Expedited Articles

Discovery of a Potent, Orally Bioavailable β_3 Adrenergic Receptor Agonist, (*R*)-*N*-[4-[2-[[2-Hydroxy-2-(3-pyridinyl)ethyl]amino]ethyl]phenyl]-4-[4-[4-(trifluoromethyl)phenyl]thiazol-2-yl]benzenesulfonamide

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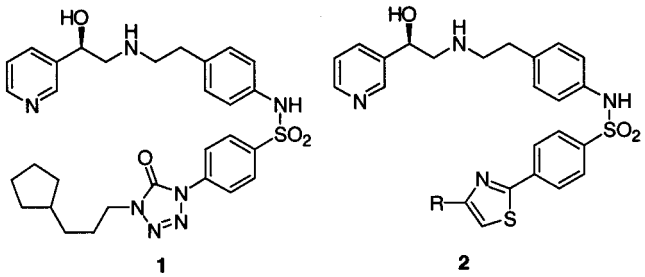
As part of our investigation into the development of orally bioavailable β_3 adrenergic receptor agonists, we have identified a series of pyridylethanolamine analogues possessing a substituted thiazole benzenesulfonamide pharmacophore that are potent human β_3 agonists with excellent selectivity against other human β receptor subtypes. Several of these compounds also exhibited an improved pharmacokinetic profile in dogs. For example, thiazole sulfonamide **2e** (R = 4-F_3C-C_6H_4) is a potent full β_3 agonist (EC_{50} = 3.6 nM, 94% activation) with >600-fold selectivity over the human β_1 and β_2 receptors, which also displays good oral bioavailability in several mammalian species, as well as an extended duration of action.

Introduction

Obesity is becoming an increasingly common disorder in the United States and other parts of the world, and is closely associated with the development of type II diabetes, coronary heart disease, and hypertension. Accordingly, the potential for therapeutic intervention has received considerable attention in the past several years, and researchers have described a number of different approaches for treating obesity.[1] One potential therapeutic approach to altering body fat composition is the enhancement of energy expenditure by increasing metabolic rate. The recognition that stimulation of β_3 adrenergic receptors (ARs) on the surface of adipocytes evokes lipolysis and activation and upregulation of the uncoupling protein UCP1, which leads to a net increase in energy utilization,[2] has led to considerable efforts to identify selective agonists of the human β_3 adrenergic receptor.[3] A recent report from our labs described the in vitro activity and pharmacokinetic properties of the pyridylethanolamine β_3 agonist **1**,[4] a tetrazolone benzenesulfonamide analogue which exhibited 27% oral bioavailability in both dogs and rats. Concurrent studies in our labs have examined a number of alternative replacements for the tetrazolone nucleus (including oxazole,[5] oxadiazole,[6] triazole,[7] pyridyl,[8] and pyrimidyl[8]), but none have been found to possess the required combination of potency, selectivity, and oral bioavailability for a clinical drug candidate.

We recently reported[9] the discovery of a class of pyridylethanolamine β_3 AR agonists possessing a substituted indoline-5-sulfonamide pharmacophore which exhibited exceptional potency and selectivity for the human β_3 AR. However, these compounds exhibited poor oral bioavailability in dogs and rats. Subsequent modifications resulted in the identification of a related series of substituted thiazole benzenesulfonamides **2** which also display excellent in vitro agonist activity and selectivity.[10] In contrast to the SAR observed in several other series of pyridylethanolamine β_3 agonists,[4,9,11] it was subsequently found that several aryl-substituted thiazoles **2** exhibited better potency, selectivity, and oral bioavailability than the alkyl-substituted analogues. Herein we detail the preparation and biological profiles of a series of thiazole sulfonamides possessing fluorinated aromatic substituents, culminating in the discovery of **2e** (R = 4-F_3C-C_6H_4), a potent and selective pyridylethanolamine β_3 agonist with an improved pharmacokinetic profile.



