# Identification of A New G-Protein-Linked Receptor for Growth Hormone Secretagogues

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**The potential application of small molecules in GH therapy has recently become a topic of increasing interest. The spiroindoline MK-0677, the benzolactam L-692,429, and the peptides, GHRP-6 and hexarelin, have been shown to possess potent and selective GH-secretory activity in several species including human. Moreover, these synthetic GH secretagogues act on a signal transduction pathway distinct from that of GHRH. A specific high affinity binding site in porcine and rat anterior pituitary membranes that mediates the activity of these secretagogues has now been identified. The binding affinity of these structurally diverse secretagogues is tightly correlated with GH-secretory activity. The binding is $Mg^{2+}$-dependent, is inhibited by GTP-γ-S, and is not displaced by GHRH and somatostatin. The receptor is distinct from that for GHRH and has the properties of a new G-protein-coupled receptor. It is speculated that these GH secretagogues mimic an unidentified natural hormone that regulates GH secretion in concert with GHRH and somatostatin. (Molecular Endocrinology 10: 57–61, 1996)**

## INTRODUCTION

The secretion of GH by the somatotropic cells of the anterior pituitary gland is under complex hormonal control (1). The hypothalamic hormones, GHRH and somatostatin, respectively, stimulate and inhibit GH secretion (2, 3). In addition to GHRH and its analogs, there has recently been an upsurge of interest in small molecule GH secretagogues (Fig. 1) that act through a mechanism different from that of GHRH. GHRP-6, a synthetic hexapeptide (His-D-Trp-Ala-Trp-D-Phe-Lys-$NH_2$) modeled from enkephalins, stimulates GH secretion of pituitary somatotrophs *in vitro* and *in vivo* in a variety of species, including rat, swine, and human (4–6). Hexarelin, a 2-methyl substitution of D-tryptophan of GHRP-6, has recently been reported to be active in humans (7). However, these peptides have the disadvantage that they are less than 1% orally bioavailable; therefore, we sought mimetics that have structures more amenable to chemical modification and optimization of oral bioavailability (8). L-692,429 (3-amino-3-methyl-N-(2,3,4,5-tetrahydro-2-oxo-1{[2′-(1H-tetrazol-5-yl)(1,1′-biphenyl)-4-yl]methyl}-1H-1-benzazepin-3(R)-yl)-butanamide), a benzolactam GH secretagogue (8–10), and L-692,585, a hydroxy propyl derivative of L-692,429 (11), both induce a transient release of GH from rat pituitary cells and in rats and dogs, and L-692,429 is also effective in humans. MK-0677 (L-163,191), being a spiroindoline, belongs to a new structural class of GHRP-6 mimetics that has high *in vitro* and *in vivo* potency (12). A particular practical advantage of using MK-0677 for the treatment of GH deficiencies is that it has high oral bioavailability and potentiates the effect of GHRH. It has therefore been selected for clinical evaluation in human. GH secretagogues of spiroindoline, benzolactam and peptide classes offer the advantage over conventional forms of GH therapy. In contrast to GH administration, GHRP-6 induces a more physiological profile of GH secretion (13). They do not bypass the feedback loops that modulate the the GH/insulin-like growth factor-I axis (14). These secretagogues stimulate somatotrophs in the pituitary gland to release GH (4, 6) and act on the arcuate nucleus in the hypothalamus to apparently cause GHRH release (15). Moreover, they potentiate the effects of GHRH and functionally antagonize somatostatin (8, 16, 17). Thus, amplification of the signal to release GH occurs at three levels and, since these compounds act across a wide variety of animal species, we speculate that these secretagogues mimic an unidentified endogenous hormone, GHRH-amplifying hormone, that amplifies and is capable of initiating pulsatile GH secretion. Critical to the identification of the natural GHRH-amplifying hormone is characterization of the receptor.

