Synthesis and Preliminary Characterization of a Novel Antiarrhythmic Compound (KB130015) with an Improved Toxicity Profile Compared with Amiodarone

Bo Carlsson,[†] B. N. Singh,[‡] Marcel Temciuc,[§] Stefan Nilsson,[†] Yi-Lin Li,[†] Charlotta Mellin,[†] and Johan Malm*[,†]

Karo Bio AB, Novum, Huddinge S-141 57, Sweden, Cardiology section (111E), VAMC of West Los Angeles, Los Angeles, California 90073, and Synthelec AB, Ideon, Lund S-223 70, Sweden

Received November 29, 2000

Recent developments in antiarrhythmic therapy have indicated that the best approach to pharmacologically controlling supraventricular arrhythmias and life-threatening ventricular tachyarrhythmias is by prolonging cardiac repolarization rather than by blocking conduction. In this context, amiodarone has emerged as the most potent compound, but its universal use has been limited by its toxicity profile. There are data to suggest that an important component of amiodarones antiarrhythmic action might be mediated via inhibition of thyroid hormone action in the heart. Therefore, a new series of carboxymethoxybenzoyl and benzyl derivatives of benzofuran has been prepared and evaluated as thyroid hormone receptor antagonists. Within this series, 2-methyl-3-(3,5-diiodo-4-carboxymethoxybenzyl)benzofuran KB130015 (**7**) was found to reveal the most promising in vitro data. It inhibits the binding of ^{125}I-T_3 to the human thyroid hormone receptors (hThR) α_1 and β_1. T_3-Antagonism was confirmed in reporter cell assays employing CHOK1 cells (Chinese hamster ovary cells) stably transfected with hTh$R\alpha_1$ or hTh$R\beta_1$ and an alkaline phosphatase reporter gene downstream a thyroid response element. The derived IC_{50} values were 2.2 μM for hTh$R\alpha_1$ and 4.1 μM for hTh$R\beta_1$. Compound **7** was selected for further characterization of chronic effects on ventricular papillary muscle by transmembrane electrophysiology after daily intraperitoneal injection of the ligand (40 mg/kg body weight) in guinea pigs. Compound **7** was found to prolong the action potential duration at 90% (APD_{90}) repolarization time (219 ± 22 ms, control: 186 ± 9 ms, $p < 0.01$) without exhibiting any reverse rate dependency of action in a manner similar to that of amiodarone. In general, preliminary tolerance experiments with **7** demonstrated an improved safety profile compared to that of amiodarone. In summary, **7** appears to be less toxic than amiodarone while maintaining its electrophysiologic properties consistent with antiarrhythmic activity. Its potential antiarrhythmic actions warrant further investigations.

Introduction

During the past decade or two, there have been considerable changes in the manner in which ventricular and supraventricular arrhythmias are being treated.[1,2] On one hand, the developments in radio frequency catheter ablation and the use of implantable devices have superseded the use of antiarrhythmic drugs in certain arrhythmias.[2] Data from controlled clinical trials have provided evidence, which has necessitated a near complete reorientation of various classes of antiarrhythmic drugs themselves.[3] The negative impact on mortality of class I agents[4] has led to the increasing focus on compounds with the property of prolonging cardiac repolarization—the so-called class III agents.[5,6] The data also support the beneficial effect of adrenergic antagonism as exemplified by β-blockers that have been shown to reduce arrhythmia mortality.[7,8] Combining these actions among numerous others,[9] amiodarone has emerged as the most potent antiarrhythmic compound for the control of a very wide spectrum of cardiac arrhythmias.[10]

Unlike most antiarrhythmic agents, amiodarone does not increase mortality,[3,9,10] and has a low proarrhythmic potential.[11] In contrast to other class III agents (complex as well as simple) studied to date, amiodarone improves ventricular function in patients with heart failure, and the compound has antiadrenergic actions without associated β-blocker side effects. Furthermore, its overall antiarrhythmic effects are synergistic with those of β-antagonists.[12] Thus, while its overall properties as an antiarrhythmic compound remain unrivalled, its side effect profile is complex; there are features such as pulmonary toxicity, ocular and skin changes, and other forms of organ toxicity that clearly limit its widespread clinical utility.[13,14] For these reasons, a compound will be of great clinical benefit if it displays the class III antiarrhythmic actions of amiodarone but with a shorter elimination half-life and an improved toxicity profile.

Many years ago, it was demonstrated that the prolongation of repolarization induced by chronic amiodarone was very similar to those produced by hypothyroidism.[6,15,16] Clinically, it is known that hyperthyroidism is associated with a high incidence of atrial fibrillation, the converse with hypothyroidism. Thus, it was considered that a compound sharing an anti-thyroid action with amiodarone should have antiarrhythmic properties.[10,17] It was also considered that the tertiary amino

* To whom correspondence should be addressed. Tel: +46-8-608 6046. Fax: +46-8-774 8261. E-mail: johan.malm@karobio.se.
[†] Karo Bio AB.
[‡] VAMC of West Los Angeles.
[§] Synthelec AB.

10.1021/jm001126+ CCC: $22.00
Published on Web 01/03/2002

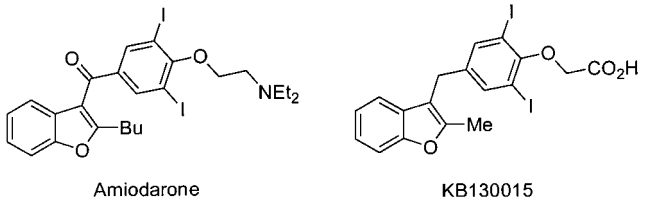


Figure 1. Chemical structures of amiodarone and KB130015 (**7**).

group of amiodarone is strongly contributing to its accumulation in the cells and to it is low clearance rate. In its neutral, lipophilic form, amiodarone easily enters cells, but becomes efficiently protonated in acidic compartments and thus trapped in, e.g., lysosomes.[18] To counteract this effect, the tertiary amino group of amiodarone can be replaced with a carboxylic acid group. Lipophilicity is also lowered if the butyl group is replaced with a methyl group.

Chronic treatment with amiodarone has been described to induce three different modes of thyroid hormone antagonism: (i) by inhibiting the enzymatic conversion of thyroxine to the active metabolite triiodothyronine T_3, (ii) by inhibiting the transport of thyroid hormones through the cell membrane, and (iii) while directly but selectively inhibiting of T_3-binding to the thyroid hormone receptors in the cell nuclei.[17] In any event, the ultimate endpoint for thyroid hormone action is the nuclear thyroid hormone receptor. Thus, our efforts have focused on the development of compounds that are T_3-antagonists at the receptor level.

