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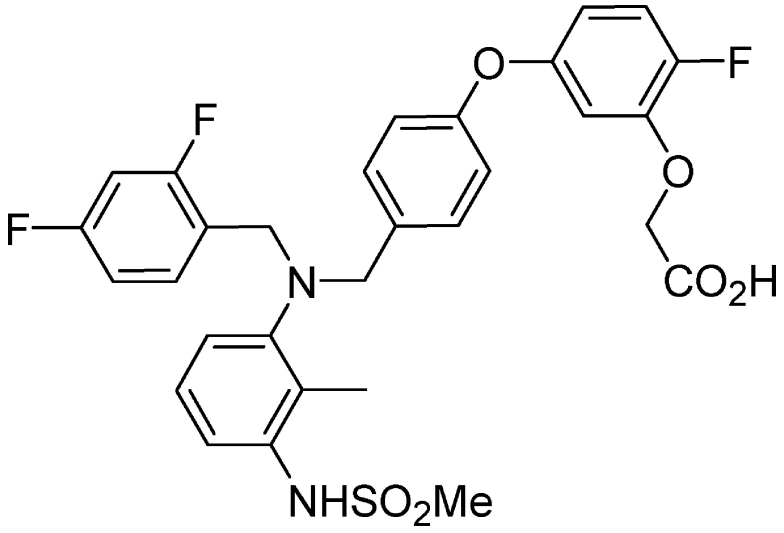

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Antidiabetic Activity of Passive Nonsteroidal Glucocorticoid Receptor Modulators

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Much has been learned about the consequences of glucocorticoid receptor antagonism by studying steroidal active antagonists such as RU-38486 (**1**). In the liver glucocorticoid receptor antagonism suppresses hepatic glucose production decreasing plasma glucose levels; however, extrahepatic antagonism produces several undesirable side effects including activation of the hypothalamic pituitary adrenal axis. A series of nonsteroidal passive *N*-(3-dibenzylamino-2-alkyl-phenyl)-methanesulfonamide glucocorticoid receptor modulators was discovered. Liver selective and systemically available members of this series were found and characterized in diabetes and side effect rodent models. A highly liver selective member of this series, acid **14**, shows efficacy in the *ob/ob* model of diabetes. It lowers plasma glucose, cholesterol, and free fatty acid concentrations and reduces the rate of body weight gain. The structurally related systemically available passive modulator **12** lowers glucose, HbA_{1c}, triglyceride, free fatty acid, and cholesterol levels. Interestingly, it did not acutely activate the hypothalamic pituitary adrenal axis in unstressed CD-1 mice or have the abortive effects observed with **1**. These results indicate that passive GR antagonists may have utility as antidiabetic agents.

Glucocorticoids are steroid hormones that mediate immune function, glucose homeostasis, fat distribution, normal growth and development, stress responses, and a multitude of other processes.[1] They exert their action via the glucocorticoid receptor (GR), which is a popular target for pharmacologic intervention.[2] Agonists, antagonists, and modulators are studied as potential therapies for multiple diseases. GR agonists have been explored primarily as antiinflammatory agents and immunosuppressants while antagonists have historically received less attention. The potent steroidal GR antagonist **1** is known for its abortifacient effects due to potent progesterone receptor (PR) antagonism (Figure 1).[3] Treatment of humans, or rodent models of diabetes, with the antagonist **1** lowers hepatic glucose production (HGP) by reducing the expression of the key gluconeogenic enzymes (PEPCK) and glucose-6-phosphatase (G6Pase).[4,5] Extrahepatic GR antagonism, however, has both desirable and undesirable consequences. Potential benefits include increased insulin sensitivity, fat redistribution, and effects on bone while detriments include activation of the hypothalamic pituitary adrenal (HPA) axis, diminution of immune response, and a decreased stress response. Recently, mice with a selective inactivation of the GR gene in hepatocytes have been created to determine the effect of hepatospecific GR antagonism.[6a] Also, improvements in hyperglycemia and hyperlipidemia after treatment with antisense oligonucleotides that cause selective reduction of the GR in liver and white adipose tissue of diabetic rodents have been reported.[6b] We have also explored a number of strategies to develop a treatment for type II diabetes by hepatoselective GR antagonism. One is the liver targeting of steroidal GR antagonists related to **1**.[7] In particular, a steroidal bile acid conjugate was discovered that gave a desirable profile in several pharmacology

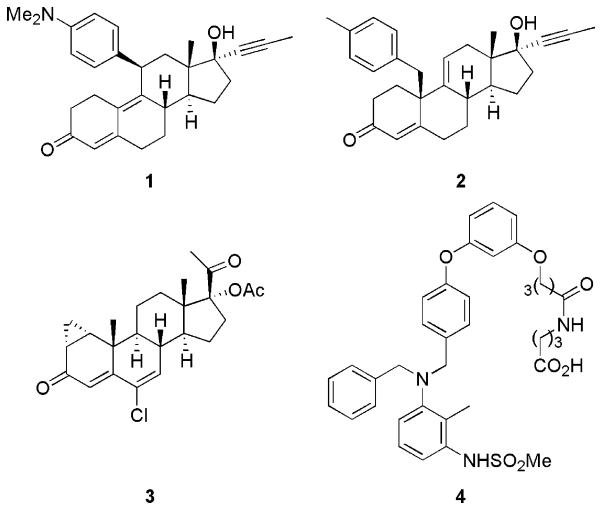

Figure 1. The GR ligands **1**, **2**, cyproterone acetate (**3**), and sulfonamide **4**.

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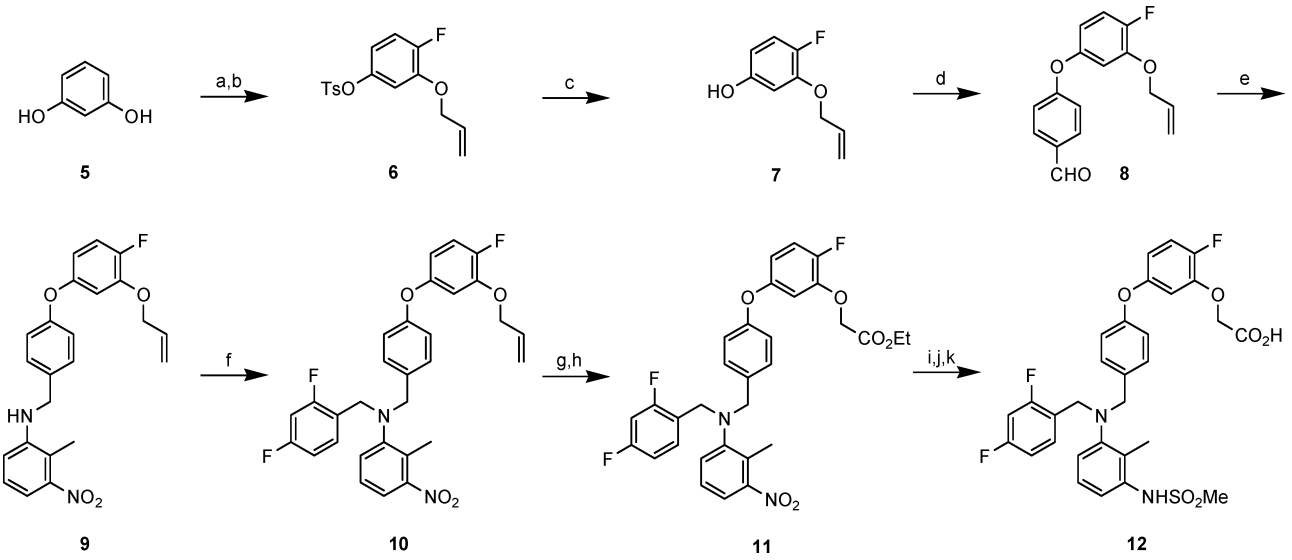
Scheme 1. GR Sulfonamide Modulator Synthesis[a]



