

Characterization of a Novel, Non-peptidyl Antagonist of the Human Glucagon Receptor*

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We have identified a series of potent, orally bioavailable, non-peptidyl, triarylimidazole and triarylpyrrole glucagon receptor antagonists. 2-(4-Pyridyl)-5-(4-chlorophenyl)-3-(5-bromo-2-propyloxyphenyl)pyrrole (L-168,049), a prototypical member of this series, inhibits binding of labeled glucagon to the human glucagon receptor with an $IC_{50} = 3.7 \pm 3.4$ nM ($n = 7$) but does not inhibit binding of labeled glucagon-like peptide to the highly homologous human glucagon-like peptide receptor at concentrations up to 10 μ M. The binding affinity of L-168,049 for the human glucagon receptor is decreased 24-fold by the inclusion of divalent cations (5 mM). L-168,049 increases the apparent EC_{50} for glucagon stimulation of adenylyl cyclase in Chinese hamster ovary cells expressing the human glucagon receptor and decreases the maximal glucagon stimulation observed, with a K_b (concentration of antagonist that shifts the agonist dose-response 2-fold) of 25 nM. These data suggest that L-168,049 is a noncompetitive antagonist of glucagon action. Inclusion of L-168,049 increases the rate of dissociation of labeled glucagon from the receptor 4-fold, confirming that the compound is a noncompetitive glucagon antagonist. In addition, we have identified two putative transmembrane domain residues, phenylalanine 184 in transmembrane domain 2 and tyrosine 239 in transmembrane domain 3, for which substitution by alanine reduces the affinity of L-168,049 46- and 4.5-fold, respectively. These mutations do not alter the binding of labeled glucagon, suggesting that the binding sites for glucagon and L-168,049 are distinct.

Glucagon is a 29-amino acid peptide that is an important counter-regulatory hormone in the control of glucose homeostasis (1). Glucagon secretion from the endocrine pancreas induces an increase in hepatic glycogenolysis and gluconeogenesis, and it attenuates the ability of insulin to inhibit these processes. As such, the overall rates of hepatic glucose synthesis and glycogen metabolism are controlled by the systemic ratio of insulin and glucagon (2, 3). Therefore, glucagon antagonists have the potential to improve hepatic insulin sensitivity and to be effective hypoglycemic agents.

Peptidyl glucagon antagonists and their hypoglycemic activity were first described over 15 years ago, and an extensive exploration of the structure/activity relationships of these glu-

cagon analogs has been reported (4–6). The hepatic receptor for glucagon was cloned recently (7, 8), confirming that it is a member of the seven-transmembrane domain, G-protein-coupled receptor superfamily. This receptor superfamily has a binding pocket for small-molecule ligands within the transmembrane domain that has made it possible to identify non-peptidyl antagonists for many receptor families in which the endogenous ligands are small peptides or proteins (9). Thus, we initiated an effort to identify non-peptidyl, orally active antagonists for the human glucagon receptor.

Collins *et al.* (10) have described a dichloroquinoxaline glucagon antagonist with weak affinity ($IC_{50} = 4$ μ M) for the rat glucagon receptor. However, there have been no subsequent reports in the patent or scientific literature describing the development of potent antagonists from this series. Our initial screening efforts identified a series of triarylimidazole and triarylpyrrole compounds with significant binding affinity for the human glucagon receptor, and efforts to evaluate the structure-activity relationships of this series have led to the identification of potent glucagon antagonists (11). In the present article, we describe the identification and characterization of a potent glucagon antagonist from this series.

MATERIALS AND METHODS

Characterization of Binding Affinity and Functional Activity—Stable CHO¹ cell lines or COS cells transiently expressing the human glucagon receptor were prepared as described previously (8, 12). Antagonist binding affinity was assessed by measuring inhibition of radiolabeled glucagon binding to CHO cell membranes. Briefly, ¹²⁵I-glucagon (58 pM) binding to the membrane preparation was measured in 20 mM Tris, pH 7.4, containing 1 mM dithiothreitol, 5 μ g/ml leupeptin, 10 μ g/ml benzamidine, 40 μ g/ml bacitracin, 5 μ g/ml soybean trypsin inhibitor, and 3 μ M *o*-phenanthroline \pm 1 μ M glucagon for 1 h at room temperature. Bound cpm were recovered by filtration using a Tomtec harvester and quantified in a γ -scintillation counter.

