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Interaction of azimilide with neurohumoral and channel receptors

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Abstract

Binding of the class III antiarrhythmic agent azimilide to brain, heart, and other organ receptors was assessed by standard radioligand binding techniques. In a survey of 60 receptors, azimilide at 10 μM inhibited binding by more than 50% at serotonin uptake (K_i: 0.6 μM), muscarinic (K_i: 0.9 to -3.0 μM), Na^+ channel site 2 (K_i: 4.3 μM), and central sigma (K_i: 6.2 μM) sites. Lesser (20–40%) inhibition was seen at adrenergic, histamine, serotonin, purinergic, angiotensin II, dopamine uptake, and norepinephrine sites and at a voltage-sensitive K^+ channel. In rat ventricle, azimilide inhibited binding to α_1- and β-adrenergic and muscarinic receptors (K_i: <5 μM) and to the L-type Ca^{2+} channel (K_i: 37.3 μM). In rat brain, azimilide blocked ligand binding to these same receptors and to a serotonin receptor, and the breadth and potency of its interaction pattern differentiated it from ten other class III antiarrhythmics. Azimilide displayed agonist and antagonist action at five muscarinic receptor subtypes in transfected NIH 3T3 cells producing receptor-sensitive mitogenesis and β-galactosidase activity. Agonist action predominated at M_2 and M_4 subtypes, and antagonist action predominated at M_1, M_3, and M_5 subtypes. The azimilide concentration for 50% maximum stimulation (EC_{50}) in M_2-expressing cells was 1.97 μM (vs 0.14 μM for carbachol). Azimilide's receptor interactions occur at concentrations from one to forty times those required to block cardiac delayed-rectifier channels but could contribute to the efficacy and safety of the drug.

Keywords: Azimilide; Class III antiarrhythmic; Radioligand binding; Adrenergic receptors; Muscarinic receptors

1. Introduction

Azimilide dihydrochloride (NE-10064, (E)-1-[[[5-(4-chlorophenyl)-2-furanyl]methylene]amino]-3-[4-(4-methyl-1-piperazinyl)butyl]-2,4-imidazolidinedione dihydrochloride) prolongs cardiac refractoriness by blocking the fast (I_{Kr}) and slow (I_{Ks}) components of the delayed-rectifier potassium current [1–3]. These *in vitro* electrophysiologic properties could account for its efficacy in rodent and dog models of ischemia-induced ventricular arrhythmia [4,5], termination of atrial arrhythmias in dogs [6,7], suppression of fibrillation in a dog model of sudden cardiac death [8], and clinical efficacy [9,10]. Azimilide blocks delayed-rectifier currents at 0.2 to 3 μM (50% inhibition *in vitro* in guinea pig) and also interacts with other ion channels, blocking cardiac Na^+ channels at 12 μM (guinea pig), an L-type Ca^{2+} channel at 18 μM (dog), and the inward rectifier channel at >50 μM (dog) [11–13].

Drugs targeted primarily at one biological receptor can exhibit some interaction with other receptors, where effects as either agonist or antagonist may contribute to efficacy or side-effects. Azimilide's block of ligand binding at 60 receptors, including neurotransmitters, ion channels, second messengers, steroids, brain and gut peptides, growth factors, and peptides was evaluated by standard protocols at the Oceanix Biosciences Corp. These receptors cover many body systems with possible targets for the drug from an efficacy or safety viewpoint. The study was designed, in particular, to identify possible targets for the compound from a side-effect perspective. A survey approach, involving 60 receptors found at many sites, was considered appropriate for identifying potentially important interactions

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Abbreviations: I_{Kr}, rapidly activating component of the delayed-rectifier current; I_{Ks}, slowly activating component of the delayed-rectifier current; EC_{50}, concentration giving 50% of the maximum stimulation; K_i, inhibition constant; and HT, hydroxytryptamine (serotonin).

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at other than the cardiac delayed-rectifier channels. Additional studies with rat heart and brain membranes compared azimilide and several class III antiarrhythmic agents for binding inhibition at adrenergic, muscarinic, and serotonin receptors. *In vitro* experiments in cells having receptor-linked mitogenic responses determined the action of azimilide at muscarinic receptor subtypes. Some of these results have been presented in abstract form [14].

2. Materials and methods

2.1. Chemicals and test articles

[^{3}H]LND-796 was obtained from the Commissariat a l'Energie Atomique. Other radioligands were purchased from Dupont-New England Nuclear. Unlabelled apamin, phentolamine, propranolol, quinidine sulfate, and veratridine were purchased from the Sigma Chemical Co., and clofilium tosylate was obtained from Research Biochemicals, Inc. Azimilide dihydrochloride, *d*-sotalol, and sematilide were synthesized. The following compounds were gifts from their manufacturers: amiodarone (Sanofi), bretylium tosylate (American Critical Care), dofetilide (Pfizer), E-4031 (Eisai Co.), glyburide (Hoechst-Roussel Pharmaceuticals, Inc.), PN 00 110 (Sandoz, Inc.), MDL-11,939 (Merrell Dow), melperone (Ferrosan), risotilide HCl (Wyeth-Ayerst), sematilide HCl (Berlex Labs), and *d*,*l*-sotalol HCl (Bristol-Myers Squibb). Solutions of class III antiarrhythmic agents were prepared fresh daily in 0.9% (w/v) saline. Several reagents used in the transfected cell analysis of muscarinic receptor function were purchased: pSV-lacZ (β-galactosidase) DNA (Promega), salmon sperm DNA (Sigma), and pcDNAI vector (Invitrogen). Other chemicals were of usual laboratory research grade and were obtained from commercial sources.

2.2. Animals

For selected work on adrenergic, muscarinic, adenosine, serotonin, and ion channel receptors in rat heart or brain, research-bred male rats were purchased from Taconic Farms. Rats were housed and fed under standard conditions in a facility approved by the Association for the Assessment and Accreditation of Laboratory Animal Care. For the general survey of receptor binding inhibition done by the Oceanix Biosciences Corp., mice, rats, guinea pigs, and rabbits were obtained from licensed suppliers of laboratory animals.

2.3. Membranes

Table 1 lists the sources of the membrane fractions used for the radioligand binding assays. Specific studies of a few target receptors used rat cardiac and brain membranes. Preparation methods involved homogenization and centrifugation and are described in the literature references of Table 1 and in sections below.

2.4. Cells and organs

Rabbit platelets were purchased from Cocalico Biologicals, Inc. Prostates isolated from rats 24 hr after castration were purchased from Zivic-Miller Labs, Inc. Kidneys were available from dogs terminated as controls in other experiments. Bovine brain and uterus were obtained from a local slaughterhouse. N1E-115 neuroblastoma, NIH 3T3 (ATCC nl. CRL 1658), PC12, HL60, and A10 rat thoracic smooth-muscle cells were purchased from the American Type Culture Collection.

