of agents showing IC_{50} inhibitory effect, line 1 represents the control.

2.2.2. Effect of amiodarone analogues on the viability of cultured cells. Viability of WRL-68 liver cells and H9C2 cardiomyocytes was detected by MTT^+ method (Fig. 4). The sensitivity of the cell lines towards amiodarone shows a difference with extracardiac cells in general, being more susceptible to amiodarone toxicity.[8]

Amiodarone **1** up to 100 μM did not decrease the viability of H9C2 cells, however in WRL-68 the viability significantly decreased above the concentration of 10 μM (Fig. 4A). In H9C2 cells **8c** and **10b** exhibited a similar effect as amiodarone, showing no or only minimal toxicity up 100 μM. Compound **10c** however proved to be toxic above 10 μM (data not shown), showing similar effect as did **7a**. When toxicity was assessed in WRL-68 cells, **8c** decreased the viability more than amiodarone above the concentration of 10 μM. In contrast to this, **10b** was less toxic in WRL-68 cells when compared to **8c** above the concentration of 10 μM (Fig. 4B). Based on the results obtained from the viability test, **8c** and **10b** proved to be non-toxic on the cardiomyocyte cells. This is also in accord with the results obtained from

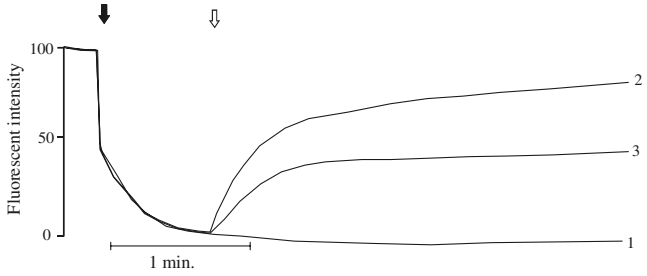


Figure 3. Mitochondrial membrane potential was monitored by the release of the fluorescent dye Rhodamine 123. Mitochondria, when added (indicated by the black arrow) sequestered the fluorescent dye (control, line 1). Following the administration of 60 μM of Ca^{2+} (indicated with the arrow) Rhodamine 123 was released from the mitochondria (line 2). Line 3 represents the fluorescent dye released following the administration of Ca^{2+} in the presence of agents exhibiting ED_{50} effect.

Table 1. IC_{50} and ED_{50} values of amiodarone analogues presenting inhibitory effect on the mitochondrial permeability transition and inducing the collapse of the mitochondrial membrane potential, respectively

Compound	IC_{50} (μM)	ED_{50} (μM)
Amiodarone **1**	3.9 ± 0.8	4.2 ± 0.7
7a	8.1 ± 1.4	20.1 ± 2.2
7c	6.1 ± 0.5	13.4 ± 3.0
7d	16.8 ± 2.1	27.8 ± 2.4
8a	15.8 ± 2.1	21.8 ± 2.2
8b	35.4 ± 5.1	51.3 ± 6.1
8c	2.1 ± 0.2	7.9 ± 0.8
9	>100	123.5 ± 9.7
10b	4.9 ± 0.5	36.2 ± 3.2
10c	3.1 ± 0.4	11.9 ± 1.8
13b	55.9 ± 6.4	29.7 ± 4.8
13c	52.7 ± 4.9	39.2 ± 4.3

the effect of these agent on the permeability transition and membrane potential in isolated mitochondria.