The ability of compound to inhibit glucagon-stimulated adenylyl cyclase was assessed as described previously (12). Briefly, cells were harvested from monolayers with enzyme-free cell dissociation solution (Specialty Media, Inc.) and were pelleted at 500 \times *g*. The cells were resuspended at 100,000 cells/100 μ l in 75 mM Tris-HCl, pH 7.4, containing 250 mM sucrose, 12.5 mM MgCl₂, 1.5 mM EDTA, 0.1 mM Ro-20-1724 (Biomol, Inc.), leupeptin (5 μ g/ml), benzamidine (10 μ g/ml), bacitracin (40 μ g/ml), soybean trypsin inhibitor (5 μ g/ml), and 0.02% bovine serum albumin. Cells were incubated for 30 min at 22 °C with increasing peptide concentrations in the presence or absence of antagonist followed by lysis by boiling. Lysates were analyzed for cAMP content versus a nonacetylated cAMP standard curve using the Amersham cAMP radioimmunoassay scintillation proximity assay kit. Data were analyzed using Packard TopCount with RIASmart and GraphPad Prism software.

¹ The abbreviations and trivial name used are: CHO, Chinese hamster ovary; GLP-1, glucagon-like peptide-1; TM, transmembrane (domain); L-168,049, 2-(4-pyridyl)-5-(4-chlorophenyl)-3-(5-bromo-2-propyloxyphenyl)pyrrole.

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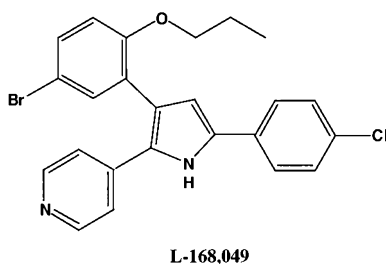


FIG. 1. Structure of the glucagon antagonist L-168,049.

Preparation and Assessment of Mutant Glucagon Receptors—The wild-type glucagon receptor was cloned into pCI-neo (Promega Corp., Madison, WI) at the *NheI* and *XbaI* sites. Mutations at amino acid residues 184 (TM2, F184A) and 239 (TM3, Y239A) of the wild-type glucagon receptor were prepared using recombinant polymerase chain reaction. The sense primers for the F184A and the Y239A mutants were 5'-TTTGCCTCCGCCGTGCTGAA-3' and 5-GTGCCAACGCCTGCTGCTGCT-3', respectively. The resulting mutant fragments were excised with either *ApaI/SacII* (F184A) or *SacII/BclI* (Y239A) and ligated into the pCI-neo wild-type glucagon receptor. Mutations were confirmed by automated sequencing. All other mutants were prepared in a similar manner using oligonucleotides specific to the production of an alanine at the indicated position with the wild-type receptor, and mutations were confirmed by sequencing. The restriction sites for the other mutations were as follows: TM1 (Tyr-145, Tyr-149, and Ser-152, digested with *ApaI/SacII*), TM2 (Phe-184 and Ser-190, digested with *ApaI/SacII*), TM3 (Gln-232, Tyr-233, Asn-238, and Tyr-239, digested with *SacII/BclI*), TM4 (Phe-278, digested with *SstII/BclI*), TM5 (Phe-312, Phe-319, and Phe-320, digested with *KspI/PflM 1*), and TM7 (Lys-381, Phe-383, Phe-384, Phe-387, Ser-390, and Phe-391, digested with *PflM 1/XbaI*).

The transfection of COS cells and the preparation of membranes for binding assays were performed as described previously (12). Compound titrations using wild-type receptor transiently expressed in COS cells were performed as controls in all experiments utilizing mutant receptors.

RESULTS

Several triarylimidazole compounds were identified from compound collection screening that inhibit labeled glucagon binding to the human glucagon receptor with potencies ranging from 300 nM to 1 μ M (11). Optimization of this activity by structural modification of these lead compounds led to the identification of the triarylimidazole glucagon antagonist, L-168,049 (Fig. 1). This compound inhibits binding of labeled glucagon with an IC_{50} of 3.7 ± 3.4 nM ($n = 7$). Surprisingly, the affinity of L-168,049 is decreased 48-fold by the inclusion of 5 mM $MgCl_2$ in the assay (Fig. 2). In the presence of 5 mM $MgCl_2$, the compound inhibits binding of labeled glucagon with an IC_{50} of 179 ± 86 nM ($n = 3$). An equivalent decrease in affinity is also observed with $MnCl_2$ and $CaCl_2$, but not with $NaCl$, indicating that the loss in affinity is divalent cation-dependent (data not shown). L-168,049 does not inhibit binding of labeled glucagon-like peptide-1 (GLP-1) to the highly homologous GLP-1 receptor at concentrations as high as 10 μ M.