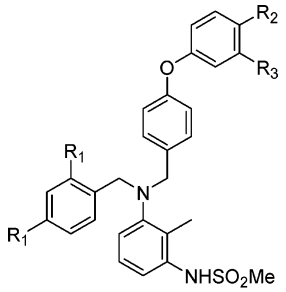
[a] (a) SELECTFLUOR, CH_3CN, reflux, 12 h; (b) TosCl, K_2CO_3, acetone, 60 °C, 12 h; allyl iodide, 60 °C, 24 h, 24% (2 steps); (c) KOH, EtOH, H_2O, 90 °C, 2 h, 68%; (d) 4-fluorobenzaldehyde, K_2CO_3, DMF, 100 °C, 14 h, 73%; (e) 2-methyl-3-nitroaniline, AcOH, DCE, rt, 4 h, then $Na(OAc)_3BH$, rt, 12 h, 88%; (f) 2,4-difluorobenzyl bromide, *i*-Pr_2NEt, DMF, 100 °C, 18 h, 100%; (g) $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2, rt, 2 h, 92%; (h) ethyl glycolate, PPh_3, di-*tert*-butyl azodicarboxylate, THF, rt, 12 h, 72%; (i) Fe, NH_4Cl, EtOH, H_2O, 80 °C, 1 h; (j) MsCl, pyridine, rt, 12 h; 100% (2 steps); (k) NaOH, H_2O, THF, EtOH, rt, 12 h, 97%.

models.[7d,e] We have also reported the development of several new classes of nonsteroidal modulators.[8] A groundbreaking novel class of modulators has also been reported for the treatment of obesity.[9] The results obtained from the in vivo evaluation of key members of a series of *N*-(3-dibenzylamino-2-alkyl-phenyl)-methanesulfonamides[8a−e] are summarized herein.

The GR receptor is a cytoplasmic nuclear hormone receptor that is normally complexed to heat shock proteins.[10] Upon ligand binding, the GR−ligand complex dissociates from these chaperones, dimerizes, and translocates to the nucleus. There it interacts with DNA sequences (termed glucocorticoid response elements [GREs]) and transcription factors to initiate, or repress, gene transcription.[11] This complex signaling pathway allows for the possibility that ligands might be differentiated on the basis of binding potency, facility/extent of dimerization, translocation, and DNA interaction of the modulator−GR complex. Ligands that compete for agonist GR binding and agonist receptor complex DNA response element binding are called "active" antagonists, while ligands that only compete for agonist GR binding have been termed "passive" antagonists.[12] Steroid **1** is an example of an active antagonist. A number of passive antagonists have been reported, including steroid (**2**)[13] and cyproterone acetate (**3**).[14] Our efforts to convert a series of GR selective nonsteroidal agonists into GR selective antagonists resulted in the discovery of subnanomolar passive modulators such as **4**.[8b,c] We sought to discover GR selective systemically available and liver selective passive GR modulators within this series to determine the potential of these types of compounds as antidiabetic agents.

Chemistry. A typical modulator synthesis is detailed in Scheme 1. Resorcinol **5** is fluorinated utilizing [1-(chloromethyl)-4-fluoro-1,4-diazoniabicyclo[2.2.2]octane-bis(tetrafluoroborate)] (SELECTFLUOR).[15] Regioselective tosylation and allylation then provide aryl tosylate **6**.[16] Basic hydrolysis removes the tosylate, and a nucleophilic aromatic substitution reaction of the resultant phenol with 4-fluorobenzaldehyde yields aldehyde **8**. Reductive amination with 2-methyl-3-nitroaniline provides aniline **9**.[17] Alkylation with 2,4-difluorobenzoldehyde yields allyl ether **10**. Palladium mediated removal of the allyl group[18] followed by Mitsunobu reaction[19] with ethyl glycolate provides nitroarene **11**. Iron mediated reduction of the nitro group, aniline mesylation, and ester hydrolysis then yields the acid **12**.

Table 1. GR Sulfonamide Modulators **12**−**15**



12-15

compd	R_1	R_2	R_3
12	F	F	OCH_2CO_2H
13	F	Br	OCH_2CO_2H
14	H	OCH_2CO_2H	H
15	F	Cl	OCH_2CO_2H

In Vitro Assays. Nuclear hormone receptor binding activity is assessed using radioligand binding assays. In the case of the GR, the radioligand is the potent agonist [^{3}H]-dexamethasone. Receptor binding selectivities against progesterone (PR), androgen (AR), estrogen (ER_α and ER_β), mineralocorticoid (MR), and thyroid hormone (TR_α and TR_β) are determined similarly. Functional activity is determined in three different assays. GRAF cells are a genetically engineered mammalian cell line. They express h-GR and a transcriptional unit composed of a GRE and core promoter

Table 2. Nuclear Hormone Assay Results for Active and Passive Antagonists **1**−**4** and **12**−**15**

	IC_{50} (nM)[a]					IC_{50} (nM)[a]			
compd	h-GR binding	GRAF	rat hepatocyte TAT (μM)[a]	VP16 EC_{50} (nM)[a]	VP16 agonism (%)	r-GR binding	h-PR binding	h-AR binding	h-MR binding
1	1.1	4.8	0.27	0.7	59	1.4	2.9	8.8	6000
2	2.4	43	7.3	7	51	2.4	2400	6500	6300
4	0.6	86	>30	NE[b]	NE	1.0	550	1600	120
12	4.8	440	1.9	NE	NE	0.2	180	ND[c]	ND
13	1.8	330	4.3	NE	NE	ND	140	ND	ND
14	5.4	255	>30	NE	NE	2.1	1700	11700	690
15	2.6	500	2.3	NE	NE	1.9	150	3600	1000

[a] The data are the geometric mean of at least two experiments. [b] NE = no effect up to 10−100 μM. [c] ND = not determined.

sequences linked to a reporter gene. The reporter gene encodes a secreted form of alkaline phosphatase (ALP). Antagonists are evaluated by their inhibition of dexamethasone-induced ALP expression in these cells. Compounds are also tested in freshly isolated rat hepatocytes for their effects on dexamethasone induced expression of the GR regulated enzyme tyrosine aminotransferase (TAT). A third functional assay is used to assess the "active" or "passive" nature of a compound. VP16 is a transcriptional activation domain that, if present in the nucleus, activates GREs. Human liver hepatoma (HuH7) cells were transiently transfected with VP16-GR fusion protein expression plasmid and a reporter (GRE-Luc). In this assay "passive" antagonists show little, or no, response while "active" antagonists robustly stimulate luciferase expression.

Results and Discussion

These assays were used to profile the GR modulators **4**, **12**−**15** (Table 1), antagonist **1**, and steroid **2**. Published data for **3** indicates a GR K_d = 45 nM and a PR K_d = 15 nM.[14] The GR modulators **4** and **12**−**15** potently inhibit GR binding (0.6−5.4 nM) and show good to excellent selectivity over other nuclear hormone receptors (Table 2). The steroids **1** and **3** have similar GR binding potency but are less selective for PR in particular. Modulators **12**, **13**, and **15**, where $R_3 = OCH_2CO_2H$, are modestly PR selective (IC_{50} = 140−180 nM) while modulator **14**, in which $R_2 = OCH_2CO_2H$, is more selective. The steroid **2** is both potent and selective. Modulator **4**, **12**−**15**, and the steroid **2** are less potent in the GRAF and rat hepatocyte TAT assays than **1**. The VP16-GR assay indicates that the steroids **1** and **2** are "active" antagonists while all of the sulfonamide modulators are "passive".

Since long duration blockade of hepatic GR is sought, robust metabolic stability is required. The metabolic profile of **1** has been thoroughly studied.[20] It undergoes rapid N-demethylation and other transformations. However, these metabolites also inhibit parent metabolism. The primary metabolites also have potent GR activity. The steroidal GR ligand (**2**) also does not have robust metabolic stability. Modulators **12** and **14** have better metabolic stability as assessed by incubation with rat and human liver microsomes and hepatocytes (Table 3). Identification of metabolites from **12** revealed that the primary metabolic event was N-debenzylation, with removal of the smaller benzyl group being the major pathway.