2.2.3. Effect of amiodarone analogues on the ischemia-reperfusion injury in Langendorff-perfused rat hearts. Based on the results obtained from the previous experiments, amiodarone analogues **8c** and **10b** were selected for further study in the perfused heart model. The results are summarized in Table 2. Amiodarone **1** proved to have beneficial effects on the level of mitochondrial high-energy phosphate metabolites in perfused hearts as detected by ^{31}P NMR.[10] During reperfusion the level of creatine phosphate returned to about 35 ± 4% of the normoxic level (Table 2). However, following the administration of 10 μM of amiodarone, the concentration of creatine phosphate returned to 74 ± 6% of the initial level (Table 2). In addition, amiodarone administration significantly ($p < 0.01$) increased the recovery of ATP levels as well (45 ± 6% vs 28 ± 5%; Table 2). Following the administration of compound **10b** in the concentration of 5 μM, it facilitated the recovery of the creatine phosphate level to 77 ± 6% of the initial value (Table 2). The rate and extent of the recovery of creatine phosphate was slightly higher compared to the one induced by amiodarone. The recovery in the level of ATP following **10c** administration was also faster and higher (54 ± 5%), compared to the effect of 10 μM of amiodarone (Table 2). When compound **8c** was administered in the concentration of 5 μM, it facilitated the recovery of creatine phosphate to 51 ± 4% of the initial value. Although this was higher compared to the control, it does not reach the level achieved by the administration of 10 μM of amiodarone (74 ± 6%). When **8c** was administered in the concentration of 10 μM, the level of creatine phosphate returned to 54 ± 4% of the initial value (Table 2). We can conclude that **8c** did not have any beneficial effect on the recovery of ATP, as it was found to be 27 ± 3% and 34 ± 3% when administered in 5 or 10 μM, respectively, compared to 28 ± 5% in the case of the control (Table 2). Based on the results obtained from the heart perfusion experiments, we found that 5 μM of **10b** had a similar effect as 10 μM of amiodarone, however **8c** did not reach the level of efficiency in facilitating the recovery of high-energy phosphate metabolites of the mitochondria compared to equimolar concentration of amiodarone **1**.

3. Conclusions

We report the synthesis of new amiadorone analogues, which were modified on benzofuran ring, for example, 2-methyl versus 2-butyl, and in aliphatic moiety of 2,6-diiodophenolether by incorporating 2,2,5,5-tetramet-2,5-dihydro-1*H*-pyrrole, 1,2,5,6-tetrahydropyridine nitroxides or their hydroxylamine or amine and precursors. It was found that 2-butylbenzofurane derivatives were less potent than 2-methylbenzofurans, although most derivatives synthesized exhibited weaker effect on mitochondrial permeability transition than amiodarone **1** itself. Compounds **8c**, **10b** and **10c** had similar inhibitory effect on mitochondrial permeability transition as amiodarone, but compound **10b** lacked the biphasic characteristic, as this agent did not induce swelling up to the concentration of 100 μM. In addition to this, compounds **7a**, **8a**, **8b** and **10b** presented a total inhibition of MPT as did 1 μM of CsA. Elevated uncoupling concentrations, indicated by the higher ED_{50} values were also a feature of most of the compounds. This would allow the administration of these agents in higher concentration without deteriorating the mitochondrial energy production by uncoupling the oxidation and the phosphorylation. Among the other parameters toxicity and restoring of the level of high-energy phosphate metabolites in perfused hearts, compound **10b** have similar effects as amiodarone **1**, however this effect could be achieved in administering this compound in a lower concentration. Compound **10b**, therefore seems a good starting point for further toxicology studies and rat pharmacokinetic studies.

4. Experimental

4.1. Chemical synthesis

Melting points were determined with a Boetius micro melting point apparatus and are uncorrected. Elemental