2.5. General receptor binding survey

Inhibition of radioligand binding by 1, 100, and 10,000 nM azimilide and three concentrations of a known agonist or antagonist was determined for 60 receptors by the Oceanix Biosciences Corp., using their standard protocols derived from the literature references in Table 1. Each concentration was assayed in duplicate. If $>$50% inhibition was caused by 10 μM azimilide, ten concentrations were tested in triplicate to determine the K_i value of binding to that receptor. In general, membranes were prepared by differential centrifugation according to published methods (Table 1) for each receptor. The receptor sources and the labeled ligands are listed in Table 1. Incubations were carried out for 10–90 min at temperatures from 0°–37° with and without an unlabeled known agonist or antagonist (to define specific binding) and terminated by rapid filtration through glass fiber filters. Radioactivity on filters was determined by liquid scintillation counting.

2.6. Studies in rat cardiac receptors

Binding to selected rat heart receptors was compared for azimilide and several class III antiarrhythmic agents at concentrations of 0.3 to 100 μM. Rats were killed with a pentobarbital overdose, and membranes were prepared from heart cells by standard techniques [15–17] and suspended at 150–200 μg/mL in ice-cold Tris-HCl buffer (50 mM, pH 7.2, at 25°). Radioligands were 0.25 nM [^{3}H]prazosin for the α_1-adrenergic receptor, 1.03 nM [^{3}H]dihydroalprenolol for the β-adrenergic receptor, 0.11 nM [^{3}H]quinuclidinyl benzilate for the muscarinic receptor, 0.08 nM [^{3}H]isradipine for the L-type Ca^{2+} channel, and 0.3 nM [^{3}H]glyburide for the ATP-sensitive K^+ channel. Incubations at 25° for 90 min were terminated by rapid filtration through glass fiber GF/C filters soaked in 5% polyethyleneimine. Specific binding was defined by inclusion of a known agonist or antagonist at 100–1000 times the K_D value of the labeled ligand.

2.7. Studies in rat brain receptors

The effect of azimilide on batrachotoxinin A binding to the neural Na^+ channel site 2 was determined using rat cerebral cortex membranes prepared by the sucrose gradient method of Hajos [18] and equilibrated with binding buffer (130 mM choline chloride, 50 mM HEPES, 5 mM glucose, 0.8 mM $MgSO_4$, 5.4 mM KCl, and 0.1% bovine serum albumin, pH 7.2, adjusted with Tris-HCl buffer). Binding was carried out at 37° with 6.7 nM [^{3}H]batrachotoxinin and 60 μg/mL of scorpion venom A for 30 min with five concentrations (10–100 μM) of azimilide and terminated by rapid filtration through glass fiber filters soaked with 5% polyethyleneimine [19]. Veratridine (300 μM) was used to define specific binding.

For other selected rat brain receptors (adenosine, adrenergic, muscarinic, serotonin, Ca^{2+} and K^+ channels), a membrane fraction was prepared from rat brains (minus cerebella) at 0–4° by homogenization and centrifugation. After homogenization (Brinkmann Polytron setting 5, 15–20 sec) in 30–40 vol. of cold 50 mM Tris-HCl buffer, the homogenate was subjected to two centrifugations (30,000 *g*, 15 min, 4° with resuspension of the pellet in fresh buffer between centrifugations. The synaptosome final pellet was suspended in 100 vol. of cold 50 mM Tris-HCl, pH 7.2, buffer. Radioligands were 0.2 nM [^{3}H]DPCPX for the A_1-adenosine receptor, 0.5 nM [^{3}H]dihydroergocryptine for nonspecific α-adrenergic receptors, 0.25 nM [^{3}H]prazosin for the α_1-adrenergic receptor, 0.6 nM [^{3}H]yohimbine for the α_2-adrenergic receptor, 1.03 nM [^{3}H]dihydroalprenolol for the β-adrenergic receptor, 0.11 nM [^{3}H]quinuclidinyl benzilate for the muscarinic receptor, 1 nM [^{3}H]lysergic acid diethylamide for the serotonin receptor, 0.2 nM [^{3}H]nitrendipine for the L-type Ca^{2+} channel, 0.004 nM [^{125}I]apamin for the apamin-sensitive K^+ channel, and 0.1 nM [^{3}H]glyburide for the ATP-sensitive K^+ channel. Binding was carried out for 30–60 min at 4° (apamin) or at 25° with several concentrations (0.1 to 100 μM) of azimilide or a class III antiarrhythmic agent and terminated by rapid glass fiber filtration. Specific binding was defined with known agonists or antagonists. If inhibition was >50% at 100 μM, an additional 4–6 concentrations were studied to determine a K_i value.

2.8. Studies in dog kidney Na/K-ATPase

Inhibition of binding of the cardiac glycoside LND-796 to the Na/K ATPase of dog kidney membranes was determined for azimilide and reference class III antiarrhythmic agents. Binding of 0.5 nM [^{3}H]LND-796 [20] for 30 min at 25° was determined with and without azimilide (100 μM) or other class III antiarrhythmic agents and with and without a known Na/K-ATPase inhibitor (LND-796 HCl) and terminated by rapid glass fiber filtration.

2.9. Agonist and antagonist actions at muscarinic receptors

To determine the functional activity of azimilide at five subtypes of muscarinic receptors, genes for these receptors were transfected along with the gene for the marker enzyme β-galactosidase into cultured NIH 3T3 cells as described [21,22]. Briefly, cells were plated 1 day before transfection using 1×10^6 cells in 10 mL medium per 10-cm plate. Cells were transfected by calcium phosphate precipitation [BR1] as described by Wigler *et al*. [23], using 5 μg of the human m1–m5 receptor plasmids [24,25] in the pcDNAI expression vector [26], 5 μg of pSV-lacZ β-galactosidase DNA, and 20 μg of salmon sperm DNA. Cells transfected with m2 or m4 were co-transfected further with 5 μg of G_q-i5 [27] in the pcDNAI vector. One day after transfection, medium was changed and after 2 days cells were trypsinized and aliquoted into the wells of a 96-well plate (100 μL/well). Azimilide (0.01 to 10 μM), the muscarinic agonist carbachol, or the muscarinic antagonist pirenzepine was added, and cells were incubated for 4 days. Agonist effects were manifested as stimulation, and antagonist effects as suppression, of cell proliferation, quantitated by the activity of β-galactosidase. Maximum cell proliferation and enzyme activity were elicited from cells expressing the M_1–M_5 receptors by carbachol concentrations of 30, 2, 10, 5, and 5 μM, respectively. For the muscarinic M_2 receptor, additional work approximated the concentration of azimilide required for 50% maximum agonist action.

2.10. Calculations and statistical methods

Radioactivity collected on filters was corrected for nonspecific binding. Effects of azimilide are presented as percent inhibition. Since only two determinations were performed for each assay in the broad screen, no statistical analyses of the results were justified. Because those receptors for which azimilide showed some affinity were reassayed at additional concentrations, an IC_{50} value (concentration for 50% inhibition of binding) was calculated by least-squares regression analysis. From the IC_{50} value, the K_i value was calculated according to the Cheng and Prusoff equation [28]. For M_2 receptors in NIH 3T3 cells, EC_{50} values (concentrations giving 50% of the maximum carbachol response) were calculated from 3-parameter sigmoid regression curves fitted to the log concentration-response plots.