The pharmacokinetic profile of acid **14** suggests a high degree of liver selectivity (Figure 2). Examination of the intravenous arm of a Sprague−Dawley rat 5 mpk PK experiment reveals that the compound has a short half-life (0.4 h), low plasma AUC, and high clearance (14.0 L/h·kg). After 5 h, plasma levels were undetectable. Liver levels at 1 h postdose were high (20 μg/g) and decreased after 7 h (3.8 μg/g) (Table 4). The corresponding oral dose had very low plasma levels and liver levels similar to those for the intravenous route of administration. Throughout the pharmacokinetic study the com-

Table 3. Metabolic Stability for Steroids **1** and **2** and Sulfonamide Modulators **12** and **14**

	$t_{1/2}$ (h)			
compd	RLM stability[a,b]	rat hepatocyte stability[c]	HLM stability[b,d]	human hepatocyte stability[c]
1	<0.33	<0.1	ND[e]	ND
2	0.33	<0.2	ND	ND
12	4.1	6.5	2.3	9.1
14	4.6	9.9	8.9	31.1

[a] RLM = rat liver microsomes. [b] 0.25 mg/mL of protein and 10 μM compound. [c] 5 x 10^5 cells/mL and 10 μM compound. [d] HLM = human liver microsomes. [e] ND = not determined.

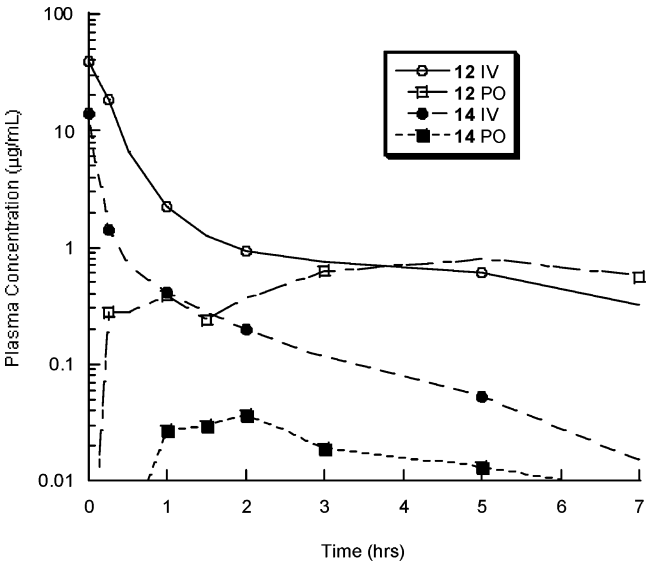


Figure 2. Sprague−Dawley rat pharmacokinetics at 5 mpk of GR modulators **12** and **14**. 3 animals per time point.

Table 4. Liver and Plasma Levels for Modulators **12** and **14** (5 mpk Dose in Sprague−Dawley Rats)[a]

compd	1 h liver level (μg/g)	1 h plasma level (μg/mL)	7 h liver level (μg/g)	7 h plasma level (μg/mL)
12 (iv)	74	1.6	40	0.65
14 (oral)	20	0.03	3.8	0.01

[a] 3 animals per time point for **12** and **14**.

Table 5. Drug Level Data from 2 Week *ob/ob* Studies for Modulators **12** and **14**

compd	dose (mpk)	liver level (μg/g)[a]	plasma level (μg/mL)[a]
12	30	20	0.82
12	100	157	6
14	10	ND[b]	0
14	100	207	1.6

[a] 2 h after the final study dose. [b] ND = not determined.

pound maintains a high liver to plasma distribution consistent with a hepatoselective profile. Although liver selective, modulator **12** also achieves high plasma levels following oral dosing at 5 mpk in Sprague−Dawley rats. Liver levels 7 h postdose were high (40 μg/g) as was the plasma AUC (3.85 μg·h/mL) from a 5 mpk oral dose. Oral bioavailability of **12** was moderate (F = 40%).

In Vivo Results. Fasted Sprague−Dawley rats are used to determine whether the modulators are able to block hepatic GR in vivo. An oral challenge with 10 mpk of the potent GR agonist prednisolone induces expression of hepatic tyrosine aminotransferase (TAT) and increases hepatic glycogen levels (as assessed in liver biopsy punches 6 h postdose). It also induces severe lymphopenia. Oral administration of **1** (100 mpk) 1 h prior to dosing completely blocks both the hepatic (TAT and glycogen) and peripheral (lymphopenic) responses. Similar administration of the passive antagonist **12** at 100 mpk does not antagonize either response. Interestingly, the passive antagonist cyproterone acetate (**3**) has been shown to inhibit transactivation of liver GR.[14] In the reported experiment, fasted male Sprague−Dawley rats were given compound (80 mpk), dexamethasone 10 min later (0.01 mg/kg), and 4 h later TAT activity was measured. It was active in this less stringent challenge model (dose of the agonist dexamethasone is significantly lower) but less active than **1** at one-half of the dose. No drug levels were reported from the study. In combination, these two experiments suggest that a passive antagonist can block hepatic GR. However, the blockade is not as strong as is observed with an active antagonist.

To determine if passive antagonists provided sufficient blockade of hepatic GR to be of potential utility as antidiabetic agents, acid modulators **12** and **14** were tested in a diabetic animal model. The *ob/ob* mouse model of diabetes was chosen since it is sensitive to GR antagonists and allows for comparison to agents with other mechanisms of action. The liver selective acid **14** was given to *ob/ob* mice orally b.i.d. for 13 days at 10 and 100 mpk. It dose dependently lowered postprandial blood glucose levels, with a 46% decrease observed at the high dose (Table 6). Body weight gain was also reduced at 100 mpk, as were circulating aspartate aminotransferase (AST)/alanine aminotransferase (ALT) enzyme levels, liver weights, cholesterol concentrations (89%), and free fatty acid (FFA) levels (39%). The decreases in AST and ALT levels are reassuring as the amount of both can increase in the presence of a hepatotoxic agent. Epididymal poststudy fat pad weights were unaltered by compound treatment. The end of study drug levels (2 h past final dose) also supported a highly liver selective profile with high liver and low plasma drug levels (Table 5). Systemically available modulator **12** also demonstrates dose dependent glucose lowering without affecting insulin levels in a 14 day study (Table 7). Decreases in liver glycogen levels and in liver TAT levels were observed. This is consistent with the proposed mechanism of action. A decrease in the rate of weight gain relative to control animals was seen. At the high dose, triglycerides and FFAs were lowered (although FFA levels were similar at both 30 and 100 mpk). End of study drug levels indicate hepatoselective distribution; however, plasma drug levels are also significant.

Due to the efficacy observed with modulator **12**, a longer 30 day study in *ob/ob* mice was conducted. At the end of the study glucose levels were lowered by 60−70% relative to vehicle leading to a 1.7% reduction in HbA_{1c} levels. In addition, triglyceride levels dropped 55%, cholesterol was lowered 33%, and free fatty acid levels decreased 25% relative to vehicle (see Table 9 for details). A reduction in ALT and AST was also observed, which suggests that **12** is not highly hepatotoxic. This is the first demonstration of antidiabetic activity in a rodent model of diabetes with a passive GR antagonist.

Table 6. 13 Day *ob/ob* Study for Vehicle, Steroid **1**, and Modulator **14** Treated *ob/ob* Mice[a]

animal	compd	po dose	glucose (mg/dL)	insulin (ng/mL)	body wt (g) initial	body wt (g) final	FFA (mequiv/L)	ALT (units/mL)	AST (units/mL)
ob/ob mice	vehicle	b.i.d.	377 ± 24	19.7 ± 2.2	45.8 ± 1.2	49.9 ± 1.3	1.3 ± 0.3	840 ± 74	474 ± 35
ob/ob mice	**14**	100 mpk b.i.d.	290 ± 19	28.1 ± 4.5	46.0 ± 0.9	46.6 ± 0.5	1.0 ± 0.3	282 ± 40	330 ± 54
ob/ob mice	**14**	10 mpk b.i.d.	333 ± 34	20.0 ± 2.6	45.4 ± 0.8	49.3 ± 0.7	1.3 ± 0.7	786 ± 200	420 ± 80
ob/ob mice	**1**	100 mpk b.i.d.	141 ± 11	7.5 ± 0.7	46.1 ± 1.3	48.2 ± 1.3	1.5 ± 0.2	1110 ± 80	310 ± 30
lean mice	vehicle	b.i.d.	190 ± 7	0.8 ± 0.1	26.3 ± 0.7	27.0 ± 0.8	0.52 ± 0.02	63 ± 11	132 ± 11

[a] 8−10 animals per time point with results reported with standard error.