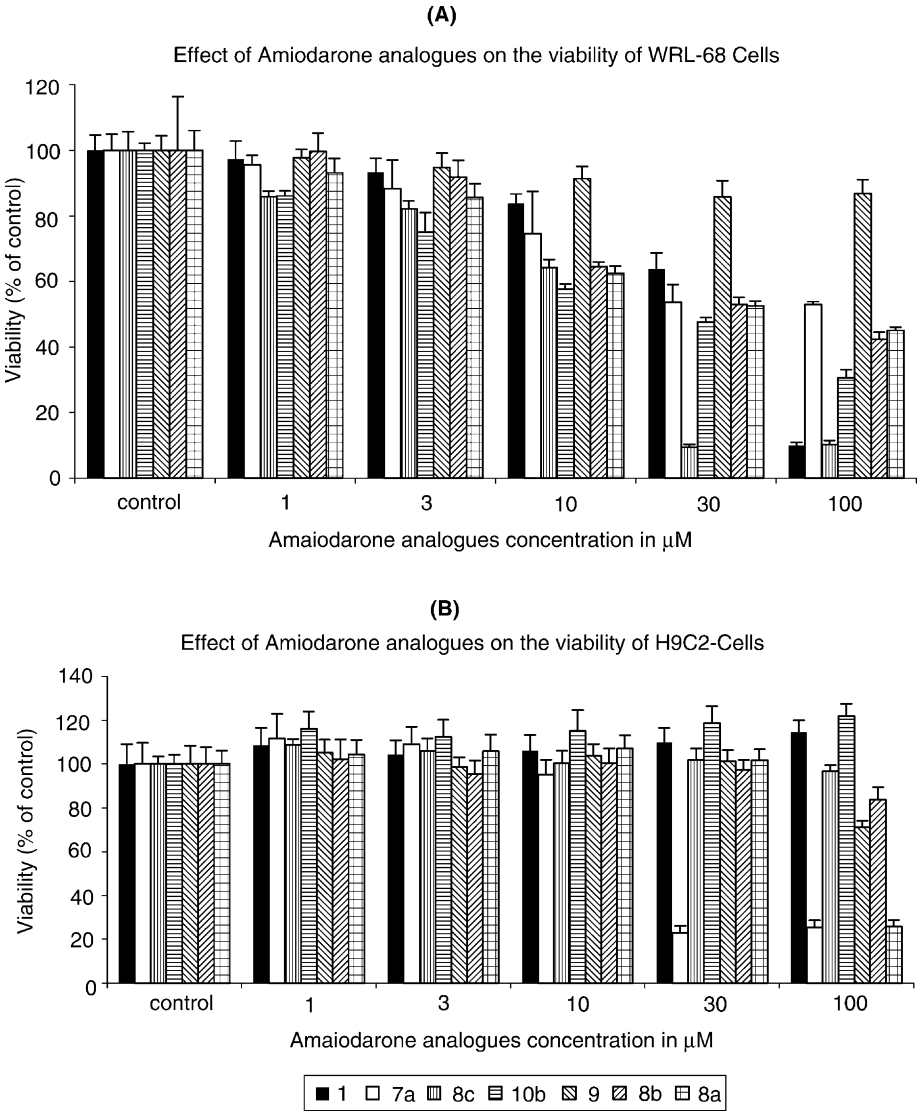


Figure 4. The concentration dependent effect of amiodarone and its analogues on the viability of WRL-68 liver (A) and H9C2 cardiomyocyte cell (B) lines following a 48 h treatment as detected by MTT^+ method.

Table 2. Effect of 10 μM of amiodarone **1**, 10 μM of **8c** and 5 μM of **10b** on the levels of creatine phosphate and ATP following ischemia-reperfusion in Langendorff-perfused rat hearts

	Control (%)	Amiodarone (%)	**8c** (%)	**10b** (%)
Creatine phosphate	35 ± 4	74 ± 6	54 ± 4	77 ± 6
ATP	28 ± 5	45 ± 6	34 ± 3	54 ± 5

analyses (C, H, N, S) were performed on Fisons EA 1110 CHNS elemental analyzer. Mass spectra were recorded on Finnigan Automass-Multi instrument in the EI mode. ^{1}H NMR spectra of diamagnetic compounds were recorded with Varian Unity Inova 400 Wb spectrometer; chemical shifts were referenced to TMS. ESR spectra were taken on Miniscope MS 200 in 10^{-4} M $CHCl_3$ solution and all monoradicals gave triplet line a_N = 14.0–14.4 G. Flash column chromatography was performed on Merck Kieselgel 60 (0.040–0.063 mm). Qualitative TLC was carried out on commercially prepared plates (20 × 20 × 0.02 cm) coated with Merck Kieselgel GF_{254}. Compounds **2**,[13] **3a**,[18] **4a**,[19] **5**[20] and **12**[3] were prepared according to published procedures.