3. Results

3.1. General receptor binding survey

The effect of azimilide on radioligand binding to 60 receptors was determined at three concentrations (1, 100, and 10,000 nM). Meaningful interaction was taken as inhi-

Table 1
Effect of azimilide on radioligand binding at neurotransmitters, ion channels, central, uptake, and second messenger sites, steroid receptors, peptides, and growth factors

Receptor	Source	Assay conditions		Binding inhibition (%) by azimilide		
		Ligand	Reference	1 nM	100 nM	10,000 nM
Neurotransmitters						
Adenosine nonselective	Rat brain cortex	[^{3}H]NECA	Mol Pharmacol 1966;29:331	3.4	8.7	4.9
Adrenergic						
α_1	Rat forebrain	[^{3}H]Prazosin	Mol Pharmacol 1981;20:295	−15.0	−11.2	1.2
α_{1A}	Rat brain cortex[a]	[^{3}H]Prazosin	Eur J Pharmacol 1988;151:333	−17.2	5.1	25.1
α_{1B}	Rat liver	[^{3}H]Prazosin	Eur J Pharmacol 1988;151:333	8.7	0.9	24.7
α_2	Rat brain cortex	[^{3}H]RX 781094	Br J Pharmacol 1983;80:155	3.8	13.9	23.6
β Nonselective	Rat brain cortex	[^{3}H]Dihydroalprenolol	Mol Pharmacol 1989;36:201	7.0	9.7	42.4
β_1[b]	Rat brain cortex	[^{125}I]Pindolol	J Neurochem 1989;53:1772	0.2	3.2	14.9
β_2[c]	Rat brain cortex	[^{125}I]Pindolol	J Neurochem 1989;53:1772	4.2	5.3	13.2
Dopamine nonselective	Rat brain striata	[^{3}H]Spiperone	Biochem Pharmacol 1978;27:307	−2.8	−0.7	11.2
Glycine	Rat brain cortex	[^{3}H]Strychnine	Mol Pharmacol 1974;10:790	18.6	14.4	19.9
Histamine						
H_1	Bovine cerebellum	[^{3}H]Pyrilamine	J Neurochem 1979;32:1653	9.9	5.8	11.5
H_2	Guinea pig striatum	[^{3}H]Tiotidine	Nature 1983;304:65	5.9	5.4	9.8
H_3	Rat forebrain	[^{3}H]*N*α-Methyl histamine	Mol Pharmacol 1990;38:610	14.8	2.4	48.1
Muscarinic						
Central nonselective	Rat brain cortex	[^{3}H]Quinuclidinyl benzilate	J Pharmacol Exp Ther 1984;228:648	7.4	17.6	87.4
Peripheral nonselective	Rat heart	[^{3}H]Quinuclidinyl benzilate	J Pharmacol Exp Ther 1984;228:648	−33.3	−10.3	74.2
M_1	Bovine striatum	[^{3}H]Pirenzepine	J Pharmacol Exp Ther 1986;237:419	8.0	6.9	87.6
M_2	Rat heart	[^{3}H]AF-DX 384	Trends Pharmacol Sci 1989;10:50	−0.3	7.5	83.1
M_3	Guinea pig ileum	[^{3}H]Methylscopalamine	Br J Pharmacol 1990;99:753	2.8	11.6	60.3
Nicotinic neuronal	Rat brain cortex	[^{3}H]*N*-Methylcarbamyl choline iodide	Eur J Pharmacol 1987;139:323	−6.3	−2.8	7.7
Purinergic P2γ	PC-12 cells	[^{35}S]ADPβS	J Biol Chem 1989;264:6202	15.9	3.9	23.8
Serotonin						
Nonselective	Rat brain striatum	[^{3}H]Lysergic acid diethylamide	Mol Pharmacol 1979;16:687	0.0	−7.8	4.5
HT_1	Rat brain cortex	[^{3}H]Hydroxytryptamine	Mol Pharmacol 1976;12:373	0.2	2.0	27.1
HT_2	Rat brain cortex	[^{3}H]Ketanserin	Mol Pharmacol 1982;21:301	4.3	6.6	22.7
HT_3	Mouse N1E-115 cells	[^{3}H]GR 65630	Eur J Pharmacol 1990;189:223	−0.1	12.9	9.8
Ion channels						
Ca^{2+} T and L	Rat brain cortex	[^{3}H]Nitrendipine	Mol Pharmacol 1984;25:235	7.5	−0.1	−12.6
Ca^{2+} N	Rat forebrain	[^{125}I]Omegaconotoxin	J Neurosci 1988;8:3354	0.7	−2.9	1.7
Cl^-	Rat brain cortex	[^{3}H]*t*-Butylbicyclo orthobenzoate	J Neurochem 1986;45:798	−4.0	−15.7	2.0
K^+ ATP-regulated	Rat brain cortex	[^{3}H]Glybenclamide	Arzneimittelforschung 1985;35:707	4.4	1.2	2.9
K^+ apamin-sensitive	Rat forebrain	[^{125}I]Apamin	J Neurosci 1987;7:565	−3.5	4.8	5.6
K^+ voltage-sensitive	Rat whole brain	[^{125}I]Charybdotoxin	J Biochem 1990;265:15564	−0.3	8.3	29.7
Na^+ site 1	Rat forebrain	[^{3}H]Saxitoxin	J Biol Chem 1986;261:6149	0.2	−2.1	−10.1
Na^+ site 2	Rat brain cortex	[^{3}H]Batrachotoxin	Mol Pharmacol 1983;23:350	5.2	4.9	52.6
Central sites						
Muscarinic CNS	Rat brain cortex	[^{3}H]Quinuclidinyl benzilate	J Pharmacol Exp Ther 1984;228:648	7.4	17.6	87.4
NMDA	Rat forebrain	[^{3}H]MK-801	Mol Pharmacol 1989;35:387	−2.0	0.4	−1.5
Opiate nonselective	Rat forebrain	[^{3}H]Naloxone	Mol Pharmacol 1974;19:968	1.9	−0.9	17.1
Phencyclidine	Rat forebrain	[^{3}H]TCP	Brain Res 1983;280:194	−14.1	−10.1	2.4
Sigma	Guinea pig brain	[^{3}H]1,3-Di-*O*(2-tolyl)guanidine	Proc Natl Acad Sci USA 1986;83: 8784	−0.1	13.7	91.2
Uptake sites						
Choline	Rat brain striatum	[^{3}H]Hemicholinium-3	Life Sci 1984;35:2335	9.0	−0.7	−17.9
Dopamine	Guinea pig brain striatum	[^{3}H]WIN 35,428	Mol Pharmacol 1989;36:518	2.4	−2.3	43.8
Norepinephrine	Rat brain striatum	[^{3}H]Desmethyl imipramine	Eur J Pharmacol 1982;78:345	13.6	14.7	21.9
Serotonin	Rat forebrain	[^{3}H]Citalopram	J Pharmacol Exp Ther 1987;242:364	59.2	75.1	95.0
Second messenger sites						
Forskolin	Rat forebrain	[^{3}H]Forskolin	Proc Natl Acad Sci USA 1984;81: 5081	4.1	−4.3	6.2
Inositol triphosphate	Rat brain cerebellum	[^{3}H]Inositol triphosphate	J Biochem (Tokyo) 1987;262:12132	−4.9	−7.0	−17.9
Protein kinase C	Mouse brain	[^{3}H]Phorbol ester dibutyrate	Cancer Res 1980;40:3635	6.5	6.6	−0.1