1 **2**

Chemistry

Thiazole benzenesulfonamides **2a**−**h** were prepared from aniline intermediate **3**[12] by the divergent route illustrated in Scheme 1. Coupling of **3** with 4-cyanobenzenesulfonyl chloride, followed by treatment with hydrogen sulfide, afforded the thioamide **4**, which was condensed with the requisite α-halo ketones to afford

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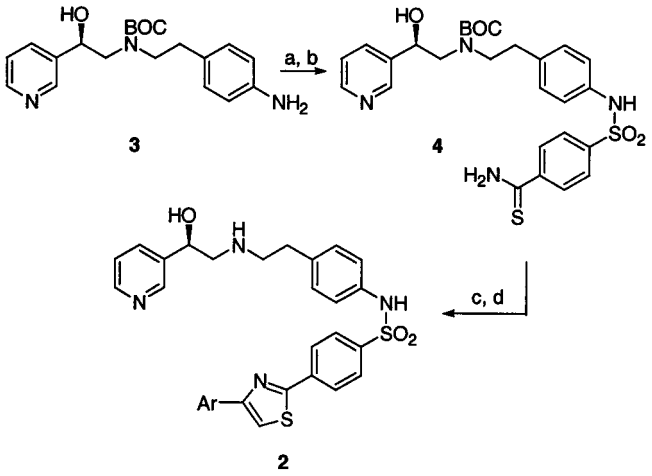
Scheme 1. Synthesis of Thiazole Sulfonamides **2**[a]



[a] Reagents: (a) 4-CNC$_6$H$_4$SO$_2$Cl, pyridine, CH$_2$Cl$_2$; (b) H$_2$S, Et$_3$N, pyridine, 25 °C; (c) ArCOCH$_2$Cl, ethanol, reflux; (d) trifluoroacetic acid, CH$_2$Cl$_2$, 25 °C.

the thiazole intermediates. Removal of the BOC protecting group afforded the final products **2**.

Results and Discussion

Compounds **2a**−**h** were evaluated for in vitro functional efficacy in stimulating increases in cAMP in Chinese hamster ovary (CHO) cells expressing the cloned human β_3 receptor. The activity of an agonist at the β_3 AR is best described by its ability to stimulate adenylyl cyclase in a functional assay, since this method measures the affinity of a compound for the high-affinity, G-protein-coupled state of the receptor. As a result, this assay accurately predicts the lipolytic potential of compounds in native adipocytes.[13] However, because the compounds under study generally have very low efficacy at β_1 and β_2 receptors, the activities of these compounds for the β_1 and β_2 ARs are better described by their binding affinities for these receptors. Thus, binding affinities to membranes prepared from CHO cells expressing the cloned human β_1 and β_2 ARs were determined, and these values permit an assessment of the selectivity of such compounds for the human β_3 receptor. The results are shown in Table 1 and indicate that all of these fluorinated aryl-substituted thiazoles behave as full agonists of the human β_3 AR (82−100% activation relative to the full agonist isoproterenol) and were much less efficacious at the human β_1 and β_2 receptors. Several of the more potent analogues (**2a**,**e**,**h**) were also evaluated for β_3 binding affinity (β_3 IC$_{50}$) and were titrated in the β_1 and β_2 functional assays for direct comparison with β_3 functional efficacies (β_3 EC$_{50}$).

Four of the most potent aryl-substituted thiazoles **2** were evaluated in vivo for their ability to evoke hyperglycerolemia in beagle dogs. Administration (10 mg/kg po) of **2a**,**d**,**e**,**h** to fasted dogs produced elevated glycerol levels, and drug levels and pharmacokinetic properties of these analogues were determined (Table 2). While **2a**,**d**,**e** exhibited comparable oral bioavailability in dogs, that of trifluoromethoxy-substituted analogue **2h** was signficantly higher. More importantly, both **2e**,**h** displayed substantially longer half-lives than the di- and trifluorophenyl analogues **2a**,**d**.