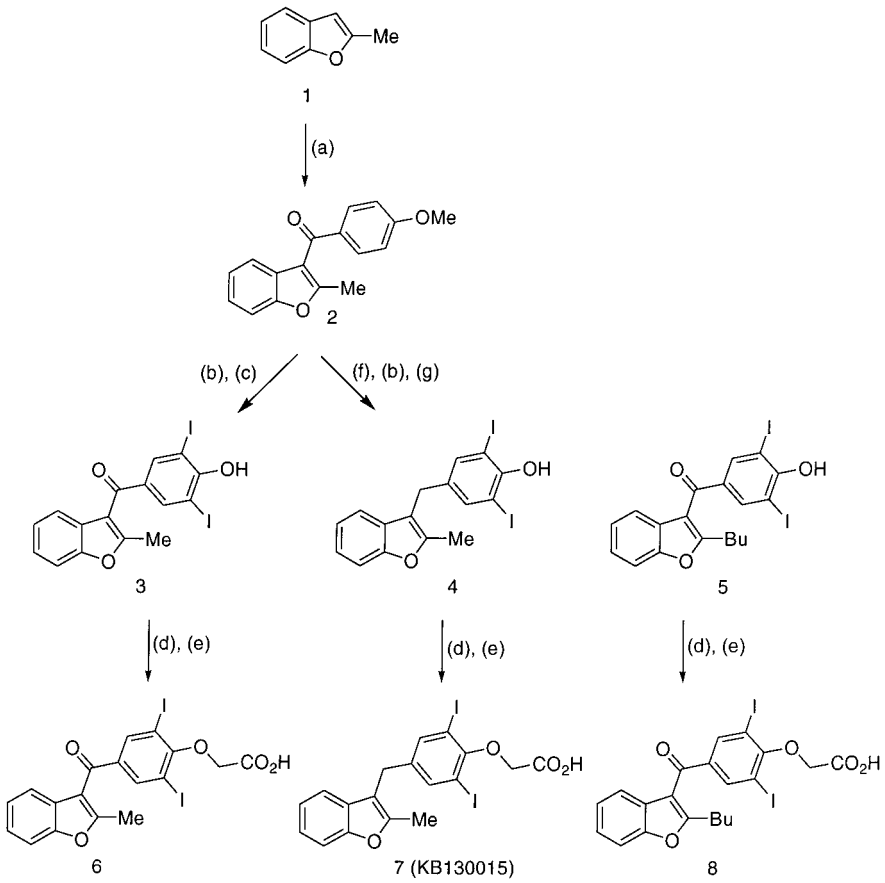
Here, we describe a novel chemical compound KB130015, 2-methyl-3-(3,5-diiodo-4-carboxymethoxybenzyl)benzofuran (**7**) (Figure 1)**,** that exhibits thyroid hormone antagonism on the receptor level which, in preliminary experiments, has the electrophysiologic properties consistent with class III antiarrhythmic potential.

Chemistry

The target carboxymethoxybenzoyl and benzyl derivatives **6**, **8**, and **7** were synthesized as depicted in Scheme 1. They were all prepared by treatment of the corresponding 4-hydroxy derivatives **3**, **4**, and **5** with α-bromoethyl acetate, subsequently followed by saponification at room temperature.

Standard organic reactions/transformations were utilized for the preparation of **3** and **4** from the starting 2-methylbenzofuran **1**, while **5** was commercially available. As **7** was selected for further in vivo studies, a feasible and effective large-scale preparation was developed. Regioselective Friedel−Crafts acylation of **1** with *p*-anisoyl chloride gave the intermediate benzoyl compound **2**, which during workup gave a pale yellow crystal mass, pure enough for next step. The next step involved reduction of the carbonyl function, where $NaBH_4$ was preferred as reducing agent over $LiAlH_4$ due to the difficulties of handling large amounts of the latter reagent in a safe and convenient way. Fusion with pyridine hydrochloride followed by iodination with ICl in morpholine gave a crystal mass, which was recrystal-

Scheme 1[a]



[a] Reagents: (a) PhCOCl(*p*-OMe), $SnCl_4$; (b) Pyr·HCl; (c) I_2, KI; (d) $BrCH_2CO_2Et$, K_2CO_3; (e) NaOH, MeOH; (f) ZnI_2, $NaBH_4$; (g) ICl, morpholine.

Table 1. Effects of Chronic Administration of Amiodarone and **8**, **6**, and **7** on Thyroid Hormone Receptors[a]

	ThRα	ThRβ	TRAFα		TRAFβ	
			agonism	antagonism	agonism	antagonism
id	IC_{50} (μM)	IC_{50} (μM)	EC_{50} (μM)	IC_{50} (μM)	EC_{50} (μM)	IC_{50} (μM)
amiodarone	0.65	0.60		>10[b]		>10[b]
8	1.4	4.0	>10	>10	>10	>10
6	0.82	0.48	0.45[c]	>10	1.7	>10
7	4.5	5.1	>10	2.2	>10	4.1

[a] EC_{50} and IC_{50} values are calculated means from duplicate measurements. The variability of the measurements is on average ±25%. [b] The MTS assay gave a toxicological response at 10 μM. [c] Partial agonist (56%).

Table 2. Effects of Chronic **7** on Guinea Pig Papillary Muscle Transmembrane Electrophysiology[a]

intervention	APA (ms)	V_{max} (V/s)	APD_{90} (ms)	APD_{50} (ms)	ERP (ms)	APD_{90}/APD_{50}
7	116 ± 2	192 ± 19	219 ± 22	182 ± 19	223 ± 19	1.22 ± 0.04
control	115 ± 2	205 ± 13	186 ± 9	168 ± 8	201 ± 9	1.10 ± 0.02
P	ns	<0.05	<0.01	<0.01	<0.01	<0.05

[a] $n = 10$ for both KB130015 treated and controls animals; ns = non significant.

lized from EtOH/H_2O to give pure **4**. It should be noted that no steps in this sequence above involved purification by chromatography.

Effects of 7 and Amiodarone on Thyroid Hormone Receptors. The results of a radioreceptor binding assay for the human thyroid hormone receptors α_1 and β_1 (ThRα and ThRβ), as well as a reporter cell assay employing CHOK1 cells (Chinese hamster ovary cells) stably transfected with hThRα_1 or hThRβ_1 and an alkaline phosphate reporter gene downstream a thyroid response element (TRAFα and TRAFβ), are summarized in Table 1. Although amiodarone exhibited a relatively strong affinity to hThR's, it also induced a significant toxicological response in the MTS/PMS assay. In an effort to evaluate the effect of a replacement of the basic moiety in amiodarone with an acidic, the diethylamine group was replaced with a carboxymethoxy group, thus producing **8**. This change gave lower binding affinity for both α_1 and β_1, accompanied by no effect in the TRAF assay. When the butyl group at the 2-position in **8** was replaced with a methyl group (**6**), binding affinity was comparable with amiodarone. Obviously a methyl group at this position was better accommodated by the receptor, but the agonist conformation of the receptor was also favored, thus producing a weak agonist. With **7** antagonism was recovered in the TRAF assay. The reason for this is unclear, especially considering that **7** is conformationally less restricted than **6**, which would normally favor agonism. The basis for selecting **7** for further studies was due to its exhibited full antagonism for the TRAF assay.