(continued on next page)

Table 1 *(continued)*

Receptor	Source	Assay conditions		Binding inhibition (%) by azimilide		
		Ligand	Reference	1 nM	100 nM	10,000 nM
Steroid receptors						
Estradiol	Bovine uterus	[^{125}I]Estradiol	Brain Res 1980;204:373	−12.5	12.1	−27.1
Progesterone	Bovine uterus	[^{3}H]Promegestone	Brain Res 1980;204:373	0.7	3.8	1.5
Testosterone	Rat prostate	[^{3}H]Methyltrienolone	Endocrinology 1986;118:1327	16.8	4.8	5.4
Brain and gut peptides						
Angiotensin II peripheral	Rat liver	[^{125}I]Angiotensin II	J Biol Chem 1976;251:7423	−3.8	0.3	34.3
Angiotensin II central	Bovine brain	[^{125}I]Angiotensin II	J Biol Chem 1976;251:7423	3.2	7.4	8.2
Bradykinin	Guinea pig ileum	[^{3}H]Bradykinin	J Pharmacol Exp Ther 1988;237:504	2.1	1.7	7.4
Endothelin A	Rat thoracic smooth muscle	[^{125}I]Endothelin	Biochem Biophys Res Commun 1989;158:195	18.7	9.2	8.4
Endothelin B	Rat cerebellum	[^{125}I]Endothelin	Biochem Biophys Res Commun 1989;158:195	2.0	2.3	−1.1
Neuropeptide Y	Bovine hippocampus	[^{125}I]Neuropeptide Y	Life Sci 1985;37:2111	−0.6	−2.4	7.0
TRH	Rat forebrain	[^{3}H](3-Methyl histidine^{2})-TRH	Life Sci 1982;30:1793	−2.4	−17.1	−18.9
VIP	Rat forebrain	[^{125}I]VIP	Peptides 1985;6:103	−5.5	−6.6	−4.3
Growth factors, peptides						
ANF-1	Rat forebrain	[^{125}I]ANF	Circ Res 1985;56:801	−3.4	−5.1	−4.7
CRF	Rat brain cortex	[^{125}I]Tyrosine-*O*-CRF	J Neurosci 1987;7:88	6.5	14.1	1.2
EGF	Rat liver	[^{125}I]EGF	J Biol Chem 1984;259:6543	−20.4	−5.6	−3.9
PAF	Rabbit platelets	[^{3}H]PAF	J Biol Chem 1985;260:15639	−5.4	−12.7	−4.2
TNF	Human monocyte HL60 cells	[^{125}I]TNFα	Immunopharmacology 1990;20:217	9.9	6.7	10.5

Binding of the indicated radioligand was carried out with and without the indicated concentrations of azimilide in duplicate. Bound ligand was collected on glass fiber filters. Abbreviations: ADF, adenosine diphosphate; ANF, atrial natriuretic factor; CRF, corticotropin-releasing factor; EGF, epidermal growth factor; HT, hydroxytryptamine (serotonin); ITP, inositol triphosphate; NECA, 5′-*N*-ethylcarboxamido-adenosine; NMDA, *N*-methyl-D-aspartate; PAF, platelet-activating factor; TCP, 1-[1-(2-thienyl)cyclohexyl]-3,4-piperidine hydrochloride; TNF, tumor necrosis factor; TRH, thyrotropin-releasing hormone; and VIP, vasoactive intestinal peptide.

[a] Rats pretreated with chlorethylclonidine.

[b] β_2 sites blocked by 120 nM ICI-118,551.

[c] β_1 sites blocked by 100 nM ICI-89406.

bition of more than 50% at the highest concentration. By this criterion, azimilide interacted with five muscarinic receptors and several central receptors (sigma, Na^+ channel site 2, and the serotonin uptake sites) (Table 1). Some evidence of interaction with azimilide (20–40% inhibition) was also seen at α- and β-adrenergic, histamine, purinergic, serotonin, and peripheral angiotensin receptors, dopamine uptake and norepinephrine uptake sites, and a voltage-sensitive K^+ channel. Lesser inhibition or increased radioligand binding was seen at other studied receptors, including Ca^{2+} and Cl^- channels, second messengers, steroid receptors, most brain and gut peptides, and growth factors and peptides (Table 1).

For the eight receptors showing a meaningful interaction with azimilide, full concentration-response curves provided an estimate of its K_i value in comparison with that of a potent reference agonist or antagonist. At M_1, M_2, and M_3 muscarinic receptors, the K_i values of azimilide ranged from 949 to 3130 nM and were at least two orders of magnitude larger than those of the reference agonists atropine and 4-diphenylacetoxy-*N*-methylpiperidine (Table 2). The K_i value of azimilide at the Na^+ channel site 2 was much closer (four times higher) to that of the reference ligand aconitine, and its values at the serotonin uptake and sigma sites were at least 1000 times larger than those of the reference ligands (Table 2).

3.2. Studies in rat cardiac receptors

Binding inhibition of azimilide, clofilium, E-4031, sematilide, and *d*,*l*-sotalol was examined at five receptors found in membranes of the rat ventricle. The compounds did not inhibit binding to the ATP-sensitive K^+ channel (data not shown). Only azimilide interacted with the L-type Ca^{2+} channel, but its K_i value of 37.3 μM indicated weak affinity. Based on K_i values, azimilide showed approximately 10-fold greater affinity for α- and β-adrenergic and muscarinic receptors than for the L-type Ca^{2+} channel (Table 3). The overall profile of azimilide was different from the profiles of four comparison class III antiarrhythmic agents: azimilide showed affinity for all four receptors with selectivity for the adrenergic and muscarinic receptors over the L-type Ca^{2+} channel (Table 3). Clofilium interacted preferentially with muscarinic receptors. E-4031 inhibited binding to the same

Table 2
Comparison of potencies of azimilide and reference ligands for inhibition of radioligand binding to selected receptors

Receptor	Reference ligand	K_i (nM)	
		Ligand	Azimilide
Muscarinic			
Central nonselective	Atropine	0.7	2030, 2240[a]
Peripheral nonselective	Atropine	0.2	2480, 3620[a]
M_1	Atropine	0.4	1970
M_2	Atropine	0.7	949
M_3	4-Diphenylacetoxy-*N*-methylpiperidine methiodide	0.7	1380, 3130[a]
Na^+ channel site 2	Aconitine	1000	4330
Serotonin uptake site	Paroxetine	0.1	588
Sigma	Haloperidol	3.6	6200

Receptor source and radioligand are given in Table 1. Several concentrations of reference ligand and ten concentrations of azimilide (1–100 μM) were tested in triplicate assays to determine K_i values.
[a] Two values from separate assays are shown.

receptors, with a preference for the α-adrenergic receptor. Sematilide was not active at any of the four receptor sites. *d,l*-Sotalol showed the expected preference for the β-adrenergic site (K_i: 2.86 μM) with affinity similar to that of azimilide (K_i: 1.89 μM). Sotalol also interacted weakly with the muscarinic receptor (Table 3).