Table 7. 14 Day *ob/ob* Data for Vehicle, Steroid **1**, and Modulator **12**[a]

animal	compd	po dose	glucose (mg/dL)	insulin (ng/mL)	body wt (g) initial	body wt (g) final	glycogen (mg/g of liver)	cholesterol (mg/dL)	liver TAT (nmol of pHB/min/mg of protein)	FFA (mequiv/L)
ob/ob mice	vehicle	b.i.d.	342 ± 21	38.2 ± 0.6.9	40.0 ± 0.9	49.5 ± 1.1	64.3 ± 3.2	223 ± 21	2.7 ± 0.3	1.26 ± 0.15
ob/ob mice	**12**	100 mpk b.i.d.	233 ± 15	32.8 ± 6.3	40.0 ± 0.7	46.9 ± 0.7	44.2 ± 4.0	134 ± 21	2.4 ± 0.3	1.01 ± 0.07
ob/ob mice	**12**	30 mpk b.i.d.	314 ± 23	25.0 ± 5.6	41.5 ± 0.5	49.2 ± 0.8	54.6 ± 1.7	225 ± 16	1.7 ± 0.3	0.99 ± 0.11
ob/ob mice	**1**	100 mpk b.i.d.	170 ± 6	14.0 ± 2.8	41.7 ± 0.5	51.2 ± 0.4	30.5 ± 1.8	366 ± 25	1.8 ± 0.2	0.84 ± 0.09
lean mice	vehicle	b.i.d.	170 ± 9	1.3 ± 0.3	23.0 ± 0.9	26.2 ± 1.2	74.2 ± 3.0	151 ± 6	2.7 ± 0.3	0.84 ± 0.09

[a] 8−10 animals per time point with results reported with standard error.

Table 8. Plasma Protein Binding of [^{3}H]-**12**[a] by Equilibrium Dialysis

species	concentration (μg/mL)	% bound
rat	2	99.9
mouse	2	99.7
human	2	99.9

[a] For the synthesis of [^{3}H]-**12** see the Experimental Section.

One of the primary side effect concerns associated with GR antagonism is activation of the HPA axis. To determine the potential of a passive GR modulator to impact the HPA axis, acid modulator **12** (which achieves significant plasma levels) was given to male CD-1 mice at 100 mpk. Two hours later, plasma levels of adrenocorticotropic hormone (ACTH) and corticosterone levels were analyzed. No change relative to vehicle was observed, indicating minimal acute impact upon the HPA axis under unstressed conditions. In contrast, the steroidal active antagonist **1** causes a dramatic increase in both plasma ACTH and corticosterone levels. Although this experiment does not demonstrate that modulator **12** has no effect on the HPA axis, it does show that passive GR modulators and antagonists can have significantly different profiles.

Steroid **1** is also an abortive agent whose effect is mediated through its PR activity. Modulator **12** was checked for in vivo effects on PR, for which it has greater in vitro selectivity relative to **1** (Table 2). At 30 and 100 mpk q.d. oral dosing for 5 days in pregnant rats, no effects on uterine weight or progesterone levels were observed. In the same experiment, **1** reduced both by 50% at 3 mpk.

Determination of the protein binding of one of the lipophilic acids using a radiolabeled variant (**12**, *c* log $P = 6.17$) indicates a low free drug fraction in rodent and human plasma (Table 8). This property may contribute to the lack of peripheral effects on the HPA axis or PR relative to **1**.

Conclusion. Starting from a nonsteroidal GR agonist a series of passive GR modulators was discovered.[8a−c] Analogues were found that are GR selective, metabolically stable, bioavailable, and hepatoselectively distributed. These passive modulators lower blood glucose levels in *ob/ob* mice. Furthermore, improvement in the lipid profile was observed. Surprisingly, even a compound such as **12** that achieves significant plasma exposure fails to acutely activate the HPA axis in unstressed CD-1 mice, whereas the active antagonist steroid **1** strongly affects it. Overall, these results suggest that passive GR antagonists may be of greater potential interest as antidiabetic agents than is currently suspected. It remains to be seen whether a GR ligand can become an antidiabetic therapy for modulating hepatic glucose production.[21]

Experimental Section

Compound Preparation. Unless otherwise specified, all solvents and reagents were obtained from commercial suppliers and used without further purification. All reactions were performed under nitrogen atmosphere unless specifically noted. Normal-phase flash chromatography was done using Merck silica gel 60 (230−400 mesh) from E.M. Science, or was performed on a hybrid system employing Gilson components and Biotage prepacked columns. Reversed-phase chromatography was performed using a Gilson 215 solvent handler-driven HPLC system (CH_3CN:0.1% TFA in H_2O or CH_3CN:NH_4OAc in H_2O) on a YMC ODS Guardpak column. Analytical LC−MS was performed on a Shimadzu HPLC system with a Zorbax SB-C8, 5 μm 2.1 × 50 mm (Agilent technologies) column, with a PE Sciex, API 150EX single quadropole mass spectrometer, at a flow rate of 1 mL/min (0.05% NH_4OAc-buffer:MeCN and 0.05% HCOOH in H_2O:MeCN). ^{1}H NMR spectra were recorded at 300 and 500 MHz; all values are referenced to tetramethylsilane as internal standard and are reported as shift (multiplicity, coupling constants, proton count). Mass spectral analysis is accomplished using fast atom bombardment (FAB-MS), electrospray (ESI-MS), or direct chemical ionization (DCI-MS) techniques. All elemental analyses are consistent with theoretical values to within +0.4% unless indicated.

4-Fluorobenzene-1,3-diol. [1-(Chloromethyl)-4-fluoro-1,4-diazoniabicyclo[2.2.2]octane bis(tetrafluoroborate)] (SELECTFLUOR) (28.4 g., 80.1 mmol) in anhydrous acetonitrile (100 mL) was treated with resorcinol (8.82 g., 80.1 mmol) and heated at 100 °C overnight. The reaction was diluted with diethyl ether, washed twice with H_2O, washed twice with saturated $NaHCO_3$, rinsed with brine, dried (Na_2SO_4), and filtered, and the filtrate was concentrated under reduced pressure to provide a mixture of isomers: ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.94 (dd, $J = 14$, 10 Hz,1 H), 6.51 (dd, $J = 8$, 3 Hz,1 H), 6.29 (m, 1 H), 5.12 (s, 1 H), 4.64 (s, 1 H); MS (DCI) *m/z* 129 $(M + H)^+$.

3-(Allyloxy)-4-fluorophenyl 4-methylbenzenesulfonate (6). *p*-Toluenesulfonyl chloride (15.2 g, 80.1 mmol) was added to a solution of 4-fluorobenzene-1,3-diol (10.2 g, 80.1 mmol) and K_2CO_3 (33.2 g, 240 mmol) in acetone (160 mL). The reaction mixture was heated at 60 °C overnight and treated with allyl iodide (7.30 mL, 80.1 mmol), and heating was continued at 60 °C for 24 h.[16] The reaction mixture was cooled to room temperature, quenched with aqueous NH_4Cl, and concentrated under reduced pressure. The crude products were diluted with ethyl acetate, extracted with H_2O, washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure. The residue was purified by flash chromatography on a prepacked Biotage column (hexane → 9:1 hexane:ethyl acetate) on silica gel to provide benzenesulfonate (**6**) (6.24 g, 24%) as a clear colorless oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.70 (d, $J =$ 12 Hz, 2 H), 7.33 (d, $J = 12$ Hz, 2 H), 6.95 (dd, $J = 14$, 12 Hz, 1 H), 6.66 (dd, $J = 12$, 3 Hz, 1 H), 6.45 (m, 1 H), 5.98 (m, 1 H), 5.33 (m, 2 H), 4.48 (m, 2 H), 2.44 (s, 3 H); MS (ESI) *m/z* 323 $(M + H)^+$.