Electrophysical Effects of 7 in Guinea Pig Papillary Muscle. (a) Chronic Effects of 7 on the Transmembrane Action Potential. Twenty days of daily intraperitoneal injection of 40 mg/kg **7** induced a significant increase of the action potential duration at 50% and 90% levels of repolarization (APD_{50} and APD_{90}) in papillary muscles prepared from 10 guinea pigs (Table 2), compared to 10 normal controls ($n = 10$, $p < 0.01$). The APD and effective refractory period (ERP) were recorded in 5 cells from each preparation. The ERP was significantly increased as well. The V_{max} of the action potential was significantly decreased ($n = 10$, $p < 0.05$). However, the action potential amplitude (APA) and the resting potential (RP) were not changed ($n = 10$, $p > 0.05$). The shape of the action potential following treatment with **7** was similar to that normally induced by amiodarone.[6]

Table 3. Thyroid Status in Rats after 2 Weeks of Daily Oral Administration of **7** and Amiodarone[a]

	TSH (ng/mL)	T_3 (nmol/L)	T_4 (nmol/L)
vehicle	11.00 ± 1.97	0.74 ± 0.15	33.1 ± 5.00
7 (30)	14.2 ± 3.19	0.52 ± 0.10*	35.1 ± 9.99
7 (100)	16.6 ± 8.33	0.51 ± 0.15*	37.1 ± 7.39
7 (300)	16.6 ± 8.33	0.52 ± 0.14*	46.6 ± 10.0*
amiodarone (30)	13.9 ± 6.48	0.66 ± 0.11	43.9 ± 4.72*
amiodarone (100)	16.1 ± 7.19*	0.66 ± 0.10	43.2 ± 7.41*
amiodarone (300)	11.2 ± 2.66	0.28 ± 0.14*	22.3 ± 5.27*

[a] Thyroid status in rats after 2 weeks of daily oral administration of **7** and amiodarone (30, 100, and 300 mg/kg body weight). Each group consisted of 5 male and 5 female Sprague−Dawley rats. Values marked with * denote significant ($p < 0.05$) difference to vehicle-treated group.

(b) The Absence of Reverse Rate Dependency of 7 on APD. The APD_{90} and APD_{50} were measured when tissue preparations were stimulated at 0.2, 0.5, 1, 2, and 3 Hz. The rate dependency curve of cells from **7**-treated (40 mg/kg/day) guinea pigs was compared with those of controls. The rate dependency curve (absolute values) for **7** was nearly parallel to that of the controls. However, the ratio ($APD_{KB}/APD_{ctrl} = f$(frequency of stimulation)) of the change in APD did not show significant frequency dependence (data not shown).

(c) Acute Effects of 7 on the Transmembrane Action Potential. When papillary muscles were acutely perfused with up to 30 μM **7**, *no* effects were seen on any of the parameters that were influenced by chronic treatment (APD_{50}, APD_{90}, ERP, and V_{max}) and neither was APA or RP changed (these parameters were not influenced by chronic treatment of **7**). However, when the papillary muscles were perfused with 100 μM **7**, the APD_{90} was shortened from 210 to 185 ms (data not shown).

Comparison of in Vivo Effects of 7 and Amiodarone in Rats after Chronic Oral (Gavage) Administration. (a) Effects on Indices of Thyroid Function. Serum concentrations of T_3 were reduced, and serum T_4 was increased in both the amiodarone-treated and the **7**-treated groups as compared to the vehicle group (Table 3). Thyrotropin (TSH) was also increased in the treated groups as compared to that in controls. There was no significant difference between amiodarone and **7** in their effects on indices of thyroid function.

(b) Serum Cholesterol and Liver Function Tests. Serum levels of cholesterol were increased in both male and female rats with medium (100 mg/kg bw) and high (300 mg/kg bw) doses of amiodarone while only the highest dose of **7** (300 mg/kg) exerted such an effect. The hyper-cholesterolaemic effects of **7** were less pronounced as compared to amiodarone, and serum transaminases (ASAT and ALAT, Table 4) were elevated to a greater extent in the case of amiodarone-treated animals compared to those treated with **7**.

(c) Comparative Tolerance Experiments Relative to Weight Changes. Weight gain in rats was not affected by **7** at doses up to 300 mg/kg/day while the

Table 4. Effects on Liver Function after 2 Weeks of Daily Oral Administration of **7** and Amiodarone[a]

	chol. (g/L) males	chol. (g/L) females	ASAT (IU/L) males	ASAT (IU/L) females	ALAT (IU/L) males	ALAT (IU/L) females
vehicle	0.67 ± 0.13	0.73 ± 0.05	111 ± 21	106 ± 18	30 ± 3	23 ± 5
7 (30)	0.63 ± 0.12	0.68 ± 0.15	104 ± 13	101 ± 12	30 ± 3	21 ± 3
7 (100)	0.78 ± 0.10	0.69 ± 0.11	111 ± 18	114 ± 19	43 ± 12	40 ± 10
7 (300)	0.91 ± 0.13*	0.93 ± 0.17	102 ± 19	115 ± 13	67 ± 41*	54 ± 22*
amiodarone (30)	0.77 ± 0.21	0.89 ± 0.17	110 ± 10	109 ± 31	35 ± 8	23 ± 6
amiodarone (100)	1.26 ± 0.13*	1.18 ± 0.09*	131 ± 20	133 ± 10*	62 ± 17*	62 ± 18*
amiodarone (300)	1.39 ± 0.25*	1.12 ± 0.30	550 ± 871	2052 ± 1807*	103 ± 100*	284 ± 201*

[a] The data shown are effects on three parameters for liver function after 2 weeks of daily oral administration of **7** and amiodarone (30, 100, and 300 mg/ kg body weight). Each group consisted of 5 male or 5 female Sprague−Dawley rats. Values marked with * denote significant ($p \leq 0.05$) difference compared to the values from the vehicle-treated group. Chol is plasma cholesterol (g/L), ASAT is aspartate aminotransferase (IU/L), and ALAT is alanine aminotransferase (IU/L).

same dose of amiodarone (300 mg/kg/day) resulted in a reduced weight gain in the treated animals (data not shown).

Ion Channel Interactions. Amiodarone at 2 μM concentration was found to inhibit 70−80% of [^{3}H]-nitrendipine binding to rat cortical membranes, implying an interaction of amiodarone with the dihydropyridine site in calcium channel type L. Amiodarone at 2 μM concentration was found to inhibit 60−70% of [^{3}H]-batrachotoxin binding to rat forebrain membranes, implying an interaction with aconitine site in sodium channel. Amiodarone was not found to interact significantly in concentrations up to 20 μM with tracer binding to any other of the ion channels tested (except for the two mentioned above). Compound **7** did not interact with tracer binding to any of the ion channels tested (data not shown).

