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CP-99,711: A NON-PEPTIDE GLUCAGON RECEPTOR ANTAGONIST

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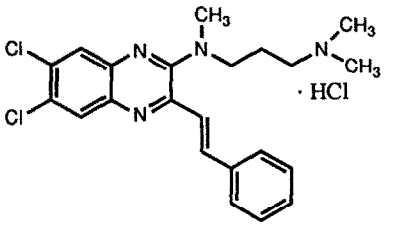
Abstract: CP-99,711, identified in a screening program, displaces [^{125}I]-glucagon from its rat liver receptor. We describe here the synthesis of this compound and its characterization as a functional glucagon receptor antagonist.

Glucagon is a 29 amino acid-containing peptide that is secreted by the α-cells in the pancreas. Binding of glucagon to its receptor in the liver leads to an increase in lipolysis, glycogenolysis and gluconeogenesis. Recent reports[1,2] have shown that in patients with type I insulin-dependent diabetes and type II non-insulin dependent diabetes mellitus, glucagon levels are high despite the concurrent basal hyperglycemia and hyperinsulinemia. Unger[2] has suggested that higher glucagon levels lead to hyperglycemia and hyperketonemia. Glucagon has also been shown to contribute more to hyperglycemia than hypoinsulinemia in an animal model of type I diabetes[3]. Thus, glucagon suppression could be a useful adjunct to conventional antihyperglycemic treatment of diabetics.

In the course of screening to discover non-peptide glucagon antagonists, we identified the quinoxaline, CP-99,711, which displaces [^{125}I]-glucagon from its rat liver receptor at low micromolar concentrations. In this paper we describe the synthesis of CP-99,711, its characterization in the binding assay, and its antagonism of glucagon-stimulated cAMP production in rat liver homogenates.

Glucagon his-ser-gln-gly-thr-phe-thr-ser-asp-tyr-ser-lys-tyr-leu-asp-ser-arg-arg-ala-gln-asp-phe-val-gln-trp-leu-met-asn-thrNH_2



CP-99,711

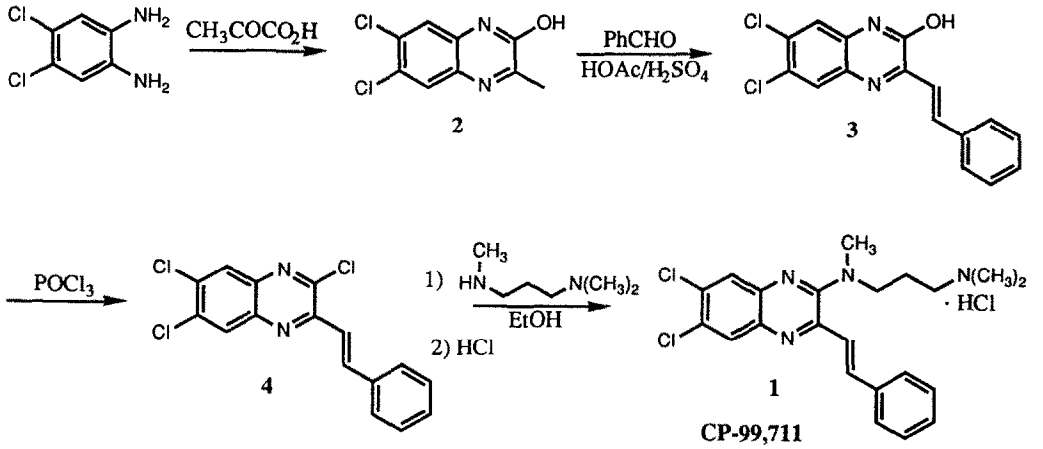
Synthesis

Preparation of CP-99,711 (1) (2-styryl-3-[3-(dimethylamino)propyl,methylamino]-6,7-dichloroquinoxaline) (Scheme 1):

Condensation of commercially available 4,5-dichloro-1,2-phenylenediamine with an excess of pyruvic acid in acetic acid-water gave quinoxaline **2** in 97% yield (needles from THF, mp > 280°C). Upon treatment with an equimolar amount of benzaldehyde in acetic acid-sulfuric acid (10 : 1) at reflux, styryl derivative **3** could be isolated in 93% yield (needles from THF, mp > 285°C). Conversion

to the trichloro species 4 was accomplished in 84% yield via $POCl_3$ addition at reflux (needles from isopropyl ether, mp 181-182°C). Reaction with excess N, N, N'-trimethylpropylenediamine in ethanol at reflux gave the amino substituted quinoxaline in 75% yield after chromatography. This compound was then converted to CP-99,711 by treatment with HCl in ether followed by recrystallization from ethanol to give yellow plates (mp 226-228 °C; ^1H NMR (300 MHz, DMSO-d_6) 8.16 (s, 1H), 7.96 (s, 1H), 7.83 (d, J = 15.7 Hz, 1H), 7.7 (m, 2H), 7.5 (m, 3H), 7.40 (d, J = 15.7 Hz, 1H), 4.3 (br s, 1H), 3.55 (t, J=7.0 Hz, 2H), 3.14 (s, 3H), 3.1 (m, 2H), 2.76 (s, 6H), 2.11 (app pent, 2H); analysis calculated for $C_{22}H_{24}N_4Cl_2 \cdot HCl$: C, 58.48; H, 5.58; N, 12.40; found: C, 58.19; H, 5.39; N,12.23).