Inclusion of increasing doses of L-168,049 increases the apparent EC_{50} for glucagon stimulation of adenylyl cyclase and decreases the maximal stimulation observed (Fig. 3). A noncompetitive antagonist should not alter maximal agonist-induced activation of the receptor, suggesting that L-168,049 is a noncompetitive antagonist of glucagon. Schild transformation of these data is linear with a slope of 0.6, which is consistent with a noncompetitive mechanism. The affinity of L-168,049, as measured by the concentration of antagonist that shifts the agonist dose-response 2-fold (K_b), is 25 nM. In the absence of exogenously added glucagon, L-168,049 does not alter intracellular cAMP levels, indicating that it has no agonist activity.

^{125}I -Glucagon dissociates from the human glucagon receptor in the presence of $MgCl_2$ with $k_{-1} = 0.013 \pm 0.001$ min $^{-1}$ ($n =$

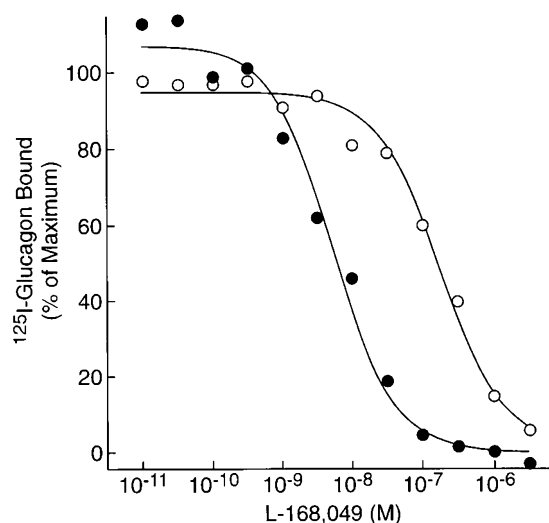


FIG. 2. Inhibition of the binding of ^{125}I -glucagon to the human glucagon receptor by L-168,049 in the absence (closed circles) or presence (open circles) of 5 mM $MgCl_2$. Assays were performed using CHO cell membranes as described under "Materials and Methods." Data are the average of three separate experiments.

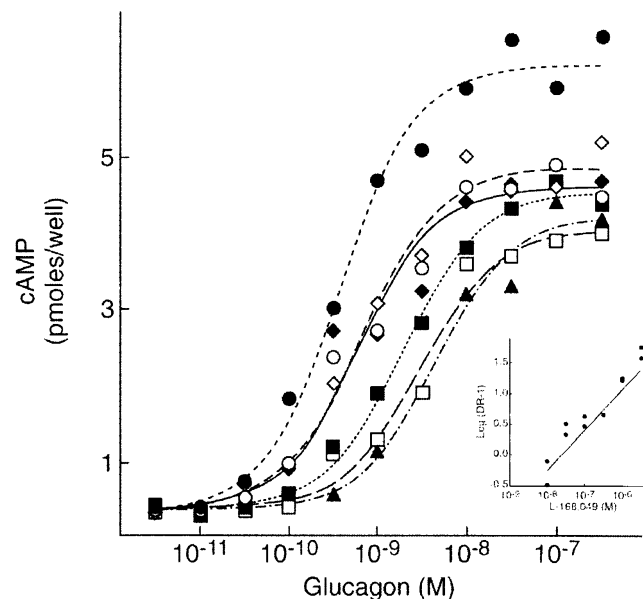


FIG. 3. Inhibition of glucagon-stimulated adenylyl cyclase activation by L-168,049. Generation of cAMP in CHO cells expressing the human glucagon receptor was measured in duplicate as described under "Materials and Methods." Glucagon dose-response curves were performed in the absence (closed circles) or presence of 10 nM (open circles), 30 nM (closed diamonds), 100 nM (open diamonds), 300 nM (closed squares), 1 μ M (open squares), or 3 μ M (closed triangles) L-168,049. Inset, Schild regression analysis of the data. K_b is the concentration of L-168,049 at which $\log(DR - 1) = 0$. DR is the ratio of the apparent EC_{50} for glucagon in the presence of a given concentration of L-168,049 over the EC_{50} in the absence of antagonist.