Discussion

There is now substantive data which supports the notion that amiodarone is a potent antiarrhythmic compound with a wide spectrum of action in ventricular and supraventricular arrhythmias.[19,20] The drug has been shown to have multiplicity of effects on the heart with a complex side effect profile. As a result, there has been much interest in the search for similar newer compounds on the basis of the drug's molecular configuration aimed at preserving its antiarrhythmic effectiveness with a lower side effect potential. The available data suggest that an important component of its antiarrhythmic action might be mediated via its cardio-selective inhibition of thyroid hormone.[6,15,17] It was for these reasons that the new series of carboxymethoxybenzoyl and benzyl derivatives of benzofuran reported in this manuscript was synthesized and evaluated as cardioselective thyroid hormone antagonists. It was found that 2-methyl-3-(3,5-diiodo-4-carboxymethoxy-benzyl)benzofuran (**7**) was the most promising on the basis of the in vitro data. It inhibited the binding of ^{125}I-T_3 to the human thyroid hormone receptors (hThR) α_1 and β_1. T_3-Antagonism was confirmed in reporter cell assays employing CHOK1 cells (Chinese hamster ovary cells) stably transfected with hThRα_1 or hThRβ_1 and an alkaline phosphatase reporter gene downstream a thyroid response element.

Some of the electrophysiological features in cardiac muscle of **7** are of particular importance. When the drug was given to guinea pigs for 3 weeks, there were significant increases in the duration of the action potential (APD) and the effective refractory period (ERP) studied by the standard microelectrode techniques in isolated papillary muscles. These effects were not accompanied by a significant change in the amplitude of the action potential; of particular interest, the effect of changes in frequency did not affect the magnitude of the APD nor of the ERP consistent with the observed reverse-use dependent effect found with other compounds that act by prolonging cardiac repolarization. The overall electrophysiology of **7** is thus identical to that originally described by Singh and Vaughan Williams[6] in the case of amiodarone and falls into the category of class III antiarrhythmic actions.[19,20]

No acute effects were observed APD prolongation with **7**, and in fact APD90 was shortened at very high concentrations (100 μM) of the compound. This indicates that **7** lacks the acute anti-arrhythmic effects of amiodarone. Also, **7** apparently differs from amiodarone by lack of interaction with the dihydropyridine site in calcium channel type L which indicates that **7** lacks class IV antiarrhythmic action (blockade of calcium channels). The interaction of amiodarone with this binding has previously been described.[21]

Amiodarone, but not **7**, also interacted with the aconitine site in the sodium channel. Aconitine is a toxin that exerts its toxic effects by slowing inactivation of Na currents through binding to the Na channel, and amiodarone has been shown to counteract aconitine-induced atrial fibrillation.[22] The absence of **7** binding to the aconitine site indicates that the compound may lack the class I effects of amiodarone. Moreover, a decreased maximum upstroke velocity (V_{max}) is one of the most consistent changes of action potential configuration induced by acute application of amiodarone (at effective doses down to 1 μM) to cardiac tissue[17] while no such effects were seen after acute KB130015 at even the highest dose tested (100 μM).

Amiodarone and **7** induced similar changes on thyroid hormone levels in the tolerance study, including decreased levels of T3, increased levels of T4, and increased TSH, though all changes were considered to be within the normal range for euthyroidism in the rat. In this context, it should be emphasized that, like amiodarone, **7** is also an iodinated compound, and therefore it is not surprising to see similar effects of the two compounds on thyroid hormone indices (Table 3), though it cannot be excluded that **7** inhibits the peripheral conversion from T4 to T3 in a similar way as has been seen with amiodarone[17] (and references within). On the other hand, our data on at least two discrete parameters of liver function indicate that **7** may exhibit a more favorable tolerance profile than amiodarone. Hypercholesterolaemic effects of amiodarone have been de-

scribed after clinical observations in patients on amiodarone therapy and from animal experiments. In the rat, this effect has been shown to be due to a decreased transcription of the T_3 responsive gene encoding for the LDL receptor, which is the same mechanism that is involved in the increase in LDL cholesterol that is seen in hypothyroidism.[23] It is also of relevance that the effects of **7** on hepatic enzymes in our studies were minor, and only mild increases were found at the highest dose in contrast to the effects of amiodarone. Clearly further toxicity studies involving other organs such as lungs need to be undertaken to precisely define the overall profile of **7**.

On the basis of the data reported herein, a number of albeit tentative conclusions can be drawn. The effects of **7** on lipid metabolism and those of liver function appear to be less severe than those reported for amiodarone but need to be confirmed in other experimental models. Since the electrophysiologic properties of **7** after chronic administration closely resemble those of amiodarone, it is possible that the antiarrhythmic profile of the drug might be similar. Thus, the effects of the compound in relevant animal models of induced cardiac arrhythmias should be defined prior to the clinical evaluation of its potential pharmacodynamic and therapeutic properties.

Experimental Section

Chemistry. General Methods. All melting points are uncorrected. The elemental analyses were carried out by Mikrokemi, Uppsala, Sweden. NMR spectra were recorded on a JEOL-270 spectrometer. Assignments of ^{13}C resonances were aided by ^{1}H-^{13}C bird-correlation spectra. The low resolution mass spectra were recorded on a Perkin-Elmer API 150Ex spectrometer, with turbo "ion spray" (LC-MS), or on a JEOL JMC-GCMATE attached to a Hewlett-Packard HP6890 with a Supelco fused silica capillary column 30 m, 0.25 mm ID, 0.25 μm film. The later MS was also used for high-resolution mass spectra (HRMS). All solvents and reagents were purchased from commercial sources in analytical grade and used without further purification. The substances were purified by flash chromatography on silica gel 60 (purchased from Merck Schucart). Amiodarone was purchased from Sigma, 2-butyl-3-(3,5-diiodo-4-hydroxybenzoyl)benzofuran (**5**) from Isochem Groupe SNPE, and 2-methylbenzofuran (**1**) from Synthelec AB.