The extended duration of action observed for the 4-substituted arylthiazole analogues **2e**,**h** led us to further examine the in vivo efficacy of these compounds in other animal models. The oral bioavailabilities of **2e**,**h** in rats (dosing 10 mg/kg po, 3 mg/kg iv) were determined to be 17% and 7%, respectively. Both compounds displayed a plasma half-life of greater than 8 h in rats. When administered to anesthetized rhesus monkeys as a series of sequential rising dose intravenous infusions, **2e**,**h** produced dose-dependent increases in serum glycerol accompanied by tachycardia, with ED$_{50}$ values for hyperglycerolemia of 0.26 and 0.14 mg/kg, respectively. The maximum extent of hyperglycerolemia elicited by both compounds was equivalent to that produced by the infusion of a maximally effective dose (100 μg/kg) of

Table 1. Comparison of β_3 AR Agonist Activity and β_1 and β_2 Binding Affinity of Thiazole Sulfonamides **2**

		β_3		β_1		β_2	
compd	Ar	EC$_{50}$, nM (% act)[a]	binding IC$_{50}$, nM[b]	EC$_{50}$, nM (% act)[a]	binding IC$_{50}$, nM[b]	EC$_{50}$, nM (% act)[a]	binding IC$_{50}$, nM[b]
2a	3,4-F$_2$C$_6$H$_3$	7.1 ± 4.7 (92)	20 ± 8	2600 (20)[c]	1700 ± 580	1800 (12)[c]	1700 ± 1200
2b	2,3-F$_2$C$_6$H$_3$	7.6 ± 3.3 (99)	nd	(6)[d]	2600 ± 540	(13)[d]	760 ± 100
2c	2,4-F$_2$C$_6$H$_3$	19 ± 12 (87)	nd	(7)[d]	2500 ± 1600	(3)[d]	2600 ± 470
2d	3,4,5-F$_3$C$_6$H$_2$	6.3 ± 4.4 (100)	nd	(20)[d]	2000 ± 1400	(12)[d]	5000 ± 4200
2e	4-F$_3$C-C$_6$H$_4$	3.6 ± 2.2 (94)	46 ± 29	4800 ± 2600 (24)	2300 ± 1000	2400 ± 950 (25)	2300 ± 700
2f	3-F$_3$C-C$_6$H$_4$	6.9 ± 3.7 (100)	nd	(17)[d]	1800 ± 410	(22)[d]	980 ± 320
2g	2-F$_3$C-C$_6$H$_4$	53 ± 42 (82)	nd	nd	1900 ± 110	nd	1800 ± 480
2h	4-F$_3$CO-C$_6$H$_4$	4.3 ± 1.6 (93)	30 ± 20	2700 ± 1500 (24)	8400 ± 4900	1400 ± 140(18)	1200 ± 1000

[a] Adenylyl cyclase activation given as % of the maximum stimulation with isoproterenol. Results are given as the mean ± SD from dose−response curves of at least two experiments ($n \geq 2$), unless otherwise indicated. [b] Receptor binding assays were carried out with membranes prepared from CHO cells expressing the cloned human receptor in the presence of [^{125}I]iodocyanopindolol. Results are given as the mean ± SD from at least two experiments ($n \geq 2$), unless otherwise indicated. nd = not determined. [c] $n = 1$. [d] Single point data, % activation at 10 μM.

Table 2. Pharmacokinetic Properties of Compounds **2a**,**d**,**e**,**h** in Dogs

comd	avg AUC$_{iv}$[a] (μg·min/mL)	avg AUC$_{po}$[b] (μg·min/mL)	Cl$_p$ (mL/min/kg)	V_{dss} (L/kg)	$t_{1/2}$ (h)	F (%)
2a	103 ± 1	124 ± 21	29.3 ± 0.3	19.0 ± 1.8	7.5 ± 0.6	36 ± 6.2
2d	148 ± 5	134 ± 76	20.5 ± 0.5	14.1 ± 0.4	8.0 ± 0.4	27 ± 14
2e	116 ± 20	136 ± 40	26.8 ± 4.6	31.3 ± 5.9	13.5 ± 0.3	38 ± 3.0
2h	212 ± 23	377 ± 57	14.3 ± 5.2	12.8 ± 2.0	10.3 ± 0.5	53 ± 2.3

[a] Dogs ($n = 2$) were dosed intravenously at 3 mg/kg test compound dissolved in vehicle (PEG400/EtOH/0.9% saline, 60/20/20 v/v/v); plasma drug levels were determined by LC/MS/MS. [b] Dogs ($n = 2$) were dosed orally at 10 mg/kg test compound dissolved in 0.5% methylcellulose/0.02% sodium lauryl sulfate containing 2 equiv of HCl; plasma drug levels were determined by LC/MS/MS.