3.3. Studies in rat brain receptors

The activity of azimilide was compared with that of ten class III antiarrhythmic compounds at nine receptors in rat brain membranes. Azimilide showed low affinity (K_i: >25 μM) for the A_1-adenosine receptor and L-type Ca^{2+} and Na^+ channels and stronger affinity (K_i: ≤ 20 μM) for adrenergic, muscarinic, and nonselective serotonin receptors (Table 4). At the α-adrenergic receptors, the affinity of azimilide increased in the order of α_1, α, and α_2 receptor subtypes.

d,l-Sotalol was the only agent besides azimilide to interact with the β-adrenergic receptor. At the rat brain β-adrenergic receptor, the affinity of *d,l*-sotalol (K_i: 0.6 μM) was several times greater than that of azimilide (K_i: 2.3 μM). Compounds that blocked binding to α-adrenergic and muscarinic receptors included amiodarone, bretylium, clofilium, LY-190147, and MDL-11,939. Amiodarone also showed affinities for the L-type Ca^{2+} channel and the A_1-adenosine site. Dofetilide was active at the α_2-adrenergic and muscarinic receptors. Sematilide lacked affinities for these receptors. Azimilide, E-4031, LY-190147, and MDL-11939 interacted (K_i: <20 μM) with the central serotonin receptors probed by lysergic acid diethylamide (Table 4). Additional work showed that quinidine and melperone also had K_i values below 20 μM at these serotonin receptors (data not shown).

3.4. Interactions with dog kidney Na/K ATPase

Neither azimilide nor any of the class III antiarrhythmics listed in Table 4, at concentrations up to 100 μM, inhibited the binding of LND-796 to dog kidney membranes.

3.5. Agonist and antagonist actions at muscarinic receptors

To determine the nature of the interaction of azimilide with muscarinic receptors, genes for these receptors were transfected into NIH 3T3 cells expressing a reporter enzyme, β-galactosidase. Agonist interaction with the expressed receptors could then be correlated with an increase

Table 3
Inhibition by class III antiarrhythmics of radioligand binding to selected rat cardiac ventricle membrane receptors

Compound	K_i at receptor (μM)			
	α_1-Adrenergic	β-Adrenergic	Muscarinic	L-type Ca^{2+} channel
Azimilide	4.16 ± 0.15	1.89 ± 0.28	1.58 ± 0.13	37.3 ± 1.7
Clofilium	2.73 ± 0.70	1.34 ± 0.26	0.29 ± 0.03	NA
E-4031	1.58 ± 0.18	16.2 ± 6.3	12.8 ± 1.0	NA
Sematilide	NA	NA	NA	NA
d,l-Sotalol	NA	2.86 ± 1.00	33.4 ± 5.0	NA

Radioactive ligands with and without test compound (several concentrations from 1 to 100 μM) were incubated with a rat ventricle membrane fraction. Membrane-bound radioactivity was collected on glass fiber filters. Values are means ± SEM for 3–4 experiments with duplicate assays at each concentration of the compound. NA, not active.

Table 4
Inhibition by class III antiarrhythmics of radioligand binding to selected rat brain membrane receptors

Compound	K_i at receptor (μM)								
	A_1-adenosine	Adrenergic				Ion channel		Muscarinic	Serotonin
		α	α_1	α_2	β	L-type Ca^{2+}	Na^+		
Amiodarone	14.5	16.4	14.1	5.8	> 28.6	0.4	ND	15.9	37.1
Azimilide	> 47.4	9.7	20.1	2.4	2.3	> 50.0	25.7	2.5	19.7
Bretylium	> 47.4	15.8	> 31.8	2.8	> 28.6	> 50.0	ND	6.2	> 100
Clofilium	> 47.4	14.1	22.5	7.2	> 28.6	23.5	4.5	1.0	77.2
Dofetilide	26.0	74.1	> 31.8	14.6	> 28.6	> 50.0	ND	3.0	39.7
E-4031	> 47.4	> 76.2	ND	ND	> 28.6	> 50.0	139.4	22.7	9.2
LY-190147	> 47.4	3.7	4.9	0.6	> 28.6	11.4	ND	0.1	1.8
MDL-11,939	> 47.4	9.1	3.5	1.5	> 28.6	28.0	ND	20.1	2.5
Risotilide	> 47.4	> 76.2	> 31.8	23.4	> 28.6	> 50.0	ND	> 23.1	> 100
Sematilide	> 50.0	> 76.2	> 31.8	69.1	> 28.6	> 50.0	NA	> 23.1	28.2
d,*l*-Sotalol	> 47.4	> 76.2	> 31.8	> 78.6	0.6	> 50.0	NA	> 23.1	> 100

Radioactive ligands with and without 1–100 μM test compound were incubated with a rat brain cortex membrane fraction. Membrane-bound radioactivity was collected on glass fiber filters. Values are means of three separate experiments each involving a concentration–response curve with 4–6 concentrations. NA, not active; ND, not determined.

in enzyme activity, and antagonist interaction, with inhibition of carbachol-stimulated enzyme activity.

Azimilide alone elicited a concentration-dependent agonist response, larger in cells expressing the M_2 and M_4 receptors than in cells expressing M_1, M_3, or M_5 receptors (Fig. 1). At 3 μM, azimilide produced 104 and 36% of the maximum carbachol response in M_2- and M_4-expressing cells, respectively (Fig. 1). Although azimilide and carbachol displayed parallel concentration-response curves for stimulating cells with the M_2 receptor subtype, azimilide had less than one-tenth the potency of carbachol (Fig. 2). The EC_{50} values of 1.97 and 0.14 μM, respectively, were estimated (Fig. 2). When azimilide was added with the agonist carbachol, a moderate concentration-dependent inhibition of enzyme activity was seen, particularly in cells expressing the M_1, M_3, or M_5 muscarinic receptors. In M_4-expressing cells, inhibition (>50%) also occurred but did not differ between cells treated with 0.1 or 3 μM azimilide. Azimilide at 3 μM allowed enzyme activity that was 61, 76, 27, 37, and 58%, respectively of the carbachol-induced maximum in cells expressing the M_1, M_2, M_3, M_4, and M_5 receptors subtypes (Fig. 1).