2-Methyl-3-(4-methoxybenzoyl)benzofuran (2). A mixture consisting of 2-methylbenzofuran (**1**) (400 g, 3.03 mol), *p*-anisoyl chloride (512 g, 3.00 mol), and dry dichloromethane (2.5 L) was stirred and cooled to 5 °C. $SnCl_4$ (1.0 kg, 3.84 mol) was added dropwise to the mixture for 2 h at a constant temperature. The reaction mixture was stirred with cooling until the starting material was consumed and then poured into cold 2 N HCl (2.0 L). The formed emulsion was broken by filtration. The organic phase was separated and the aqueous phase further extracted once with CH_2Cl_2 (1.5 L). The collected organic phases were washed with water (2.0 L) and twice with 0.8 N NaOH (2 × 1.8 L) and with brine. The organic phase was dried over Na_2SO_4. After filtration and evaporation, the residue was solved in diethyl ether (0.5 L). To the obtained crystal mass was added *n*-heptane (0.7 L), and the crystals were filtered and washed with a mixture of *n*-heptane and diethyl ether (2:1). This gave 499 g (63%) of **2** as a pale yellow solid. The analytical sample was further purified by column chromatography (silica gel, *p*-ether/EtOAc 8:1): mp 68−69 °C; ^{1}H NMR (CD_3COCD_3) δ 2.49 (s, 3H, CH_3), 4.02 (s, 3H, OCH_3), 7.08 (d, 2H, H-3′ and H-5′, J = 8.9 Hz), 7.20−7.55 (m, 4H, aromatic), 7.83 (d, 2H, 3H, H-2′ and H-6′, J = 8.7 Hz); ^{13}C NMR (CD_3COCD_3) δ 13.31 (CH_3), 54.92 (OCH_3), 110.89, 121.21, 123.64, 124.57 (aromatic), 113.91 (C-3′ and C-5′), 131.65 (C-2′ and C-6′), 189.93 (CO); GC-MS (EI, 70 eV) *m*/*z* (%) 266(M+, 100), 265(94), 249(21), 235(70), 159(48), 135(70), 103(20), 92(31); HRMS (EI, 70 eV) for $C_{17}H_{14}O_3$ (M^+) calcd 266.0943, found 266.0943. Anal. Calcd. for $C_{17}H_{14}O_3$: C, 76.68; H, 5.30. Found: C, 75.6; H, 5.2.

2-Methyl-3-(3,5-diiodo-4-hydroxybenzoyl)benzofuran (3). A mixture of **2** (1.9 g, 7.13 mmol) and pyridine hydrochloride (4.95 g, 42.8 mmol) was gently refluxed for 30 min. When the temperature was below 100 °C, hydrochloric acid (25 mL, 1 M) was added. The obtained precipitate solidified after standing overnight. After filtration and drying in air, the solid mass was mixed under stirring with ammonium hydroxide (50 mL, 25%). A solution of iodine (3.9 g, 15.4 mmol) and potassium iodide (3.8 g, 22.9 mmol) in water was added dropwise to the mixture during 15 min. The reaction mixture was stirred at room temperature for 48 h and acidified with ice cooled sulfuric acid (15%). The resultant precipitate was collected, washed with water, and dried to give a red solid, which was further purified by column chromatography (silica gel, *p*-ether/EtOAc 4:1). This gave 3.2 g (89%) of **3** as a pale yellow solid: mp 171−172 °C; ^{1}H NMR (CD_3COCD_3) δ 2.54 (s, 3H, CH_3), 7.2−7.6 (m, 4H, aromatic), 8.22 (s, 2H, H-2′ and H-6′); ^{13}C NMR (CD_3COCD_3) δ 13.41 (CH_3), 110.99, 121.13, 123.80, 124.72 (aromatic), 141.16 (C-2′ and C-6′), 186.93 (CO); MS (ES-1) *m*/*z* 504(20), 503($M^+ - 1$, 100), 502(19), 127(16). Anal. ($C_{16}H_{10}I_2O_3$) C, H.

2-Methyl-3-(3,5-diiodo-4-carboxymethoxybenzoyl)benzofuran (6). To a mixture of **3** (1.0 g, 1.98 mmol) and potassium carbonate (0.56 g, 4 mmol) in dry acetone (100 mL) was added α-bromoethyl acetate (1.0 g, 12 mmol) during 5 min. The reaction mixture was stirred for 24 h at 50 °C. Dichloromethane (200 mL) was added, and the solution was washed with water (100 mL). The organic phase was evaporated to dryness, and the yellow residue was dissolved in a mixture of methanol (50 mL) and sodium hydroxide (50 mL, 1 M). The reaction mixture was then heated at 50 °C for 15 h, extracted with dichloromethane (3 × 75 mL), and dried ($MgSO_4$). Concentration of the organic phase gave 1.1 g (99%) which was further purified by column chromatography (silica gel, $CHCl_3$/MeOH/AcOH 90:10:1): mp 167−170 °C; ^{1}H NMR (CD_3COCD_3) δ 2.54 (s, 3H, CH_3), 4.74 (s, 2H, CH_2), 7.2−7.6 (m, 4H, aromatic), 8.26 (s, 2H, H-2′ and H-6′); ^{13}C NMR (CD_3COCD_3) δ 13.70 (CH_3), 68.30 (CH_2), 111.06, 121.22, 124.05, 124.96 (aromatic), 140.90 (C-2′ and C-6′), 187.66 (CO); MS (ES-1) *m*/*z* (%) 562($M^+ - 1$, 9), 503(100), 504(22), 317(16). Anal. ($C_{18}H_{12}I_2O_5$) C, H.

2-*n*-Butyl-3-(3,5-diiodo-4-carboxymethoxybenzoyl)benzofuran (8). This compound was prepared in analogy with **6** from 2-butyl-3-(3,5-diiodo-4-hydroxybenzoyl)benzofuran (**5**) and gave 90% yield. The crude product was purified by column chromatography (silica gel, $CHCl_3$/MeOH/AcOH 90:10:1): mp 130−132 °C; ^{1}H NMR (CD_3COCD_3) δ 0.90 (t, 3H, CH_3), 1.36, 1.78 (m, 2H, CH_2), 2.86 (t, 2H, $ArCH_2$), 4.73 (s, 2H, OCH_2), 7.25−7.58 (m, 4H, aromatics); ^{13}C NMR (CD_3COCD_3) δ *12.71* (CH_3), 21.94 (CH_2), 27.1 (CH_2), 29.9 (CH_2), 68.32 (OCH_2), 111.1, 121.4, 124.0, 125.0 (C-4, C-5, C-6, C-7), 140.8 (C-2′ and C-6′), *187.75 (CO);* MS (ES-1) *m*/*z* (%) 603($M^+ - 1$, 13), 546(17), 545-(100), 493(56), 471(13). Anal. ($C_{21}H_{18}I_2O_5$) C, H.

2-Methyl-3-(3,5-diido-4-hydroxybenzyl)benzofuran (4). To a homogeneous solution of **2** (420 g, 1.577 mol) in dry 1,2-dichloroethane (1.8 L) was added zinc(II) iodide (201 g, 0.631 mol) in one portion while stirring. To the heterogeneous reaction mixture was added $NaBH_4$ (60 g, 1.58 mol) in three equal portions (slightly exothermic). The reaction mixture was heated slowly, with caution, to 70 °C (the reaction becomes very vigorous with further heating). Addition of $NaBH_4$ (3 × 20 g) continued every 45 min. The reaction mixture was allowed to reach room temperature, poured out with caution on ice−water under stirring, and acidified with an aqueous solution of HCl (10%). The phases were quickly separated, and the water phase was extracted with dichloromethane (2 × 0.40 L). The combined organic phases were washed with an aqueous solution of sodium thiosulfate (10%) and brine. The organic layer was dried (Na_2SO_4), filtered, and concentrated in vacuo to provide a brown oil. To this oil was added a melt of pyridine