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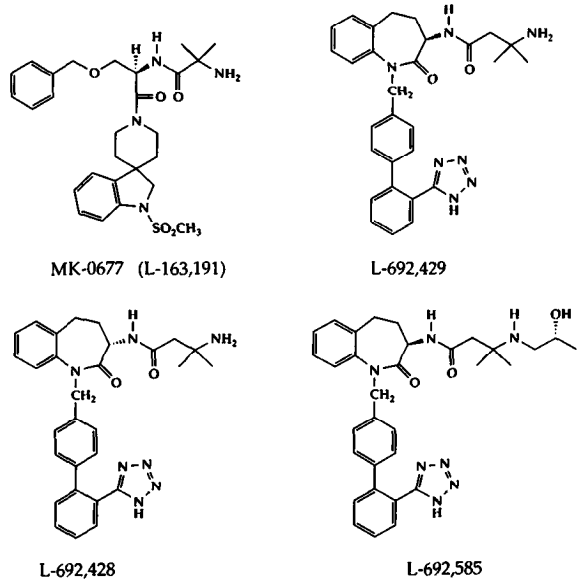


His-D-Trp-Ala-Trp-D-Phe-Lys-$NH_2$

GHRP-6

His-D-2-Methyl-Trp-Ala-Trp-D-Phe-Lys-$NH_2$

Hexarelin

**Fig. 1.** Structures of Spiroindoline, Benzolactam, and Peptide GH Secretagogues

Until now, identification of a discrete receptor for small and nonpeptide GH secretagogues has been elusive. The receptor appears to be distinct from that of the well characterized natural GH secretagogue GHRH. GHRH stimulates the activity of adenylate cyclase and increases the production of cAMP (3) while L-692,429, MK-0677, and GHRP-6 cause depolarization and inhibition of potassium channels in rat somatotrophs, activate protein kinase C, and increase phosphatidylinositol hydrolysis (4, 6, 8, 12, 16–19). Furthermore, desensitization is more rapid compared with GHRH, and cells that are desensitized by prolonged exposure to L-692,429, MK-0677, and GHRP-6 still response to GHRH (8, 12, 20). GH secretion induced by GHRP-6 and L-692,429, but not GHRH, is blocked by selective inhibitors *in vitro* (8) and by L-756,867 ($H_2N$-D-Arg-Pro-Lys-Pro-D-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Leu-$NH_2$) *in vitro* and *in vivo* (21). Indirect evidence therefore implies the existence of a new receptor(s) mediating the effects of GHRP-6, hexarelin, L-692,429, and MK-0677.

Very early in our discovery program we developed binding assays using [$^3$H]GHRP-6 and $^{125}$I-labeled analogs of GHRP-6; similar assays were reported by others (22, 23). However, we were frustrated by the findings that the binding specificity did not correlate with GH-secretory activity of peptide, benzolactam, and spiroindoline secretagogues. The binding was also of relatively low affinity and high capacity. We attributed the lack of correlation of binding and GH-secretory activity to masking of the receptor binding site because of the relatively low specific activity and "stickiness" of the radiolabeled ligands. We therefore sought an alternative ligand. $^{35}$S-Labeled MK-0677 was synthesized and used to detect the specific binding sites for GH secretagogues.

## RESULTS AND DISCUSSION

A RRA using high specific activity (700–1,100 Ci/mmol) $^{35}$S-labeled MK-0677 as ligand was developed. Saturable, high affinity binding was detected in porcine anterior pituitary membranes (Fig. 2A). Scatchard analysis (Fig. 2B) indicates the presence of a single class of high affinity sites with an apparent dissocia-