isoproterenol, indicating that both **2e**,**h** act as full agonists for hyperglycerolemia in the rhesus monkey. Furthermore, the doses of **2e**,**h** which produced a 15% elevation in heart rate (ED_{15HR}) under the same experimental conditions were determined to be 10 and 2.5 mg/kg, respectively. Trifluoromethyl analogue **2e** thus exhibits a significantly better therapeutic index for hyperglycerolemia over cardiovascular effects (ED_{50gly}/ED_{15HR}) than the trifluoromethoxy analogue **2h**. In addition, compound **2e** is >100-fold selective for β_3 AR agonist activity when tested against a panel of receptors and ion channels, except for the human dopamine D2 and D3 receptors, where the selectivity is 61- and 26-fold, respectively. Since the D2 and D3 receptors are located within the CNS, and **2e** displays poor brain penetration, this level of cross-reactivity is not likely to be biologically significant.

Conclusions

The introduction of a range of fluorinated aryl substituents onto the thiazole ring of the 3-pyridylethanolamine benzenesulfonamide **2** results in a number of exceptionally potent and selective β_3 agonists, several of which exhibit improved pharmacokinetic profiles. The 4-(trifluoromethyl)phenyl analogue **2e**, in particular, is a potent (EC_{50} 3.6 nM) full agonist which displays good oral bioavailability in both dogs (%F = 38) and rats (%F = 17), as well as an exceptionally long half-life ($t_{1/2}$ > 8h) in all species tested. On the basis of these data and favorable safety assessment, compound **2e** was selected for phase I clinical studies, the results of which will be reported elsewhere.

Experimental Section

Chemistry. General Methods. Proton magnetic resonance spectra were recorded on a Varian XL 400 spectrometer. Low-resolution mass spectral analyses were obtained with a LKB 9000 mass spectrometer at an ionizing voltage of 70 eV. All reagents, solvents and drying agents were obtained from commercial sources and used without further purification unless otherwise noted. Precoated plates (silica gel F254, 250 mM; Analtech, Inc., Newark, DE) were used for TLC. Flash chromatography was carried out utilizing silica gel 60 (E. Merck). Elemental analyses were performed by Robertson Microlit Laboratories, Inc., Madison, NJ.

(*R*)-*N*-[4-[2-[*N*-(1,1-Dimethylethoxycarbonyl)-*N*-[2-hydroxy-2-(3-pyridinyl)ethyl]amino]ethyl]phenyl]-4-(aminothiocarbonyl)benzenesulfonamide (4). To a solution of 6.5 g (18.2 mmol) of (*R*)-*N*-[2-(4-aminophenyl)ethyl]-2-hydroxy-2-(3-pyridyl)ethylcarbamic acid 1,1-dimethylethyl ester (**3**) (Fisher et. al. U.S. Patent 5,561,142, Oct 1, 1996) in 70 mL of methylene chloride were added 2.1 mL of pyridine and 5.0 g of 4-cyanobenzenesulfonyl chloride. The reaction mixture was stirred at room temperature overnight. TLC (3:1 methylene chloride:acetone) on silica gel indicated the formation of a major fast moving (R_f 0.48) spot. Purification by flash chromatography (silica gel, 3:1 methylene chloride:acetone) gave 8.7 g of (*R*)-*N*-[4-[2-[*N*-(1,1-dimethylethoxycarbonyl)-*N*-[2-hydroxy-2-(3-pyridyl)ethyl]amino]ethyl]phenyl]-4-cyanobenzenesulfonamide as a white solid: ^{1}H NMR ($CDCl_3$) δ 8.53−8.44 (m, 2H), 7.78 (d, J = 8.7 Hz, 2H), 7.72−7.68 (m, 1H), 7.67 (d, J = 8.7 Hz, 2H), 7.3−7.23 (m, 1H), 7.1−6.9 (m, 4H), 4.8 (m, 1H), 3.5−2.6 (m, 6H), 1.42 (s, 9H).