3-(Allyloxy)-4-fluorophenol (7). Potassium hydroxide (10.9 g, 194 mmol) was added to a solution of 3-(allyloxy)-4-fluorophenyl 4-methylbenzenesulfonate (6.24 g, 19.4 mmol) in EtOH (20 mL) and H_2O (20 mL). The reaction mixture was heated to 90 °C for 2 h, cooled to room temperature, and concentrated under reduced pressure. The residue was purified by flash chromatography (9:1 → 4:1 hexane:ethyl acetate) on silica gel to provide the phenol (**7**) (2.20 g, 68%): ^{1}H NMR (300

Table 9. 30 Day *ob/ob* Data for Vehicle and Modulator **12**[a]

animal	compd	po dose	HbA_{1c} (%)	triglycerides (mg/dL)	FFA (mequiv/L)	cholesterol (mg/dL)	AST ALT (units/mL)
ob/ob mice	vehicle	b.i.d.	9.5 ± 0.4	272 ± 33	1.32 ± 0.09	291 ± 17	322 ± 30
ob/ob mice	**12**	100 mpk b.i.d.	7.7 ± 0.3	163 ± 22	1.27 ± 0.05	239 ± 11	303 ± 45
ob/ob mice	**12**	30 mpk b.i.d.	8.9 ± 0.4	214 ± 82	0.99 ± 0.13	266 ± 18	395 ± 53
lean mice	vehicle	b.i.d.	4.8 ± 0.2	73 ± 12	0.56 ± 0.04	133 ± 5	33 ± 4

[a] 9-12 animals per time point with results reported with standard error.

MHz, $CDCl_3$) δ 6.93 (dd, J = 16, 10 Hz, 1 H), 6.49 (dd, J = 10, 3 Hz, 1 H), 6.36 (m, 1 H), 6.06 (m, 1 H), 5.37 (m, 2 H), 4.61 (s, 1 H), 4.57 (m, 2 H); MS (DCI) *m/z* 169 $(M + H)^+$.

4-(3-(Allyloxy)-4-fluorophenoxy)benzaldehyde (8). 4-Fluorobenzaldehyde (1.41 mL, 13.1 mmol) was added to a solution of 3-(allyloxy)-4-fluorophenol (2.20 g, 13.1 mmol) and K_2CO_3 (5.40 g, 39.3 mmol) in DMF (13 mL). The reaction mixture was heated to 100 °C for 14 h and was cooled to room temperature. The crude products were diluted with ethyl acetate, washed with H_2O (2x) and brine, dried (Na_2SO_4), and concentrated under reduced pressure. The residue was purified by flash chromatography (40:1 → 9:1 hexane:ethyl acetate) on silica gel to provide aldehyde (**8**) (2.59 g, 73%): MS (DCI) *m/z* 273 $(M + H)^+$.

***N*-(4-(3-(Allyloxy)-4-fluorophenoxy)benzyl)-*N*-(2-methyl-3-nitrophenyl)amine (9).** 2-Methyl-3-nitroaniline (1.45 g, 9.52 mmol) and 4-(3-(allyloxy)-4-fluorophenoxy)benzaldehyde (2.59 g, 9.52 mmol) in dichloroethane (10 mL) were treated with glacial acetic acid (2.18 mL, 38.1 mmol). The yellow reaction mixture was stirred for 4 h at room temperature, treated with sodium triacetoxyborohydride (4.04 g, 19.0 mmol), and stirred overnight at room temperature. The mixture was poured into saturated aqueous $NaHCO_3$ (50 mL) and extracted with ethyl acetate (50 mL). The organic phase was washed with brine (50 mL), dried (Na_2SO_4), and filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (40:1 → 6:1 hexane:ethyl acetate) on silica gel to provide nitroarene (**9**) (3.41 g, 88%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32 (d, J = 10 Hz, 2 H), 7.15 (m, 2 H), 7.02 (m, 2 H), 6.94 (d, J = 10 Hz, 2 H), 6.78 (m, 1 H), 6.67 (dd, J = 9, 3 Hz, 1 H), 6.52 (m, 1 H), 6.03 (m, 1 H), 5.34 (m, 2 H), 4.56 (m, 2 H), 4.39 (s, 2 H), 2.22 (s, 3 H); MS (ESI−) *m/z* 407 $(M-H)^-$.

***N*-(4-(3-(Allyloxy)-4-fluorophenoxy)benzyl)-*N*-(2,4-difluorobenzyl)-*N*-(2-methyl-3-nitrophenyl)amine (10).** *N*-(4-(3-(Allyloxy)-4-fluorophenoxy)benzyl)-*N*-(2-methyl-3-nitrophenyl)amine (3.41 g, 8.36 mmol) and diisopropylethylamine (2.91 mL, 16.7 mmol) in DMF (10 mL) were treated with 2,4-difluorobenzyl bromide (1.29 mL, 10.0 mmol) and heated for 18 h at 100 °C. After cooling to room temperature, the mixture was diluted with ethyl acetate (50 mL). The mixture was washed with saturated ammonium chloride (50 mL), water (2 × 25 mL), and brine (25 mL), dried (Na_2SO_4), and filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (silica gel, 19:1 → 9:1 hexane:ethyl acetate) to provide dibenzylaniline (**10**) as a yellow solid (4.46 g, 100%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.52 (dd, J = 10, 2 Hz, 1 H), 7.15 (m, 5 H), 7.02 (dd, J = 16, 12 Hz, 1 H), 6.89 (d, J = 12 Hz, 2 H), 6.75 (m, 2 H), 6.65 (dd, J = 10, 3 Hz, 1 H), 6.48 (m, 1 H), 6.01 (m, 1 H), 5.31 (m, 2 H), 4.53 (m, 2 H), 4.14 (s, 2 H), 4.09 (s, 2 H), 2.53 (s, 3 H); MS (ESI+) *m/z* 535 $(M + H)^+$.

5-(4-(((2,4-Difluorobenzyl)(2-methyl-3-nitrophenyl)-amino)methyl)phenoxy)-2-fluorophenol. *N*-(4-(3-(Allyloxy)-4-fluorophenoxy)benzyl)-*N*-(2,4-difluorobenzyl)-*N*-(2-methyl-3-nitrophenyl)amine (4.46 g, 8.36 mmol) and $Pd(PPh_3)_4$ (1.00 g, 0.836 mmol) in CH_2Cl_2 (10 mL) were treated with phenylsilane (2.08 mL, 16.7 mmol) and stirred for 2 h at room temperature. The reaction mixture was concentrated under reduced pressure, and the residue was purified by flash chromatography (silica gel, 19:1 → 6:1 hexane:ethyl acetate) to provide the phenol as a yellow oil (3.87 g, 92%).

Ethyl (5-(4-(((2,4-Difluorobenzyl)(2-methyl-3-nitrophenyl)amino)methyl)phenoxy)-2-fluorophenoxy)acetate (11). A solution of 5-(4-(((2,4-difluorobenzyl)(2-methyl-3-nitrophenyl)-amino)methyl)phenoxy)-2-fluorophenol (3.87 g, 7.73 mmol), triphenylphosphine (4.05 g, 15.5 mmol), and di-*tert*-butyl azodicarboxylate (2.67 g, 11.6 mmol) in anhydrous THF (16 mL) was treated with ethyl glycolate (0.92 mL, 9.67 mmol). The reaction mixture was stirred overnight at room temperature. The mixture was diluted with ethyl acetate, washed with H_2O and brine, dried (Na_2SO_4), and filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (silica gel, 1:1 → 7:3 methylene chloride:hexanes) to provide the ester (**11**) (3.25 g, 72%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.53 (dd, J = 8, 2 Hz, 1 H), 7.15 (m, 5 H), 7.03 (dd, J = 16, 12 Hz, 1 H), 6.88 (d, J = 12 Hz, 2 H), 6.75 (m, 2 H), 6.61 (dd, J = 10, 3 Hz, 1 H), 6.55 (m, 1 H), 4.62 (s, 2 H), 4.22 (q, J = 9 Hz, 2 H), 4.14 (s, 2 H), 4.08 (s, 2 H), 2.54 (s, 3 H), 1.26 (t, J = 10 Hz, 3 H); MS (ESI+) *m/z* 581 $(M + H)^+$.