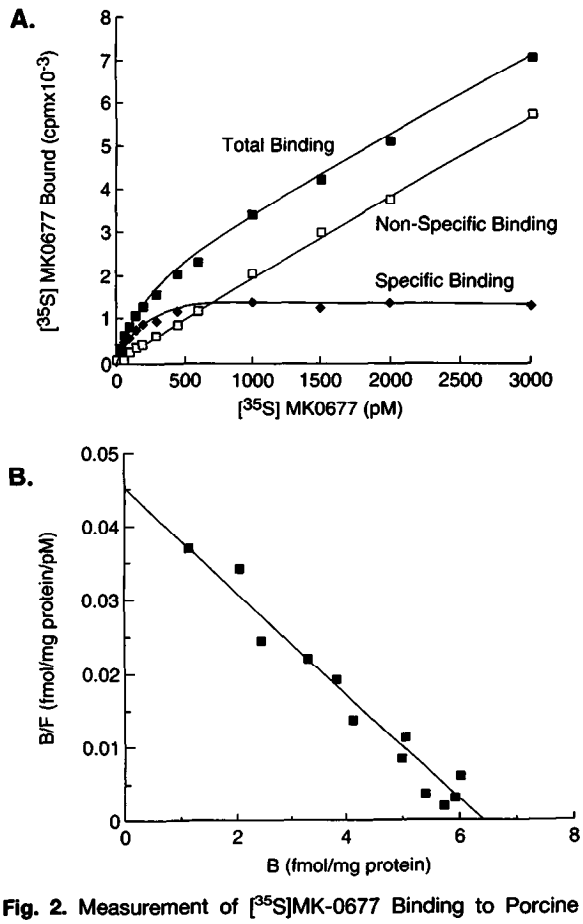


**Fig. 2.** Measurement of [$^{35}$S]MK-0677 Binding to Porcine Anterior Pituitary Membranes

Saturation experiments were performed by incubating a fixed concentration of membranes (150 $\mu$g protein) with various concentrations of [$^{35}$S]MK-0677 (20–3,000 pM) (A). Saturation isotherms of specific [$^{35}$S]MK-0677 binding were analyzed by Scatchard analysis (B).

tion constant ($K_D$) of 161 ± 11 pM and a concentration ($B_{max}$) of 6.3 ± 0.6 fmol/mg of protein (n = 4). A similar specific high affinity binding was detected in rat pituitary membranes indicating a $K_D$ value of 180 ± 9 pM and $B_{max}$ of 2.3 ± 1.1 fmol/mg protein (n = 3).

Competition binding studies show that relative binding affinity is predictive of the GH-secretory activity of both our nonpeptides and the GHRP-6 class of peptide GH secretagogues first described by Bowers *et al.* (4, 5). The specificity of [$^{35}$S]MK-0677 binding was established by determining the ability of GH secretagogues to compete with the radioligand for the binding sites (Fig. 3). Unlabeled MK-0677 completely displaced [$^{35}$S]MK-0677 from specific binding sites with an inhibition constant, $K_i$, of 240 pM, which is similar to the $K_D$ value determined by Scatchard analysis. L-692,585 ($K_i$ 0.8 nM), GHRP-6 ($K_i$ 6.3 nM), peptide antagonist L-765,867 ($K_i$ 38 nM), and L-692,429 ($K_i$ 63 nM) had affinities of 30, 3.8, 0.6, and 0.4%, respectively, of that of MK-0677. L-692,428, the biologically inactive stereoisomer of L-692,429, competed poorly with [$^{35}$S]MK-0677 binding. Saturation isotherm for [$^{35}$S]MK-0677 binding analyzed by double reciprocal plot showed that GHRP-6 inhibition was overcome by increasing concentration of [$^{35}$S]MK-0677 (Fig. 4). This result suggests that GHRP-6 interacts competitively with MK-0677 in the same binding site. Similarly, L-692,429 was shown to be a competitor of [$^{35}$S]MK-0677 binding. GH-secretory activity in primary cultures of rat pituitary cells is directly correlated with [$^{35}$S]MK-0677 binding affinity (Table 1). The most potent agonists had the highest affinities for pituitary receptor sites. Compounds inactive on binding of [$^{35}$S]MK-0677 at 1 μM included GHRH, somatostatin, met-enkephalin, substance P, galanin, GnRH, TRH, gastrin releasing peptide, PHM-27, MSH, pituitary adenylate cyclase activating polypeptide-38, phenoxybenzamine, dopamine, bromocriptine, methoxamine, benoxathian, isoproterenol, propanolol, and clonidine.

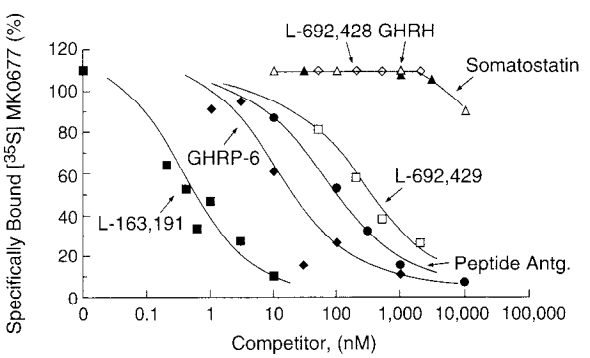


**Fig. 3.** Inhibition of [$^{35}$S]MK-0677 Binding to Porcine Anterior Pituitary Membranes by Various Compounds

Pituitary membranes were added to mixtures containing the test compounds and [$^{35}$S]MK-0677 (100 pM). Aliquots were incubated for 60 min at 20 C, and the results are expressed as percent of inhibition of [$^{35}$S]MK-0677 specifically bound.