A steady stream of hydrogen sulfide was bubbled into a solution of 10.2 g of the product from the previous step and triethylamine (2.9 mL) in 100 mL of pyridine at 25 °C for 15 min. The green solution was stirred for 2.5 h, and then nitrogen was bubbled through the solution for 30 min. The reaction mixture was concentrated under reduced pressure, and the residue was purified by flash chromatography on silica gel (8% methanol in dichloromethane eluant), affording 9.31 g of **4** as a bright yellow foam: ^{1}H NMR (CD_3OD) δ 8.45 (m, 2H), 7.87 (d, J = 8.5 Hz, 2H), 7.80 (m, 1H), 7.70 (m, 2H), 7.01 (overlapping s, 4H and m, 1H), 4.84 (m, 1H), 3.15−3.45 (m, 4H), 2.7 (m, 2H), 1.30 (s, 9H).

General Procedure for Preparation of Thiazole Benzenesulfonamides (2): (*R*)-*N*-[4-[2-[[2-Hydroxy-2-(3-pyridinyl)ethyl]amino]ethyl]phenyl]-4-[4-(3,4-difluorophenyl)thiazol-2-yl]benzenesulfonamide (2a). A mixture of 62 mg (0.111 mmol) of **4** and 24 mg (0.126 mmol) of 2-chloro-3′,4′-difluoroacetophenone in absolute ethanol (1 mL) was warmed at reflux for 4 h. The cooled reaction mixture was concentrated under reduced pressure, and the residue was dissolved in 1 mL of dichloromethane and 1 mL of trifluoroacetic acid (TFA). After stirring for 1 h at ambient temperature, the solution was concentrated under reduced pressure. Residual TFA was removed by azeotropic distillation with dichloromethane, and the residue was purified by flash chromatography (9:1 dichloromethane/10% NH_4OH in methanol eluant), affording **2a** (48 mg) as a light yellow foam: ^{1}H NMR (CD_3OD) δ 8.49 (d, J = 2.1 Hz, 1H), 8.40 (dd, J = 5.0 and 1.5 Hz, 1H), 8.09 (d, J = 8.6 Hz, 2H), 7.94 (s, 1H), 7.91 (m, 1H), 7.75−7.85 (overlapping d, J = 8.6 Hz, 2H and m, 2H), 7.25−7.38 (m, 2H), 7.09 (d, J = 8.6 Hz, 2H), 7.04 (d, J = 8.6 Hz, 2H), 4.80 (dd, J = 7.3 and 5.7 Hz, 1H), 2.90−2.70 (m, 6H); EIMS m/z 593 (M^+ + H). Anal. ($C_{30}H_{26}F_2N_4O_3S_2$) C, H, N.

(*R*)-*N*-[4-[2-[[2-Hydroxy-2-(3-pyridinyl)ethyl]amino]ethyl]phenyl]-4-[4-(2,3-difluorophenyl)thiazol-2-yl]benzenesulfonamide (2b). Prepared by general procedure outlined for the preparation of **2a**: ^{1}H NMR (CD_3OD) δ 8.49 (d, J = 1.9 Hz, 1H), 8.40 (dd, J = 4.9 and 1.6 Hz, 1H), 8.12 (d, J = 8.4 Hz, 2H), 8.03 (m, 1H), 8.01 (d, J = 2.2 Hz, 1H), 7.82 (d, J = 8.4 Hz, 2H), 7.78 (m, 1H), 7.36 (dd, J = 7.9 and 5.0 Hz, 1H), 7.25 (m, 2H), 7.09 (d, J = 8.5 Hz, 2H), 7.04 (d, J = 8.5 Hz, 2H), 4.78 (m, 1H), 2.90−2.70 (m, 6H); EIMS m/z 593 (M^+ + H). Anal. ($C_{30}H_{26}F_2N_4O_3S_2$) C, H, N.

(*R*)-*N*-[4-[2-[[2-Hydroxy-2-(3-pyridinyl)ethyl]amino]ethyl]phenyl]-4-[4-(2,4-difluorophenyl)thiazol-2-yl]benzenesulfonamide (2c). Prepared by general procedure outlined for the preparation of **2a**: ^{1}H NMR (CD_3OD) δ 8.48 (d, J = 2.2 Hz, 1H), 8.39 (dd, J = 4.9 and 1.6 Hz, 1H), 8.27 (m, 1H), 8.10 (d, J = 8.6 Hz, 2H), 7.86 (d, J = 2.4 Hz, 1H), 7.81 (d, J = 8.6 Hz, 2H), 7.77 (m, 1H), 7.34 (dd, J = 7.9 and 5.0 Hz, 1H), 7.05 (m, 6H), 4.77 (m, 1H), 2.90−2.70 (m, 6H); EIMS m/z 593 (M^+ + H). Anal. ($C_{30}H_{26}F_2N_4O_3S_2$) C, H, N.