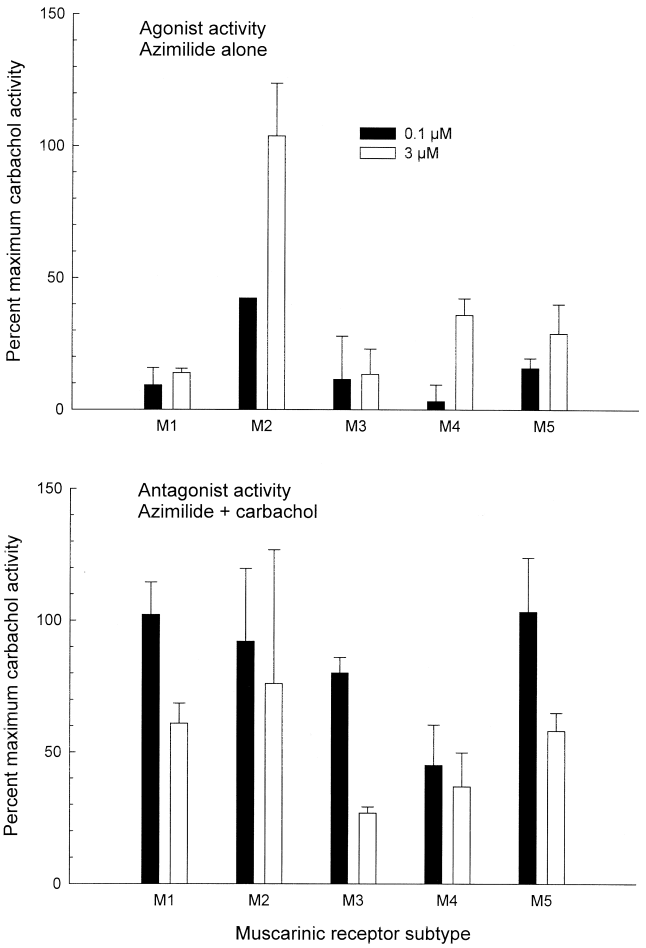

Fig. 1. Agonist (top) and antagonist (bottom) effects of azimilide in NIH 3T3 cells expressing muscarinic receptors whose stimulation caused mitogenesis and increased β-galactosidase activity. Cells were incubated for 4 days with azimilide at 0.01 μM (closed bars) and 3 μM (open bars), without (top) and with (bottom) carbachol. Activity is presented as the mean percent of the maximum produced at that receptor by carbachol alone. Range bars are shown for two (M_1, M_3, and M_5) and SEM bars for three (M_2 and M_4) determinations.

4. Discussion

Azimilide at micromolar concentrations interacts with several biological receptors. A general survey demonstrated that radioligand binding to muscarinic receptors and several central receptors (sigma, a Na^+ channel, and the serotonin uptake site) was inhibited more than 50% by 10 μM azimilide. Additional studies in rat heart tissue indicated interactions with α- and β-adrenergic receptors, muscarinic receptors, and the L-type Ca^{2+} channel. In rat brain, azimilide also reduced binding to adrenergic and muscarinic receptors and showed affinity for a central Na^+ channel site, and the

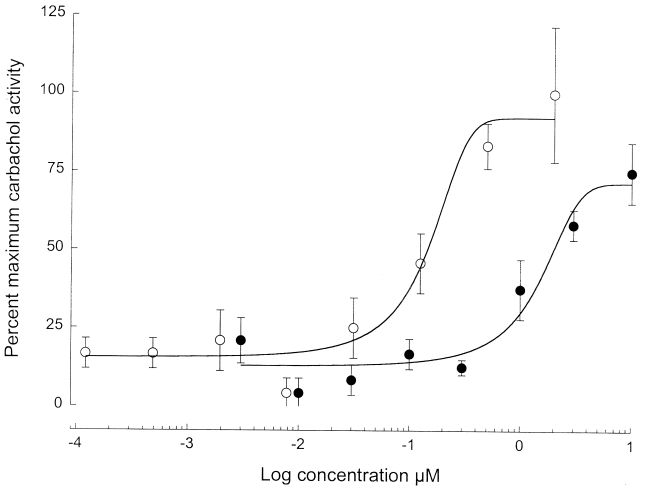

Fig. 2. Agonist actions of carbachol (○) and azimilide (●) in NIH 3T3 cells expressing muscarinic M_2 receptors whose stimulation caused mitogenesis and increased β-galactosidase activity. Cells were incubated for 4 days with carbachol or azimilide. Values are presented as a percentage of the maximum activity produced by carbachol alone. Means and SEM bars are shown for three plate wells at each agonist concentration. The EC_{50} values (concentrations giving 50% of the maximum carbachol response derived from the regression-fitted 3-parameter sigmoid equations) were 0.14 μM for carbachol and 1.97 μM for azimilide.

serotonin uptake site. In a transfected cell assay, azimilide exhibited mixed agonist and antagonist properties at muscarinic receptors, with agonism predominant at M_2 and M_4 subtypes and antagonism predominant at M_1, M_3, and M_5 subtypes. This dichotomy corresponds to the differentiation of even and odd number muscarinic subtypes based on coupling to phospholipase C and adenylate cyclase, respectively, as second messenger systems [29]. Although the affinities of azimilide for α- and β-adrenergic, muscarinic, Ca^{2+} channel, sigma, and serotonin uptake sites were two or more orders of magnitude less than for established agonists or antagonists, the concentrations at which azimilide could inhibit receptor binding were only two to ten times the concentration required for interaction with its primary target, the cardiac delayed-rectifier channel.

The pattern and affinities of interactions with rat brain receptors suggest that azimilide has a unique profile as compared with ten other class III antiarrhythmic agents. While those drugs may affect one or more of the receptors examined in brain, azimilide has a broader affinity for diverse receptors and a good affinity (K_i: $\leq$20 μM) at four important receptors, i.e., α- and β-adrenergic, muscarinic, and serotonin. Most similar to azimilide in the rat brain receptor interaction profile were amiodarone, LY-190147, and MDL-11,939. However, none of these compounds had the affinity for the β-adrenergic receptor seen with azimilide, and LY-190147 had greater relative affinities for the muscarinic and serotonin receptors.

Interaction of class III drugs with various receptors other than cardiac ion channels can be part of their mechanism of action. In atrial fibrillation, class III drugs can slow conduction through the atrioventricular node by activating muscarinic and A_1-adenosine receptors or by blocking β-adrenergic receptors [30]. Even where a direct link to antiarrhythmic actions is not clear, antiarrhythmic agents are known to have affinities for the receptors identified for azimilide. Bretylium binds to high-affinity muscarinic sites in heart and brain, and its anticholinergic action is correlated with antiarrhythmic activity [31]. Amiodarone down-regulates A_1-adenosine receptors [32], blocks both α- and β-adrenergic receptors, and interacts with muscarinic receptors of heart and brain [33]. Both *d,l*-sotalol and MS-551 block muscarinic receptors, which reduces the acetylcholine-induced K^+ current [34]. Binding of class III antiarrhythmics to the sigma 2 receptor of rat cerebral cortex has been correlated with potency for increasing action potential duration in Purkinje fibers [35]. Conversely, several compounds whose primary target is a noncardiac receptor have antiarrhythmic efficacy. Examples include the selective sigma-receptor antagonist DuP 734 and the 5-HT_2-selective antagonist ketanserin [36,37].