4.1.1. Alkylation of diiodophenols, general procedure (7a, 8a, 9, 10a, 13a). A mixture of compound **2** (1.0 g, 2.0 mmol) or **5** (1.09 g, 2.0 mmol) finely powdered K_2CO_3 (203 mg, 2.2 mmol or 580 mg, 4.2 mmol in case of **5**), 18-crown-6 in acetone (30 mL) was stirred for 10 min, then alkyl-halogenide or mesylate **3a**, **4a**, **6a** (2.2 mmol) dissolved in acetone (10 mL) or HCl salt of compound **5** (2.2 mmol) was added in one portion and the mixture was stirred and refluxed for 24 h. After cooling the inorganic salts were filtered off, the acetone was evaporated, the residue was dissolved in EtOAc (20 mL), the organic phase was washed with brine (10 mL), separated, dried ($MgSO_4$), filtered and evaporated. The remaining oil was purified by flash column chromatography (hexane/Et_2O or hexane/EtOAc) to give amiodarone derivatives in 35–62% yield.

4.1.1.1. 2-Methyl-3-[3,5-diiodo-4-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)oxybenzoyl]benzofuran, radical (7a). 551 mg (42%), mp 179–182 °C. Anal. Calcd for $C_{25}H_{24}I_2NO_4$: C, 45.75; H, 3.69; N, 2.13. Found: C, 45.71; H, 3.64; N, 2.05.

4.1.1.2. 2-Methyl-3-[3,5-diiodo-4-(1-oxyl-2,2,6,6-tetramethyl-1,2,5,6-tetrahydropyridine-4-ylmethyl)oxybenzoyl]benzofuran, radical (8a). 469 mg (35%), mp 167–168 °C. Anal. Calcd for $C_{26}H_{26}I_2NO_4$: C, 46.59; H, 3.91; N, 2.09. Found: C, 46.55; H, 3.88; N, 1.94.

4.1.1.3. 2-Methyl-3-{3,5-diiodo-4-[2-(2,2,6,6-tetramethylpiperidin-1-yl)ethyl]oxybenzoyl}-benzofuran, radical (9). 698 mg (52%), mp 151–153 °C (HCl). Anal. Calcd for HCl salt $C_{27}H_{32}ClI_2NO_3$: C, 45.82; H, 4.56; N, 1.98. Found: C, 45.76; H, 4.52; N, 1.82.

^{1}H NMR ($CDCl_3$): δ 1.42 (6H, s, CH_3), 1.60 (2H, m, CH_2), 1.77 (2H, m, CH_2), 1.79 (6H, s, CH_3), 2.57 (3H, s, CH_3), 2.73 (2H, m, CH_2), 3.45 (2H, t, J = 8.6 Hz, CH_2), 4.72 (2H, t, J = 8.6 Hz, CH_2), 7.29 (2H, m, aromatic CH), 7.46 (2H, m, aromatic CH), 8.18 (2H, s, aromatic CH).

4.1.1.4. 2-Methyl-3-(3,5-diiodo-4-{2-[*N*-ethyl,*N*-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)ethyl]}oxybenzoyl)benzofuran, radical (10a). 843 mg (58%), oil. Anal. Calcd for $C_{29}H_{33}I_2N_2O_4$: C, 47.89; H, 4.57; N, 3.85. Found: C, 47.81; H, 4.55; N, 3.71.