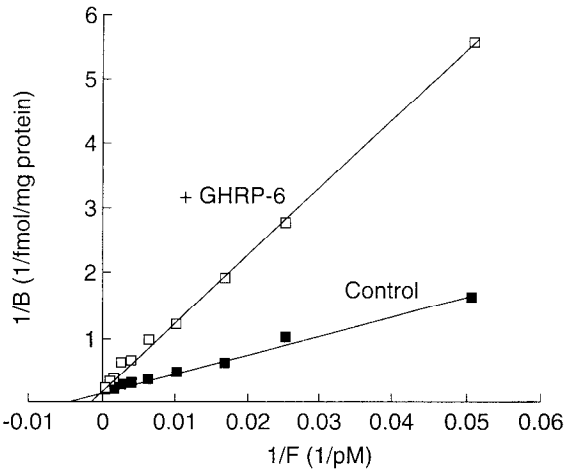


**Fig. 4.** Effect of GHRP-6 on Specific [$^{35}$S]MK-0677 Binding to Porcine Anterior Pituitary Membranes at Equilibrium

Saturation isotherms for [$^{35}$S]MK-0677 binding were performed as described in Fig. 2. Membranes were incubated with increasing concentrations of [$^{35}$S]MK-0677 in the absence or presence of 10 nM GHRP-6 at 20 C. The specific binding data were analyzed by double reciprocal plot.

**Table 1.** Comparison of Relative Biological Potencies and Binding Affinities of Different GH Secretagog Agonists and Antagonists

| | Concentration (nM) | |
|---|---|---|
| | $K_i$ (binding)[a] | $EC_{50}$ (secretion)[b] |
| MK-0677 | 0.24 | 1.3 |
| L-692,585 | 0.8 | 3 |
| L-692,429 | 63 | 60 |
| L-692,428 | >5,000 | >5,000 |
| Hexarelin | 0.3 | 1 |
| GHRP-6 | 6 | 10 |
| L-765,867 | 38 | (50)[c] |

[a] $K_i$ were determined by using the formula $k_i = IC_{50}/1 + [L]/K_D$; ligand concentration [L] + 100 pM.

[b] Biological potencies were determined by their abilities to stimulate GH secretion in rat pituitary cell cultures according to Cheng *et al.* (6).

[c] Inhibition of GH secretion induced by 100 nM GHRP-6.

To study whether the [$^{35}$S]MK-0677 specific binding site was G-protein linked, the effects of stable GTP analogs GTP-γ-S and guanylyl-imidodiphosphate (GMP-PNP) on [$^{35}$S]MK-0677 binding were studied. GTP-γ-S and GMP-PNP were found to be potent inhibitors of [$^{35}$S]MK-0677 binding with $IC_{50}$ values of 30 and 110 nM, respectively (Fig. 5). ATP-γ-S was ineffective. In addition, in the absence of $Mg^{2+}$, only 15–25% of specific binding of [$^{35}$S]MK-0677 binding was detected in comparison with control (10 mM $Mg^{2+}$), suggesting that the specific binding of [$^{35}$S]MK-0677 required the presence of $Mg^{2+}$.

In summary, L-692,585, L-692,429, GHRP-6, hexarelin, and a peptide antagonist L-756,867 inhibited [$^{35}$S]MK-0677 binding at their physiologically relevant

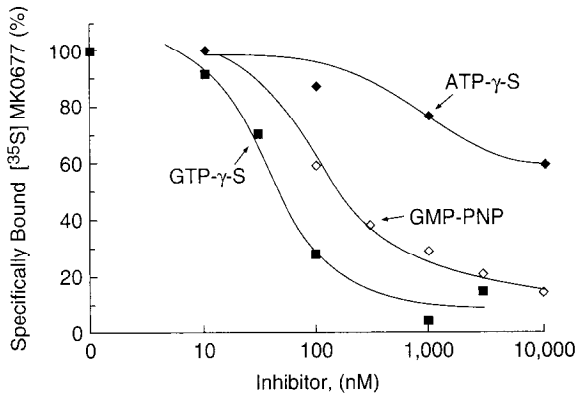


**Fig. 5.** Effects of GTP-γ-S and Nucleotides on the Specific [$^{35}$S]MK-0677 Binding to Porcine Anterior Pituitary Membranes

Membranes were added to mixtures containing the test compounds and 100 pM [$^{35}$S]MK-0677 and incubated for 60 min at 20 C. Results are expressed as percent of [$^{35}$S]MK-0677 specifically bound.