The interactions of azimilide at sites where other class III agents have affinity raises the possibility that some of its activity may involve receptors other than the primary target of the cardiac delayed rectifier. Direct actions at cardiac ion channels other than I_{Kr} and I_{Ks} may contribute to antiarrhythmic activity or cardiac safety. Block of a cardiac Na^+ channel could help slow conduction and interrupt a reentrant arrhythmia. Induction of the I_{KAch} channel by a muscarinic agonist activity could promote repolarization, limit action potential prolongation, and diminish the threat of torsade de pointes arrhythmias. Conversely, azimilide, like other class III drugs, has been reported to inhibit carbachol-induced I_{KAch} in guinea pig atrial cells, perhaps by interaction with muscarinic M_2 receptors [34,38]. Adrenergic interactions are important for hemodynamic actions. Azimilide is known to decrease heart rate and, at higher doses in animals, to reduce blood pressure, which might depend on antagonist actions at adrenergic receptors. Previous work has characterized azimilide as an antagonist at the β-adrenergic receptor [39]. However, its lack of effect on hemodynamic changes wrought by isoproterenol in rats is not consistent with a role of β-adrenergic blockade in modifying hemodynamics [40].

The actions of azimilide at muscarinic receptors could also mediate both hemodynamic effects and ion channel actions. Muscarinic receptors, sometimes via adrenergic actions, can be involved in changes in rate, conduction, contraction, and heterogeneity of repolarization. Receptor subtypes with odd numbers are linked to phospholipase C and may regulate a Na^+ current and heart rate. M_3 receptor stimulation has been reported to activate a delayed-rectifier K^+ current in canine atrial myocytes [41]. Muscarinic receptors are known to affect cardiac ion currents, i.e., by activation of a delayed-rectifier K^+ current [41], inhibition of a Ca^{2+} current [42] and the pacemaker current [43] of the sinoatrial node, and induction of I_{KAch} in nodal tissue and

the atria [44]. Indirect muscarinic effects (elevated parasympathetic tone) can also have antiarrhythmic actions [45,46].

The radioligand binding data in our studies indicate azimilide interaction with several receptors of the central nervous system (serotonin and serotonin uptake, sigma, and Na^+ channel site 2). However, there is little or no evidence from animal or human studies that azimilide has any central effects.

At its primary target, the cardiac delayed-rectifier channel, azimilide acts at a concentration of about 1 μM. Its animal and clinical efficacy occurs at plasma concentrations of 1–5 μM. Thus, it is more potent as a prolonger of cardiac refractoriness at this site than at most other targets, and its interaction at receptors other than the cardiac K^+ channel should be small at antiarrhythmic doses. The relative lesser potency of azimilide at most of those other receptors may restrict their role to minor effects or actions under conditions of augmented sensitivity. In clinical studies no action has been reported that might clearly be ascribed to interaction at targets other than the cardiac delayed rectifier. Nevertheless, the micromolar affinities of azimilide for a variety of important biological receptors, having similarities to the amiodarone profile, open the possibility that one or more may play a role in the efficacy and safety profile of this compound.

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References

[1] Busch AE, Malloy K, Groh WJ, Varnum MD, Adelman JP, Maylie J. The novel class III antiarrhythmics NE-10064 and NE-10133 inhibit I_{sK} channels expressed in *Xenopus* oocytes and I_{Ks} in guinea pig cardiac myocytes. Biochem Biophys Res Commun 1994;202:265–70.

[2] Fermini B, Jurkiewicz NK, Jow B, Guinosso PJ, Baskin EP, Lynch JJ, Salata JJ. Use-dependent effects of the class III antiarrhythmic agent NE-10064 (azimilide) on cardiac repolarization: block of delayed rectifier potassium and L-type calcium currents. J Cardiovasc Pharmacol 1995;26:259–71.

[3] Salata JJ, Brooks RR. Pharmacology of azimilide dihydrochloride (NE-10064). Cardiovasc Drug Rev 1997;15:137–56.

[4] Brooks RR, Carpenter JF, Miller KE, Maynard AE. Efficacy of the class III antiarrhythmic agent azimilide in rodent models of ventricular arrhythmia. Proc Soc Exp Biol Med 1996;212:84–93.

[5] Drexler AP, Micklas JM, Brooks RR. Suppression of inducible ventricular arrhythmias by intravenous azimilide in dogs with previous myocardial infarction. J Cardiovasc Pharmacol 1996;28:848–55.

[6] Nattel S, Liu L, St-Georges D. Effects of the novel antiarrhythmic agent azimilide on experimental atrial fibrillation and atrial electrophysiologic properties. Cardiovasc Res 1998;37:627–35.

[7] Restivo M, Hegazy M, Caref EB, Avitable MJ, Assadi MA, el-Hamami M, Yin H, Piracha M, Brooks RR, el-Sherif N. Effects of azimilide dihydrochloride on circus movement atrial flutter in the canine sterile pericarditis model. J Cardiovasc Electrophysiol 1996;7:612–24.

[8] Black SC, Butterfield JL, Lucchesi BR. Protection against programmed electrical stimulation-induced ventricular tachyarrhythmia and sudden cardiac death by NE-10064, a class III antiarrhythmic drug. J Cardiovasc Pharmacol 1993;22:810–8.

[9] Pritchett E, Page R, Connolly S, Marcello S, Schnell D, Wilkinson W, ASAP Investigators. Azimilide treatment of atrial fibrillation. Circulation 1998;98(Suppl):I-633.

[10] Page RL, Connolly SJ, Marcello SR, Schnell DJ, Wilkinson WE, Pritchett ELC. Dose response for azimilide treatment of paroxysmal supraventricular tachycardia. Pacing Clin Electrophysiol 1999;22:770.

[11] Conder ML, Hess TA, Smith MA, D'Alonzo AJ, McCullough JR. The effects of NE-10064 on cardiac sodium channels. FASEB J 1994;8:A609.

[12] Yao J-A, Tseng G-N. Azimilide (NE-10064) can prolong or shorten the action potential duration in canine ventricular myocytes: dependence on blockade of K, Ca, and Na channels. J Cardiovasc Electrophysiol 1997;8:184–98.

[13] Yao J-A, Tseng G-N. Effects of azimilide (NE-10064) on cardiac K channels. J Am Coll Cardiol 1995;Special Issue:176A.

[14] Pong SF, Kinney CM, Moorehead TJ. Binding profile of NE-10064, a novel class III antiarrhythmic agent, to rat brain receptors. FASEB J 1993;7:A474.

[15] Wei XY, Luchowski E, Rutledge A, Su CM, Triggle DJ. Pharmacologic and radioligand binding analysis of the actions of 1,4-dihydropyridine activator-antagonist pairs in smooth muscle. J Pharmacol Exp Ther 1986;239:144–53.

[16] Wei XY, Rutledge A, Triggle DJ. Voltage-dependent binding of 1,4-dihydropyridine Ca^{2+} channel antagonists and activators in cultured neonatal rat ventricular myocytes. Mol Pharmacol 1989;35:541–52.