4.1.1.5. 2-Butyl-3-(3,5-diiodo-4-{2-[*N*-ethyl-*N*-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)-ethyl]}oxybenzoyl)benzofuran, radical (13a). 600 mg (39%), mp 31–33 °C. Anal. Calcd for $C_{32}H_{39}I_2N_2O_4$: C, 49.95; H, 5.11; N, 3.64. Found: C, 49.85; H, 5.03; N, 3.56.

4.1.2. General procedure for *N*-hydroxylamine salt formation (7b, 8b, 10b, 13b). A solution of radicals **7a** or **8a** or **10a** or **13a** (1.0 mmol) was refluxed in EtOH (saturated with HCl) for 30 min. The solvent was evaporated off, the residue was crystallized with acetone or Et_2O to give white solids in 55–71% yield.

4.1.2.1. 2-Methyl-3-[3,5-diiodo-4-(1-hydroxy-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)oxybenzoyl]benzofuran, HCl salt (7b). 471 mg (68%), mp 164–166 °C. Anal. Calcd for $C_{25}H_{26}ClI_2NO_4$: C, 43.28; H, 3.78; N, 2.02. Found: C, 43.31; H, 3.70; N, 1.95.

4.1.2.2. 2-Methyl-3-[3,5-diiodo-4-(1-hydroxy-2,2,6,6-tetramethyl-1,2,5,6-tetrahydropyridine-4-ylmethyl)oxybenzoyl]benzofuran, HCl salt (8b). 424 mg (60%), mp 201–203 °C. Anal. Calcd for $C_{26}H_{28}ClI_2NO_4$: C, 44.12; H, 3.99; N, 1.98. Found: C, 44.06; H, 3.91; N, 1.86.

4.1.2.3. 2-Methyl-3-(3,5-diiodo-4-{2-[*N*-ethyl-*N*-(1-hydroxy-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)ethyl]}oxybenzoyl)benzofuran, 2HCl salt (10b). 504 mg (63%), mp 127–130 °C. Anal. Calcd for $C_{29}H_{36}Cl_2I_2N_2O_4$: C, 43.4; H, 4.53; N, 3.50. Found: C, 43.40; H, 4.45; N, 3.39.

4.1.2.4. 2-Butyl-3-(3,5-diiodo-4-{2-[*N*-ethyl-*N*-(1-oxyl-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)-ethyl]}oxybenzoyl)benzofuran, 2HCl salt (13b). 463 mg (55%), mp 112–114 °C. Anal. Calcd for $C_{32}H_{42}Cl_2I_2N_2O_4$: C, 45.57; H, 5.02; N, 3.32. Found: C, 45.40; H, 4.93; N, 3.22.

4.1.3. General procedure for reduction of nitroxides to amines (7c, 8c, 10c, 13c). To a solution of nitroxide **7a** or **8a** or **10a** or **13a** (1.0 mmol) in AcOH (8 mL) Fe powder (560 mg, 10 mmol) was added and the mixture was warmed up to 70 °C until the reaction started. The mixture was stirred at room temperature for 1 h, diluted with water (15 mL), decanted and the decanted aq solution made alkaline with solid K_2CO_3. The mixture was extracted with $CHCl_3$ (3 × 15 mL), dried ($MgSO_4$), filtered, evaporated and after chromatographic purification ($CHCl_3$/MeOH) we got the title amines in 36–51% yield.

4.1.3.1. 2-Methyl-3-[3,5-diiodo-4-(2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)oxybenzoyl]benzofuran (7c). 256 mg (40%), mp 222–224 °C (HCl). Anal. Calcd for $C_{25}H_{26}ClI_2NO_3$: C, 44.30; H, 3.87; N, 2.07. Found: C, 44.21; H, 3.75; N, 1.91. ^{1}H NMR (DMSO-d_6): δ 1.55 (6H, s, CH_3), 1.66 (6H, s, CH_3), 2.48 (3H, s, CH_3), 4.60 (2H, s, CH_2), 6.05 (1H, s, =CH), 7.30 (1H, m, aromatic CH), 7.37 (1H, m, aromatic CH), 7.45 (1H, m, aromatic CH), 7.66 (1H, m, aromatic CH), 8.19 (2H, s, aromatic CH), 9.28 (2H, br s, NH HCl).