hydrochloride (630 g, 5.5 mol), and the stirred reaction mixture was heated at 190 °C for approximately 2 h. The mixture was cooled to 100 °C, poured slowly into ice−water (3 L) under vigorous stirring, and extracted with ethyl acetate (3 × 1 L). The combined organic phases were washed with 0.5 N HCl (1 L) and brine (1 × 0.3 L). The organic layer was dried (Na_2SO_4), filtered, concentrated in vacuo. The residue was filtered on a pad of silica gel, first eluted with *n*-heptane and then with a mixture of *n*-heptane and diethyl ether (3:1). The *n*-heptane fraction was discarded, but the heptan/diethyl ether fraction was collected and concentrated in vacuo. The residue was solved in methanol (2 L) under stirring. Morpholine (195 mL, 2.22 mol) was added, followed by ICl·tetramethylammonium chloride complex (560 g, 2.08 mol), which was added in portions to the mixture. The reaction temperature was maintained at 20−30 °C during the addition. After approximately 1 h of stirring, the resultant precipitate was collected, washed with water, and dried to give 429 g (56%) of a yellow solid, which was used directly in the next step. The analytical sample was further purified by recrystallization from EtOH/H_2O, followed by column chromatography (silica gel, *p*-ether/EtOAc 4:1): mp 197.5−198.5 °C; ^{1}H NMR (CD_3COCD_3) δ 2.47 (s, 3H), 3.49 (s, 2H), 7.10−7.41 (m, 4H, aromatics), 7.67 (s, 2H, H-2′ and H-6′); ^{13}C NMR (CD_3COCD_3) δ 11.22 (CH_3), 27.07 (CH_2), 110.51, 119.12, 122.36, 123.43 (aromatics), 139.31 (C-2′ and C-6′); MS (ES-1) *m*/*z* (%) 489-($M^+ - 1$, 100). Anal. ($C_{16}H_{12}I_2O_2$) C, H.

2-Methyl-3-(3,5-diiodo-4-carboxymethoxybenzyl)benzofuran (7). To a solution of **4** (388 g, 0.79 mol) in DMF (1250 mL) was added K_2CO_3 (160 g, 1.65 mol), and the suspension was cooled on an ice−water mixture. Ethylbromo acetate (138 g, 0.83 mol) was added during 15 min, with the temperature below 20 °C. The reaction mixture was stirred for another 30 min. The mixture was poured out in 3500 mL of water under vigorous stirring, and the obtained crystal mass was filtered and washed with large amounts of water. The crystal mass was dried in air atmosphere overnight and then added in one portion to warm methanol (4 L). A solution of 37.5 g of NaOH in 400 mL of water was added quickly, and the temperature was increased to 50 °C. After the starting material was consumed (2 h), the reaction was cooled to room temperature and acidified with HCl (10%, 300 mL). The obtained crystals were filtered, washed with water, and dried. The crystals were recrystallized with ethyl acetate to give KB130015 (333 g, 77%) as white crystals: mp 197.5−198.5 °C; ^{1}H NMR (CD_3COCD_3) δ 2.49 (s, 3H, CH_3), 4.02 (s, 2H, CH_2), 4.56 (s, 2H, CH_2), 7.1−7.5 (m, 6H, aromatics), 7.78 (s, 2H, J = 1.6 Hz, 3H, H-2′ and H-6′); ^{13}C NMR δ 10.76 (CH_3), 26.8 (CH_2), 68.29 (CH_2), 110.63, 119.18, 122.59, 123.66 (aromatics), 140.07 (C-2′ and C-6′), 167.99 (CO_2); LC-MS (ES-1) *m*/*z* 547($M^+ - 1$, 39), 503(21), 490-(26), 489(100), 127(37). Anal. ($C_{18}H_{14}I_2O_4$) C, H.

ThR-Binding Assay. This assay has been described previously.[24,25] All compounds tested, except amiodarone, were dissolved to 10 mM stock solutions, and serial dilutions thereof were obtained in DMSO. A 4 μL aliqout of the compound solution was mixed with radioactive triiodothyronine (^{125}I-T3, final concentration = 200 pM) and recombinant human thyroid hormone receptor (ThR, final concentration = 20 pM) diluting to a final incubation volume of 204 μL with an aqueous buffer containing 400 mM KCl, 17 mM K_2HPO_4, 3 mM $KHPO_4$, 1 mM $MgCl_2$, 0.5 mM EDTA, 9% glycerol, and 6 mM monothioglycerol. Amiodarone was dissolved in 50% EtOH /5 mM HCl to a concentration of 10 mM stock solution. Serial dilutions of amiodarone were performed in 5% EtOH/1 mM HCl and further treated as described above. Samples were incubated overnight at 4 °C after which the fraction of ThR-bound compound was separated from free compound on prepacked G25 columns. The concentration of each compound required to inhibit 50% of binding of ^{125}I-T_3 to hThR (IC_{50}) is presented in Table 1. Recombinant hThR was obtained from nuclear extracts from SF9 cells infected with baculovirus vectors encoding for ThRα1 or ThRβ1. The competition binding experiments were evaluated by a nonlinear four-parameter logistic model

$$b = ((b_{max} - b_{min})/(1 + (I/IC_{50})^S)) + b_{min}$$

where b_{max} is the total concentration of binding sites, b_{min} is the nonspecific binding, I is the added concentration of binding inhibitor, IC_{50} is the concentration of binding inhibitor at half-maximal binding, and S is a slope factor.[26]

Vector Constructs, Generation of Reporter Cell Lines (TRAF), and Assay Procedure. The cDNAs encoding the full length human ThRα1 and ThRβ1 were cloned in the mammalian expression vector pMT-hGH.[27−29] The pDR4-ALP reporter vector contains one copy of the direct repeat sequence AGGTCA nnnnAGGTCA, fused upstream of the core promoter sequences of the mouse mammary tumor virus long terminal repeat (MMTV), replacing the glucocorticoid response elements. The DR4-MMTV promoter fragment was then cloned 5′ of the cDNA encoding human placental alkaline phosphatase (ALP),[30] followed in the 3′-end by the polyA-signal sequence of the human growth hormone gene.[29] Chinese hamster ovary (CHO) K1 cells (ATCC no. CCL 61) were transfected in two steps, first with the receptor expression vectors pMT-hThRα_1 and pMT-ThRβ_1, respectively, and the drug resistance vector pSV2-Neo,[31] and in the second step, with the reporter vector pDR4-ALP and the drug resistance vector pKSV-Hyg.[32] Individual drug resistant clones were isolated and selected based on T_3 inducibility. One stable reporter cell clone each of CHO/hThRα_1 and CHO/hThRβ_1 were chosen for further study in response to various thyroid hormone agonists and antagonists. The procedure for characterization of agonism/antagonism of ligands has previously been described in detail.[28] Toxicity was assessed by microscopic evaluation of cell morphology and by the MTS/PMS assay (CellTiter 96 cell proliferation assay), in which the mitochondrial formation of a colored tetrazolium salt is measured spectrophotometrically at 492 nm (Promega Corporation, technical bulletin no. 169). The absorbance is directly proportional to the number of living cells in culture.