2). Inclusion of L-168,049 (1 μ M) increases the rate of dissociation 4-fold (Fig. 4). These data confirm that L-168,049 is a noncompetitive antagonist of glucagon. The nonhydrolyzable guanine nucleotide analog, guanosine 5'-(β , γ -imido)-triphosphate, which reduces glucagon affinity by uncoupling the receptor from G-protein, increases the rate of dissociation 29-fold. These data suggest that L-168,049 does not inhibit glucagon binding by this mechanism. Although the data shown are in the presence of divalent cation, L-168,049 also increases the rate of dissociation of ^{125}I -glucagon 4-fold in the absence of divalent cation.

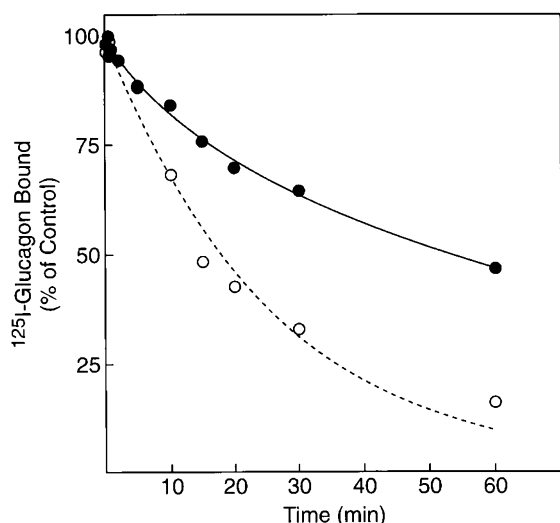


FIG. 4. Effect of L-168,049 on the dissociation of ^{125}I -glucagon from the human glucagon receptor. Membranes were incubated for 40 min with ^{125}I -glucagon in the presence of 5 mM MgCl_2 as described under "Materials and Methods" before the addition of an excess (2 μM) of unlabeled glucagon to initiate dissociation of labeled ligand in the presence (open circles) or absence (closed circles) of L-168,049 (1 μM). Data shown are the average of two separate experiments. Data analysis was performed using GraphPad Prism software.

To confirm that L-168,049 binds within the transmembrane domain of the glucagon receptor, 19 residues predicted to be within this region (8) were separately mutated to alanine, and the binding affinities of these mutants for glucagon and L-168,049 were determined following transient transfection into COS cells. These include Tyr-145, Tyr-149, Ser-152, Phe-184, Ser-190, Gln-232, Tyr-233, Asn-238, Tyr-239, Phe-278, Phe-312, Phe-319, Phe-320, Lys-381, Phe-383, Phe-384, Phe-387, Ser-390, and Phe-391. Alanine substitution of these individual residues does not alter affinity for labeled glucagon. Whereas most of these substitutions also do not alter affinity for L-168,049, the substitution of alanine for Phe-184 and Tyr-239 results in a 46- and a 4.5-fold reduction in the affinity for L-168,049, respectively (Table I). Inclusion of divalent cation reduced the affinity of the compound for both the wild-type and mutant receptors, indicating that these residues were not involved in mediating the effect of divalent cations on compound affinity (data not shown).

The triarylpyrrole glucagon antagonists have poor affinity for the rat, guinea pig, and rabbit glucagon receptors (>1 μM). However, L-168,049 inhibits labeled glucagon binding to the murine and canine glucagon receptors with $\text{IC}_{50} = 63 \pm 44$ nM ($n = 3$) and 60 nM, respectively. As with the human receptor, these affinities are reduced ≥ 20 -fold in the presence of 5 mM MgCl_2 ($\text{IC}_{50} = 331 \pm 122$ nM ($n = 3$)) and >1 μM for the murine and dog receptors, respectively.