Ethyl (5-(4-(((2,4-Difluorobenzyl)(2-methyl-3-((methylsulfonyl)amino)phenyl)amino)methyl)phenoxy)-2-fluorophenoxy)acetate. Ethyl (5-(4-(((2,4-difluorobenzyl)(2-methyl-3-nitrophenyl)amino)methyl)phenoxy)-2-fluorophenoxy)acetate (3.25 g, 5.60 mmol) and NH_4Cl (208 mg, 3.92 mmol) in ethanol (10 mL) and water (3 mL) were treated with iron powder (2.19 g, 39.2 mmol) and heated at 80 °C for 1 h. The reaction mixture was cooled to room temperature, diluted with CH_2Cl_2 (100 mL), and filtered through Celite with CH_2Cl_2 washes, and the filtrate was concentrated under reduced pressure to provide the aniline compound, which was used without further purification. The residue was dissolved in anhydrous pyridine (10 mL), and to this was added methanesulfonyl chloride (0.52 mL, 6.72 mmol). The reaction mixture was stirred overnight at room temperature. The mixture was diluted with ethyl acetate, washed with H_2O, 1 N H_3PO_4, and brine, dried (Na_2SO_4), and filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (9:1 → 6:4 hexane:ethyl acetate) on silica gel to provide the sulfonamide (3.52 g, 100%): ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.22−7.00 (m, 6 H), 6.92 (m, 1 H), 6.86 (d, J = 10 Hz, 2 H), 6.73 (m, 2 H), 6.56 (m, 2 H), 6.19 (s, 1 H), 4.62 (s, 2 H), 4.22 (q, J = 9 Hz, 2 H), 4.07 (s, 2 H), 4.02 (s, 2 H), 2.95 (s, 3 H), 2.30 (s, 3 H), 1.25 (t, J = 9 Hz, 3 H); MS (ESI+) *m/z* 629 $(M + H)^+$.

(5-(4-(((2,4-Difluorobenzyl)(2-methyl-3-((methylsulfonyl)amino)phenyl)amino)methyl)phenoxy)-2-fluorophenoxy)acetic Acid (12). A solution of ethyl (5-(4-(((2,4-difluorobenzyl)(2-methyl-3-((methylsulfonyl)amino)phenyl)amino)-methyl)phenoxy)-2-fluorophenoxy)acetate (3.52 g, 5.60 mmol) in THF (10 mL) was treated with aqueous 4 N sodium hydroxide (0.70 mL, 28.0 mmol) and ethanol (2 mL) and stirred overnight at room temperature. The mixture was concentrated under reduced pressure, diluted with water, and extracted with diethyl ether. The aqueous phase was acidified with 1 N aqueous H_3PO_4 and extracted with chloroform. The chloroform phase was dried (Na_2SO_4) and filtered, and the filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (9:1 methylene chloride:methanol) on silica gel to provide the acid (**12**) (3.25 g, 97%) as a light brown solid: ^{1}H NMR (500 MHz, DMSO) δ 8.95 (s, 1 H), 7.26 (d, J = 8.4 Hz, 2 H), 7.21 (m, 2 H), 7.11 (m, 1 H), 7.05 (d, J = 7.8 Hz, 1 H), 7.00 (d, J = 7.5 Hz, 1 H), 6.95 (m, 2 H), 6.88 (d, J = 8.4 Hz, 2 H), 6.82 (m, 1 H), 6.48 (m, 1 H), 4.75 (s, 2 H), 4.07 (s, 2 H), 4.05 (s, 2 H), 2.90 (s, 3 H), 2.33 (s, 3 H); MS (ESI+) *m/z* 601 $(M + H)^+$. Anal. Calcd for $C_{30}H_{27}F_3N_2O_6S\cdot 1H_2O$: C, 58.25; H, 4.73; N, 4.53. Found: C, 58.47; H, 4.78; N, 4.30. Analytical HPLC using a YMC CombiScreen ODS-A analytical column and 20% acetonitrile/0.1% TFA aqueous buffer to 100% acetonitrile gradient showed >95% purity.

Compounds **13**−**15** and the [H^3]-**12** precursor **16** were prepared using similar methods and conditions.

(2-Bromo-5-(4-(((2,4-difluorobenzyl)(2-methyl-3-((methylsulfonyl)amino)phenyl)amino)methyl)phenoxy)phenoxy)acetic Acid (13). Light brown solid: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.48 (d, J = 8.7 Hz, 1 H), 7.35 (dd, J = 15, 8.4 Hz, 1 H), 7.28 (dd, J = 7.8, 1.2 Hz, 1 H), 7.21 (m, 2 H), 7.09 (s, 1 H), 7.03 (d, J = 8.4 Hz, 2 H), 6.78 (dd, J = 8.4, 2.2 Hz, 1 H), 6.73 (m, 1 H), 6.63 (dd, J = 8.6, 2.7 Hz, 1 H), 6.55 (d, J = 2.5 Hz, 1 H), 4.64 (s, 2 H), 4.35 (s, 2 H), 4.20 (s, 2 H), 2.89 (s, 3 H), 2.01 (s, 3 H); MS (ESI+) *m/z* 663 $(M + H)^+$; HRMS calcd for $C_{30}H_{27}N_2O_6S_1Br_1F_2$ 660.0741, found 660.0762. Analytical HPLC using a YMC CombiScreen ODS-A analytical column and 20% acetonitrile/0.1% TFA aqueous buffer to 100% acetonitrile gradient showed >95% purity.

(4-{4-[(Benzyl{2-methyl-3-[(methylsulfonyl)amino]-phenyl}amino)methyl]phenoxy}phenoxy)acetic Acid (14).

Light brown solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.25 (m, 6 H), 7.15 (m, 3 H), 6.95 (m, 5 H), 6.83 (d, J = 8.8 Hz, 2 H), 6.29 (s, 1 H), 4.66 (s, 2 H), 4.05 (s, 2 H), 4.01 (s, 2 H), 2.94 (s, 3 H), 2.33 (s, 3 H); MS (ESI+) *m/z* 547 (M + H)$^+$; HRMS calcd for $C_{30}H_{31}N_2O_6S_1$ 547.1897, found 547.1899. Anal. Calcd for $C_{30}H_{30}N_2O_6S\cdot1.5\ H_2O$: C, 62.71; H, 5.81; N, 4.88. Found: C, 62.91; H, 5.77; N, 4.63. Analytical HPLC using a YMC CombiScreen ODS-A analytical column and 20% acetonitrile/0.1% TFA aqueous buffer to 100% acetonitrile gradient showed >95% purity.

(2-Chloro-5-{4-[((2,4-difluorobenzyl){2-methyl-3-[(methylsulfonyl)amino]phenyl}amino)methyl]phenoxy}-phenoxy)acetic Acid (15). Light brown solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32 (d, J = 8.8 Hz, 2 H), 7.25 (m, 3 H), 7.20 (m, 2 H), 6.96 (d, J = 8.5 Hz, 2 H), 6.81 (d, J = 8.8 Hz, 2 H), 6.77 (s, 1 H), 6.75 (m, 1 H), 6.62 (d, J = 2.7 Hz, 1 H), 4.65 (s, 2 H), 4.17 (s, 2 H), 3.98 (s, 2 H), 2.91 (s, 3 H), 1.97 (s, 3 H); MS (ESI+) *m/z* 617 (M + H)$^+$. Anal. Calcd for $C_{30}H_{27}Cl_1F_2N_2O_6S\cdot1.5H_2O$: C, 55.94; H, 4.69; N, 4.35. Found: C, 56.02; H, 4.35; N, 4.25. Analytical HPLC using a YMC CombiScreen ODS-A analytical column and 20% acetonitrile/0.1% TFA aqueous buffer to 100% acetonitrile gradient showed >95% purity.

The synthesis of compound **4** has been reported previously (by methods similar to those for the preparation of sulfonamide **12**).[8c] A procedure that is not reported herein and characterization information are included below.