4.1.3.2. 2-Methyl-3-[3,5-diiodo-4-(2,2,6,6-tetramethyl-1,2,5,6-tetrahydropyridine-4-ylmethyl)oxybenzoyl]benzofuran (8c). 235 mg (36%), mp 231–233 °C (HCl). Anal. Calcd for $C_{26}H_{26}ClI_2NO_3$: C, 45.14; H, 4.08; N, 2.02. Found: C, 45.03; H, 4.00; N, 1.94. ^{1}H NMR (DMSO-d_6): δ 1.45 (6H, s, CH_3), 1.50 (6H, s, CH_3), 2.41 (2H, br s, CH_2), 2.48 (3H, s, CH_3), 4.48 (2H, s, CH_2), 5.94 (1H, s, =CH), 7.29 (1H, m, aromatic CH), 7.37 (1H, m, aromatic CH), 7.44 (1H, m, aromatic CH), 7.65 (1H, m, aromatic CH), 8.18 (2H, s, aromatic CH), 8.88 (2H, br s, NH HCl).

4.1.3.3. 2-Methyl-3-(3,5-diiodo-4-{2-[*N*-ethyl-*N*-(2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)ethyl]}-oxybenzoyl)benzofuran (10c). 363 mg (51%), mp 132–134 °C (2HCl). Anal. Calcd for $C_{29}H_{36}Cl_2I_2N_2O_3$: C, 44.35; H, 4.62; N, 3.57. Found: C, 44.31; H, 4.55; N, 3.44. ^{1}H NMR ($CDCl_3$): δ 1.10 (3H, t, J = 7.0 Hz, CH_3), 1.29 (6H, s, CH_3), 1.34 (6H, s, CH_3), 2.56 (3H, s, CH_3), 2.68 (2H, q, J = 7.0 Hz, CH_2), 3.06 (2H, t, J = 6.5 Hz, CH_2), 3.16 (2H, br s, CH_2), 4.12 (2H, t, J = 6.5 Hz, CH_2), 5.56 (1H, s, =CH), 7.24 (1H, m, aromatic CH), 7.30 (1H, m, aromatic CH), 7.42 (1H, m, aromatic CH), 7.47 (1H, m, aromatic CH), 8.20 (2H, s, aromatic CH).

4.1.3.4. 2-Butyl-3-(3,5-diiodo-4-{2-[*N*-ethyl-*N*-(2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)ethyl]}-oxybenzoyl)benzofuran (13c). 294 mg, (39%), mp 127–130 °C (2HCl). Anal. Calcd for $C_{32}H_{42}Cl_2I_2N_2O_3$: C, 46.45; H, 5.12; N, 3.39. Found: C, 46.36; H, 4.99; N, 3.24. ^{1}H NMR ($CDCl_3$): δ 0.90 (3H, t, J = 7.5 Hz,

CH_3), 1.09 (3H, t, $J = 7.0$ Hz, CH_3), 1.25 (6H, s, CH_3), 1.29 (6H, s, CH_3), 1.35 (2H, m, CH_2), 1.75 (2H, q, $J = 7.5$ Hz, CH_2), 2.67 (2H, q, $J = 7.0$ Hz, CH_2), 2.83 (2H, t, $J = 7.5$ Hz, CH_2), 3.05 (2H, t, $J = 6.6$ Hz, CH_2), 3.15 (2H, s, CH_2), 4.11 (2H, t, $J = 6.6$ Hz, CH_2), 5.54 (1H, s, =CH), 7.22 (1H, m, aromatic CH), 7.28 (1H, m, aromatic CH), 7.40 (1H, m, aromatic CH), 7.46 (1H, m, aromatic CH), 8.19 (2H, s, aromatic CH).