(*R*)-*N*-[4-[2-[[2-Hydroxy-2-(3-pyridinyl)ethyl]amino]ethyl]phenyl]-4-[4-(3,4,5-trifluorophenyl)thiazol-2-yl]benzenesulfonamide (2d). Prepared by general procedure outlined for the preparation of **2a**: ^{1}H NMR (CD_3OD) δ 8.50 (d, J = 2.0 Hz, 1H), 8.42 (dd, J = 4.9 and 1.6 Hz, 1H), 8.07 (d, J = 8.6 Hz, 2H), 8.01 (s, 1H), 7.81 (d, J = 8.6 Hz, 2H), 7.76 (m, 3H), 7.37 (dd, J = 7.8 and 4.7 Hz, 1H), 7.11 (d, J = 8.6 Hz, 2H), 7.06 (d, J = 8.6 Hz, 2H), 4.80 (m, 1H), 2.90−2.70 (m, 6H); EIMS m/z 611 (M^+ + H). Anal. ($C_{30}H_{25}F_3N_4O_3S_2$) C, H, N.

(*R*)-*N*-[4-[2-[[2-Hydroxy-2-(3-pyridinyl)ethyl]amino]ethyl]phenyl]-4-[4-[4-(trifluoromethyl)phenyl]thiazol-2-yl]benzenesulfonamide (2e). Prepared by general procedure outlined for the preparation of **2a**: ^{1}H NMR (CD_3OD) δ 8.49 (d, J = 2.1 Hz, 1H), 8.40 (dd, J = 5.0 and 1.5 Hz, 1H), 8.18 (d, J = 7.9 Hz, 2H), 8.12 (d, J = 8.6 Hz, 2H), 8.09 (s, 1H), 7.82 (d, J = 8.6 Hz, 2H), 7.77 (m, 1H), 7.71 (d, J = 8.6 Hz, 2H), 7.35 (dd, J = 7.9 and 5.0 Hz, 1H), 7.09 (d, J = 8.6 Hz, 2H), 7.04 (d, J = 8.6 Hz, 2H),), 4.80 (dd, J = 7.3 and 5.7 Hz, 1H), 2.90−2.70 (m, 6H); EIMS m/z 625 (M^+ + H). Anal. ($C_{31}H_{27}F_3N_4O_3S_2$) C, H, N.

(*R*)-*N*-[4-[2-[[2-Hydroxy-2-(3-pyridinyl)ethyl]amino]ethyl]phenyl]-4-[4-[3-(trifluoromethyl)phenyl]thiazol-2-yl]benzenesulfonamide (2f). Prepared by general procedure outlined for the preparation of **2a**: ^{1}H NMR (CD_3OD) δ 8.49 (d, J = 2.3 Hz, 1H), 8.39 (dd, J = 4.9 and 1.6 Hz, 1H), 8.31 (s, 1H), 8.25 (d, J = 6.5 Hz, 1H), 8.13 (d, J = 8.5 Hz, 2H), 8.09 (s, 1H), 7.83 (d, J = 8.5 Hz, 2H), 7.77 (m, 1H), 7.62 (m, 2H), 7.36 (dd, J = 7.9 and 5.0 Hz, 1H), 7.09 (d, J = 8.6 Hz, 2H), 7.05 (d,

J = 8.6 Hz, 2H), 4.80 (m, 1H), 2.90−2.70 (m, 6H); EIMS m/z 625 (M^+ + H); ESI HRMS m/z 625.1558 (M + H) ($C_{31}H_{27}F_3N_4O_3S_2$ requires 625.1549). Anal. ($C_{31}H_{27}F_3N_4O_3S_2$) H, N; C: calcd, 59.60; found, 59.00.