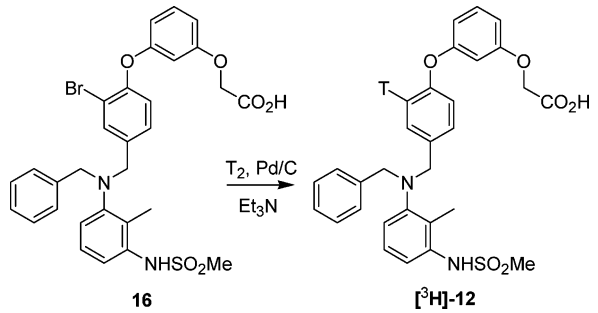
Ethyl 4-[3-(4-{[Benzyl(2-methyl-3-nitrophenyl)amino]-methyl}phenoxy)phenoxy]butanoate. A solution of *N*-(3-{benzyl[4-(3-hydroxyphenoxy)benzyl]amino}-2-methyl-phenyl)-methanesulfonamide (0.100 g, 0.227 mmol) in DMF (0.57 mL) was treated with NaH (0.011 g, 0.27 mmol, 60% dispersion). After 15 min, ethyl 4-bromobutyrate (0.039 mL, 0.27 mmol) was added and the reaction mixture was stirred overnight. The crude products were diluted with diethyl ether, washed with saturated NH_4Cl, extracted twice with H_2O, washed with brine, dried (Na_2SO_4), and concentrated under reduced pressure. The residue was purified by flash chromatography on silica gel (hexane → 7:1 hexane:ethyl acetate) to provide ethyl 4-[3-(4-{[benzyl(2-methyl-3-nitrophenyl)amino]methyl}-phenoxy)phenoxy]butanoate as a yellow oil.

4-(3-{4-[(Benzyl{2-methyl-3-[(methylsulfonyl)amino]-phenyl}amino)methyl]phenoxy}phenoxy)butanoic Acid. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.97 (s, 1 H), 7.23 (m, 8 H), 6.88−7.08 (m, 5 H), 6.68 (dd, J = 8.3, 2.5 Hz, 1 H), 6.49 (m, 2 H), 4.06 (s, 2 H), 4.02 (s, 2 H), 3.93 (t, J = 6.4 Hz, 2 H), 3.33−3.61 (br s, 1 H), 2.91 (s, 3 H), 2.40 (s, 3 H), 2.35 (t, J = 7.3 Hz, 2 H), 1.90 (m, 2 H). MS (ESI): *m/z* 575 (M + H$^+$).

4-{[4-(3-{4-[(Benzyl{2-methyl-3-[(methylsulfonyl)amino]-phenyl}amino)methyl]phenoxy}phenoxy)butanoyl]-amino}butanoic Acid (4). ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.97 (s, 1 H), 7.83 (t, J = 5.8 Hz, 1 H), 7.13−7.34 (m, 8 H), 6.87−7.09 (m, 5 H), 6.67 (m, 1 H), 6.47 (m, 2 H), 4.27−4.62 (br s, 1 H), 4.06 (s, 2 H), 4.03 (s, 2 H), 3.91 (t, J = 6.4 Hz, 2 H), 3.03 (dd, J = 12.5, 6.8 Hz, 2 H), 2.91 (s, 3 H), 2.40 (s, 3 H), 2.19 (t, J = 7.5 Hz, 4 H), 1.88 (m, 2 H), 1.59 (m, 2 H). MS (APCI): *m/z* 660 (M + H$^+$). HRMS calcd for $C_{36}H_{42}N_3O_7S_1$ 660.2738, found 660.2739. Analytical HPLC using a YMC CombiScreen ODS-A analytical column and 20% acetonitrile/0.1% TFA aqueous buffer to 100% acetonitrile gradient showed >95% purity.

[^{3}H]-12 (Scheme 2). The brominated precursor, **16** (4.0 mg, 0.00589 mmol), was taken up in methanol (1.0 mL) in a 5 mL flask. Triethylamine (0.010 mL, 0.072 mmol) and 10% Pd/C (8 mg) were added. The flask was attached to the apparatus for tritium gas reactions, frozen in liquid nitrogen, and evacuated. It was thawed, frozen in liquid nitrogen, and evacuated again. Tritium gas (1.3 Ci, 0.0069 mmol, ~58 Ci/mmol) was generated and admitted to the reaction flask. It was warmed to room temperature and the reaction mixture stirred for 1.5 h. The volatiles were removed under vacuum, and then the reaction mixture was filtered. It was evaporated to dryness in vacuo, taken up in methanol (~1 mL), and then evaporated again. This process was repeated two times and the residue again taken up in methanol (1 mL) and assayed

Scheme 2. Synthesis of [^{3}H]-**12**



(total activity = 69.5 mCi). Radio-HPLC analysis (from 0.1% TFA:60% CH_3CN to 75% CH_3CN in 15 min on Phenomenex Luna C18(2), 5 μm, 4.6 × 250 mm at 1 mL/min, 220 nM UV) showed ~89% radiochemical purity. The UV trace showed about equal amounts of starting material and product. About 6 mL of [^{3}H]-**12** was evaporated to dryness, and the residue was dissolved in 10 mL of acetonitrile:water:TFA (52:48:0.1). Approximately 2 mL per injection was made onto a Phenomenex Synergi Max-RP column (4 μm, 250 mm × 21.2 mm i.d.) using a Shimadzu HPLC system. [^{3}H]-**12** was eluted at a flow rate of approximately 20 mL/min by an isocratic mobile phase consisting of acetonitrile:water:TFA (56:44:0.1). Peak detection and chromatograms were obtained using a Shimadzu variable wavelength UV detector set at 220 nm and Shimadzu Class-VP software. The fractions containing [^{3}H]-**12** were collected at approximately 24 min using a Shimadzu fraction collector. Fractions were combined and the solvents were evaporated in vacuo. The residue was dissolved in 200 proof ethanol (20 mL).

Determination of Purity and Specific Activity. [^{3}H]-**12** was assayed using an Agilent 1100 series HPLC system consisting of a quaternary pump, an autosampler, and a photodiode array UV detector. A Packard Radiomatic A 500 radioactivity detector was connected to the HPLC system. For radiodetection, a 500 μL flow cell and a 3:1 ratio of Ultima-Flo M scintillation cocktail to HPLC mobile phase were used. The analyses were performed using a Phenomenex Synergi, Max-RP column (4 μm, 250 mm × 4.6 mm i.d.). The mobile phase was isocratic at 52% B for 25 min followed by ramping to 90% B in 1 min and holding at 90% B for 9 min, where mobile phase A = 0.1% TFA/water and mobile phase B = 0.1% TFA/acetonitrile. The flow rate was set at approximately 1 mL/min, and the UV detection was set at 220 nm.

The radiochemical purity of [^{3}H]-**12** was found to be >99%. Confirmation of the identity of [^{3}H]-**12** was obtained when **12** had an identical retention time. The specific activity was determined to be 17.7 Ci/mmol by measuring the mass and radioactivity concentrations of [^{3}H]-**12** dissolved in the mobile phase. The concentration of radioactivity was determined by liquid scintillation counting of an accurately measured aliquot. The mass concentration was measured by comparing the HPLC−UV peak area of an accurately measured aliquot to a standard solution.

Assay Protocols. GR Binding Assay. ^{3}H-Dexamethasone (TRK 645, "^{3}H-dex") was purchased from Pharmacia Amersham, Uppsala, Sweden. Dexamethasone ("dex") was purchased from Sigma. The Costar 96-well polypropylene plates (3794 or 3365) were purchased from Life Technologies AB, Täby, Sweden. The GF/B filter (1450-521), filter cassette (1450-104), MeltiLex scintillating wax (1450-441), sample bag (1450-42), Microbeta 1450-PLUS, and Microsealer 1495-021 were all purchased from Wallac Oy, Turkku, Finland. Human glucocorticoid receptors were extracted from Sf9 cells infected with a recombinant baculovirus transfer vector containing the cloned h-GR gene. Recombinant baculovirus was generated utilizing the BAC-TO−BAC expression system (Life Technologies) in accordance with instruction from the supplier. The h-GR coding sequences were cloned into a baculovirus transfer vector by standard techniques. The recombinant baculoviruses expressing h-GR were amplified and used to infect Sf9 cells.

Infected cells were harvested 48 h postinfection. The receptors were extracted from the cell pellet with a phosphate buffer (1 mM EDTA, 20 mM KPO_4 (pH 8), 8.6% glycerol, 12 mM MTG, 20 mM Na_2MoO_4). The concentration of h-GR in the extract was measured via specific binding of ^{3}H-dexamethasone ("^{3}H-dex") with the G25-assay[22] and estimated to approximately 25 nM. The extract was aliquoted and stored at −70 °C.