concentrations. The binding is stereospecific since L-692,428, the stereoisomer of L-692,429, was inactive. Importantly, double reciprocal plots demonstrate that the peptide GHRP-6 binds to the same site as MK-0677. Competition binding studies and titration of the concentration of binding sites for MK-0677 on pituitary cell membranes argue that these GH secretagogues bind to a different receptor than that defined for GHRH. Apparently, MK-0677 binds to a new receptor important in the control of GH release since other compounds, such as galanin, somatostatin, substance P, and adrenergic agents that are known to regulate GH release *in vivo* do not bind to the MK-0677 site. Because GTP-γ-S inhibits MK-0677 binding and $Mg^{2+}$ is required for binding, it is likely that the receptor mediating the GH-secretory activities of the benzolactam and spiroindoline secretagogues and GHRP-6 is G-protein linked; a receptor with identical properties has also been characterized in hypothalamic membranes from rats and pigs (data not shown). Other studies are consistent with activation of G-protein pathways coupled to potassium channels (16, 17) and phospholipase C (18, 19). Through our previous identification of selective antagonists of this class of GH secretagogue, and through our understanding of the signal transduction pathway for GHRP-6, L-692,429, and MK-0677 compared with GHRH, the development of a receptor-binding assay for MK-0677 provides the additional tool necessary for identifying a natural hormone for which we believe MK-0677, L-692,429, and GHRP-6 are mimetics.

## MATERIALS AND METHODS

### Preparation of High Specific Activity Radioligand [$^{35}$S]MK-0677

[$^{35}$S]MK-0677 was prepared from an appropriate precursor, N-[1(R)-[(1,2-dihydrospiro[$^{3}$H-indole-3,4′-piperidin]-1′-yl)-carbonyl]-2-(phenylmethyloxy)ethyl]-2-amino-t-butoxycarbonyl-2-methylpropanamide, using methane [$^{35}$S]sulfonyl chloride as described (24). Purification by semipreparative HPLC [Zorbax SB-phenyl column, 68% MeOH/water, 0.1% trifluoroacetic acid, 5 ml/min]) was followed by N-t-BOC cleavage using 15% trifluoroacetic acid in dichloromethane (25 C, 3 h) to give [methylsulfonyl-$^{35}$S]MK-0677 in near quantitative yield. HPLC purification (Hamilton PRP-1 4.6x250 mm column, linear gradient of 50–75% methanol-water with 1 mM HCl over 30 min, 1.3 ml/min) provided the ligand in greater than 99% radiochemical purity. The structure was established by HPLC coelution with unlabeled MK-0677 and by mass spectral analysis (D. C. Dean, R. P. Nargund, S-S. Pong, L-YP. Chaung, R. Chen, P. Griffin, D. G. Melillo, L. Van der Ploeg, A. A. Patchett, and R. G. Smith, manuscript in preparation). The latter method also indicated a specific activity of approximately 1000 Ci/mmol.

### Preparation of Pituitary Membranes

Frozen anterior pituitary glands from male swine (50–80 kg) or from Wistar male rats (150–200 g) were homogenized in a tissue homogenizer in ice-cold buffer (50 mM Tris-HCl buffer, pH 7.4, 5 mM $MgCl_2$, 2.5 mM EDTA, 0.1% BSA, and 30 μg/ml bacitracin). The homogenates were centrifuged for 5 min at 1,400 × *g*, and the resulting supernatants were then centrifuged at 34,000 × *g* for 20 min. The pellets were resuspended in the same buffer to a 1,500 μg protein/ml and stored at −80 C. Protein was determined by a Bio-Rad method (Bio-Rad Laboratories, Richmond, CA).

### Receptor Binding Assay

Standard binding solution contained: 400 μl of 25 mM Tris-HCl buffer, pH 7.4, 10 mM $MgCl_2$, 2.5 mM EDTA, and 100 pM [$^{35}$S]MK-0677. Pituitary membranes (100 μl, 150 μg protein) were added to initiate the binding reaction. Aliquots were incubated at 20 C for 60 min, and bound radioligand was separated from free by filtration through GF/C filters pretreated with 0.5% of polyethylenimine in a Brandel cell harvester. The filters were washed three times with 3 ml ice-cold buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 2.5 mM EDTA, and 0.015% Triton X-100), and the radioactivity on the filters was counted in Aquasol 2. Specific binding was defined as the difference between total binding and nonspecific binding assayed in 500 nM unlabeled MK-0677. Specific bindings were 65–85 and 45–60% of total binding, in porcine and rat membranes, respectively. Assays were carried out in triplicate and experiments repeated at least three times.

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