(*R*)-*N*-[4-[2-[[2-Hydroxy-2-(3-pyridinyl)ethyl]amino]ethyl]phenyl]-4-[4-[2-(trifluoromethyl)phenyl]thiazol-2-yl]-benzenesulfonamide (2g). Prepared by general procedure outlined for the preparation of **2a**: ^{1}H NMR (CD_3OD) δ 8.55 (d, J = 2.1 Hz, 1H), 8.46 (dd, J = 4.9 and 1.6 Hz, 1H), 8.08 (d, J = 8.6 Hz, 2H), 7.84 (m, 2H), 7.82 (d, J = 8.6 Hz, 2H), 7.69 (m, 3H), 7.61 (m, 1H), 7.41 (m, 1H), 7.15 (d, J = 8.5 Hz, 2H), 7.08 (d, J = 8.5 Hz, 2H), 4.85 (m, 1H), 3.10−2.80 (m, 6H); EIMS m/z 625 (M^+ + H); ESI HRMS m/z 625.1565 (M + H) ($C_{31}H_{27}F_3N_4O_3S_2$ requires 625.1549).

(*R*)-*N*-[4-[2-[[2-Hydroxy-2-(3-pyridinyl)ethyl]amino]ethyl]phenyl]-4-[4-[4-(trifluoromethoxy)phenyl]thiazol-2-yl]-benzenesulfonamide (2h). Prepared by general procedure outlined for the preparation of **2a**: ^{1}H NMR (CD_3OD) δ 8.52 (d, J = 2.0 Hz, 1H), 8.43 (dd, J = 4.9 and 1.6 Hz, 1H), 8.12 (d, J = 8.5 Hz, 2H), 8.10 (d, J = 8.6 Hz, 2H), 7.98 (s, 1H), 7.83 (overlapping d, J = 8.5 Hz, 2H and m, 1H), 7.38 (dd, J = 7.9 and 5.0 Hz, 1H), 7.33 (d, J = 8.6 Hz, 2H), 7.12 (d, J = 8.5 Hz, 2H), 7.07 (d, J = 8.5 Hz, 2H), 4.85 (dd, J = 8.8 and 4.2 Hz, 1H), 2.7−3.1 (m, 6H); EIMS m/z 641 (M^+ + H). Anal. ($C_{31}H_{27}F_3N_4O_4S_2$) C, H, N.

Biological Methods. 1. In Vitro Functional Assays. The human β_3 receptor was obtained from Dr. J. Grannemann (Wayne State University), and other receptors were cloned as previously described.[14,15] Human β_1, β_2 and β_3 receptors were expressed in mammalian cell lines for the primary screening assays. CHO cells, stably transfected with the cloned β-adrenergic receptors, were harvested in enzyme-free dissociation media (Specialty Media, Lavallette, NJ) 3 days after plating. Cells were counted and distributed in the assay tubes, after being resuspended in ACC buffer (75 mM Tris, pH 7.4, 250 mM sucrose, 12.5 mM $MgCl_2$, 1.5 mM EDTA), containing the antioxidant sodium metabisulfite at a concentration of 0.2 mM and a phosphodiesterase inhibitor (0.6 mM IBMX). The cAMP production reaction was initiated by mixing cells with 20 μL of a 6X stock of the ligand to be tested. Tubes were shaken at 275 rpm for 45 min at room temperature, and the reaction was stopped by boiling the tubes for 3 min. The cAMP produced in response to the ligand was measured in the lysate by competing against [^{125}I]cAMP for binding to a cAMP-directed antibody using an automated RIA machine (ATTOFLO, Atto Instruments, Baltimore, MD). The cAMP level was determined by comparison to a standard curve.

2. Binding Assays. CHO cells expressing the cloned human and rhesus β receptors were grown in selective media for 3 days and membranes prepared by hypotonic lysis in 1 mM Tris, pH 7.2. Receptor binding assays were carried out in a final volume of 250 μL containing 5−10 μg of membrane protein, the radioligand [^{125}I]cyanopindolol at a concentration of 45 pM, and the compound of interest at various concentrations. Binding reactions were carried out for 1 h at 23 °C and terminated by filtration over GF/C filters using a 96-well cell harvester from Inotech (Lansing, MI).