4.1.4. 2-Methyl-3-[3,5-diiodo-4-(1,2,2,5,5-pentamethyl-2,5-dihydro-1*H*-pyrrol-3-ylmethyl)oxybenzoyl]benzofuran (7d). To a stirred solution of amine **7c** (712 mg, 1.0 mmol), K_2CO_3 (276 mg, 2,0 mmol) in $CHCl_3$ (20 mL), MeI (710 mg, 5.0 mmol) was added and the mixture was stirred and refluxed for 24 h. After cooling the inorganic salt was filtered off, the solvent was evaporated, the residue was dissolved in $CHCl_3$ (20 mL), washed with brine, separated, dried ($MgSO_4$), filtered, evaporated and the residue was purified by flash column chromatography ($CHCl_3$/Et_2O) to give compound 288 mg (44%) **7d**, mp 157–159 °C (HCl salt). Anal. Calcd for $C_{26}H_{28}ClI_2NO_3$: C, 45.14; H, 4.08; N, 2.02. Found: C, 45.25; H, 4.01; N, 1.96. ^{1}H NMR ($CDCl_3$): δ 1.22 (6H, br s, CH_3), 1.31 (6H, br s, CH_3), 2.35 (3H, br s, CH_3), 2.56 (3H, s, CH_3), 4.59 (2H, s, CH_2), 5.95 (1H, s, =CH), 7.23 (1H, m, aromatic CH), 7.30 (1H, m, aromatic CH), 7.41 (1H, m, aromatic CH), 7.47 (1H, m, aromatic CH), 8.21 (2H, s, aromatic CH).

4.1.5. 3-{[Ethyl-(2-hydroxyethyl)-amino]-methyl}-2,2,5,5-tetramethyl-2,5-dihydro-1*H*-pyrrol-1-yloxyl, radical (11). A solution of 2-(ethylamino)ethanol (891 mg, 10.0 mmol), compound **3a** (2.54 g, 11.0 mmol) and K_2CO_3 (1.51 g, 11.0 mmol) was stirred and refluxed in $CHCl_3$ (25 mL) for 4 h. After cooling the inorganic salt was filtered off, the filtrate washed with brine (10 mL), the organic phase was separated, dried ($MgSO_4$), filtered, evaporated the residue was purified by flash column chromatography ($CHCl_3$/MeOH) to give the title compound **11** as a yellow oil 1.8 g (75%). MS (*m*/*z*, %): 241 (M^+, 4), 211 (5), 210 (7), 138 (28), 102 (100). Anal. Calcd for $C_{13}H_{25}N_2O_2$: C, 64.69; H, 10.44; N, 11.61. Found: C, 64.60; H, 10.32; N, 11.51.

4.1.6. Procedure for synthesis of mesylate (6a). To a stirred solution of alcohol **11** (1.20 g, 5.0 mmol) and Et_3N (550 mg, 5.5 mmol) in CH_2Cl_2 (10 mL) methanesulfonyl chloride (630 mg, 5.5 mmol) dissolved in CH_2Cl_2 (2 mL) was added dropwise at 0 °C and the mixture was stirred at ambient temperature for 1 h. The reaction mixture was washed with water (5 mL), the organic phase was separated, dried ($MgSO_4$), filtered and evaporated to give the crude mesylate **6a** 1.06 g (66%), which were not purified further, but dissolved in acetone and immediately used for the synthesis of compounds **10a** and **13a**.

4.2. Biology

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

4.2.2. Isolation of mitochondria. Liver and heart mitochondria were prepared according to standard protocol.[25] Only difference among the organs were in the primary homogenization protocol; liver ($n = 5$) was squeezed through a liver press, while pooled heart tissue from five rats ($n = 15$) was minced with a blender. All isolated mitochondria were purified by Percoll gradient centrifuging.[26] All experiments were repeated at least three times from the same pool of mitochondria.