[17] Bolger GT, Gengo P, Klockowski R, Luchowski E, Siegel H, Janos RA, Triggle AM, Triggle DJ. Characterization of binding of the Ca^{2+} channel antagonist, [^{3}H]nitrendipine, to guinea pig ileal smooth muscle. J Pharmacol Exp Ther 1983;225:291–309.

[18] Hajos F. An improved method for the preparation of synaptosomal fractions in high purity. Brain Res 1975;93:485–9.

[19] Creveling CR, McNeal ET, Daly JW, Brown GB. Batrachotoxin-induced depolarization and [^{3}H]batrachotoxinin-A 20α benzoate binding in a vesicular preparation from guinea pig cerebral cortex. Inhibition by local anesthetics. Mol Pharmacol 1983;23:350–8.

[20] Maixent J-M, Fenard S, Kawamoto RM. Tissue localization of active Na,K-ATPase isoenzymes by determination of their profile of inhibition with ouabain, digoxin, digitoxigenin and LND 796, a new aminosteroid cardiotonic. J Recept Res 1991;11:687–98.

[21] Messier TL, Dorman CM, Brauner OH, Eubanks D, Brann MR. High throughput assays of cloned adrenergic, muscarinic, neurokinin, and neurotrophin receptors in living mammalian cells. Pharmacol Toxicol 1995;76:308–11.

[22] Brauner-Osborne H, Brann MR. Pharmacology of muscarinic acetylcholine receptor subtypes (m1–m5): high throughput assays in mammalian cells. Eur J Pharmacol 1996;295:93–102.

[23] Wigler M, Silverstein S, Lee LS, Pellicer A, Cheng Y, Axel R. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell 1977;11:223–32.

[24] Bonner TI, Young AC, Brann MR, Buckley NJ. Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. Neuron 1988;1:403–10.

[25] Bonner TI, Buckley NJ, Young AC, Brann MR. Identification of a family of muscarinic acetylcholine receptor genes [published erratum appears in Science 1987;237:1556]. Science 1987;237:527–32.

[26] Okayama H, Berg P. A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. Mol Cell Biol 1983;3:280–9.
[27] Conklin BR, Farfel Z, Lustig KD, Julius D, Bourne HR. Substitution of three amino acids switches receptor specificity of $G_q\alpha$ to that of $G_i\alpha$. Nature 1993;363:274–6.
[28] Cheng Y-C, Prusoff WH. Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. Biochem Pharmacol 1973; 22:3099–108.
[29] De Ferrari GM, Zaza A. Drugs acting on cholinergic receptors. In: Singh BN, Dzau VJ, Vanhoutte PM, Woosley RL, editors. Cardiovascular pharmacology and therapeutics. New York: Churchill Livingstone, 1994. p. 125–44.
[30] Grant AO. Mechanisms of atrial fibrillation and action of drugs used in its management. Am J Cardiol 1998;82:43N–9N.
[31] Schreiber G, Sokolovsky M. Muscarinic receptor heterogeneity revealed by interaction with bretylium tosylate. Different ligand-receptor conformations versus different receptor subclasses. Mol Pharmacol 1985;27:27–31.
[32] El-Ani D, Jacobson KA, Shainberg A. Regulation of A_1 adenosine receptors by amiodarone and electrical stimulation in rat myocardial cells *in vitro*. Biochem Pharmacol 1997;54:583–7.
[33] Cohen AM, Schreiber G, Sokolovsky M. Interaction of the antiarrhythmic drug amiodarone with the muscarinic receptor in rat heart and brain. J Cardiovasc Pharmacol 1984;6:1148–55.
[34] Uemura H, Hara Y, Endou M, Mori K, Nakaya H. Interaction of class III antiarrhythmic drugs with muscarinic M_2 and M_3 receptors: radioligand binding and functional studies. Naunyn Schmiedebergs Arch Pharmacol 1995;353:73–9.
[35] Jeanjean AP, Mestre M, Maloteaux J-M, Laduron PM. Is the σ_2 receptor in rat brain related to the K^+ channel of class III antiarrhythmic drugs? Eur J Pharmacol 1993;241:111–6.
[36] Coker SJ, Ellis AM. Ketanserin and ritanserin can reduce reperfusion-induced but not ischaemia-induced arrhythmias in anaesthetised rats. J Cardiovasc Pharmacol 1987;10:479–84.
[37] Maslov LN, Krylatov AV, Lishmanov IB. [The antiarrhythmic effect of the selective sigma-receptor antagonist DuP 734 [1-(cyclopropylmethyl)-4-(2′-(4″-fluorophenyl)-2′-oxoethyl)piperidine HBr]]. Eksp Klin Farmakol 1997;60:24–6.
[38] Nishida A, Uemura H, Ogura T, Furusawa Y, Yabana H, Nakaya H. Inhibitory effects of azimilide on K^+ currents in isolated guinea-pig cardiomyocytes. Jpn J Pharmacol 2000;82:96P.
[39] Miller KE, Carpenter JF, Brooks RR. Inhibition of isoproterenol-induced tachycardia by azimilide in the isolated perfused guinea pig heart. Cardiovasc Drugs Ther 1998;12:83–91.
[40] Mittelstadt SW, Maynard AE, Benn DR, Lowe ER, Kostreva DR. Effects of azimilide and *d*,*l*-sotalol on the heart rate and blood pressure response to isoproterenol in anesthetized rats. Cardiovasc Drugs Ther 1997;11:591–8.
[41] Shi H, Wang HZ, Wang ZG. M-3 muscarinic receptor activation of a delayed rectifier potassium current in canine atrial myocytes. Life Sci 1999;64:PL251–7.
[42] Ye C, Sowell MO, Vassilev PM, Milstone DS, Mortensen RM. $G\alpha_{i2}$, $G\alpha_{i3}$ and $G\alpha_o$ are all required for normal muscarinic inhibition of the cardiac calcium channels in nodal/atrial-like cultured cardiocytes. J Mol Cell Cardiol 1999;31:1771–81.
[43] DiFrancesco D, Tromba C. Muscarinic control of the hyperpolarization-activated current (i_f) in rabbit sino-atrial node myocytes. J Physiol (Lond) 1988;405:493–510.
[44] Renaudon B, Bois P, Bescond J, Lenfant J. Acetylcholine modulates I_f and $I_{K(ACh)}$ via different pathways in rabbit sino-atrial node cells. J Mol Cell Cardiol 1997;29:969–75.
[45] Lynch JJ, Nelson SD, MacEwen SA, Driscoll EM, Lucchesi BR. Antifibrillatory efficacy of concomitant *beta* adrenergic receptor blockade with dilevalol, the *R*,*R*-isomer of labetalol, and muscarinic receptor blockade with methylscopolamine. J Pharmacol Exp Ther 1987;241:741–9.
[46] Rosenshtraukh L, Danilo P, Anyukhovsky EP, Steinberg SF, Rybin V, Brittain-Valenti K, Molina-Viamonte V, Rosen MR. Mechanisms for vagal modulation of ventricular repolarization and of coronary occlusion-induced lethal arrhythmias in cats. Circ Res 1994;75:722–32.