3. In Vivo Efficacy and Oral Bioavailability in Beagle Dogs. Test compounds for oral gavage were prepared as 10 mg/mL solutions in a vehicle consisting of methylcellulose (0.5%) and sodium lauryl sulfate (0.2%) containing 2 equiv of 2 N HCl. The test compound was dosed by oral gavage to animals in a dose volume of 1 mL/kg thus providing a dose of 10 mg/kg. Blood samples were collected prior to dosing and at 15 and 30 min and 1, 2, 6, 8, and 24 h postdosing, for determination of plasma glycerol concentrations and, in some cases, plasma drug concentrations. Glycerol levels were determined on samples of cell-free plasma prepared by centrifugation of the blood sample. Glycerol was assayed using a Sigma triglyceride (GPO-TRINDER) assay kit. Plasma drug concentrations were determined by adding sodium carbonate (0.2 mL of 0.1 M, pH = 10) and ethyl acetate (4 mL) to 100 μL of plasma sample. The mixture was shaken and then centrifuged. The ethyl acetate layer was transferred to 13- × 100-mm borosilicate tubes and then evaporated to dryness. The residue was reconstituted in 100 μL of 50% acetonitrile/water, vortexed, and then sonicated in an ultrasonic agitator for 10 min. The mixture was transferred to autosampler vials with low-volume micro inserts. Sample analysis was performed by APCI-LC/MS/MS in the positive ion mode using a PhaseSep SCN (4.6- × 150-mm) column. A mobile phase containing 60% acetonitrile/water, 20 mM ammonium acetate and 0.4% trifluoroacetic acid was used at a flow rate of 1.0 mL/min at ambient temperature. Data reduction was performed using Sciex MacQuan software. Area under the curve (AUC) calculations were performed by the trapezoid method using Kaleidagraph.

Test compounds for intravenous administration were prepared as 10 mg/mL solutions in a vehicle consisting of poly(ethylene glycol) 400 (PEG400), ethanol and normal saline (60/20/20 v/v/v). The test compound was dosed to animals via an accessible peripheral vein in a dose volume of 0.3 mL/kg thus providing a dose of 3 mg/kg. Blood samples were collected prior to dosing and at 5, 15, and 30 min and 1, 2, 4, 6, 8, and 24 h postdosing, for determination of plasma glycerol concentrations and plasma drug concentrations. Oral bioavailability (F) was calculated according to the following equation: F (%) = ($AUC_{oral} \times dose_{iv}$) ÷ ($AUC_{iv} \times dose_{oral}$).

4. Measurement of Lipolysis and Heart Rate in Rhesus Monkeys. Due to the small signal-to-noise ratio in conscious animals, this assay was run in anesthetized rhesus monkeys, which effectively precludes oral dosing. Male lean rhesus monkeys (4−7 kg body weight, age 3−5 years; n = 2−4/group) were fasted for 24 h and were lightly anesthetized with ketamine (Fort Dodge Labs, Fort Dodge, IA; 10 mg/kg, im). A 22-gauge intraveneous catheter (Becton Dickinson & Co., Sandy, UT) was placed in a saphenous vein for the administration of test compounds after which the animals were administered Nembutal (Abbott Labs, North Chicago, IL; 25 mg/kg, iv). A 20-gauge angiocatheter, connected to a TNF-R pressure transducer (Ohmeda Medical Device Systems, Madison, WI), was placed in a femoral artery for monitoring blood pressure. ECG leads were connected for the continuous measurement of heart rate. Heart rate and blood pressure were monitored for approximately 30 min until stable baseline values were obtained, at which time animals were administered a series of rising dose infusions (0.1 mL/min) of test compound or an equivalent volume of vehicle over a 15-min period. Infusion periods were separated by an interval of approximately 20 s. Blood samples (2 mL) were collected from the femoral artery 1 min prior to the initiation of infusions and 14 min into each infusion period. Serum glycerol was measured using an enzymatic colorimetric assay and serum potassium was determined using an ion-specific electrode. Under these conditions, for isoproterenol the ED_{50} for hyperglycerolemia is 3 μg/kg and the ED_{50} for tachycardia is 0.2 μg/kg.[9]

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