Transmembrane Electrophysiologic Effects in Guinea Pig Myocardial Ventricular Papillary Muscle. The electrophysiological effects of treatment with **7** were elucidated in rabbit papillary muscles. The animals were killed by a blow on the neck, and the hearts were quickly excised and immersed in cool oxygenated Tyrode's solution. The papillary muscles, 2 to 3 mm in length and about 1 mm in diameter, were isolated from the right ventricle and fixed in a tissue bath of 2 mL in volume. The preparation was driven at 1 Hz unless otherwise mentioned and superfused with 100% O_2 aerated Tyrode's solution at a rate of 6 mL/min. The Tyrode's solution was of the following composition (in mM): NaCl 140; KCl 4.0; $CaCl_2$ 1.2; $MgSO_4$ 0.5; glucose 10.0; HEPES 5.0; pH 7.4 and temperature 37 °C. The transmembrane action potentials were recorded with standard glass microelectrodes filled with 3 M KCl (15−25 MOhm DC resistance). The microelectrodes were coupled through an Ag−AgCl wire to the input of a high impedance, capacitance neutralized amplifier. The V_{max} of the action potential was obtained by electronic differentiation. The action potential and V_{max} were displayed on a storage oscilloscope and recorded with a Polaroid camera for later analysis. Effective refractory periods (ERP) were determined by premature stimulus with increasing coupling interval after the 10th basic stimulus until an evoked potential was observed. The shortest interval of the premature stimulus that evoked a conducted action potential was defined as the ERP. Presented results are from preparations in which continuous microelectrode implements could be maintained throughout the whole experimental procedure. Results are expressed as mean ± SD.

The effects of chronic treatment of **7** was elucidated after daily treatment of the animals for 20 days with intraperitoneal injections of compound dissolved in DMSO (and corresponding vehicle control) in a daily dosage of 40 mg/kg bodyweight for 20 days to guinea pigs weighing 250−400 g.

To elucidate the acute effects of **7**, the compound was added to the perfusion buffer in a range of concentrations (final concentrations between 10^{-8} to 10^{-4} Molar). The papillary muscle preparations were perfused with the solution for 4 h before transmembrane action potentials were recorded.

Ion Channel Interactions. A standard in vitro test of interactions with ion channels targets was provided by NovaScreen (Hanover, MD), in accordance with the company's quality assurance specifications. Amiodarone and **7** were tested in three concentrations (0.2, 2, and 20 μM) for inhibition of binding of radioactive labeled tracers to 10 identified ion channel targets. An interaction of the test compounds with the target was considered to have occurred in the cases where more than 50% of the tracer was displaced by the test compound. Interactions found were confirmed by repeated tests.

Interaction was found for amiodarone with the dihydropyridine binding site in calcium channel type L as an inhibition of binding of [^{3}H]-nitrendipine (70−87 Ci/mmol and a final concentration of 0.2 nM in 50 mM Triz-HCl (pH 7.7) at 25 °C for 60 min) to rat cortical membranes.[33] Amiodarone was also found to interact with sodium channel site-2 (aconitine site) seen as an inhibition of [^{3}H]-batrachotoxin (30−60 Ci/mmol, final concentration of 2.0 nM in 50 mM Triz-HCl (pH 7.4) + 130 mM choline chloride at 37 °C for 45 min) to rat forebrain membranes[34] while KB130015 did not interact with any of the ion channels tested.

In Vivo Effects of 7 and Amiodarone in Rats after Oral (Gavage) Administration. Selected data describing blood clinical chemistry and body weight after 2 weeks of treatment with **7** and amiodarone are presented. The data are extracted from a standardized tolerance evaluation of **7** and amiodarone performed by Chrysalis, Lyon, France. The evaluation was carried out in accordance with OECD Principles of Good Laboratory Practice. Blood clinical chemistry was analyzed at Department of Endocrinology, National Veterinary School, Lyon, France. Amiodarone and **7** were dissolved in propyleneglycol and administered per os as gavage to Sprague−Dawley rats for 14 consecutive days. The doses selected were 30 100 and 300 mg/kg/day of each compound and vehicle control (propyleneglycol alone). Each of the resulting seven groups consisted of five males and five females. Individual body weights were recorded twice weekly, and food consumption was measured weekly for each cage of animals. Clinical pathology investigations were performed after two weeks of treatment. Blood samplings for blood clinical chemistry were performed on day 15 in the males and on day 16 in the females. The effects of the treatments on thyroid status were evaluated from measurements of serum levels of TSH (thyroid stimulating hormone), T_3 (triiodothyronine), and T_4 (thyroxin) by using commercial assay kits [Amerlex-MT3 and Amerlex-MT4 (J&J Clinical Diagnostics) for T_3 and T_4 and the rTSH/^{125}I assay system (Amersham), according to instructions from the manufacturers]. The effects of the treatments on liver function[6] were assessed from plasma cholesterol levels and ASAT and ALAT activity. Plasma cholesterol was measured after hydrolysis by cholesterolesterase of esterified cholesterol and after oxidation of free cholesterol with cholesterol-oxidase according to instructions from the manufacturer of the reagents and instrumentation (MONARCH Instrument Laboratory). Aspartate aminotransferase (ASAT) and Alanine aminotransferase (ALAT) were both measured by kinetic determination at 37 °C (MONARCH Instrument Laboratory).

Supporting Information Available: (i) The absence of reverse rate-dependency of KB130015 on APD; (ii) ion channel experiments of KB130015 and amiodarone; and (iii) synopsis from study number 847/019 entitled "KB 130015-14 day oral (gavage) dose range-finding study in the rat". This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Singh, B. N. Current Antiarrhythmic Drugs. An Overview of Mechanisms of Action and Potential Clinical Utility. *J. Cardiovasc. Electrophysiol.* **1999**, *10*, 283−301.

(2) Camm, A. J.; Zipes D. P. (Eds). Antiarrhythmic Drugs at the Crossroads: From the Cell to the Bedside. *J. Cardiovasc. Electrophysiol.* **1998**, *10*, 267−317.

(3) Singh, B. N. Antiarrhythmic drugs: A Reorientation in Light of Recent Developments in the Control of Disorders of Rhythm. *Am. J. Cardiol.* **1998**, *81* (6A), 3D−13D.

(4) The Cardiac Arrhythmia Suppression Trial II Investigators: Effect of the Antiarrhythmic Agent Moricizine on Survival after Myocardial Infarction. *N. Engl. J. Med.* **1992**, *327*, 227−233.

(5) Singh, B. N.; Vaughan Williams, E. M. A third class of antiarrhythmic action. Effects on atrial and ventricular intracellular potentials, and other pharmacological actions on cardiac muscle, of MJ1999 and AH 3474. *Br. J. Pharmacol.* **1970**, *39*, 675−687.