Scheme 1



Biological Methods : Ligand Binding

Fresh liver tissue from adult male Sprague-Dawley rats was homogenized (Polytron) in cold 50 mM Tris•HCl buffer, pH 7.2 (at room temperature), and diluted to 4 g wet tissue/80 mL. Following centrifugation (30,000g, 15 min), the pellet was resuspended in 80 mL of the same buffer, recentrifuged, and finally resuspended at 0.005 g (original wet tissue weight)/mL 50 mM Tris•HCl buffer, pH 7.2, and kept at 0°C. [^{125}I]-glucagon (2200 Ci/mmol, Amersham) was diluted to 0.1 μCi/mL in 50 mM Tris•HCl buffer, pH 7.2, containing 1 mg/mL BSA and 0.1 mg/mL bacitracin.

Assays were carried out in 96-well microtiter plates, with each well containing 50 μL radioligand, 100 μL test compound in 50 mM Tris•HCl buffer, pH 7.2 containing bacitracin (0.1 mg/mL), BSA (1 mg/mL) and 0.7% v/v DMSO, and 50 μL tissue suspension. Following a 45 min incubation (room temperature, with gentle orbital shaking), unbound ligand was removed with a Skatron harvester (50 mM Tris pH 7.6, 0° wash) and the bound ligand, collected on fiberglass mats (LKB preprinted filtermats) pretreated with 0.2% polyethyleneimine, was counted in a BetaPlate scintillation counter (LKB). Non-specific binding was estimated by the addition of glucagon (Sigma; 0.5 μM final concentration) to control wells.

Biological Methods : Functional Characterization

CP-99,711 was tested for functional activity vs. glucagon-stimulated cAMP production as follows: rat liver homogenates were prepared as described in the ligand binding section above and resuspended at 0.02 g (original wet tissue weight)/mL 100 mM HEPES buffer, pH 7.2, and kept at 0°C. The reaction tubes contained 50 µL of this liver homogenate plus glucagon (10 nM) and test compound or vehicle in a reaction buffer media containing 1 mM EGTA, 2 mM $MgCl_2$, 10 mM creatine phosphate, 1 mM ATP, 0.5 mM IBMX, 50 µM GTP, 0.01% bacitracin, 0.002 mg/mL aprotinin, 100 units/mL creatine phosphokinase and 0.18% DMSO in a final volume of 200 µL. After incubation for 20 min, 30°, in a shaking water bath, the reactions were stopped by the addition of 10 µL of a 50 mM EDTA solution and the tubes were placed in a 95°C water bath for 3 minutes. The tubes were centrifuged at 14,000 rpm in an Eppendorf 5415 centrifuge for 3 minutes and the supernatant used for cAMP analysis. The amount of cAMP present in each tube was determined using the Amersham [^{3}H]cAMP assay kit. Fifty percent maximal stimulation was seen with 2 nM glucagon with a 6-fold increase in cAMP over basal observed at maximal glucagon concentration (100 nM). Assays with CP-99,711 contained 10 nM glucagon (5 times the EC_{50}).

Results and Discussion

As shown in Figure 1, CP-99,711 displaced radiolabelled glucagon from its rat liver receptor with an IC_{50} value of 4 ± 1 µM. Measurement of glucagon-stimulated cAMP production in rat liver homogenates showed that CP-99,711 is a functional antagonist with an IC_{50} value of 7 ± 1 µM (Figure 1). In the absence of glucagon, CP-99,711 (up to 32 µM) led to no increase in cAMP levels, indicating no measurable agonist activity.

Unson et al. have shown that modification of glucagon can lead to potent receptor antagonism[4,5]. Substitution of Asp^9 with Glu and elimination of the N-terminal histidine residue destroys agonist activity, giving a potent (2-fold less potent than glucagon) peptide antagonist. However, without knowledge of the bioactive conformation of glucagon, it is presumptive to specu-

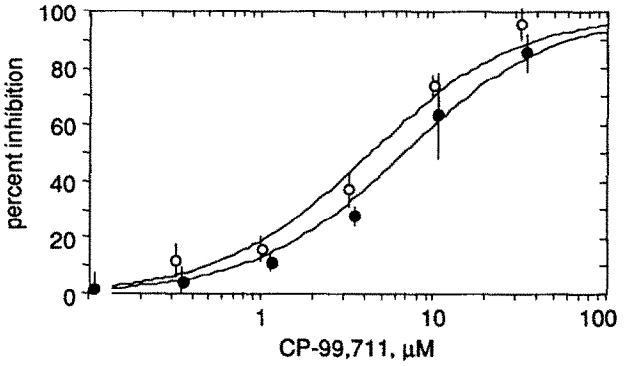


Figure 1 : [^{125}I]-Glucagon Displacement (○) and Functional Activity (•) of CP-99,711

Radioligand binding and cAMP production experiments were performed as described in the text.

late which functional groups of the peptides are mimicked by CP-99,711. While CP-99,711 represents a significant advance, it is important to note that other members of this chemical family are not specific glucagon receptor antagonists. Further examination has shown that other styryl quinoxalines inhibit the binding of other radioligands to their respective receptors. These receptors are all G-protein linked 7-transmembrane spanning proteins (eg. adrenergic), of which the glucagon receptor is a member. Thus, one can speculate that the styryl quinoxalines are binding to a common motif in G-protein linked receptors which is most likely distinct from the ligand-binding domain.

In conclusion, CP-99,711 joins the list of non-peptide antagonists of G-protein coupled receptors which have peptide agonists[6]: the Substance P antagonist CP-96,345 and GRP antagonists CP-70,030 and CP-75,998 recently described[7,8], CCK-A and CCK-B antagonists[9], angiotensin II antagonists[10], vasopressin antagonists[11], oxytocin antagonists[12], and C5a antagonists[13].

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