Dilution series of the test compounds and dexamethasone ("dex") as reference were made from 10 mM (1 mM dex) stock solutions in DMSO. Ten microliters of each dilution was added in duplicate to the wells. The cell extracts were diluted 10-fold in EPMo + MTG buffer (1 mM EDTA, HPO_4 20 mM (pH 8), 6 mM MTG). The diluted extract was added to the wells (110 μL). ^{3}H-Dex was diluted from the stock solution to 10 nM in EPMo + MTG buffer. Then 110 μL aliquots of the diluted ^{3}H-dex were added to the wells. The final concentration of h-GR in the experiment was estimated to 1 nM. All preparations were made in ambient temperature (20−25 °C) on ice and with buffers at +4 °C. The plates were incubated overnight at +4 °C (15−20 h).

The incubation was stopped by filtration through a GF/B filter on a Tomtec CellHarvester. The GF/B filters were dried for at least 1 h at 65 °C. A MeltiLex scintillation wax was melted onto filters with the Microsealer. Filters were placed in a sample bag, which was thereafter trimmed with scissors to fit the filter cassette. The cassettes were placed in the Microbeta and measured for 1 min/position, returning ccpm (corrected counts per minute).

For compounds able to displace the ^{3}(H)-dexamethasone from the receptor an IC_{50} value (the concentration required to inhibit 50% of the binding of ^{3}(H)-dex) was determined by a nonlinear four parameter logistic model:

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

where I is added concentration of binding inhibitor, IC_{50} is the concentration for inhibitor at half-maximal binding, and S is a slope factor. For determinations of the concentration of ^{3}H-dex in the solutions, regular scintillation counting in a Wallac Rackbeta 1214 was performed using the scintillation cocktail Supermix (Wallac).

The Microbeta instrument generates the mean cpm (counts per minute) value/minute and corrects for individual variations between the detectors thus generating corrected cpm values. Counting efficiency between detectors differed by less than 5%.

Similar protocols were employed to measure affinity of the compounds for progesterone receptor (PR), mineralocorticoid receptor (MR), androgen receptor (AR), estrogen receptors (ER_α and ER_β), and thyroid hormone receptors (TR_α and TR_β).

GRAF Assay. Chinese hamster ovarian cells (CHO-K1) were stably transfected with an expression plasmid encoding hGR and a reporter construct containing a glucocorticoid response element driving expression of alkaline phosphatase (ALP) to produce the GRAF cell line. GRAF cells were grown in HAM's F12 medium supplemented with 10% FCS and 1% L-glutamine. Induction medium was Opti-MEM with 1% L-glutamine, 50 μg/mL gentamycin. GRAF cells were seeded in growth medium in 96-well plates at ~50 000 cells per well. After 24 h of incubation, the cells were induced with test compounds as well as dexamethasone, as a positive control, at serial dilutions, to measure agonist response. In order to examine antagonist effects GRAF cells were treated with increasing amounts of test compounds in the presence of 5 nM dexamethasone. After 48 h of induction, disodium 3-(4-methoxyspiro{1,2-dioxetane-,2′-(5′-chloro)tricyclo[3.3.1.13.7]decan}-4-yl)phenylphosphate (CSPD; Applied Biosystems) was added to the medium, and levels of secreted alkaline phosphatase were analyzed by chemiluminescence on a MicroBeta Trilux.

Hepatocyte TAT Assay. Primary rat hepatocytes were plated in 96-well collagen-coated plates at 50 000 cells per well. Cells were incubated in DMEM with 10% charcoal-stripped serum at 37 °C for 4 h. Cells were pretreated with serial diluted compounds for 30 min, followed by 100 nM of prednisolone for 4 h. Cells were then washed with PBS and lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 10 μg/mL PMSF and 0.1% Lubrol PX). The tyrosine aminotransferase assay was performed according to standard protocols.[23] Basically, the substrate mixture containing 6mM L-tyrosine, 11 μM α-ketoglutarate and 56 μM pyridoxal-5′ phosphate in potassium phosphate buffer was added to the lysates and incubated for 30 min at 37 °C. The reaction was stopped by 10N KOH and plates were read on a Spectrophotometer at 340 nm. Wells that contained substrate without compound or Dex were used as the background, while the wells that contained substrate and Dex without any compound were considered as maximal signal. Percent inhibition of each compound was calculated relative to the maximal signal and IC_{50} curves were generated.

In Vivo Pharmacology. Rat Prednisolone Challenge Model. Overnight fasted 150 g male Sprague−Dawley rats are orally dosed with vehicle, **1** (30−100 mg/kg), or selected glucocorticoid receptor antagonists (30−100 mg/kg), 60 min prior to an oral challenge with prednisolone at 10 mg/kg. Six hours following the prednisolone challenge, rats are euthanized with CO_2, and bled via cardiac puncture for evaluation of blood lymphocytes and plasma drug levels. Seven millimeter liver biopsy punches are harvested for evaluation of tyrosine aminotransferase (TAT), hepatic glycogen, and GR antagonist levels. Additional liver tissue, retroperitoneal fat, skeletal muscle, kidney, and skin from an ear biopsy are also removed for isolation an evaluation of mRNA. Prednisolone increases hepatic TAT and glycogen levels, and induces severe lymphopenia during the 6 h challenge interval. At doses of 100 mpk, **1** completely antagonizes these hepatic and peripheral responses.

Mouse Hypothalamic−Pituitary−Adrenal (HPA) Activation Model. Nonfasted CD-1 male mice, weighing approximately 25 g, are dosed with vehicle, **1** (30−100 mg/kg), or GR antagonist (30−100 mg/kg) at 0800 h when corticosterone levels are low. Two hours later, mice are euthanized with CO_2 and bled by cardiac puncture, and the plasma is analyzed for corticosterone by mass spectroscopy and for adrenocorticotropic hormone (ACTH) levels by ELISA. Brains and plasma are also removed for analysis of GR antagonist levels. At doses of 100 mpk, **1** significantly increases ACTH and corticosterone levels compared to vehicle controls.

***ob/ob* Mouse Efficacy Model.** Male B6.VLep$^{ob(-/-)}$ (*ob/ob*) mice and their lean littermates (Jackson Laboratory) were group housed and allowed free access to food (Purina 5015) and water. Mice were 6−7 weeks old at the start of each study. On day 0 animals were weighed and postprandial glucose levels determined (Precision X glucometer, Abbott Laboratories). Mean glucose levels did not differ significantly from group to group (n = 10) at the start of the studies. Animals were weighed and postprandial glucose measurements were taken weekly throughout the study. On the last day of the study 16 h postdose the mice were euthanized and blood samples (EDTA) were taken by cardiac puncture and immediately placed on ice. Whole blood measurements for HbA1c were taken with hand held meters (A1c NOW, Metrika Inc., Sunnyvale, CA). Blood samples were then spun, and plasma was removed and frozen until further analysis. Plasma parameters were run according to instructions by the manufacturer and consisted of ALT/AST for hepatic enzymes (GO-& GP-transaminase kit, Sigma Diagnostics, St. Louis, MO), free fatty acids (NEFA C kit, Wako Chemicals, Neuss, Germany), triglyceride and cholesterol lipid measurements (Infinity kits, Sigma Diagnostics, St. Louis, MO), and insulin and ACTH hormones by ELISA (American Laboratory Products Co., Windham, NH). Tissue samples were collected, weighed, and snap frozen.

Liver biopsies were taken for hepatic tyrosine aminotransferase activity. The TAT activity was determined by the spectrophotometric method of Diamondstone and normalized for hepatic protein content by the method of Lowry.[24,25] Glycogen content was measured by known methods.[26]

13 Day *ob/ob* Study with Modulator 14, Mice were dosed orally twice a day for 13 consecutive days with compound and

vehicle controls. Control *ob/ob* mice and lean littermates were dosed with vehicle (1 equiv of NaOH, 5% ethanol, 10% PEG 400, 85% saline) that was also used to formulate **14**.

14 Day *ob/ob* Study with Modulator 12. Mice were dosed orally twice a day for 14 consecutive days with compound and vehicle controls. Control *ob/ob* mice and lean littermates were dosed with vehicle (0.2% hydroxypropyl methylcellulose) that was also used to formulate **12**.

30 Day *ob/ob* Study with Modulator 12. Mice were dosed orally twice a day for 30 consecutive days with compound and vehicle controls. Control *ob/ob* mice and lean littermates were dosed with vehicle (0.2% hydroxypropyl methylcellulose) that was also used to formulate **12**.

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