DISCUSSION

We have identified a structural class of non-peptidyl glucagon antagonists. Preliminary data indicate that these compounds are absorbed systemically after oral administration (11), suggesting that they have the potential to be useful, orally active hypoglycemic agents. The prototype of this class, L-168,049, is a high affinity, noncompetitive antagonist of the human glucagon receptor, which inhibits glucagon-stimulated adenylyl cyclase with a K_b of 25 nM. This compound has poor affinity for the highly homologous GLP-1 receptor and for a panel of other G-protein-coupled receptors, indicating that it is a selective glucagon antagonist.

The observation that inclusion of L-168,049 alters both the

TABLE I

Analysis of the affinity of L-168,049 for glucagon receptor mutants

The affinity was determined after transient transfection of either wild-type or mutant receptors into COS cells as described under "Materials and Methods."

Receptor	Inhibition of I-glucagon binding (IC_{50}) ^a
	<i>nM</i>
Wild type	1.3 \pm 0.8 (3)
F184A	46 \pm 45 (2)
Y239A	4.5 \pm 0.5 (2)

^a Each value is mean \pm S.D. The number of experiments is shown in parentheses.

apparent affinity and maximal receptor activation of glucagon suggests that these compounds are noncompetitive glucagon antagonists. The demonstration that L-168,049 increases the dissociation rate of labeled glucagon confirms this hypothesis, because these data suggest that both glucagon and L-168,049 are able to bind to the receptor at the same time.

Whereas mutagenesis data suggest that the binding site for glucagon is within the amino-terminal domain and other extracellular domains of the glucagon receptor (13–15), our data suggest that the L-168,049 binding site is within the transmembrane domain. The binding of glucagon to the 19 transmembrane domain mutant receptors is unaltered, suggesting that these mutations did not dramatically effect overall receptor conformation. Phenylalanine 184 and tyrosine 239, the two mutations with altered affinity for L-168,049, are predicted to be within the second and third transmembrane domains, respectively. Thus, these compounds appear to bind in the same common binding pocket that has been postulated for other G-protein-coupled receptors (9). These data, and the observation that L-168,049 is a noncompetitive antagonist of glucagon, suggest that the compound allosterically modulates the binding of glucagon to the receptor.

Divalent cations have been shown to allosterically enhance the binding of agonists to many types of G-protein-coupled receptors, including the binding of glucagon to the human glucagon receptor (12), but divalent cation effects on antagonist binding affinity are less well documented. However, inclusion of 5 mM divalent cation decreases the affinity of L-168,049 48-fold. The activity of antagonists in the cellular functional assays that are performed under more physiologically relevant buffer conditions correlates more closely with the binding affinity measured in the presence of divalent cation. More importantly, because circulating levels of MgCl_2 are in the low mM range, the decrease in affinity observed in the presence of divalent cation would be expected to be important for the activity of antagonists *in vivo*.

We previously showed that the deletion of residues 252–259 in the second intracellular domain of the glucagon receptor attenuates the effects of divalent cation on the binding affinity for glucagon (12). However, this mutation does not significantly alter the affinity for L-168,049, and inclusion of divalent cation reduces affinity of L-168,049 for this mutant protein 30-fold. Thus, the effects of divalent cation on agonist and antagonist binding appear to be distinct.

Because L-168,049 has poor affinity for all of the species orthologs of the glucagon receptor tested to date, it has been difficult to test its *in vivo* efficacy. However, L-168,049 has the highest affinity for the murine receptor of any compound in this series identified to date, and high levels of compound (3 μM) increase the apparent EC_{50} for glucagon-stimulated adenylyl cyclase activation in murine liver membranes by >10-fold.

These data suggest that the compound is an antagonist of glucagon activity in a physiological tissue preparation. However, the compound (50 mg/kg, *per os*) does not inhibit glucagon

gon-stimulated increases in the blood glucose in mice, suggesting that its activity at the murine receptor is insufficient to be a useful tool in this species. Thus, identification of a suitable animal model is required for the analysis of the effects of this class of orally active glucagon antagonists on glucose homeostasis *in vivo*. Recently we have generated a murine line in which the native murine glucagon receptor has been replaced with the human receptor by homologous recombination, and preliminary data suggest that the activity of compounds in liver membranes from these mice correlates with their affinity as measured using the cloned human receptor.² Our current efforts are focused on expanding this murine line to extend these observations and to determine the effect of this class of compounds on glucose homeostasis.

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