(6) Singh, B. N.; Vaughan Williams, E. M. The effect of amiodarone, a new anti-anginal drug, on cardiac muscle. *Br. J. Pharmacol.* **1970**, *39*, 657−667.

(7) MERIT-HEFT Study Group. Effect of metoprolol CR/XL in chronic heart failure: Metoprolol CR/XL randomized intervention trial in congestive heart failure (MERIT-HF). *Lancet* **1999**, *353*, 2001−2007.

(8) CIBIS-II Investigators and Committees. The cardiac insufficiency bisoprolol study II (CIBIS II). *Lancet* **1999**, *353*, 9−13.

(9) Singh, B. N. Expanding indications for the use of class III antiarrhythmic agents in patients at high risk for sudden death. *J. Cardiovasc. Electrophysiol.* **1995**, *6*, 887−900.

(10) Singh, B. N. Antiarrhythmic actions of amiodarone: A profile of a paradoxical agent. *Am. J. Cardiol.* **1996**, *78*, 41−53.

(11) Hohnloser, S.; Klingenheben, T.; Singh, B. N. Amiodarone-associated proarrhythmic effects: a review with special reference to torsades de pointes tachycardia. *Ann. Int. Med.* **1994**, *121*, 529−537.

(12) Boutitie, F.; Boissel, J. P.; Connolly, S. J.; Camm, A. J. Amiodarone interaction with beta-Blockers: analysis of the merged EMIAT and CAMIAT databases. *Circulation* **1999**, 2286−2275.

(13) Pollak, T. M. Clinical organ toxicity of antiarrhythmic compounds: Ocular and pulmonary manifestations. *Am. J. Cardiol.* **1999**, *84*, 37R−45R.

(14) Wiersinga, W. M. Amiodarone and the Thyroid. In *Handbook of Experimental Pharmacology*; Weetman A. P., Grossman, A., Eds.; Springer-Verlag: Berlin, 1977; Vol. 128, Chapter 10.

(15) Latham, K. R.; Seletti, D. F.; Goldstein, R. E. The effects of amiodarone and desethylamiodarone with solubilized nuclear thyroid hormone receptors. *J. Am. Coll. Cardiol.* **1987**, *7*, 472−450.

(16) Norman, M. F.; Lavin, T. A. Competition of thyroid hormone action by amiodarone in rat pituitary tumor cells. *J. Clin. Invest.* **1989**, *83*, 306−313.

(17) Kodama, I.; Kamiya, K.; Toyama, J. Cellular electropharmacology of amiodarone. *Cardiovasc. Res.* **1997**, *35*, 13−29.

(18) De Duve, C.; De Barsky, T.; Poole, B.; Trouet, A.; Tulkens, P.; Van Hoof, F. Lysosomotropic agents. *Biochem. Pharmacol.* **1974**, *23*, 2495−2531.

(19) Singh, B. N. Arrhyhmia control by prolonging repolarization: the concept and its potential therapeutic impact. *Eur. Heart J.* **1993**, *14*, 14−23.

(20) Singh, B. N. (Ed). Approaches to Controlling Cardiac Arrhythmias: Focus on Amiodarone, the last 15 Years. *Am. J. Cardiol.* **1999**, *84*, 1−171.

(21) Nokin, P.; Clinet, M.; Swillens, S.; Delisee, C.; Meysmans, L.; Chatelain, P. Allosteric modulation of of [^{3}H]-Nitrendipine binding to cardiac and cerebral cortex membranes by amiodarone. *J. Cardiovasc. Pharmacol.* **1986**, *8*, 1051−1057.

(22) Winslow, E. Hemodynamic and arrhytmogenic effects of aconitine applied to the left atria of anesthetized cats. Effects of amiodarone and atropine. *J. Cardivasc. Pharmacol.* **1981**, *3*, 87−100.

(23) Hudig, F.; Bakker, O.; Wiersinga, W. M. Triiodothyronine prevents the amiodarone-induced decrease in the expression of liver low-density lipoprotein receptor gene. *Endocrinology* **1997**, *152*, 413−421.

(24) Greenidge, P. A.; Carlsson, B.; Bladh, L. G.; Gillner, M. Pharmacophores Incorperating Numerous Excluded Volymes Defined by X-ray Christallographic Structure in Three-Dimensional Database Searching: Application to the Thyroid Hormone Receptor. *J. Med. Chem.* **1998**, *14*, 2503−2512.

(25) Barkhem, T.; Carlsson, B.; Simons, J.; Moeller, B.; Berkenstam, A.; Gustafsson, J.-Å.; Nilsson, S. High level expression of functional full-length human thyroid hormone receptor β1 in insect cells using a recombinant baculovirus. *J. Steroid Biochem. Mol. Biol.* **1991**, *38*, 667−75.

(26) Schults, J. R.; Ruppel, P. I.; Johnson, M. A. In *Biopharmaceutical Statistics for Drug Development*; Peace, K. E., Ed.; Dekker: New York, 1988; pp 21−82.

(27) Alksnis, M.; Barkhem, T.; Stroemstedt, P. E.; Ahola, H.; Kutoh, E.; Gustafsson, J. Å.; Poellinger, L.; Nilsson, S. High level expression of functional full length and truncated glucocorticoid receptor in Chinese hamster ovary cells. Demonstration of ligand-induced down-regulation of expressed receptor mRNA and protein. *J. Biol. Chem.* **1991**, *266*, 10078−10085.

(28) Barkhem, T.; Carlsson, B.; Nilsson, Y.; Enmark, E.; Gustafsson, J.-Å.; Nilsson, S. Differential response of estrogen receptor α and estrogen receptor β to partial estrogen agonists/antagonists. *Mol. Pharmacol.* **1998**, *54*, 105−112.
(29) Friedman, J. S.; Cofer, C.; Anderson, C. L.; Kushner, J. A.; Gray, P. P.; Chapman, G. E.; Lazarus, L.; Shine, J.; Kushner, P. J. High expression in mammalian cells without amplification. *Biotechnology* **1989**, *7*, 359−362.
(30) Berger, J.; Hauber, J.; Hauber, R.; Geiger, R.; Cullen, B. R. Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. *Gene* **1988**, *66*, 1−10.
(31) Southern, P. J.; Berg, P. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* **1982**, *1*, 327−341.
(32) Gritz, L.; Davies, J. Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in *Escherichia coli* and *Saccharomyces cerevisiae*. *Gene* **1983**, *25*, 179−188.
(33) Ehlert, F. J.; Roeske, W. R.; Yamamura, H. I. The binding [^{3}H]-nitrendipine to Receptors for Calcium Channel Antagonists in the Heart, Cerebral Cortex and Ileum of Rats. *Life Sci.* **1982**, *30*, 2191−2202.
(34) Creveling, C. R. Batrachotoxin-Induced Depolarization and [^{3}H]-Batrachotoxin-A 20a-Benzoate Binding in a Vesicular Preparation from Guinae Pig Cerbral Cortex. *Mol. Pharmacol.* **1983**, *23*, 350−358.

JM001126+