4.2.3. Mitochondrial permeability transition. Mitochondrial permeability transition was monitored by following the accompanying large amplitude swelling via the decrease in absorbance at 540 nm[27] measured at room temperature by a Perkin–Elmer fluorimeter (London, UK) in reflectance mode. Briefly, mitochondria at the concentration of 1 mg protein/mL were pre-incubated in the assay buffer (70 mM sucrose, 214 mM mannitol, 20 mM *N*-2-hydroxyethyl piperasine-*N*′-2-ethanesulfonic acid, 5 mM glutamate, 0.5 mM malate, 0.5 mM phosphate) containing the studied substances for 60 s. Mitochondrial permeability transition was induced by the addition of either 60 μM of Ca^{2+}, or amiodarone or desethylamiodarone at the indicated concentration. Decrease of E_{540} was detected for 20 min. The results are demonstrated by representative original registration curves from at least five independent experiments, each repeated three times using mitochondria prepared from the same liver or pool of rat hearts, respectively. The results are demonstrated by representative original registration curves from five independent experiments, each repeated three times using mitochondria prepared from the same liver or pool of rat hearts, respectively.

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

4.2.5. Cell culture. WRL-68 liver cells and H9C2 mouse cardiomyocytes cells were from American Type Culture Collection. WRL-68 and H9C2 cells were cultured in DMEM containing 1% antibiotic–antimycotic solution

(Sigma) and 10% FCS. Cells were passaged at intervals of three days.

4.2.6. Cell viability assay.[28] WRL-3A and H9C2 cells were seeded into 96-well plates at a starting density of 2×10^4 cells/well and cultured overnight. The following day, amiodarone and amiodarone analogues at the indicated concentrations were added to the medium. Twenty-four hours later, 0.5% of the water-soluble mitochondrial dye, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT^+) was added. Incubation was continued for three more hours, the medium was removed, and the water insoluble blue formasan dye formed stochiometrically from MTT^+ was solubilized by acidic isopropanol. Optical densities were determined by an ELISA reader (Anthos Labtech 2010) at 550 nm wavelength. All experiments were run in at least four parallels and repeated three times.

4.2.7. Heart perfusion. Studies were performed with isolated, Langendorff-perfused rat hearts ($n = 12$), which were either treated with amiodarone analogues **8c** ($n = 4$) or **10b** ($n = 5$) or untreated ($n = 3$) as control group. Hearts were perfused via the aorta as described before.[29] After a washout (non-recirculating period of 15 min), hearts were perfused under normoxic conditions for 10 min; the flow was subsequently discontinued for 30 min by inflating a balloon (ischemia), which was followed by 15 min of reperfusion. Levels of high-energy phosphate intermediates were monitored in the magnet of a ^{31}P NMR spectroscope during the entire perfusion.

4.2.8. NMR spectroscopy. NMR spectra were recorded with a Varian Unity Inova 400 Wb instrument. ^{31}P measurements (161.90 MHz) of perfused hearts were run at 37 °C in a Z·SPEC 20-mm broadband probe (Nalorac Co. Martinez, CA), applying WALTZ proton decoupling ($\gamma B_2 = 1.6$ kHz) during the acquisition only. Field homogeneity was adjusted by following the ^1H signal ($w_{1/2} = 10$–15 Hz). Spectra were collected with a time resolution of 3 min by accumulating 120 transients in each free induction decay. Flip angle pulses (45°) were used after a 1.25-s recycle delay, and transients were acquired over a 10-kHz spectral width in 0.25 s, and the acquired data points (5000) were zero-filled to 16384. Under the above circumstances, relative concentrations of the species are proportional to the corresponding peak areas, since interpulse delays exceeded 4–5 times the T_1 values of the metabolites that were analyzed in the ^{31}P experiments. Data were acquired from five independent experiments for each drug.

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